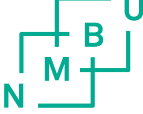

Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Production Animal Clinical Sciences

Philosophiae Doctor (PhD)
Thesis 2020-34

Tick-borne encephalitis virus – a One Health approach

Skogflåttencefalittvirus – i én-helse perspektiv

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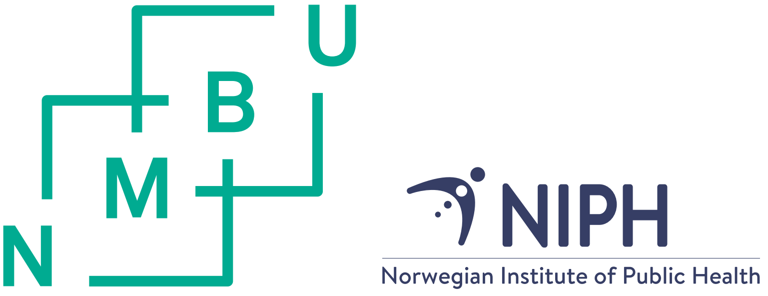

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Oslo 3/3-20

Katrine Mørk Paulsen

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List of papers

This Ph.D. - thesis is based on the following papers:

Paper 1: Cervids as sentinel-species for tick-borne encephalitis virus in Norway - A serological study

Katrine M. Paulsen*, Carlos G. das Neves*, Benedikte N. Pedersen, Knut Madslien, Erik G. Granquist, Snorre Stuen, Rose Vikse, Mara Rocchi, Ellie Laming, Karin Stiasny and Åshild K. Andreassen (2019)

*Both authors contributed equally to this work

Zoonoses and Public Health. Published online December 19. 2019.

DOI: 10.1111/zph.12675

Paper 2: Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway

Katrine M. Paulsen, Snorre Stuen, Carlos G. das Neves, Faisal Suhel, Deepa Gurung, Arnulf Soleng, Karin Stiasny, Rose Vikse, Åshild K. Andreassen* and Erik G. Granquist*

*Both authors contributed equally to this work

Zoonoses and Public Health. 2019 Mar;66(2):216-222.

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Paper 3: Experimental infection of lambs with of tick-borne encephalitis virus and co-infection with *Anaplasma phagocytophilum*

Katrine M. Paulsen, Erik G. Granquist, Wenche Okstad, Rose Vikse, Karin Stiasny, Åshild K. Andreassen* and Snorre Stuen* (2019).

*Both authors contributed equally to this work

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Abbreviations and definitions

16S rRNA	16S ribosomal RNA is the component of the 30S small subunit of a prokaryotic ribosome.
Amplifying host	A host in which infectious agents, e.g. TBEV, multiply to. higher concentrations
BHK-21 cells	Baby hamster kidney cells
Bp	Base pair
C	Capsid protein
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
Co-feeding	When an infected tick and non-infected tick(s) are feeding close together or soon after each other on the same area of a host. Transmission of TBEV by co-feeding is independent of the host's viraemia.
Counties	Names of Norwegian counties used in this thesis are based on the counties as they were in 2018. Since then, counties have been merged. As of 1st of January 2020 these counties have had changed borders:

Old counties:	**New counties:**
Hordaland and Sogn og Fjordane	Vestland
Aust-Agder and Vest-Agder	Agder
Vestfold and Telemark	Vestfold og Telemark
Oppland and Hedmark	Innlandet
Buskerud, Akershus and Østfold	Viken
Troms and Finnmark	Troms og Finnmark

Ct	Cycle threshold

DNA	Deoxyribonucleic acid
E	Envelope protein
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immunosorbent assay
Endemic area	A geographical region where a particular disease is prevalent. According to ECDC classification for the risk for TBE, an area is endemic if TBE cases have been shown to occur regularly in subsequent seasons.
FFU	Focus forming units
Foci	TBEV exist in natural foci, which are small restricted areas where TBEV is circulation among its vectors and reservoir hosts. The size of a TBEV foci may vary from approximately 5,000 to 10,000 m^2.
FOTS	The Norwegian Animal Research Authority (Norwegian Food Safety Authority)
gltA	Citrate synthase gene
HI	Haemagglutination inhibition test
HOP	Norwegian health monitoring program for deer and muskox
Host	Tick hosts include all species that ticks feed on in nature. See also reservoir host.
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LB	Lyme borreliosis
LGTV	Langat virus
LIV	Louping-ill virus
NCR	Non-coding region

NS	Non-structural proteins
M	Membrane protein
MSIS	Norwegian Surveillance System for Communicable diseases
NIPH	Norwegian Institute of Public Health
PCR	Polymerase chain reaction
ORF	Open reading frame
RNA	Ribonucleic acid
RNase	Ribonuclease
RT PCR	Reverse transcriptase polymerase chain reaction
SNT	Serum neutralisation test
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TBEV-Eu	Tick-borne encephalitis virus, European subtype
TBEV-FE	Tick-borne encephalitis virus, Far Eastern subtype
TBEV-Sib	Tick-borne encephalitis virus, Siberian subtype
TBF	Tick-borne fever
Vector	To be considered a vector, a tick species must: (i) feed on infectious vertebrates; (ii) be able to acquire the pathogen during the blood meal; (iii) maintain it through one or more life stage (transstadial passage); and (iv) pass it through to other hosts when feeding again.
qPCR	Quantitative polymerase chain reaction
Reservoir host	Reservoir hosts are proven natural hosts of vector ticks, and ticks may become infected while feeding on them. The

	reservoir of an infectious agent, e.g. TBEV, is the habitat in which the agent normally lives, grows, and multiplies.
Sequelae	A pathological condition resulting from a prior disease, injury or attack. It may be a chronic condition that is a complication which follows a more acute condition. It is different from, but is a consequence of, the first condition.

Summary

Tick-borne encephalitis virus (TBEV) is a *Flavivirus* which is transmitted to humans and animals by tick bites, and in rare cases by consumption of unpasteurised milk and dairy products. The virus can cause the disease tick-borne encephalitis (TBE), and lead to severe infection of the central nervous system in humans and animal species such as dogs and horses. TBEV is widespread in Europe and Asia, and an increasing number of TBE cases have been reported in the recent decades, including in Norway. The aims of this Ph.D.-thesis were to study the occurrence of: antibodies against TBEV in deer and cows, TBEV in unpasteurised cow's milk in Norway, and to study co-infection of TBEV and the bacterium *Anaplasma phagocytophilum* in lambs by an experimental study.

TBEV antibodies were found in moose (*Alces alces*), roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) from areas both with and without reported human TBE cases. The study showed that seroprevalence studies in cervids can be used to identify possible new distribution areas for TBEV. Up-to-date information on the prevalence of TBEV is important in a public health perspective, as it may prevent future cases of TBE.

TBEV was found for the first time in unpasteurised cow's milk at three farms in Norway. As in the cervid study, the findings were from areas both with and without reported TBE cases. Antibodies against TBEV were found at one farm, located in an area where several human TBE cases have been reported. Detection of TBEV in unpasteurised milk shows that it may be important to pasteurise the milk in areas where TBEV is distributed to prevent infection. Future studies should investigate whether the virus found in unpasteurised milk in Norway can cause TBE in humans.

The experimental study showed that a single infection with TBEV (Hochosterwitz strain) did not give clinical signs in lambs. In contrast to co-infection with louping-ill virus and *A. phagocytophilum,* which is known for the possibility to cause severe disease in sheep, co-infection with TBEV and *A. phagocytophilum* did not seem to have an impact on the clinical symptoms in lambs. In our study, a significantly higher TBEV antibody response was found in the group of lambs co-infected with TBEV and *A. phagocytophilum* than in the groups with a single infection of TBEV.

Sammendrag (Norwegian summary)

Skogflåttencefalittvirus (TBEV) er et *Flavivirus* som overføres til mennesker og dyr via flåttbitt, og i sjeldne tilfeller fra inntak av upasteurisert melk og andre meieriprodukter. Viruset kan forårsake sykdommen skogflåttencefalitt (TBE) og føre til alvorlig infeksjon i sentralnervesystemet hos mennesker og dyr som hund og hest. TBEV er utbredt i Europa og Asia, og det har blitt rapportert om et økende antall TBE-tilfeller de siste tiårene, også i Norge. Hensikten med denne doktorgradsavhandlingen var å studere forekomsten av: antistoffer mot TBEV i hjortedyr og kyr, TBEV i upasteurisert kumelk i Norge, og å studere koinfeksjon av TBEV og bakterien *Anaplasma phagocytophilum* i lam i en eksperimentell studie.

TBEV-antistoffer ble funnet i elg (*Alces alces*), rådyr (*Capreolus capreolus*) og hjort (*Cervus elaphus*) fra områder både med og uten rapporterte humane TBE-tilfeller. Studien viste at seroprevalensstudier i hjortedyr kan brukes til å identifisere mulige nye utbredelsesområder for TBEV. Oppdatert informasjon om utbredelsen til TBEV er viktig i et folkehelseperspektiv, da det kan hindre fremtidige tilfeller av TBE.

TBEV ble for første gang påvist i upasteurisert kumelk fra tre gårder i Norge. Som i hjortedyrstudien, var funnene fra områder både med og uten meldte TBE-tilfeller. Antistoffer mot TBEV ble funnet fra én gård, i et område hvor flere humane TBE-tilfeller har blitt rapportert. Påvisning av TBEV i upasteurisert melk viser at det kan være viktig å pasteurisere melken i områder hvor TBEV er utbredt for å forhindre smitte. Fremtidige studier bør undersøke om viruset som ble funnet i upasteurisert melk i Norge kan forårsake TBE hos mennesker.

Den eksperimentelle studien viste at infeksjon med kun TBEV (Hochosterwitz-stammen) ikke ga kliniske sykdomstegn hos lam. I motsetning til koinfeksjon med louping-ill virus og *A. phagocytophilum*, som er kjent for å forårsake alvorlig sykdom hos sau, så det ikke ut til at koinfeksjon med TBEV og *A. phagocytophilum* hadde noen effekt på de kliniske symptomene hos lam. I vår studie ble det funnet en signifikant høyere antistoffrespons i gruppen med lam koinfisert med TBEV og *A. phagocytophilum*, sammenlignet med gruppene infisert med kun TBEV.

1. Introduction

1.1. General background

Tick-borne diseases, such as tick-borne encephalitis (TBE), have been an increasing challenge in Europe during the last decades. This could have several reasons, such as increased awareness of tick-borne diseases among the public, improved diagnostics and reporting of TBE cases, increased recreational activity in areas where ticks are abundant, and changes in climatic conditions and tick hosts affecting tick habitats (Jaenson et al., 2012; Lindquist & Vapalahti, 2008).

Tick-borne encephalitis virus (TBEV) is the causative agent of TBE, a severe neurological disease in humans and a few other mammals (Lindquist & Vapalahti, 2008; Suss, 2011). TBEV is an arthropod-borne virus (arbovirus) transmitted by hard ticks of the family Ixodidae and is distributed in large areas of Europe and Asia. The epidemiology of TBE depends on a number a factors, such as climate, virus genotypes, vector density and transmission hosts (Jaenson et al., 2012; Jaenson et al., 2018; Randolph, 2004). There are more than 10,000 cases of TBE reported annually in Europe and Asia (Erber et al., 2019; Suss, 2011). TBE has been a mandatory reportable disease to the European Centre for Disease Prevention and Control (ECDC) since 2012.

TBE was first described by Hans Schneider in 1931 (Schneider, 1931). The causative agent of TBE was unknown until 1937, when Levkovich Zilber, the head of the first medical virological laboratory in the former Soviet Union, led an expedition in Far Eastern Russia. The background for this expedition was that in the 1930s, a number of inhabitants, living in the Taiga region in the Far East became ill with an unknown causative pathogen, giving severe neurological disease. During the expedition, TBEV was isolated and characterised. The team isolated the virus from human patients, mice, and ticks feeding on the mice. In this way, the team was able to identify the causative agent and its vector, *Ixodes persulcatus.* The team also warned the local inhabitants to avoid tick bites, resulting in a declining number of human cases (Zilber, 1939; Zlobin et al., 2017).

Taxonomically, TBEV is a member of the genus *Flavivirus* within the family *Flaviviridae.* Other medically important viruses in the same genus are the mosquito-borne viruses; dengue

virus, Japanese encephalitis virus, West Nile virus, yellow fever virus and Zika virus (Figure 1) (Gould & Solomon, 2008). Flaviviruses are known to pose a threat to both public and animal health (Hollidge et al., 2010; Wilson, 2013).

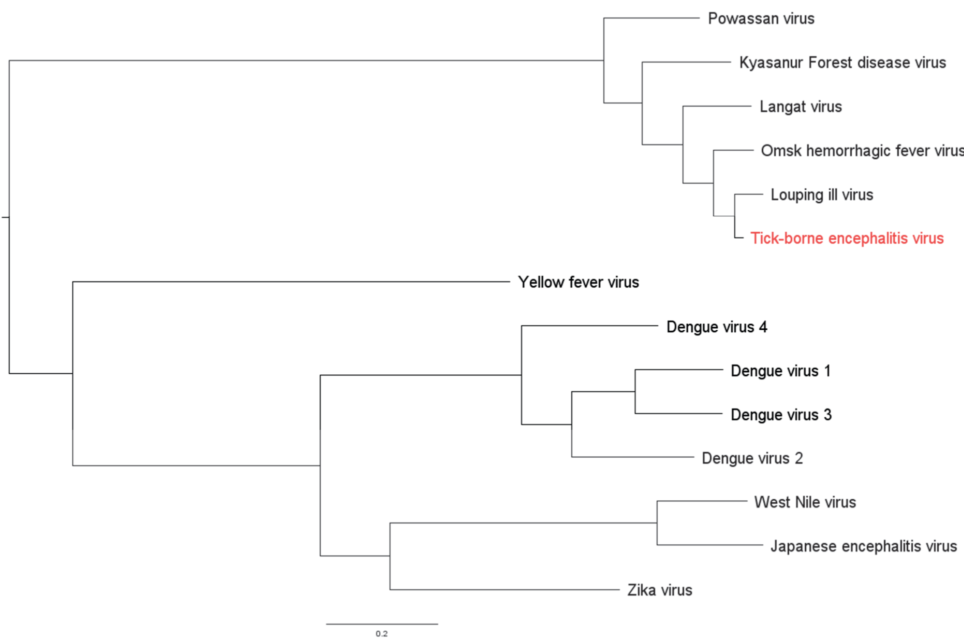

Figure 1: Phylogenetic tree of the TBEV complex and mosquito-borne flaviviruses (midpoint rooted maximum likelihood tree made in Geneious Prime v. 2019 1.1).

1.2. The tick as a vector

Ticks are distributed in most terrestrial regions of the word, and are considered one of the most important vectors of pathogens that can cause diseases in humans and animals in Europe (Barker & Murrell, 2008; Beugnet & Marie, 2009; Granstrom, 1997). Ticks belong to the class Arachnida and the subclass Acari, which are further divided into the three following families: Ixodida, Argasidae and Nuttalliellidae. These families consist of more than 900 species (Barker & Murrell, 2008).

The sheep tick (*Ixodes ricinus*), also called the castor bean tick, is considered an important vector for human and animal pathogens in Europe (Charrel et al., 2004; Granstrom, 1997; Parola & Raoult, 2001), and is together with the taiga tick (*Ixodes persulcatus*), the main

vector for TBEV in Europe and Asia (Lindquist & Vapalahti, 2008). Other tick species which may transmit TBEV are *Ixodes arborcola*, *Ixodes gibbosus*, *Dermacentor marginatus*, *Dermacentor reticulatus*, *Haemaphysalis concinna* and *Haemaphysalis inermis.* TBEV has been detected in numerous other tick species, but it is not known if they function as vectors for the virus (Chitimia-Dobler et al., 2019; Hubalek & Rudolf, 2012; Kozuch & Nosek, 1971, 1980; Lichard & Kozuch, 1967; Nosek et al., 1972).

I. ricinus is a three-host tick species and is feeding on a wide range of animal species. The life cycle of *I. ricinus* consists of four stages: egg, larva, nymph and adult (Figure 2). The active stages; larva, nymph and adult, are feeding on different hosts. The larvae are mostly feeding on smaller vertebrates such as rodents and ground feeding birds. Nymphs and adults are feeding on larger vertebrates, such as hares and cervids (Jaenson et al., 1994; Jaenson et al., 2018; Talleklint & Jaenson, 1997). The life cycle of *I. ricinus* usually lasts two to three years, but may last up to six years (Gray et al., 2016) .

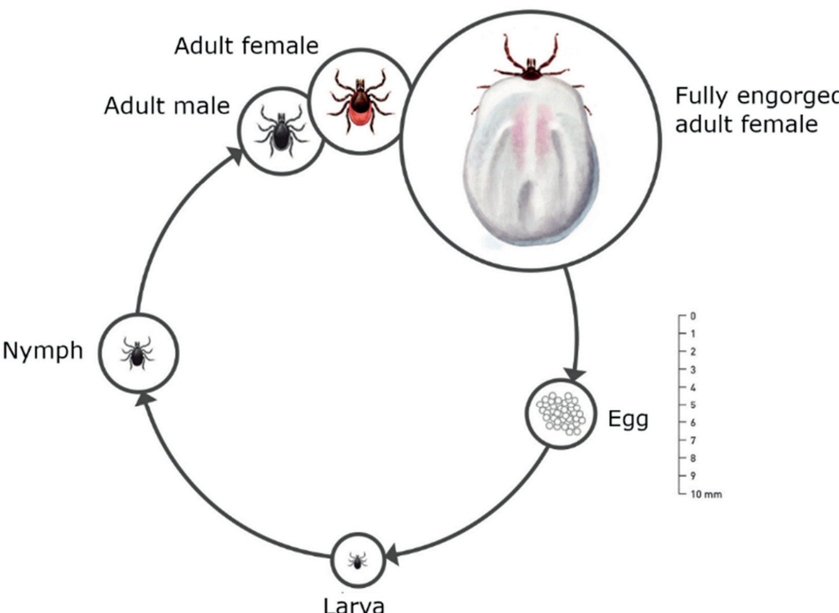

Figure 2: The life cycle of *Ixodes ricinus* consists of four stages: egg, larva, nymph and adult (Preben Ottesen and Hallvard Elven, NIPH. Modified and used with permission).

The main vector for TBEV in Norway is *I. ricinus* (Kjaer et al., 2019; Vikse et al., 2020). Norway represents part of the northern border of the geographical distribution range of *I. ricinus*. The tick species has primarily been found in coastal regions of Norway, from the county of Østfold in the southeast to Brønnøy in the county of Nordland in the north (Hvidsten et al., 2014; Larsson et al., 2018; Mehl, 1983; Soleng et al., 2018; Tambs-Lyche, 1943). There are reports of ticks further north than Brønnøy, however, the tick population densities reported in field studies have been low (Jenkins et al., 2012; Soleng et al., 2018). The northernmost location of a permanent *I. ricinus* population in Norway has recently been found on the island of Dønna at 66.2°N (Hvidsten et al., 2020).

I. persulcatus has not been documented in Norway (Kjaer et al., 2019). However, established populations of *I. persulcatus* have been detected in Finland and recently in northern Sweden, which may indicate a north-western spread of this species (Jaaskelainen et al., 2006; Jaenson et al., 2016; Laaksonen et al., 2017).

1.3. Tick-borne encephalitis virus

1.3.1. Genomic structure

TBEV has an approximately 11 kilobases long positive-sense single stranded RNA genome (Heinz & Mandl, 1993). The genome encodes one open reading frame (ORF), which is flanked by 5′ and 3′ non-coding regions (NCR). The ORF encodes one large polyprotein of about 3400 amino acids and is divided into the structural proteins capsid (C), membrane (M) and envelope (E), the non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, and the peptide 2K (Figure 3). Infectious TBE virons are spherical particles approximately 50 nm in diameter. The nucelocapsid consists of one viral RNA genome and multiple copies of the C-proteins (Slavik et al., 1970).

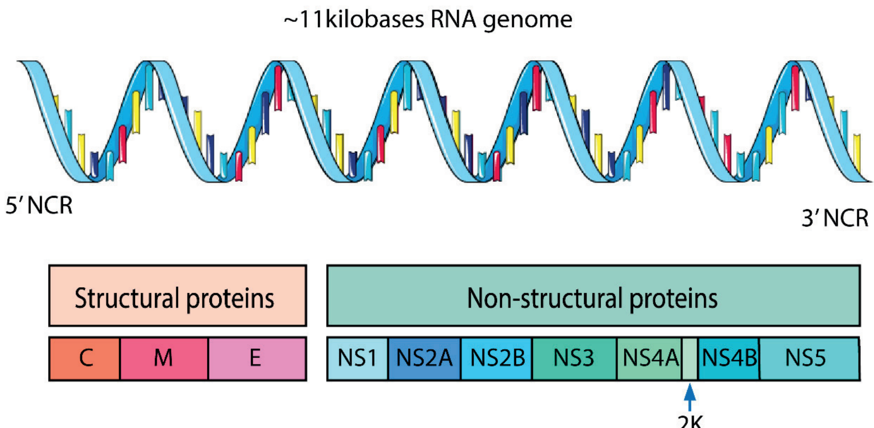

Figure 3: Schematic representation of the genome of tick-borne encephalitis virus (TBEV). C=capsid, M=membrane, E=envelope, NS=non-structural (NS) proteins (Ruzek et al., 2019). The figure is not to scale. The figure is created using Servier Medical art (www.servier.com).

The structural proteins are mainly involved in formation of the TBEV particle (Heinz & Mandl, 1993). The C protein interacts with viral RNA genome and represents a structural component of the virus and is known to have low sequence homology between different flaviviruses (Lindenbach & Rice, 2003). The E glycoprotein contains the major viral antigens and is the main target for neutralising antibodies. The E protein is also responsible for specific binding to receptors and penetration of the virus into the host cells, and is believed to be a main determinant of TBEV virulence (Gritsun et al., 1995). The PrM protein is a glycosylated precursor of the M-protein, and a conserved region in this protein is found to be a critical molecular dominant for the assembly and secretion of the virus (Yoshii et al., 2012). The NS proteins are multifunctional and are responsible for viral RNA replication and viral assembly (Lindenbach & Rice, 2003). The 5′ and 3′ NCR consists of elements that are essential for genome cyclisation, resulting in initiation of RNA synthesis (Khromykh et al., 2001; Kofler et al., 2006; Muto et al., 2018; Ng et al., 2017). The 3′ NCR has low sequence conservation, repeating sequences and an internal polyA that varies in size (Asghar et al., 2016; Gritsun & Gould, 2007a, 2007b; Wallner et al., 1995). The length range in the variable region of 3′ NCR has been related to the number of laboratory passages of viral strains (Mandl et al., 1998).

1.3.2. Subtypes and distribution

TBEV has a patchy geographical distribution in large areas of Europe and Asia. The virus has traditionally been divided into three main subtypes: the European (TBEV-Eu), the Siberian (TBEV-Sib) and the Far Eastern (TBEV-FE). At least three other subtypes have been proposed: the Baikalian (also named 886-84), the Himalayan and 178-79 (Adelshin et al., 2019; Dai et al., 2018; Demina et al., 2010; Dobler et al., 2012; Kovalev & Mukhacheva, 2017).

The different subtypes of TBEV differ in geographical distribution, vector, virulence, and in nucleotides and amino acids in their polyprotein. TBEV-Eu is distributed in Europe, from the United Kingdom, France and the Netherlands in the west to European parts of Russia in the east (Figure 4) (Dekker et al., 2019; Dobler et al., 2012; Holding et al., 2020; Ruzek et al., 2019; Velay et al., 2018). In addition, TBEV-Eu has also been found in South-Korea (Im et al., 2019; Yun et al., 2011). TBEV-FE and TBEV-Sib are mainly found in Asia. In some regions, such as the Baltic States, Siberia and Ukraine, the TBEV-Eu, TBEV-Sib and TBEV-FE subtypes are known to coexist (Beauté et al., 2018; Ecker et al., 1999; Lundkvist et al., 2001). TBEV-Sib has also been found in Finland (Jaaskelainen et al., 2006; Kuivanen et al., 2018). The Baikalian and 178-79 subtypes have both been found near lake Baikal in the Irkutsk region in Russia, while the Himalayan subtype has been found in Himalaya (Dai et al., 2018; Kovalev & Mukhacheva, 2017).

The TBEV-Eu subtype is primarily transmitted by *I. ricinus,* while TBEV-Sib and TBEV-FE are mainly transmitted by *I. persulcatus* (Lindquist & Vapalahti, 2008). However, TBEV-Eu and TBEV-Sib seem to co-circulate in *I. persulcatus* and *I. ricinus* in some countries, such as Finland (Jaaskelainen et al., 2011; Jaaskelainen et al., 2016).

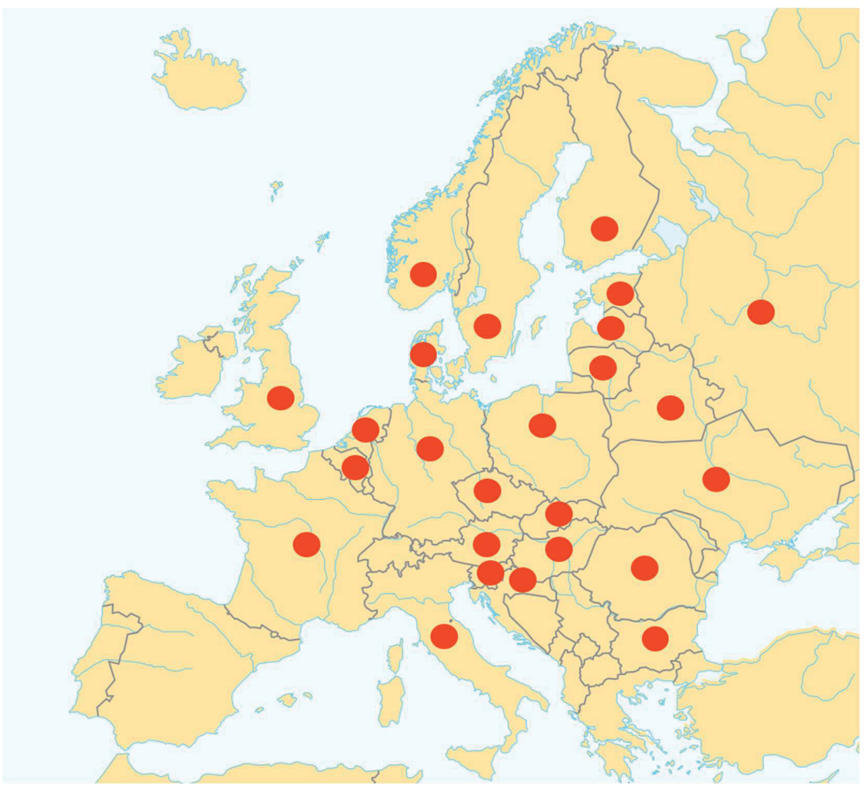

Figure 4: Countries with confirmed cases of tick-borne encephalitis (TBE) in Europe are indicated with red dots (Dobler et al., 2019). Infection sites are not indicated in the map (Kartverket, Creative Commons BY 4.0).

Within a subtype, the degree of variation in amino acid is low, up to 2.2 % in the polyprotein, and the variations between subtypes is found to vary from 5.0 % to 7.3 % sequence difference in the polyprotein. The difference between the subtypes in the nucleotides has been found to be between 14.6 % and 16.5 % (Adelshin et al., 2019; Demina et al., 2010; Ecker et al., 1999).

The disease caused by the different subtypes of TBEV varies in severity and mortality (Gritsun et al., 2003b). Human infection with the TBEV-Eu subtype usually results in mild forms of TBE with a mortality rate of less than 2 %. TBEV-Sib causes similar symptoms although more severe disease outcomes have been reported (Gritsun et al., 2003c). TBEV-FE is the subtype with the highest reported mortality rate of up to between 20 % and 40 % (Dorrbecker et al., 2010). Fatality rates are, however, difficult to find. An epidemiological

report by ECDC indicated a TBE fatality rate of about 0.6 % in Europe in 2018 (Russia not included) (European Centre for Disease Prevention and Control 2019).

1.3.3. TBE and TBEV in Norway

In Norway, TBE has been a mandatory notifiable disease to the Norwegian Surveillance System for Communicable Diseases (MSIS) since 1975. The first reported human case of TBE in Norway occurred in 1997 (Skarpaas et al., 2004). Phylogenetical analysis of parts of the E-protein showed that the strain belonged to the TBEV-Eu subtype (Skarpaas et al., 2006). The patient was assumed to be infected at Tromøy located in the county of Aust-Agder in southern Norway (Skarpaas et al., 2002). Skarpaas and co-workers also analysed questing *I. ricinus* ticks collected from the neighbouring county of Vest-Agder, and found a TBEV-prevalence of 0.2 % to 0.3 % (Skarpaas et al., 2006).

The viral strain "Mandal 2009" is the only whole genome sequence of TBEV retrieved from Norway. Phylogenetically, "Mandal 2009" was found to belong to the Scandinavian group of the TBEV-Eu subtype. However, "Mandal 2009" revealed a shorter form of the TBEV genome within the 3' NCR, similar to the highly virulent Hypr-strain (Asghar et al., 2014).

The first suggested isolate of TBEV in Norway was described in 1978. The isolate originated from questing *I. ricinus* ticks collected in Sogn og Fjordane county in western Norway. Suspensions of the ticks were injected to mice brain. Serum samples from mice that showed clinical signs were analysed by haemagglutination inhibition tests and five positive samples with close serological relationship to the TBEV complex were detected (Traavik et al., 1978). In 1979, Traavik detected a 19.6 % TBEV seroprevalence by haemagglutination inhibition tests in humans from the same area (Traavik, 1979).

An overview of studies that have detected TBEV in Norway is given in Table 1. In 2012, Andreassen et al. published a study on the prevalence of TBEV in questing *I. ricinus* nymphs in southern Norway. This study found a prevalence ranging from 0.1 % to 1.2 % in the study areas (Andreassen et al., 2012). In addition to the findings of TBEV in questing ticks in southern Norway (Andreassen et al., 2012; Kjelland et al., 2018; Skarpaas et al., 2006), TBEV has also been detected in questing *I. ricinus* nymphs and adults from coastal areas in the counties of Akershus, Østfold, Telemark, Buskerud, Vestfold, Rogaland, Hordaland, Sogn og Fjordane Møre og Romsdal, Trøndelag, and Nordland (Figure 6) (Larsen et al., 2014; Paulsen

et al., 2015; Soleng et al., 2018; Vikse et al., 2020). Furthermore, a study on blood donors has been published from the county of Østfold where a seroprevalence of 0.65 % was confirmed by a neutralisation test (Larsen et al., 2014).

There is limited information on TBE in animals in Norway. A seroprevalence of 17.3 % has previously been detected in bovines by haemagglutination inhibition tests in southern and western Norway (Traavik, 1973). A study on TBEV IgG antibodies in dog serum found a seroprevalence of 16.4 % in southern Norway (Csango et al., 2004). Additionally, a seroprevalence study on cervids in the counties of Vest-Agder and Møre og Romsdal found a prevalence of 41 % and 1.6 %, respectively (Ytrehus et al., 2013).

Table 1: Overview of studies and prevalence of TBEV in Norway

Area	Reported human TBE cases	Species	Method	Prevalence range % (# of pos. samples/ total) *	Reference
Østfold	No	*I.ricinus* nymphs	RT real-time PCR, pyrosequencing	0-0.4	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-15	Vikse et al. 2020
		I.ricinus nymphs	RT real-time PCR, pyrosequencing	0.1 (1/740)	Larsen et al. 2014
		blood donors	ELISA and SNT	0.65 (3/461)	Larsen et al. 2014
Vestfold	Yes	*I. ricinus* nymphs	RT real-time PCR, pyrosequencing	0-0.9	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	1.9-20	Vikse et al. 2020
Akershus	No	*I.ricinus* nymphs	RT real-time PCR, pyrosequencing	0-0.4	Vikse et al. 2020
Buskerud	Yes	*I. ricinus* adults	RT real-time PCR, pyrosequencing	0-8.6	Vikse et al. 2020
Telemark	Yes	*I. ricinus* nymphs	RT real-time PCR, pyrosequencing	0-0.6	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	1.4-9.5	Vikse et al. 2020
Vest-Agder	Yes	*I. ricinus* nymphs	Conventional RT PCR, Sanger sequencing	0.2-0.3 (1/450 and 1/360)	Skarpaas et al. 2006
		I. ricinus nymphs	RT real-time PCR, pyrosequencing	0.2-1.2 (2/940-9/780)	Andreassen et al. 2012
		I. ricinus nymphs	RT real-time PCR, pyrosequencing	0.5 (4/740)	Kjelland et al. 2018
		I. ricinus adults	RT real-time PCR	0-20 (0/95-3/15)	Sidorenko et al. 2018
		I. ricinus nymphs	RT real-time PCR, pyrosequencing	0-1.1	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-5.6	Vikse et al. 2020
		cervids	ELISA, SNT	41.0 (22/54)	Ytrehus et al. 2013
		dogs	ELISA, verification by a second ELISA	16.4 (52/317)	Csango et al. 2004
Aust-Agder	Yes	*I. ricinus* nymphs	RT real-time PCR, pyrosequencing	0.1-0.7 (1/900-4/620)	Andreassen et al. 2012
		I. ricinus nymphs	RT real-time PCR, pyrosequencing	0-0.7	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-12.5	Vikse et al. 2020
		I. ricinus nymphs	RT real-time PCR	0-3.3 0/130-1/30	Sidorenko et al. 2018
		I. ricinus adults (female)	RT real-time PCR	0-20 (0/95-13/65)	Sidorenko et al. 2018

Rogaland	No	*Ixodes ricinus* nymphs	RT real-time PCR, pyrosequencing	0-3.5	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-20.6	Vikse et al. 2020
Hordaland	No	*I. ricinus* nymphs	RT real-time PCR, pyrosequencing	0-1.3	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-15.4	Vikse et al. 2020
Sogn og Fjordane	No	*Ixodes ricinus*	Cultivation in mice brain, HI and CFT	-	Traavik et al. 1978
		patients examined for viral diseases	HI	19.6 (67/341)	Traavik 1979
		Ixodes ricinus nymphs	RT real-time PCR, pyrosequencing	0.1-0.6	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-1	Vikse et al. 2020
Møre og Romsdal	No	*I. ricinus* adults	RT real-time PCR, pyrosequencing	3.1 (2/65)	Paulsen et al. 2015
		Ixodes ricinus nymphs	RT real-time PCR, pyrosequencing	0-0.6	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-4.7	Vikse et al. 2020
		cervids	ELISA and SNT	1.6 (1/64)	Ytrehus et al. 2013
Trøndelag	No	*I. ricinus* nymphs	RT real-time PCR, pyrosequencing	0.41 (6/97)	Paulsen et al. 2015
		I. ricinus nymphs	RT real-time PCR, pyrosequencing	0.1 (1/740)	Soleng et al. 2018
		I. ricinus adults	RT real-time PCR, pyrosequencing	8.6 (9/105)	Soleng et al. 2018
		I. ricinus nymphs	RT real-time PCR, pyrosequencing	0.1-0.4	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-8.6	Vikse et al. 2020
Nordland	No	*I. ricinus* nymphs	RT real-time PCR, pyrosequencing	0.1-3.0 (1/740 and 1/40)	Soleng et. al 2018
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-9.0 (0/94 and 10/111)	Soleng et. al 2018
		Ixodes ricinus nymphs	RT real-time PCR, pyrosequencing	0.1-1.2	Vikse et al. 2020
		I. ricinus adults	RT real-time PCR, pyrosequencing	0-17	Vikse et al. 2020
Southern and western Norway	Yes/No	Bovine	HI	17.3 (14/81)	Traavik 1973

*number of positive samples/number of analysed samples. For further details from Vikse et al. 2020, see the paper. Abbreviations: CFT - complement fixation test, ELISA - Enzyme-linked immunosorbent assay, HI - haemagglutination inhibition test, RT PCR - reverse transcription polymerase chain reaction, SNT - Serum neutralisation test

Even though TBEV has been detected in several areas in Norway, the number of human TBE cases is low, with a total of 167 reported autochthonous cases between 1997 and 2019 (Figure 5). In addition, 30 cases were infected abroad or have an unknown infection history (MSIS, data per 03.02.2020).

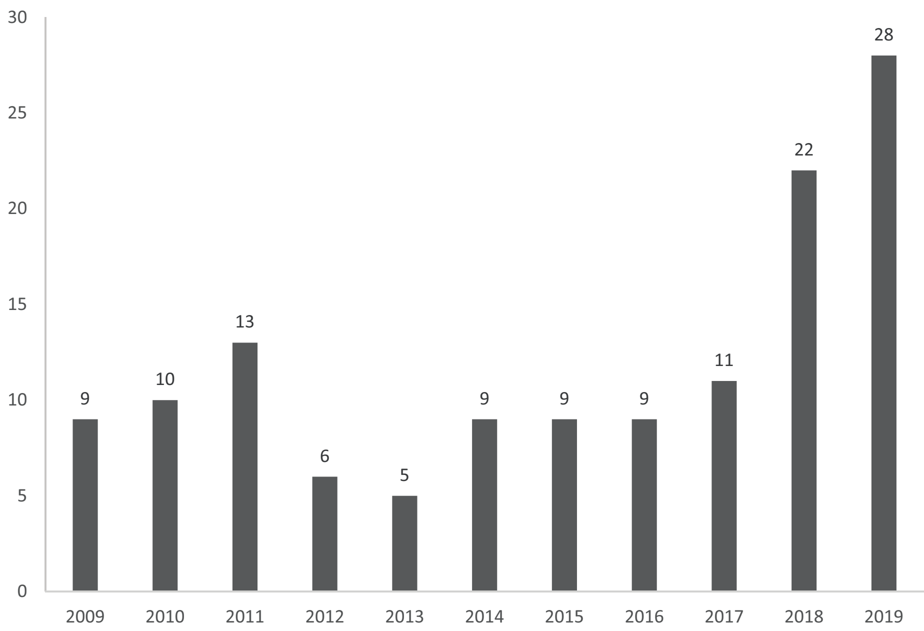

Figure 5: Number of autochthonous TBE cases reported in Norway from 2009 to 2019 according to MSIS. Data per 03.02.2020.

The reported cases were limited to southern and south-eastern Norway in the counties of Vest-Agder, Aust-Agder, Vestfold, Telemark, and Buskerud (Figure 6). During the period from 2009 to 2019, there have been between five and 28 registered domestic cases annually, which corresponds to an annual incidence rate of 0.2 to 0.5 per 100,000 inhabitants. Interestingly, an increased number of cases was observed in 2018 and 2019, particularly in the counties of Vestfold and Telemark (MSIS, 2020).

Figure 6: Map of Norway showing counties with reported human TBE cases in dark orange (MSIS, 2020). Counties with TBEV positive *Ixodes ricinus* are in green. Areas with TBEV positive ticks are indicated with a red star, where one star may represent more than one sampling site (Larsen et al., 2014; Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020). All human TBE cases and TBEV positive ticks are from coastal areas. The map is based on the counties of Norway as of December 2019, see definitions (Kartverket, Creative Commons BY 4.0.).

1.3.4. Reservoir hosts and transmission

Transmission of TBEV is dependent on several factors, such as the interaction between the virus, ticks, and hosts (Nuttall & Labuda, 2003).

Small mammals, such as rodents and shrews, have an essential role in the maintenance of TBEV foci because they are important hosts for larval and nymphal *Ixodes* ticks. Species in the genus of *Myodes* and *Apodemus* have been identified as reservoir hosts of TBEV (Mlera & Bloom, 2018). Other mammals and birds are also important hosts for *Ixodes* ticks (Hasle, 2013; Jaenson et al., 1994; Jaenson et al., 2018; Talleklint & Jaenson, 1997). Birds can be infested with ticks, and may transport ticks infected with TBEV over long distances (Hasle, 2013; Waldenstrom et al., 2007). However, there is a lack of information on wild birds' role in spread and circulation of the virus. TBEV prevalences of above 50 % have been found in some bird species, such as fieldfares (*Turdus pilaris*), bramblings (*Fringilla montifrigilla*), and the common redstart (*Phoenicurus phoenicurus*), which indicates that they may play a role as reservoirs, or amplifying hosts for TBEV (Mikryukova et al., 2014). TBEV viraemia has been found in mallards (*Anas platyrhynchos*) in an experimental study (van Tongeren, 1983).

Large mammals, such as cervids, play an important role for tick populations by providing blood meals for adult ticks. These animals may also be involved in spreading of the virus to new sites (Carpi et al., 2008). Seroprevelance studies in wild cervids in different European countries have identified previously unknown foci and suggested that cervids can be used as sentinel species for the distribution of TBEV (Andersen et al., 2019; Balling et al., 2014; Gerth et al., 1995; Imhoff et al., 2015; Kiffner et al., 2012; Skarphedinsson et al., 2005; van der Poel et al., 2005).

Ixodes ticks are considered both vector and reservoir for TBEV, and they can transmit the virus to the host via saliva shortly after they start feeding and during the blood meal (Kazimírová et al., 2017). Ticks may be infected with TBEV during feeding on a viraemic host, or when infected ticks and non-infected ticks are feeding close together or soon after each other on the same area of a host. The latter type of transmission is called co-feeding, and is independent of the host's viraemia (Labuda et al., 1993; Labuda et al., 1997; Randolph, 2011). The adult, nymph and larval tick can be infected with TBEV (Karbowiak et al., 2018) and it is estimated that approximately 0.1 % to 5 % of the *I. ricinus* ticks in endemic areas are infected with the virus (Andreassen et al., 2012; Pettersson et al., 2014; Suss, 2003).

Seasonal synchronised co-feeding by TBEV infected nymphs and uninfected larvae seems to be an important mechanism for maintenance of the virus in some areas (Randolph et al., 1999).

TBEV is transmitted transstadially, but may also be transmitted transovarially (Benda, 1958; Danielová & Holubová, 1991; Rehacek, 1962). Only a few eggs in egg batches have been found to be infected with TBEV, as low as 0.2 % to 0.8 % (Danielová & Holubová, 1991). Although transovarial transmission usually is less than 1 %, Nuttall et al. (1994) suggested that this type of transmission may be important for maintaining natural TBEV foci. Sexual transmission of TBEV from a male tick to a female tick has been suggested as another type of transmission (Chunikhin et al., 1983). Further, vertical transmission of the virus between generations of small rodents has been found by experimental studies and has been suggested to possibly ensure long-term persistence of the virus in populations of mammalian hosts without involving the arthropod vectors (Bakhvalova et al., 2009). In addition, transmission of TBEV through transplantation of solid organs and blood transfusion in humans has been reported (Leiby & Gill, 2004; Lipowski et al., 2017).

Although humans are mainly infected with TBEV by bites from TBEV infected ticks, transmission by consumption of unpasteurised infected goat, sheep and cow's milk and other dairy products has been reported. In most of the reported alimentary TBE cases, the virus is transmitted through unpasteurised milk from goat (Balogh et al., 2010; Caini et al., 2012; Gresikova et al., 1975; Holzmann et al., 2009; Hudopisk et al., 2013; Markovinovic et al., 2016). The largest registered outbreak of alimentary TBE occurred in Slovakia in 1951-52, where at least 660 people were infected after consumption of unpasteurised cow milk mixed with goat milk (Kerlik et al., 2018).

Infected domestic ruminants do not normally display clinical symptoms, but they may develop a viraemia with a duration of approximately one week (van Tongeren, 1955). In milk samples from infected ruminants, TBEV has been detected for up to 19 days post infection (Balogh et al., 2012). Another study found TBEV in goat milk for five to 25 days post TBEV infection (Gritsun et al., 2003a). Detectable antibodies in ruminants have been found for at least 28 months (Klaus et al., 2014).

Historically, the disease caused by TBEV contaminated milk was called "biphasic milk fever" because the disease could be divided into two phases. The incubation period is shorter (3-4 days) compared to TBEV infection from tick-bites (7-14 days) (Bogovic & Strle, 2015). The first phase includes fever, fatigue, body pain and headache. The second phase may occur after an asymptomatic period of two to 14 days, and involves inflammation of the central nervous system (CNS) (Ruzek et al., 2010).

TBEV has been found to be stable for up to two hours in gastric juice (pH 2 to 7) taken from humans after a meal. When milk is consumed, it takes approximately one and a half to two hours for all the milk to pass from the stomach to the duodenum. TBEV may then bind to the microfold cells of the Peyers's patches in the ileum (Gritsun et al., 2003a).

A laboratory study on milk infected with Langat virus (LGTV), a closely related virus, found the virus to be stable for more than 74 hours at 4 °C, while at ambient temperature (22 °C), the virus was present after 24 hours of incubation, and it declined to undetectable levels by 48 hours. The virus has been found to be completely inactivated under high temperature conditions, such as high-temperature short-time pasteurisation, which is heating the milk to about 72 °C for 15 seconds, followed by rapid cooling to about 4 °C (Offerdahl et al., 2016). Pasteurisation of milk is therefore recommended in TBEV risk areas (Hudopisk et al., 2013; Offerdahl et al., 2016).

1.4. Tick-borne encephalitis in humans

1.4.1. Clinical manifestation

The clinical outcome of TBEV-Eu infection ranges from asymptomatic to severe infection in the central nervous system (CNS) (Kaiser, 2012). Studies have found that approximately 70 % to 90 % of all infections are asymptomatic. These numbers are, however, uncertain, due to the fact that patients with mild symptoms rarely get diagnosed (Gustafson et al., 1992; Kaiser, 2008).

The incubation period of TBE ranges from two to 28 days (average seven to ten days) after a bite by a TBEV infected tick. However, the exact incubation period is difficult to establish because tick-bites often remain unnoticed. Alimentary TBEV infections have been found to have a shorter incubation period, usually three to four days (Bogovic & Strle, 2015; Dumpis

et al., 1999). The clinical manifestation caused by TBEV-Eu is usually biphasic. In the first phase, febrile symptoms are common during the viraemia. The first viraemic phase lasts for approximately two to four days. The most frequently observed symptoms during this phase are fever and headache, but symptoms such as fatigue, myalgia, decreased appetite, nausea and vomiting have also been reported (Kaiser, 2012; Ruzek et al., 2010). About 30 % of the patients develop further symptoms in the second phase, which normally occurs after approximately one week (range 1 to 21 days) without symptoms. The second phase includes more severe symptoms, which involve the CNS, and includes either meningitis, meningoencephalitis, meningoencephalomyelitis, encephaloradiculitis, or a mixed form of these (Bogovic et al., 2010; Kaiser, 2012; Ruzek et al., 2010). Studies have found that up to 46 % of the patients that undergo the second phase also develop long term sequelae (Haglund & Gunther, 2003; Kaiser, 2012).

1.4.2. Diagnosis and treatment

Because the symptoms of TBE typically are non-specific with fever, laboratory analyses are needed to make and confirm a diagnosis. Information on patient history with regards to tick bites is beneficial to make a diagnosis due to the lack of specific symptoms (Holzmann, 2003; Kaiser, 2012).

Enzyme-linked immunosorbent assay (ELISA) is the most used serological test to detect antibodies against TBEV (Hofmann et al., 1983; Roggendorf et al., 1981). The ELISA test is generally of high sensitivity, however, because of cross-reactions to other flaviviruses or vaccines against flaviviruses, the ELISA test has a moderate specificity (Holzmann et al., 1996; Litzba et al., 2014; Reusken et al., 2019). In the ELISA, immunoglobulin M and G (IgM/IgG) are detected. The IgM antibodies are normally present before CNS symptoms occur (second phase) and can normally be detected for about six weeks after the onset of CNS symptoms. IgG antibodies to TBEV are generally present at the onset of CNS symptoms. They reach a maximum titre after approximately six weeks, and persist for years (Holzmann, 2003; Kaiser & Holzmann, 2000).

Another widely used serological test is the neutralisation test, which identifies the antibodies' capacity to neutralise infectious viruses. Normally, neutralising antibodies appear around two weeks after infection and vaccination. In situations where it is necessary

to distinguish between specific antibody response to TBEV and other flaviviruses, a neutralisation test is recommended. Tick- and mosquito-borne viruses are closely related genetically and antigenically, and antibodies against one virus may cross-react with antibodies against other tick- and mosquito-borne viruses. In areas where more than one tick- and mosquito borne virus circulate, the possibilities of cross-reactions should be considered (Ergunay et al., 2016; Vene et al., 1998).

Detection of the TBEV RNA is also possible by reverse transcription polymerase chain reaction (RT PCR) (Andreassen et al., 2012; Schwaiger & Cassinotti, 2003). RT PCR has no major role as a diagnostic test for TBE because viral RNA is usually only present in the first phase of infection. RT PCR may, however, be useful in the first phase, and for confirmation of TBE in fatal cases (Saksida et al., 2005). Furthermore, RT PCR may be of importance as a diagnostic method in immunosuppressed patients that do not develop antibodies to TBEV. In such situations, the viral RNA may be present over a longer period than the first febrile phase of infection (Caracciolo et al., 2015). In addition, TBEV RNA might be detected by RT PCR in urine samples from patients for up to 19 days after the start of neurological symptoms (Veje et al., 2014).

The treatment is usually symptomatic in nature, including pain management and fluid therapy (Bogovic & Strle, 2015). There is no specific curative treatment against TBE, but high doses of intravenous immunoglobulins have been used for treatment of severe cases (Elsterova et al., 2017). However, TBE can be successfully prevented by vaccines (Kaiser, 2008).

1.4.3. Prevention and vaccine recommendations

Prevention of tick-bites is important to avoid TBEV infection. When traveling in areas where ticks are abundant and TBEV is prevalent, it is recommended to limit exposure to ticks by appropriate clothing, such as long trousers that cover the ankles. Furthermore, it is recommended to avoid habitats that have high tick densities, e.g. by walking on cleared paths instead of through long grass, ferns, heather and bushes. In addition, use of insect repellents on skin and clothes may reduce the risk of tick-bites and thereby also TBEV infection. Examples of protective substances in repellents are: DEET (N,N-diethyl-3-methylbenzamide), Icaridin ((2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl

ester)) and EBAAP (3-[N-butyl-N-acetyl]-aminopropionic acid, ethyl ester) (Buchel et al., 2015). Inspection of the body for tick bites immediately after visiting risk areas for ticks and TBEV, and removal of ticks as quickly as possible are important measures (Banzhoff et al., 2008; Rendi-Wagner, 2004).

TBE can successfully be prevented by vaccination, and there are a few preventive vaccines available (Erber & Schmitt, 2018; Heinz et al., 2013). In Europe, the vaccines FSME-IMMUN/TicoVac (Pfizer, New York, USA) and Encepur (GlaxoSmithKline, Brentford, Great Britain) are available. Additionally, there are other vaccines available in Russia and China (Amicizia et al., 2013).

In Norway, the vaccine TicoVac is used and the vaccine recommendations apply to people who have experienced several tick bites and spend a lot of time outdoors in areas with a known risk of TBEV infection. People who may be exposed to TBEV infected samples from animals and humans should also consider vaccination (Norwegian Institute of Public Health, 2019).

In Europe, the vaccination rate and recommendations vary between countries (Erber & Schmitt, 2018). Austria has a national vaccine program that is recommended for the entire population. The vaccination rate in Austria is approximately 84 % and there are about 60 to 80 cases of TBE reported annually. Before the vaccine recommendation applied to the entire population, the number of annually reported cases was close to 700 (Heinz et al., 2007). Recommendations for other countries vary, but are usually linked to risk areas, age, and/or to persons that have an increased risk of tick-bites (Erber & Schmitt, 2018; Erber et al., 2019).

Vaccine failures have been reported, but these are rare (Hansson et al., 2019; Lotric-Furlan et al., 2017). A recent retrospective study from Sweden found a total of 5 % failed vaccines within the study period from 2006 to 2015 in Stockholm county. The study suggested adding an extra dose of the vaccine for individuals above the age of 50 (Hansson et al., 2019). The illness in patients with a failed vaccination may be more severe than in unvaccinated patients (Lotric-Furlan et al., 2017).

1.5. Tick-borne encephalitis in animals

The knowledge on TBE in animals is limited. Most animals seem to not develop symptomatic disease when infected with TBEV either naturally or experimentally, but several animal species have been found to seroconvert after exposure to the virus. TBE in dogs and horses has been described with neurological symptoms (Klaus et al., 2013; Leschnik et al., 2002; Muller et al., 2006; Weissenbock et al., 1998). In addition, single cases of TBE have been described in moose, monkey, sheep, goat and mouflon (*Ovis ammon musimon*) (Bago et al., 2002; Bohm et al., 2017; Suss et al., 2007; Suss et al., 2008; Svedmyr et al., 1965; Zindel & Wyler, 1983).

1.5.1. TBE in dogs

Dogs are the animal species with the most reported clinical TBEV infections. Less than 50 cases with clinical TBEV infection in dogs have been reported (Pfeffer & Dobler, 2011; Pfeffer et al., 2019). However, seroprevalence studies in TBE endemic areas in Europe have reported that between 1 % and 40 % of dogs are seropositive. Even though dogs are often highly exposed to ticks, and a high TBEV seroprevalence in dogs has been found in several endemic areas, most dogs do not develop clinical symptoms of TBE (Csango et al., 2004; Garcia-Bocanegra et al., 2018; Klimes et al., 2001; Leschnik et al., 2013; Levanov et al., 2016).

In dogs with clinical symptoms, a high proportion of the cases have fatal outcome. The incubation period for dogs seems to be the same as in humans, between one and two weeks after the tick bite (Leschnik et al., 2002; Reiner et al., 1999). The first symptoms have been found to be non-specific, such as fever and change in behaviour (e.g. aggressiveness, reduced appetite, nervousness or apathy). TBE has been found to give neurological disorder in the cerebrum and the brain stem of dogs (Leschnik et al., 2002; Reiner et al., 1999).

1.5.2. TBE in horses

The available information on TBE in horses is limited compared to TBE in dogs, but TBE in horses seems to be rare with less than ten cases reported. The clinical symptoms of TBE in horses have been described as neurological. A few seroprevalence studies on TBE in horses have been published. The seroprevalence found in these studies ranged from 0 % to 26 % (Klaus et al., 2013).

1.5.3. TBE in small ruminants

Small ruminants rarely display any clinical symptoms with TBEV infection, not even from experimental infections (Balogh et al., 2012). There is, however, one report of a TBEV infected lamb with neurological disease in Germany, one goat infected with TBE in Switzerland, and one TBEV infected mouflon (*Ovis aries musimon*) in Austria (Bago et al., 2002; Bohm et al., 2017; Zindel & Wyler, 1983). The lamb with TBE was found in 2015 in Bavaria, a TBE endemic area in Germany, and it displayed acute neurological signs. The lamb was euthanised, and TBEV RNA was detected from brain tissue by quantitative RT PCR and sequencing (Bohm et al., 2017). The TBEV infected goat was found in 1982 in the Prättigau region in Switzerland. The goat was diagnosed with TBE based on the neurological symptoms and titres from haemagglutination inhibition test (Zindel & Wyler, 1983). The TBEV infected mouflon was found in a moribund condition in Burgenland, a TBEV endemic area in Austria, in 1994. The animal was euthanised, and TBEV was detected from brain tissue by RT PCR and sequencing analysis (Bago et al., 2002).

1.5.4. TBE in monkeys

A single case of TBE in monkey (*Macaca sylvanus*) has been described from Germany. This species is not native in Eurasia, despite a small population in Gibraltar on the Iberian Peninsula. The monkey was living in a zoo in a TBEV endemic area in Germany, and displayed severe neurological symptoms. The monkey was euthanised, and subsequently the diagnosis was demonstrated by immunohistochemistry, RT PCR and virus isolation (Suss et al., 2007; Suss et al., 2008). Other monkeys in the zoo seroconverted without clinical signs (Klaus et al., 2010a).

1.6. Other important tick-borne pathogens in Norway

I. ricinus is the main vector of several other tick-borne pathogens affecting human and/or animal health in Norway, such as *Anaplasma phagocytophilum*, *Babesia divergens* and *Borrelia burgdorferi* sensu lato (s. l.) (Hasle et al., 2010; Kjelland et al., 2010; Kjelland et al., 2018; Oines et al., 2012; Stuen et al., 2013). Additionally, a virus closely related to TBEV, namely the louping-ill virus (LIV), has been detected in sheep (Norwegian Veterinary Institute., 2019; Stuen et al., 1996; Ulvund et al., 1983).

A. phagocytophilum, formerly known as *Ehrlichia phagocytophila*, is one of the most widespread tick-borne pathogens in Europe, infecting a wide range of animal species (Stuen, 2007; Stuen et al., 2013). In Norway, it is prevalent in the areas where *I. ricinus* is abundant (Henningsson et al., 2015; Jenkins et al., 2001; Stuen et al., 2002a). *A. phagocytophilum* is a small, obligate intracellular, gram negative bacterium, and belongs to the genus *Anaplasma*, family *Ehrlichiacea* and the order Rickettsiales. The bacterium is a multi-host species, and is the cause of tick-borne fever (TBF) in animals such as sheep, cattle, horses and dogs and of granulocytic anaplasmosis in humans (Stuen et al., 2013). *A. phagocytophilum* has a great negative impact on sheep farming, and it has been estimated that more than 300,000 lambs are infected by *A. phagocytophilum* annually in Norway (Stuen & Bergstrom, 2001). *A. phagocytophilum* infects and replicates mainly in neutrophil granulocytes (Choi et al., 2004). In contrast to for TBEV, co-feeding ticks may not be important for maintenance of *Anaplasma* in an area (Kocan & de la Fuente, 2003), as *A. phagocytophilum* can persist in several mammalian hosts (Stuen, 2003). Multiple genetic variants of *A. phagocytophilum* have been characterised, and they may cause different clinical manifestations and have different degrees of persistency in animals (Granquist et al., 2010a; Granquist et al., 2010b; Tuomi, 1967).

Infection with *A. phagocytophilum* in domestic ruminants may include high fever, depression, reduced appetite, sudden drop in milk yield and reduced weight gain (Grova et al., 2011; Stuen et al., 2002b; Tuomi, 1967; Woldehiwet, 2008). The fever response has been found to vary with the age of the animal, genetic variants of the bacterium, host species and the immunological status of the host. The infection may cause severe immune suppression, especially in three to six weeks old lambs, and high lamb losses may therefore occur due to secondary infections such as *Bibersteinia trehalosi*, *Mannheimia haemolytica* and *Staphylococcus aureus* (Stuen, 2003). Most humans infected with *A. phagocytophilum* are assumed asymptomatic, and the number of reported human anaplasmosis cases in Norway is limited (Stuen & Bergstrom, 2008), but in the USA more than 10,000 cases have been reported (Bakken and Dumler 2015).

Other tick-borne pathogens, such as *B. divergens* infection in cattle, is widespread in the coastal areas of southern Norway, and has been known for several decades (Hasle et al., 2010; Oines et al., 2012; Tambs-Lyche, 1943), while only one human case of babesiosis has

so far been verified (Morch et al., 2015). In addition, *Borrelia burgdorferi* s. l. is common in the distribution area of *I. ricinus* (Kjelland et al., 2010; Soleng & Kjelland, 2013) and approximately 200-400 cases of LB (not including cases that only showed erythema migrans) are reported annually (MSIS, 2020). Some animal species, such as dogs, may display symptoms such as fever, reduced appetite, and joint pain when infected with *B. burgdorferi* s.l. (Azuma et al., 1993; Krupka & Straubinger, 2010).

LIV is a tick-borne *Flavivirus* in the *Flaviviridae* family. The virus may cause severe disease in sheep and red grouse (*Lagopus lagopus scoticus*), and is mainly distributed in upland areas of Great Britain and Ireland (Jeffries et al., 2014). Sheep, mountain hares (*Lepus timidus*) and red grouse have been identified to be important transmission hosts for LIV (Gilbert et al., 2000; Gilbert, 2016; Jeffries et al., 2014; Reid et al., 1978; Reid et al., 1986). In contrast to TBEV, only a few human cases of LIV infection have been reported (Davidson et al., 1991). Stockmen, abattoir workers, butchers, veterinarians and laboratory workers who have either worked with the virus or had contact with sheep or other potentially infected species are most at risk of LIV infection (Davidson et al., 1991; Reid et al., 1972). In Norway, clinical LIV infections in sheep were reported from the west coast in the 1980s and early 90s, but no clinical cases have been reported since (Norwegian Veterinary Institute., 2019; Ulvund et al., 1983).

1.6.1. Co-infection of tick-borne pathogens

The risk of infection with multiple pathogens after a tick bite depends on the occurrence of tick-borne pathogens in different locations (Diuk-Wasser et al., 2016). A recently published study investigated infection with TBEV, *B. burgdorferi* s. l., *Borrelia miyamotoi*, *A. phagocytophilum* and *N. mikurensis* in questing *I. ricinus* nymphs collected from five islands popular as recreational sites in Norway. Several tick-borne pathogens were detected, which indicates a risk of infection by multiple pathogens after tick bites in these locations. The same study found co-infection of *Borrelia afzelii* and *N. mikurensis* in 3.3 % of the nymphs (Kjelland et al., 2018). A study on co-infections in TBE patients showed that the patients were co-infected with *Borrelia* spp. (27 % / 30/110), A*. phagocytophilum* (10.9 % / 12/110) and with *Babesia* spp. (0.9 % / 1/110). Triple infections with TBEV, *Borrelia* spp. and *A. phagocytophilum* were observed in three (2.7 % / 3/110) patients (Moniuszko et al., 2014).

Little information is available on the medical consequences of co-infections in humans or wild and domestic animals. However, there are some reports of a changed clinical outcome (Alekseev et al., 2001; Czupryna et al., 2011; Diuk-Wasser et al., 2016; Krause et al., 1996). In a retrospective study of more than 600 patients with TBE, two percent were diagnosed with TBE and neuroborreliosis. The TBE patients without neuroborreliosis more frequently showed symptoms such as headaches, vertigo and nausea, compared to the group co-infected with both pathogens. However, the latter group more frequently displayed neurological symptoms of greater severity (Czupryna et al., 2011). Alekseev et al. suggested that *Borrelia* spp. might suppress viral replication in ticks and in TBE susceptible individuals (Alekseev et al., 2001). In other studies of co-infection in patients with TBEV and *Borrelia* spp., it could not be proved that additional symptoms occurred due to interaction between these two pathogens (Logina et al., 2006; Moniuszko et al., 2014). Co-infection of *A. phagocytophilum* and *Borrelia* spp may contribute to the severity, dissemination and possible sequelae of the disease (Grab et al., 2007).

In sheep, co-infection with LIV and *A. phagocytophilum* is known to give a more severe outcome than single infection with LIV. An experimental study showed that co-infection of LIV and *A. phagocytophilum* gave a greater and prolonged viraemia compared to a single LIV infection. The LIV antibody response in co-infected sheep has been shown to either not establish, or being delayed compared to single LIV infection (Reid et al., 1986). It has been speculated if the same outcome may occur for co-infection of TBEV and *A. phagocytophilum* due to the close genetic relationship between TBEV and LIV.

1.7. Knowledge gaps

TBEV is distributed in large areas of Europe and Asia, and several studies on a variety of aspects of the virus are already published. However, there is a general lack of information on the veterinary aspect of TBEV. Furthermore, updated information on the distribution and transmission of TBEV is important for the public health. This Ph.D.-project is divided into the three following topics related to the knowledge gaps.

1. Because TBEV may cause severe neurological disease in humans, updated knowledge on the distribution of the virus is important for vaccine recommendation and the public health. The current information on TBEV in Norway is limited to reported human cases,

two blood donor studies, surveillance in ticks, and a few geographically restricted serological studies in animals. There is a lack of information on sentinel animals for TBEV in Norway, but studies from other countries have suggested that surveillance of cervid populations may be useful for identifying TBEV risk areas.

2. TBEV is mainly transmitted to humans by TBEV infected ticks. However, unpasteurised milk has been found to be another transmission route. The trend of less-processed products such as unpasteurised milk and other dairy products seems to be increasing in both Norway and other European countries. There are no reports of alimentary TBE from Norway, and the information on TBE in animals in the country is limited. Updated information on risk areas for TBEV transmission from both ticks and unpasteurised dairy may prevent future cases of TBE.

3. There is a general lack of knowledge on TBE in animals. Although clinical cases have been reported in a few mammals, TBE mainly seems to be a human disease. It has, however, been speculated if co-infection of TBEV and *A. phagocytophilum* may give clinical disease in sheep. The background for these hypotheses is that LIV, which is closely related to TBEV, has previously been found to give more severe disease in sheep which are co-infected with *A. phagocytophilum.*

2. Aim of the study

The aim of the Ph.D.-project was to provide updated knowledge on the biology, infection dynamics and epidemiology of TBEV that may reduce the risk of infection in humans and animals.

Specific aims:

1. Estimate the seroprevalence of antibodies to TBEV in cervids in Norway and the possible emergence of new foci, and evaluate if cervids in Norway can serve as possible sentinel animals for the distribution of TBEV as a supplement to surveillance of TBEV in ticks (**Paper 1**).

2. Provide updated information on antibodies to TBEV in cows and analyse TBEV RNA in unpasteurised milk samples in Norway (**Paper 2**).

3. Investigate infection of TBEV and co-infection of TBEV and *A. phagocytophilum* in lambs (**Paper 3**).

3. Summary of individual papers

3.1. Paper 1

Cervids as sentinel-species for tick-borne encephalitis virus in Norway - a serological study

In this paper, the seroprevalence of TBEV antibodies in cervids in Norway and the possible emergence of new TBEV foci in the country was investigated. A second aim was to evaluate if cervids can function as possible sentinel animals for the distribution of TBEV in Norway in addition to surveillance in ticks. Serum samples from 286 moose, 148 roe deer, 140 red deer and 83 reindeer across Norway were collected by hunters in the autumn of 2013. All samples were screened for TBEV immunoglobulin G (IgG) antibodies with a modified commercial enzyme-linked immunosorbent assay, and positive and borderline samples were re-tested by a TBEV-specific serum neutralisation test. The overall seroprevalence of the TBEV-complex in the cervid specimens from Norway was 4.3 %. The highest number of TBEV seropositive cervids was found in south-eastern Norway, but seropositive cervids were also detected in southern and central Norway. Most of the positive samples in the present study originated from areas where human cases of TBE have been reported in Norway. Antibodies against TBEV were detected by SNT in 9.4 % of the analysed samples from moose, 1.4 % in red deer and 0.7 % in roe deer. The study represents the first comprehensive screening of cervid species in Norway for viruses in the TBE complex and shows that cervids may serve as sentinel animals for the distribution of TBEV in the country as a supplement to existing surveillance of TBEV in *I. ricinus* ticks. Furthermore, the results indicate that TBEV may be spreading northwards in Norway. This information supports previous studies of TBEV in *I. ricinus* ticks and may be of relevance for public health considerations.

3.2. Paper 2

Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway

TBEV is mainly transmitted to humans and animals by bites from TBEV infected ticks. However, alimentary TBE after consumption of unpasteurised dairy products has been reported from other European countries. The epidemiology of TBEV in domestic ruminants in Norway has not been fully established, and human alimentary TBE has not been reported. The aims of this paper were to investigate the occurrence of TBEV RNA in unpasteurised milk from dairy cows in Norway and to study the seroprevalence of neutralising antibodies to TBEV in the same animals. Milk and serum samples from a total of 112 cows from farms located in the municipalities of Mandal, Arendal, Skedsmo, Finnøy and Brønnøy were included in the study. TBEV RNA was detected in unpasteurised milk from farms located in the municipalities of Mandal, Skedsmo and Brønnøy. Neutralising antibodies to TBEV were only detected in Arendal. Together with other studies in ticks, humans and cervids, the study indicates that TBEV is distributed in a greater area in Norway compared to where human cases have been reported. The study was the first report of TBEV in unpasteurised milk in Norway, and further studies on TBEV in milk should be performed to conclude if TBEV found in unpasteurised milk in Norway is infectious to humans.

3.3. Paper 3

Experimental infection of lambs with tick-borne encephalitis virus and co-infection with *Anaplasma phagocytophilum*

Although TBEV is known to cause severe disease in humans, the knowledge on TBE in animals is limited. The objectives of this paper were to investigate if TBEV causes disease in sheep, and if co-infection with TBEV and *A. phagocytophilum* have an effect of the clinical outcome. A total of 30 lambs, at the age of five to six months, of the breed "Norwegian white sheep", were used. The experimental study was divided into two parts. In part one, pre- and post-infection of TBEV or *A. phagocytophilum* was investigated (group 1 to 4), while in part two, co-infection of TBEV and *A. phagocytophilum* was investigated (group 5 and 6). The lambs inoculated with TBEV displayed no clinical TBE symptoms or fever and had a short or non-detectable viraemia by RT real-time PCR. All lambs inoculated with TBEV developed neutralising antibodies. The study indicates that TBEV rarely causes symptomatic disease in ruminants, which is in accordance with previous studies. All the lambs that were inoculated with *A. phagocytophilum* developed fever and clinical symptoms of tick-borne fever, and *A. phagocytophilum* was present in the blood samples of all infected lambs, shown by qPCR. Significantly higher mean TBEV antibody titre was detected in the group co-infected with TBEV and *A. phagocytophilum*, compared to the groups that were pre- or post-infected with *A. phagocytophilum*. These results indicate that co-infection with TBEV and *A. phagocytophilum* in sheep stimulates an increased TBEV antibody response.

4. Materials and methods

In this Ph.D.-project, standard virology methods such as virus cultivation, enzyme-linked immunosorbent assay, serum neutralisation test, RNA and DNA extraction, reverse transcription of RNA and real-time PCR, have been used. The materials and methods for **Paper 1**, **2** and **3** are described in further details in the respective papers, and are discussed in the results and discussion section of this thesis.

4.1. Ethical approval

Paper 1:

Samples from the Norwegian health monitoring program for deer and muskox (HOP) were used. HOP documents the health status of the game populations in Norway by mapping and monitoring various diseases of moose (*Alces alces*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), wild reindeer (*Rangifer tarandus*) and muskox (*Ovibos moschatus*). The program regularly organises the collection of different samples from these species during the hunting season with the help of hunters and local hunting organisations. For the samples used in this study, hunters were asked to collect blood from the thoracic cavity. The work presented in this paper required no specific ethical approvals.

Paper 2:

All blood and milk samples were considered diagnostic and were collected by trained veterinarians. Vaccination of calves, to serve as positive controls, was authorised by the Norwegian Animal Research Authority (Norwegian Food Safety Authority, FOTS ID 8135).

Paper 3:

The study was authorised by the Norwegian Animal Research Authority (FOTS ID 8632 and 8135). This study was conducted at the Norwegian University of Life Sciences in Sandnes, Norway. All lambs were observed during daytime throughout the experiment.

4.2. Study material and experimental design

4.2.1. Study material

Paper 1:

A total of 657 samples were collected across Norway by hunters in the regular hunting season in 2013 (Figure 7). These samples were used for serological analysis of antibodies to TBEV and LIV.

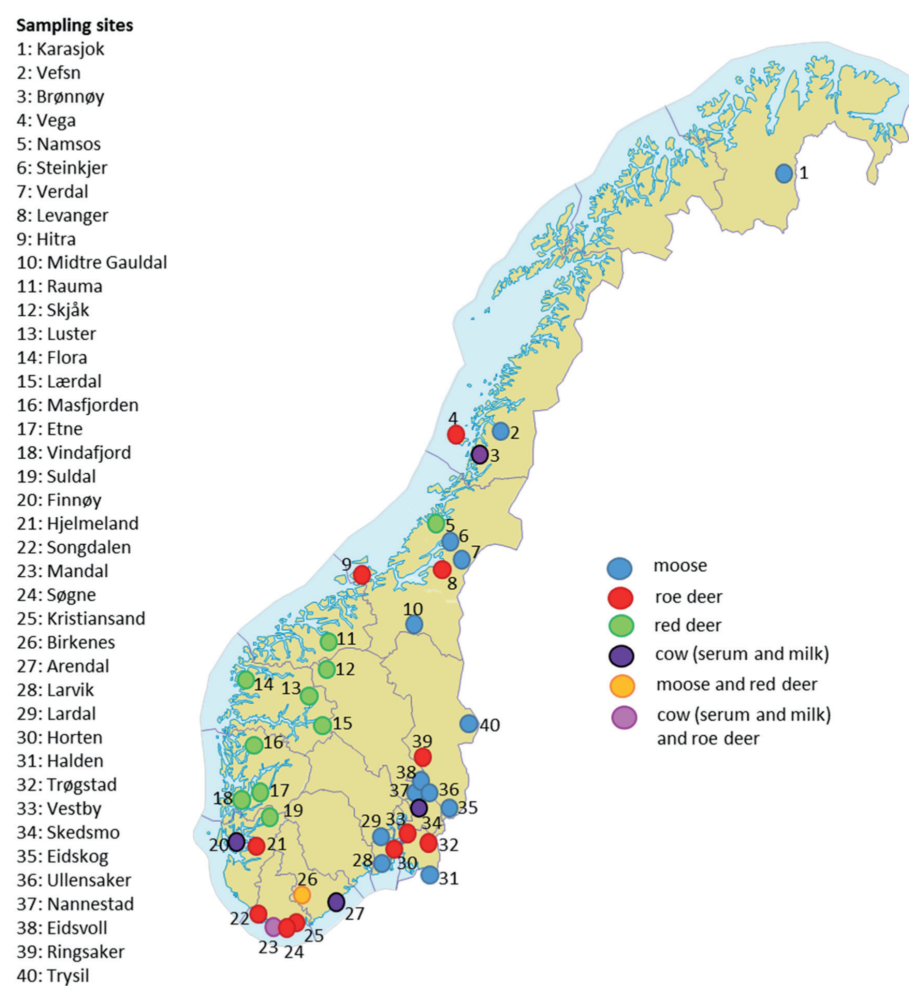

Figure 7: Sampling sites for moose, red deer and roe deer collected by hunters in 2013, presented in Paper 1, and sampling sites for cow serum and milk collected by local veterinarians between 2014 and 2017 in Paper 2. Reindeer sampling sites (Paper 1) are not indicated in the map, but sampling occurred in mountain areas in the following reindeer districts: Setesdal/Ryfylke, Setesdal Austhei and Snøhetta. Map from Kartverket, Creative Commons BY 4.0.

Paper 2:

For the paper on TBEV in cows and unpasteurised cow's milk, blood and milk samples were collected by local veterinarians from 112 dairy cows from farms in the municipalities of Akershus, Arendal, Mandal, Finnøy and Brønnøy (Figure 7). The farms were selected on the basis of where human cases have been detected in Norway, where TBEV has previously been detected in ticks, and in areas where there is no information on TBEV. Serum samples were analysed for antibodies to TBEV, and milk samples for TBEV RNA.

4.2.2. Experimental design – Paper 3

The experimental study on TBEV and *A. phagocytophilum* in sheep was divided into two parts. A total of 30 lambs, at the age of five to six months, of the breed "Norwegian white sheep", were used. All lambs were observed daily, including measurement of rectal temperature, throughout the experimental period of three to six weeks. The lambs were used to handling before the start of the study. The experimental design is summarised in Table 2.

Part one, pre- and post- infection of TBEV or *A. phagocytophilum*

Part one included only male lambs, and it was performed in the autumn of 2017. The lambs were randomly divided into four groups of five lambs (group 1 to 4). On day 0, lambs in group 1 were inoculated subcutaneously with 1 ml of the TBEV-Eu strain Hochosterwitz (approximately 6.5×10^6 focus forming units per ml (FFU/ml)). Furthermore, lambs in group 2 and 3 were inoculated intravenously with 1 ml of a strain of *A. phagocytophilum* corresponding to the accession number M73220 (approximately 1×10^6 infected cells). The lambs in group 4 were negative controls, and they were inoculated subcutaneously with uninfected cell medium from the virus cultivation. On day 21, lambs in group 1 were inoculated with the same strain of *A. phagocytophilum*, and lambs in group 3 with the same strain of TBEV as described above. Lambs in group 2 served as *A. phagocytophilum* controls (Table 2). Blood samples were drawn from *Vena jugularis* on day 0, 2, 4, 6, 8, 10, 14, 18, 21, 25, 31, 35, 39 and 42. All lambs from part one of the study were euthanised, and brain samples were obtained for PCR analysis.

Part two, co-infection of TBEV and *A. phagocytophilum*

Part two consisted entirely of ewe lambs and was carried out in the autumn of 2018. The lambs were randomly divided into two groups (group 5 and 6). The same strains and batches of TBEV and *A. phagocytophilum* as in part one were inoculated to group 1 on day 0, while physiological saline water was used as negative control and inoculated to group 2 on day 0 (Table 2). Blood samples were drawn from all animals on day 0, 2, 4, 6, 8, 10, 14, 18 and 21.

Table 2: Overview of the study groups and the experimental design of part one and part two of the experimental study with infection of tick-borne encephalitis virus (TBEV) and *Anaplasma phagocytophilum* in lambs. Group 1 to 4 consisted of male lambs and group 4 to 6 consisted of female lambs.

Part one: pre- and post- infection of TBEV and *A. phagocytophilum*					**Part two: co-infection of TBEV and *A. phagocytophilum***	
Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
0	Inoculated with TBEV[a]	Inoculated with *A. Phago-cytophilum*[b]	Inoculated with *A. phago-cytophilum*[b]	Negative controls[c]	Inoculated with TBEV and *A. phago-cytophilum,*[a,b]	Negative controls[c]
21	Inoculated with *A. phago-cytophilum*[b]	Inoculated with uninfected cell medium[c]	Inoculated with TBEV[a]	Negative controls[c]	End of experiment	
42	End of experiment				-	

[a]TBEV was inoculated subcutaneously (1 ml of the strain Hochosterwitz, approximately 6.5×10^{6} FFU/ml.

[b]*A. phagocytophilum* was inoculated intravenously (0.4 ml of heparinised sheep blood stabilised with 10 % demethyl sulphoxide (DMSO), approximately 1×10^{6} infected cells, GenBank accession number M73220).

[c]1 ml negative cell medium or physiological saline solution was inoculated subcutaneously.

4.3. Laboratory methods

4.3.1. Enzyme linked immunosorbent assay

A modified commercial direct ELISA (ELISA, Enzygnost® Anti-TBE virus IgG, Siemens, Eschborn, Germany) was performed to screen serum samples for TBEV immunoglobulin G (IgG) antibodies in **Paper 1**, **2** and **3**. The ELISA was modified by changing the conjugate to the species of interest; Anti-Bovine IgG, Anti-deer IgG, Anti-sheep IgG (TriChem ApS-interkemi, Skanderborg, Denmark KLP, Gaithersburg, USA). The conjugates were labelled with the horseradish peroxidase enzyme, which reacts with the chromogen solution in the next step and turns the solution blue. The reaction is stopped by adding sulphuric acid, which causes a colour change to yellow. The intensity of the yellow colour produced is a measure of the immunochemical reactivity of the TBEV IgG antibodies in the sample. The results were calculated by a correction factor that was found by dividing a lot specific nominal value (from the kit) by the mean value of the reference positive controls. Cut-off values were calculated by adding a margin of 0.2 to the mean of the negative controls.

4.3.2. Haemagglutination inhibition test

All positive and borderline samples from TBEV ELISA were analysed by haemagglutination inhibition test (HI) to detect IgG antibodies to LIV in cervid serum samples in **Paper 3**. The analyses were performed at Moredun Research Institute in Scotland, UK, according to Clarke and Casals (1958). The HI tests are used in routine testing of many animal species, including deer. Samples giving a result of HI ≥ 20 were considered positive. Samples with a titre of 10 were inconclusive, and titres < 10 were considered negative.

4.3.3. Serum neutralisation test

Serum neutralisation test (SNT) was performed to confirm TBEV antibodies found by ELISA in **Paper 1**, **2** and **3**, and LIV antibodies detected by HI in **Paper 1**.

The serum samples were analysed for TBEV neutralising antibodies at the Centre for Virology of the Medical University of Vienna, as described by Stiasny et al. (2009). In short, the test was performed in 96-well plates, and serial dilutions of heat-inactivated serum samples were incubated with TBEV (strain Neudoerfl). This mixture was added to baby hamster kidney cells (BHK-21). The neutralisation titre was defined as the reciprocal of the sample

dilution that showed a 90 % reduction in the absorbance compared to the control without antibodies. Samples with titres ≥ 10 were defined as TBEV seropositive and samples with a titre < 10 were defined as negative.

LIV SNT was performed using the constant virus varying serum method performed at Moredun Research Institute in Scotland (Grist et al. 1966). The test is modified to be performed in 96-well plates and used BHK-21 cells and the LIV strain L31 using 30–300 median tissue culture infective dose (TCID50) per well. Serum samples with a titre ≥ 15 were interpreted as IgG positives against LIV by SNT. Samples with a titre < 15 were defined as negative in the test.

4.3.4. RNA extraction and reverse transcription

Viral RNA was extracted from serum samples in **Paper 2** and **3**, and from milk samples in **Paper 2** by QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. Briefly, viral RNA was extracted using the selective binding properties of a silica-based membrane. The viral RNA was eluted by RNase free water and 0.04 % sodium azide (provided by the kit).

Total RNA was extracted from brain samples from sheep in **Paper 3** using RNeasy mini kit (QIAGEN, GmbH, Hilden, Germany) according to the manufacturer's instructions. This protocol used the same silica-based membrane technology as the QIAamp Viral RNA mini kit. In the last step, high-quality RNA was eluted in water or one mM tris-buffer (pH 8.0).

RNA was transcribed into cDNA directly after the RNA extraction by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™ Foster City, California, USA). The reverse transcription was performed using random primers, the recombinant Moloney Murine Leukemia Virus (MMLV) enzyme (MultiScribe™ Reverse Transcriptase) and RNase inhibitors, all provided by the kit.

4.3.5. DNA extraction

DNA was extracted from EDTA blood samples in **Paper 3** by MagNA Pure 96 DNA and Viral NA Large Volume Kit following the manufacturer's protocol (Roche LifeScience, Basel, Switzerland). In short, the nucleic acid isolation procedure is based on the technology of nucleic acid binding to magnetic glass particles.

4.3.6. Real-time polymerase chain reaction

Real-time polymerase chain reaction (real-time PCR) and quantitative PCR (qPCR) were used to detect TBEV from cDNA samples and *A. phagocytophilum* from DNA, in **Paper 2** and **3**.

The real-time PCR for TBEV was performed according to Andreassen et al. (2012), and was designed to detect a 54-base pair (bp) fragment on the E-gene of the virus.

For *A. phagocytophilum,* a qPCR method described by Henningsson et al. (2015) was performed. The *A. phagocytophilum* qPCR was designed to detect a 64 bp fragment of the *gltA* gene.

4.4. Statistics

For **Paper 1** and **3**, the clinical and laboratory data were collected into Microsoft Excel (2016) spreadsheets and transferred to Stata 14.2 for Windows (StataCorp, 4905 Lakeway Drive. College Station, Texas 77845) for statistical analysis. $p<0.05$ was considered significant.

Spearman correlation was used in **Paper 1** to assess the relationships between SNTs. The squared value ρ^2 can be interpreted in terms of predictive power (explained variability) of one SNT ranks by the other SNT ranks.

In **Paper 3**, the quality of data and distributions were analysed using tabulations and histograms. Initial analyses included multilevel linear regression modelling of each of the continuous variables; rectal temperature, neutrophils, lymphocytes, monocytes, quantitative PCR of *A. phagocytophilum* and TBEV titre. Predictors were group (exposure) and day of infection and the random effects variable were individual lambs. The statistical analyses were performed on day 0 to day 21 post inoculation with TBEV and *A. phagocytophilum*. Residuals were estimated and visualised in quantile plots.

In addition, descriptive statistical analyses were performed in Excel and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California).

5. Results and discussion

TBEV is known to cause severe disease in humans, and it is recognised as an important tick-transmitted virus in Europe and Asia. For this reason, updated knowledge on the distribution, susceptible hosts and transmission routes of TBEV is important for the public health (Ruzek et al., 2019). In contrast to in humans, little information on the impact of TBEV in animals is available. The three papers that form the basis for this Ph.D.-thesis are investigations of the occurrence of: antibodies to TBEV in cervids (**Paper 1**) and cattle (**Paper 2**), TBEV in unpasteurised cow's milk (**Paper 2**), and TBEV infection and co-infection of TBEV and *A. phagocytophilum* in lambs (**Paper 3**). In the following sections, the results from each paper included in this thesis are presented and discussed.

5.1. Antibodies to TBEV in cervids from Norway

In **Paper 1**, a total of 657 serum samples from cervids collected across Norway were analysed for antibodies to TBEV, and the overall seroprevalence was 4.6 % (30/657). Neutralising antibodies were detected in 9.4 % (27/286) of the moose samples, 1.4 % (2/140) of the red deer samples, and 0.7 % (1/148) of the roe deer samples. None of the reindeer samples were confirmed positive by SNT (Table 3). It is worth mentioning that the natural habitat of wild reindeer is in alpine areas, where ticks are not normally found. Disregarding the samples from reindeer gives an overall prevalence of 5.2 % (Table 3).

Table 3: Seroprevalence of tick-borne encephalitis virus in moose, roe deer, red deer and reindeer hunted in Norway in 2013 (Paper 1).

Species	Total analysed serum samples	TBEV positive by ELISA (borderline)	Confirmed TBEV positive by serum neutralisation test	Seroprevalence %
moose	286	29 (3)	27	9.4
roe deer	148	0 (1)	1	0.7
red deer	140	2 (0)	2	1.4
reindeer	83	1 (2)	0	0
total	657	32 (6)	30	4.6
total excluding reindeer	574	31 (4)	30	5.2

Seroprevalence studies in cervids have been found to be a relevant tool for mapping the distribution of TBEV and may function as an early warning system for the occurrence of TBEV in areas where human cases have not been reported (Esser et al., 2019). Varying prevalences of antibodies to TBEV have been found in previous studies in Europe. A seroprevalence study in moose from endemic and non-endemic areas in Finland found an overall prevalence of 0.74 % (9/1213) (Tonteri et al., 2016), which is lower than the prevalence of 4.6 % in the present study. A recent study from Denmark found an overall prevalence of 6.9 % (51/736) in roe deer (Andersen et al., 2019). Our study detected a low prevalence of 0.7 % (1/148) in the same species, but we found a comparable overall prevalence of 4.6 % in cervids. A previous study from Norway found higher prevalences of 32 % (8/25) and 56 % (14/25) with the same methods in roe deer and moose, respectively. The higher prevalences in that study could be explained by them having only two sampling sites, both of which had high abundance of ticks (Ytrehus et al., 2013).

Most of the positive samples in **Paper 1** originated from areas in southern and south-eastern Norway with reported human TBE cases (MSIS, 2020). The highest prevalence of TBEV antibodies in cervids in the current study was from Vestfold county (38.1 % / 24 of 63) located in south-eastern Norway. Seropositive cervids were also detected in the following counties: Vest-Agder (4.5 % / 1 of 22) and Aust-Agder (25.0 % / 2 of 8) located in southern Norway, Østfold (2.0 % / 1 of 49) located in south-eastern Norway, Rogaland (2.3 % / 1 of 44) located in south-western Norway and Trøndelag (1.7 % / 1 of 59) located in central Norway. TBEV positive samples from ticks and blood donors have been found outside the areas with reported TBE cases in Norway previously, in the counties of Akershus, Østfold, Rogaland, Hordaland, Sogn og Fjordane, Møre og Romsdal, Trøndelag and Nordland (Larsen et al., 2014; Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020).

The highest number of positive samples was from moose (Table 3). Moose have a preference for foraging in wet/lake areas. This may have contributed to the higher prevalence, as the presence of bodies of water and well-connected forests of oak, birch and/or pine has been identified as important factors for tick abundance (Zeimes et al., 2014). One of the positive moose samples was from Steinkjer in central Norway, and it represents the northernmost detection of a TBEV seropositive large animal in Norway. Although TBEV has been detected in *I. ricinus* ticks in this area and further north previously (Paulsen et al., 2015; Soleng et al.,

2018; Vikse et al., 2020), human cases have not been reported in central Norway (MSIS, 2020). These findings are in accordance with a previous study on TBEV antibodies in cervids which found one seropositive roe deer sample in Molde in north-western Norway (Ytrehus et al., 2013). The detection of TBEV positive samples outside the areas where human TBE cases have been registered may be explained by migratory birds transporting TBEV infected ticks over long distances and across geographical barriers (Hasle et al., 2009; Hasle, 2013; Waldenstrom et al., 2007). Additionally, large and medium sized animals can transport infected ticks over shorter distances (Debeffe et al., 2019; Klitgaard et al., 2017; Talleklint & Jaenson, 1997). The TBEV positive moose sample from Steinkjer was from a male. It is known that male moose generally migrate greater distances, and this moose might have migrated from TBE endemic areas (Debeffe et al., 2019; Qviller et al., 2013; Singh et al., 2012). Because TBEV is known to be focally distributed, even in endemic areas, animals which roam further have an increased chance of being exposed to TBEV infected ticks (Lindquist & Vapalahti, 2008).

TBEV neutralising antibodies were found in two red deer and one roe deer in addition to the positive moose samples. One seropositive roe deer and red deer originated from areas with reported human TBE cases (Vest-Agder and Aust-Agder counties) (MSIS, 2020). The second TBEV antibody positive red deer (titre 20) was from Vindafjord. This sample was also positive for LIV neutralising antibodies (titre 128). Vindafjord is located in the county of Rogaland in south-western Norway which is near the area with reported LIV cases in sheep in the 1980s and early 90s (Norwegian Veterinary Institute., 2019; Ulvund et al., 1983). Furthermore, a study from 1996 found antibodies to LIV or related viruses in sheep herds in Hordaland county in western Norway (Stuen et al., 1996), and a study published in 2013 by Ytrehus and co-workers found antibodies against TBEV and LIV in Farsund in southern Norway in samples collected during the autumn of 2005 (Ytrehus et al., 2013). However, no reports of clinical LIV-cases among sheep in Norway have been reported since it was last diagnosed in Vanse, Vest-Agder, in 1991 (Norwegian Veterinary Institute., 2019; Ulvund et al., 1983). Additionally, 7,615 *I. ricinus* ticks have been analysed for LIV in Norway, all of which were LIV negative (Paulsen et al., 2019). The lack of reported LIV cases in Norway for more than 20 years might be due to asymptomatic infections, low focus on this pathogen in Norway, or that the virus has diminished in the country. Furthermore, farms that experienced

problems with LIV infections have changed pastures or have quit sheep farming (personal communication, Snorre Stuen, NMBU). Future studies should investigate LIV distribution on sheep pastures, as sheep is one of the most ecologically important transmission hosts for LIV (Gilbert, 2016)

There is limited information on TBEV in western Norway, and human TBE cases have not been reported in this region. The first suggested TBEV isolate in Norway was based on haemagglutination inhibition test titres, and originated from questing *I. ricinus* ticks collected in Sogn og Fjordane county in western Norway in 1978 (Traavik et al., 1978). In addition, two seroprevalence studies on human and bovine sera from western Norway have been published with TBEV positive results (Traavik, 1973, 1979). The study in human serum found a prevalence of 19.16 % (67/341) and the study in bovine serum found a prevalence of 17.3 % (14/81). However, these results were from haemagglutination inhibition tests, which are less specific compared to neutralisation tests. A more recently published seroprevalence study (based on SNT) in healthy blood donors in Sogn og Fjordane county could not verify any positive samples (Hjetland et al., 2015).

The reindeer serum samples which were found to be positive for antibodies to TBEV by ELISA in **Paper 1**, could not be confirmed by SNT. The reason why these and the other ELISA positive serum samples could not be confirmed by SNT might be due to unspecific binding in the ELISA and cross-reactions to other closely related flaviviruses (Calisher et al., 1989; Klaus et al., 2014). In addition, wild reindeer in Norway live in mountain areas where they are not normally exposed to ticks. They are, however, exposed to biting insects, such as mosquitoes.

In **Paper 1,** it is difficult to connect the positive observations of TBEV antibodies in animal hosts to exact endemic foci, as it is possible that some of the antibody-positive animals have migrated from endemic areas. Our results are supported by previous detections of TBEV in *I. ricinus*, which indicate that TBEV is also sporadically present in western, north-western, central and northern Norway (Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020).

Another limitation in **Paper 1** is the limited and varying number of available samples from each municipality and county, as the samples were collected by hunters for biobank

purposes, and not specifically for this project. The small number of samples available from each municipality makes it difficult to compare prevalences from different areas of Norway.

5.2. TBEV in cows and unpasteurised cow's milk in Norway

In **Paper 2**, we investigated antibodies to TBEV in cows and the presence of the virus in unpasteurised cow's milk. A total of 112 milk and blood samples from five farms were collected from grazing dairy cows between 2014 and 2017. TBEV RNA was detected in 5.4 % (6/112) of the analysed unpasteurised milk samples. The positive samples from individual cows were found from farms in the municipalities of Mandal (26.8 %), Skedsmo (13.6 %) and Brønnøy (2.1 %) (Table 4). Bulk tank milk was also collected. The bulk tank milk samples from Mandal were positive, while the remaining tank samples were negative.

Table 4: Prevalence of tick-borne encephalitis virus in 112 unpasteurised milk samples collected from cows at five farms in Norway (Paper 2).

Location (municipality, county, date)	No. of samples	Positive milk samples by RT PCR and pyrosequencing (%)	Positive serum samples by SNT (%)
Skedsmo, Akershus, 02.06.2014	22	3 (13.6)	0
Arendal, Aust-Agder, 05.10.2015	17	0	15 (88.2)
Mandal, Vest-Agder, 01.10.2014	7	2 (28.6)	0
Finnøy, Rogaland, 11.09.2017	19	0	0
Brønnøy, Nordland, 02.09.2015	47	1 (2.1)	0
Total	112	6 (5.4)	15 (13.4)

TBEV RNA was found in unpasteurised milk collected from areas both with and without reported human TBE cases. The TBEV positive milk from Mandal were the only positive samples in our study from areas with reported human TBE cases in Norway (MSIS). Skedsmo and Brønnøy are, on the other hand, located in areas with no reported cases. These results are supported by previous studies on TBEV in *I. ricinus* ticks and healthy blood donors, which have reported TBEV to be distributed in a wider geographic area in Norway than the human

cases reported by MSIS (Larsen et al., 2014; Paulsen et al., 2015; Soleng et al., 2018). In the municipality of Skedsmo, no studies on TBEV have previously been published, but ticks are found on livestock (personal communication with the farmer).

A comparable study in Poland found a higher overall TBEV prevalence of 11.1 % in unpasteurised cow milk by RT PCR (Cisak et al., 2010) compared to the prevalence of 5.4 % in our study. In the study from Poland, all samples were collected from endemic areas, in contrast to our study where regions with no previously reported TBE cases were included. Furthermore, the TBE incidence is higher in Poland than in Norway. From 2000 to 2018, the annual TBE incidence rate in Poland varied from 0.3 to 0.92 per 100,000 inhabitants. In the same period, the incidence rate in Norway varied from 0.1 to 0.4 per 100,000 inhabitants (Pancer & Gut, 2019; Paulsen et al., 2019).

From the serological analysis, 13.4 % (15/112) of the samples were positive by SNT (Table 4). However, all positive samples were from the farm located in Arendal (15/17 samples were positive). The high proportion of the positive animals from Arendal indicates that ticks and TBEV is prevalent in the area. These findings are in accordance with annual reports of human TBE cases from the area (MSIS, 2020). None of the cows with confirmed TBEV positive milk samples had detectable antibodies in serum by SNT.

Further investigation is needed to find out why the milk was found positive for TBEV by PCR in three herds without any seroconverted animals. TBEV RNA positive milk samples indicates that the cows have been exposed to the virus, and it is likely to think that some of the other animals would have detectable antibodies. However, the herds included in this study had a low number of animals (7 to 47), and there is limited information on the duration of the antibody response in ruminants. A study in goats detected antibodies 28 months past primary TBEV infection (Balogh et al., 2012). Further, it is unclear if all animals exposed to TBEV by infected ticks develop an immune response, and the antibody response might also vary between animal species and TBEV strains (Klaus et al., 2010b; Klaus et al., 2012). One reason why no antibodies against TBEV were found in the herds in Mandal, Skedsmo and Brønnøy might be that the exposed animals did not develop antibodies, possibly because of low viral load in the TBEV infected ticks. In **Paper 3** of this thesis, all lambs developed neutralising antibodies six to eight days after infection, however, the study did not provide

information on the longevity of the antibodies beyond 21 days. Furthermore, the antibody response after a smaller infectious dose is not known.

The time of sample collection may be a part of the explanation for finding TBEV positive milk when no animals had seroconverted. In a previous study, immunised goats did not shed TBEV through the milk (Balogh et al., 2012). The same might apply for cows, and this might be the reason why no TBEV positive milk samples were detected in Arendal. The milk and serum samples from Arendal were collected in October. The cows could have been infected during spring or summer, or in previous tick seasons, and hence, would no longer shed TBEV through the milk when the samples were collected in October. The samples from Skedsmo, Brønnøy and Mandal were collected in June, September and October, respectively. It is not known if these animals developed neutralising antibodies to TBEV post infection.

A limited number of cow milk and blood samples from five medium sized farms were included in the study. A greater sample size together with a broader geographical study area in Norway should be considered for future studies. Repeated sampling to investigate if the cows with TBEV positive milk developed antibodies after infection should also be done.

5.3. Detection of TBEV outside the area of reported human TBE cases

In **Paper 1** and **2**, TBEV RNA and antibodies were detected outside the geographical area of reported TBE cases in Norway. This has recently also been documented in studies in ticks, cervids and blood donors in Norway (Larsen et al., 2014; Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020; Ytrehus et al., 2013). There might be several reasons why TBE cases are only reported from the southern parts of the country. It is possible that TBE cases go unrecognised by patients and general practitioners, leading to underdiagnosis of the disease. As a zoonotic agent, TBEV circulates among wildlife, and most likely, the virus has greater presence in various animal species than reflected by the number of human TBE cases (Randolph & Sumilo, 2007).

Different genetic variants of TBEV are known to vary in their virulence (Asghar et al., 2017; Romanova et al., 2007; Ruzek et al., 2008). The only TBEV strain from Norway that is whole genome sequenced, "Mandal 2009", had a deletion in the 3' NCR, similar to the highly virulent "Hypr" strain. This type of deletion might influence the virulence of TBEV currently

circulating in Norway (Asghar et al., 2017). TBEV strains found in southern Norway, where human cases are reported, may be more pathogenic than those found elsewhere. A recently published study reported that some TBEV strains gave mild gastrointestinal symptoms rather than CNS symptoms (Dobler et al., 2016). Such rare or non-typical symptoms complicate the diagnosis, and patients who do not display neurological symptoms are less likely to be diagnosed with TBE. This could also be applicable to the strains circulating in Norway. A study by Kurhade et al. (2018) did not detect gastrointestinal symptoms in mice infected with a gastrointestinal strain (MucAr HB171/11), however, the mice showed a delayed neuroinvasiveness and low neurovirulence, which might explain the lack of neurological symptoms in humans (Kurhade et al., 2018).

The abundance of nymphs in an area may be associated with the incidence of human cases of tick-borne diseases (Stafford et al., 1998). In Norway, *I. ricinus* is abundant in coastal areas from Østfold county in the east to approximately Brønnøy in Nordland county in the north (Hvidsten et al., 2014; Hvidsten et al., 2020; Mehl, 1983; Soleng et al., 2018; Tambs-Lyche, 1943; Vikse et al., 2020). *I. ricinus* has been found to be abundant in Brønnøy for more than 60 years (Hvidsten et al., 2020; Mehl, 1983; Tambs-Lyche, 1943), and the *Borrelia* spp. prevalence in *I. ricinus* has been found to be comparable to the prevalences found in southern Norway (Soleng & Kjelland, 2013). However, the density of ticks varies greatly between locations, but generally, based on tick flagging over a ten-year period, the abundance seems to be higher in southern parts of Norway compared to western and northern parts (personal communication Arnulf Soleng, NIPH). Although the abundance of ticks cannot be precisely measured by single flagging sessions, repeated flagging may give good indications of the tick abundance (Randolph, 2008). Lower abundance of ticks decreases the risk of human tick bites, which may help explain the lack of human cases in western and northern Norway. The population densities in northern Norway are lower than in the south, which may further reduce the number of people being bitten by ticks in the north. Further, the "summer season" is shorter in the north of Norway.

Single findings of TBEV positive ticks do not prove that TBEV is endemic in an area. TBEV RNA has been detected in cow's milk from one farm in this study, and in *I. ricinus* nymphal and adult ticks from three separate locations (Soleng et al., 2018). Through flagging, all stages of *I. ricinus* have been detected in Brønnøy for more than two years. Synchronised activity of

larvae and nymphs may be a prerequisite for co-feeding, an important mechanism for maintenance of TBEV foci (Randolph, 2011).

5.4. Infection of TBEV and co-infection of TBEV and *A. phagocytophilum* in lambs

In **Paper 3,** we investigated infection of TBEV and co-infection of TBEV and *A. phagocytophilum* in lambs. The background of the study was that co-infection of LIV and *A. phagocytophilum* has been shown to give a greater and prolonged viraemia compared to a single LIV infection (Reid et al., 1986). It has been speculated if the same may occur for TBEV and *A. phagocytophilum.*

The study showed that all lambs infected with TBEV (group 1, 3 and 5) developed neutralising antibodies to the virus (Figure 8), but none of the lambs displayed clinical TBE symptoms. Furthermore, the infected lambs had a short- or non-detectable viraemia by RT real-time PCR. A significantly higher mean TBEV titre was found in the group co-infected with TBEV and *A. phagocytophilum* than in the other groups (Figure 8). All of the analysed brain samples were negative.

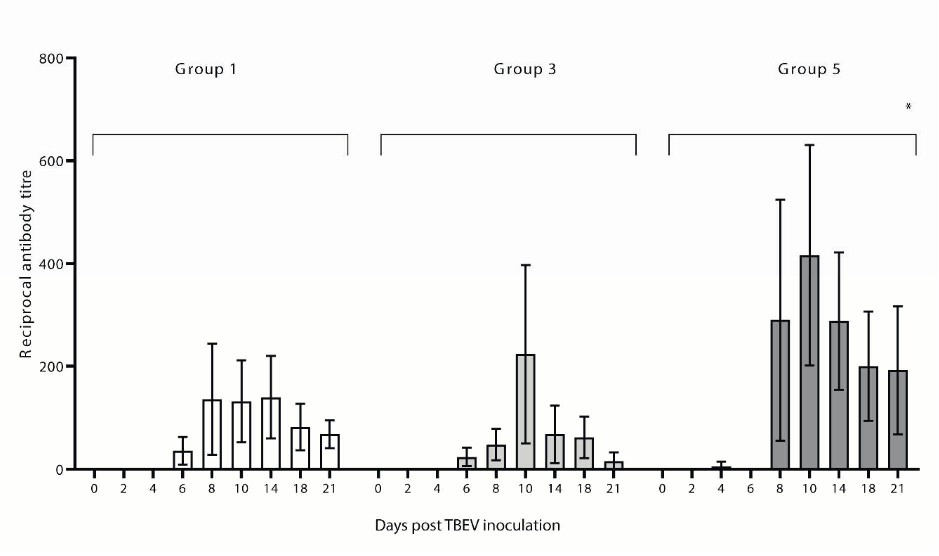

Figure 8: Mean reciprocal TBEV antibody titre in lambs post TBEV infection TBEV titres (Y axis) were measured by serum neutralisation test in group 1, 3 and 5 on day 0 to day 21 post inoculation with

TBEV (X axis). Group 5 had significantly higher mean TBEV titre values than group 1 and 3, indicated with asterisk (*). Standard deviations (SD) are illustrated with error bars (Paper 3).

The results indicate that co-infection of TBEV and *A. phagocytophilum* in lambs may give an increased antibody response compared to TBEV infected lambs which were pre or post infected with *A. phagocytophilum* (**Paper 3**). The reason for the increased antibody titres in the co-infected lambs is, however, unknown. While *A. phagocytophilum* is known to cause a neutropenia derived immunosuppression in lambs, which may lead to secondary infections (Woldehiwet, 2008), this did not seem to have an effect on the TBEV infection in the lambs. One reason for this might be that TBEV replicates poorly in sheep cells (Mansfield et al., 2016), resulting only in an asymptomatic TBEV infection in sheep.

Although there have been few reported cases of TBE in ruminants, the results in this study are in accordance with previous studies, which indicate that TBEV rarely causes symptomatic disease in sheep (Bago et al., 2002; Bohm et al., 2017; Mansfield et al., 2016).

A recent study on infection of TBEV and LIV sheep by Mansfield and colleagues found comparable results as presented in **Paper 3**: no clinical symptoms post TBEV infection with the TBEV strain Neudoerfl, but neutralising antibodies were detected in all infected sheep (Mansfield et al., 2016). The same study found lower TBEV titres compared to the LIV titres after infection.

The possibility of humans being alimentary infected with TBEV shows that ruminants develop a viraemia after TBEV infection (Balogh et al., 2010; Caini et al., 2012; Gresikova et al., 1975; Holzmann et al., 2009; Kerbo et al., 2005; Kohl et al., 1996; Kriz et al., 2009; Rieger et al., 1998). A short-lived TBEV-viraemia was found in **Paper 3**. An experimental study in goats detected TBEV in milk samples for up to 19 days after infection with the viral strain "Kem I” (Balogh et al., 2012). The reason for the prolonged secretion detected in goats compared to sheep is unknown, but it might indicate that goats are more susceptible to TBEV infection than sheep, or that there are differences in the pathogenicity of the viral strains.

The lambs were inoculated with only one TBEV-Eu strain. Different TBEV-Eu strains are known to vary in their virulence (Asghar et al., 2014; Asghar et al., 2016; Mandl et al., 1998).

Other, and possible more virulent TBEV-strains, e.g. the strain “Hypr”, which is considered a pathogenic strain, should also be tested to confirm the effects of co-infections and to investigate if other strains may give clinical symptoms of TBE in lambs. TBEV is known to adapt to different hosts, and may change its virulence after passages in cell lines (Mandl et al., 1998). The “Hochosterwitz” strain used in our study could be considered a “laboratory strain”, since it had been cultivated in mice brain and passaged in mammalian cell lines. However, the “Hochosterwitz” strain has been shown to be pathogenic in mice (personal communication Åshild K. Andreassen, NIPH). Additionally, the study by Mansfield et al. (2016) used the strain “Neudoerfl”, which is also considered a strain causing low pathogenicity (Mandl et al., 1998).

The significant difference of the mean antibody titre to TBEV could have been affected by the difference of the gender of the lambs in part one, which consisted entirely of males, and part two, which consisted entirely of females. A previous study on *A. phagocytophilum* infection in laboratory mice found that infected male mice had an increased *A. phagocytophilum* DNA load (Naimi et al., 2018). However, no differences between genders have been found previously in *A. phagocytophilum* infected lambs (Stuen, 2003), and in our study, no significant difference was found in the mean *A. phagocytophilum* DNA load.

5.5. Methodological considerations

5.5.1. Sample collection

Proper treatment of samples is crucial for further analyses and accurate results. In this Ph.D.-project, the samples were collected by hunters, farmers and veterinarians. The sample quality may vary due to e.g. contamination in the field and the samplers’ experience.

The serum samples from cervids (**Paper 1**) were collected by hunters in the regular hunting season (autumn) in Norway. The hunters in general have less experience in sample handling, thus, the samples collected by hunters may have lower quality. Contamination from one carcass to another could occur by use of the same equipment (e.g. a contaminated knife). However, the hunters collected blood from the thoracic cavity with sterile single-use plastic Pasteur pipettes and transferred it to full blood collections tubes. The blood samples were sent to the Norwegian Veterinary Institute at ambient temperature, within 1 to 3 days after

collection. Due to the blood sampling method from the hunted animals, some samples were haemolysed. However, the ELISA results are not affected by haemolysis according to the manufacturer's information. Furthermore, since these samples only was analysed by serological methods and not PCR, the transportation time in ambient temperature is not as critical because IgG antibodies are relatively stable (Henriksen et al., 2014). The milk and blood samples (**Paper 2** and **3**) were collected by local farmers and experienced veterinarians, respectively. Sterile tubes and gloves were used to avoid contamination. The blood samples were centrifuged within two hours post sampling, and serum was stored at -80 °C until analysis to prevent degradation of the RNA. The milk samples were stored and transported to the lab under refrigerator conditions, and then stored at -80 °C until analysis.

5.5.2. Serological methods

The serological results presented in **Paper 1** were screened by a commercial TBEV antibody ELISA. Diagnostic use of ELISA for detection of TBEV antibodies is known to have high sensitivity, but only moderate specificity. The sensitivity and specificity vary between the different commercial kits. A recent study on five commonly used commercial kits found that three of the five tested TBEV ELISA kits had an acceptable sensitivity of 94 % for IgG, while all showed a low specificity when testing other flaviviruses. For IgM, the five ELISA kits had a sensitivity of 94 to 100 % (Reusken et al., 2019).

The moderate IgG specificity in the TBEV ELISA kits is caused by cross-reactions to other closely related flaviviruses. For this reason, verification of the ELISA results with a TBEV-specific SNT is recommended, especially in cases where more than one *Flavivirus*, e.g. TBEV and LIV, is known to circulate (Klaus et al., 2011; Rieille et al., 2017). SNT is considered the gold standard as a serological test for TBEV. However, the results presented in **Paper 1** showed that the TBEV and LIV cross-reacted in the SNTs. It is therefore difficult to distinguish if the antibody response is from a previous TBEV or LIV infection. However, as discussed previously, TBEV is most likely to have been the causative agent, due to the slightly higher titres in TBEV SNT compared to LIV SNT for most of the samples. Additionally, TBEV seems to be more widespread, as TBEV has been found in ticks where LIV has not, and the number of clinical LIV cases is limited. Other laboratory methods, such as PCR, could have been performed to gain more specific results compared to the results from SNT. However, little is known about TBEV viraemia in cervids, except for one report of a moose with TBEV infection

in Sweden in the 60s (Svedmyr et al., 1965). Further, the sample handling with transportation in ambient temperature before storage at -80 °C was not ideal for PCR analyses.

5.5.3. Molecular biological methods

The results on TBEV RNA in unpasteurised milk samples and TBEV RNA in the experimental study were based on an in-house real-time PCR. PCR is a well-known laboratory method for making multiple copies of a specific DNA fragment by designing specific PCR primers. Several factors might influence the PCR results, such as the quality of the RNA or DNA, properties of the enzyme used, and properties and specificity of the primers. For this reason, optimisation of PCR methods is important. Both real-time PCR methods were published and optimised prior to this study (Andreassen et al., 2012; Henningsson et al., 2015).

RNA is easily degraded by RNases. RNases are present in the environment due to being natural parts of skin and mucosa (Green & Sambrook, 2019). For this reason, it is important to work with the RNA in an RNase free environment. Furthermore, the use of RNase inhibitors and keeping the RNA at low temperature is essential. To avoid degradation of RNA, the RNA was placed on ice after the extraction and transcribed into cDNA the same day. To avoid contamination in RNA/DNA extraction and in the PCR reactions, separate rooms were used for pre and post PCR. Preparation of PCR master mixes were performed in a "clean room", and extraction of RNA was performed in a biosafety level 2 cabinet, and the RNA was added to the PCR tubes in a PCR hood. UV-light was used post all analyses.

All TBEV positive milk samples had high Ct. values close to the detection limit of the PCR (Ct. approx. 30-35), and unfortunately duplicate runs in the PCR were not performed. However, only samples confirmed positive by pyrosequencing were considered true positive. The PCR and sequencing methods were designed to be TBEV specific and to not amplify other related viruses such as LIV, and to detect very low viral load of TBEV (Andreassen et al. 2012). The method has been validated at the Department of Virology at NIPH for use in diagnostics and evaluated by inter-laboratory comparison. A limitation of the TBEV PCR method is, however, that it was designed to be specific for the Norwegian TBEV strain "Norway-1" (GenBank accession no. EF565947). Therefore, it may not detect all other European TBEV strains, which may result in false negative results.

The study on TBEV RNA in milk did not provide any information regarding the pathogenicity. Other methods, such as cultivation of the virus and performing a focus forming assay to measure the virus concentration, should have been performed to check if there was active virus in the samples. However, these methods are time consuming and require a biosafety level 3 laboratory. Further studies should investigate this.

5.5.4. The experimental study

One limitation of the experimental study (**Paper 3**) is the number of lambs included in the study. Due to this, individual variations of the lambs can affect the results. The statistical power depends on the sample size and including more lambs in the study would give a higher probability of finding true effects. However, the number of lambs used was limited due to ethical guidelines including the three R's, replacement, reduction and refinement (FOTS ID 8632).

Additionally, the study divided the animals in groups based on gender to avoid disturbances due to rutting behaviour in young males. However, no differences have previously been observed between genders with regards to experimental *A. phagocytophilum* infection in lambs, as mentioned earlier (Stuen, 2003).

6. Main conclusions

Paper 1 represents the largest serological TBEV study on cervids in Norway. The study found antibodies to TBEV in areas with and without reported human TBE cases, and supports previous findings of TBEV in ticks, which indicate that TBEV is distributed in a broader geographical area compared to the areas where human TBE cases have been reported in Norway. The study showed that cervids may be used as sentinels for the distribution of TBEV in Norway, as a supplement to prevalence studies of TBEV in ticks and reports of human cases. Early updated information on emerging pathogens in areas where no human cases have been reported is important to prevent new cases. Such information can be used for risk assessments.

Paper 2 was the first study to detect TBEV in unpasteurised cow's milk from Norway. TBEV was detected in unpasteurised milk collected from areas both with and without reported human TBE cases. Further studies on TBEV in milk should be performed to conclude if TBEV found in unpasteurised milk in Norway is infectious to humans. The study recommended a risk assessment to evaluate the consequences of consuming unpasteurised milk from domestic ruminants in Norway.

In **Paper 3,** lambs were experimentally infected with TBEV and *A. phagocytophilum*. None of the lambs infected with TBEV displayed any clinical symptoms related to TBE. However, all TBEV infected lambs developed neutralising antibodies. The *A. phagocytophilum* infected lambs had a fever reaction. Lambs co-infected with TBEV and *A. phagocytophilum* were found to have an increased mean TBEV titre compared to TBEV infected lambs which were either pre- or post- infected with *A. phagocytophilum.* For future experimental studies other and possibly more virulent TBEV strains should be considered.

7. Further work

This Ph.D.-thesis has provided information on TBEV antibodies in cervids and cattle, and TBEV RNA in unpasteurised cow's milk in Norway. Furthermore, it has provided information on TBEV infection and co-infection of TBEV and *A. phagocytophilum* in lambs. Some suggestions for further research project related to the studies in this Ph.D.-thesis follow:

In **Paper 1** and **2**, TBEV was found to be distributed in a wider geographical area in Norway than the area with reported human TBE cases. One reason for this might be that different TBEV strains with different pathogenicity may circulate in Norway. Study of whole genome sequences of Norwegian TBEV strains is therefore recommended to increase the understanding of the phylogeographical relationships between TBEV strains. Another approach may be to analyse samples from human patients with encephalitis with an unknown causative agent in Norway, especially for patients outside the area of reported TBE cases. The awareness of TBE among medical doctors may be lower in areas without previous reports of TBE compared to TBE endemic areas.

The distribution of LIV in Norway should be elucidated in future studies, as discussed in **Paper 1**. Prevalence studies of LIV in ticks and sheep are recommended, since updated information on LIV in Norway is lacking. This is important because LIV and TBEV cross react in serological assays.

For the results in **Paper 2**, information on the pathogenicity of TBEV detected in the milk is not known, and future studies should aim to investigate whether the TBEV RNA found in milk samples in Norway is infectious to humans. Furthermore, studies on the relationship between TBEV antibodies in serum and TBEV RNA in milk should be investigated.

In **Paper 3**, no symptoms related to TBE in the lambs were found, but there are a few reported cases in ruminants with clinical TBE symptoms. Other, and possibly more virulent TBEV strains should be tested in future studies to confirm the seemingly minimal effect of co-infection with TBEV and *A. phagocytophilum* and that TBEV rarely gives symptomatic disease in ruminants.

In the light of climatic changes, the distribution of both TBEV and its main vector, *I. ricinus*, may change in Norway, and this should be investigated in future studies. Recently, *I. persulcatus* was detected in Sweden (Jaenson et al., 2016). Future studies in Norway should follow up this finding, as *I. persulcatus* may bring other more pathogenic TBEV strains and subtypes such as TBEV-Sib and TBEV-FE to the country.

8. References

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9. Appendix - scientific papers 1-3

PAPER 1

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ORIGINAL ARTICLE

WILEY

Cervids as sentinel-species for tick-borne encephalitis virus in Norway - A serological study

Katrine M. Paulsen[1,2] | **Carlos G. das Neves**[3] | **Erik G. Granquist**[2] | **Knut Madslien**[3] | **Snorre Stuen**[5] | **Benedikte N. Pedersen**[1,4] | **Rose Vikse**[1] | **Mara Rocchi**[6] | **Ellie Laming**[6] | **Karin Stiasny**[7] | **Åshild K. Andreassen**[1]

[1]Department of Virology, Division for Infection Control and Environmental Health, Norwegian Institute of Public Health, Oslo, Norway

[2]Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway

[3]Norwegian Veterinary Institute, Oslo, Norway

[4]Department of Natural Science and Environmental Health, University of South-Eastern Norway, Bø, Norway

[5]Department of Production Animal Clinical Sciences, Section of Small Ruminant Research and Herd Health, Norwegian University of Life Sciences, Sandnes, Norway

[6]Virus Surveillance Unit, Moredun Research Institute, Penicuik, Scotland, UK

[7]Center for Virology, Medical University of Vienna, Vienna, Austria

Correspondence
Carlos G. das Neves, Norwegian Veterinary Institute, PO Box 750 Sentrum, N0106 Oslo, Norway.
Email: carlos.dasneves@vetinst.no

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Abstract

Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis (TBE). TBEV is one of the most important neurological pathogens transmitted by tick bites in Europe. The objectives of this study were to investigate the seroprevalence of TBE antibodies in cervids in Norway and the possible emergence of new foci, and furthermore to evaluate if cervids can function as sentinel animals for the distribution of TBEV in the country. Serum samples from 286 moose, 148 roe deer, 140 red deer and 83 reindeer from all over Norway were collected and screened for TBE immunoglobulin G (IgG) antibodies with a modified commercial enzyme-linked immunosorbent assay (ELISA) and confirmed by TBEV serum neutralisation test (SNT). The overall seroprevalence against the TBEV complex in the cervid specimens from Norway was 4.6%. The highest number of seropositive cervids was found in south-eastern Norway, but seropositive cervids were also detected in southern- and central Norway. Antibodies against TBEV detected by SNT were present in 9.4% of the moose samples, 1.4% in red deer, 0.7% in roe deer, and nil in reindeer. The majority of the positive samples in our study originated from areas where human cases of TBE have been reported in Norway. The study is the first comprehensive screening of cervid species in Norway for antibodies to TBEV, and shows that cervids are useful sentinel animals to indicate TBEV occurrence, as supplement to studies in ticks. Furthermore, the results indicate that TBEV might be spreading northwards in Norway. This information may be of relevance for public health considerations and supports previous findings of TBEV in ticks in Norway.

KEYWORDS
cervids, sentinel animals, seroprevalence, tick-borne encephalitis virus

Katrine M. Paulsen and Carlos G. das Neves are contributed equally to this work

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1 | INTRODUCTION

Tick-borne encephalitis virus (TBEV) is a vector borne disease that cause tick-borne encephalitis (TBE) in humans and animals. The virus is widespread throughout Europe and consists of five known subtypes: European, Siberian, Far Eastern, Baikalian and Himalayan (Dai, Shang, Lu, Yang, & Xu, 2018; Dobler, Gniel, Petermann, & Pfeffer, 2012; Kovalev & Mukhacheva, 2017). TBEV is a positive sense single stranded RNA virus belonging to the *Flaviviridae* family, and is a part of a complex of related viruses known as the TBEV complex. In addition to TBEV, this complex includes Louping ill virus, Langat virus, Powassan virus, Omsk hemorrhagic fever virus, Kyasanur Forest disease virus, Spanish sheep encephalomyelitis virus and Greek goat encephalomyelitis virus (Grard et al., 2007). The main vectors for transmission of TBEV in Eurasia are the *Ixodes ricinus* and *Ixodes persulcatus*. It is estimated that TBEV is one of the most important neurological pathogens transmitted by tick bites in Central and Eastern Europe, as well as Russia, with significant impact on the public health (Ruzek et al., 2019). In the past decades, a rapid increase in the incidence of TBE has been observed in many European countries where TBE is endemic, simultaneously with the emergence of new foci (Jaenson, Hjertqvist, Bergstrom, & Lundkvist, 2012; Ruzek et al., 2019; Suss, 2011).

In Norway, *I. ricinus* ticks are mainly distributed along the coastline from Østfold county in the southeast up to near the Arctic Circle (66°33′47.5″N) in Nordland county (Hvidsten et al., 2014; Jenkins et al., 2012; Mehl, 1983; Soleng et al., 2018; Tambs-Lyche, 1943). TBEV has been documented in ticks, where *I.ricinus* is abundant (Andreassen et al., 2012; Paulsen et al., 2015; Soleng et al., 2018). Consistently, the distribution of TBE has also been shown in a blood donor and tick study in Østfold county in eastern Norway. (Larsen et al., 2014). Although studies have found that TBEV in ticks is distributed from southern to northern Norway, the number of human cases of TBE in the country is low, with a total of 139 reported autochthonous cases (incidence ranges from < 0.1–0.4 per 100,000 inhabitants per year) since the first case occurred in 1997. These cases are limited to the southern and south-eastern parts of the country (Norwegian Surveillance System for Communicable Diseases (MSIS), 2019).

Another flavivirus, closely related to TBEV, is the louping-ill virus (LIV). TBEV and LIV are maintained by different reservoirs: TBEV mainly by ticks and rodents, LIV by ticks and mountain hare (Gilbert, Jones, Hudson, Gould, & Reid, 2000; Labuda & Randolph, 1999; Norman, Ross, Laurenson, & Hudson, 2004). TBEV is known to cause infections in humans, horses and dogs, whereas LIV is known to cause severe neurological disease in sheep and red grouse (Gordon, Brownlee, Wilson, & Macleod, 1932; Jeffries et al., 2014; Kaiser, 2012; Klaus, Horugel, Hoffmann, & Beer, 2013; Reid, Duncan, Phillips, Moss, & Watson, 1978; Weissenbock, Suchy, & Holzmann, 1998). LIV has not been detected in ticks in Norway previously, and the last reported case of LIV infection in sheep in Norway was in 1991 (Norwegian Veterinary Institute., 2019; Paulsen et al., 2017).

Impacts

- The study is the first comprehensive screening of tick-borne encephalitis (TBE) antibodies in cervid species in Norway.
- The study shows that cervids are useful sentinel animals for distribution of tick-borne encephalitis virus (TBEV) in Norway as a supplement to data on human TBE cases and prevalence of TBEV in ticks.
- This study supports previous findings of TBEV in ticks, which indicate that TBEV is distributed in Norway more widely than suggested by human TBE cases.

However, a previous study in cervids shows that both viruses may circulate in Norway (Ytrehus, Vainio, Dudman, Gilray, & Willoughby, 2013).

Apart from climatic variables and human drivers, many studies have clearly shown the important role of large wildlife species in TBEV epidemiology, as recently summarized by Esser and colleagues (Esser et al., 2019). The use of these cervids as sentinels has been documented in different countries with variable results, but there is a consistent conclusion that these animals represent a relevant epidemiological tool in understanding and mapping the distribution of TBEV, as well as potentially functioning as an early warning system for the presence of these viruses in areas where human cases have not yet been reported.

Deer and moose can serve as transient hosts for TBEV, perhaps with a more relevant role in maintaining tick populations rather than being a relevant reservoir for TBEV (Carpi, Cagnacci, Neteler, & Rizzoli, 2008). The most plausible direct contribution of cervids to TBEV transmission is the non-viremic transmission from infected ticks to naïve ticks co-feeding on the same host (Jaenson et al., 2018; Mlera & Bloom, 2018; Randolph, 2011). Cervid species usually exhibit low or no viremia post TBEV infection, but show a low titre antibody response that can be measured over time (Gerth, Grimshandl, Stage, Doller, & Kunz, 1995; Imhoff et al., 2015). Given that the TBEV prevalence in ticks usually is low (Andreassen et al., 2012; Pettersson, Golovljova, Vene, & Jaenson, 2014), cervid sampling can be an important supporting tool as the TBE antibodies will reflect TBEV circulation. Several studies in wild cervids in different European countries have confirmed the transmission of TBEV within the sampling region, as indicated by records of human TBE. These studies have also helped identify previously unknown foci and confirmed that wildlife mammals can be used as sentinel species for TBEV (Balling, Plessow, Beer, & Pfeffer, 2014; Kiffner, Vor, Hagedorn, Niedrig, & Ruhe, 2012; van der Poel et al., 2005; Skarphedinsson, Jensen, & Kristiansen, 2005).

In Norway, there are four major free-ranging species in the deer family (Cervidae): roe deer (*Capreolus capreolus*), red deer (*Cervus*

elaphus), euroasian reindeer (*Rangifer tarandus tarandus*, both wild and semi-domesticated) and moose (*Alces alces*) (Morellet, Klein, Solberg, & Andersen, 2010). The total number of wild cervids in Norway has been rising during the last decades and was estimated to approximately 450,000 individuals in 2009 (Solberg et al., 2010). Roe deer, red deer, reindeer and moose are all subject to licensed hunting during autumn. In Norway, these species have varying geographical distributions and population densities, as well as different habitat preferences (Apollonio, Andersen, & Putman, 2010). Wild reindeer migrate and feed at high altitudes in the southern part of Norway (Apollonio et al., 2010), mostly above the current altitude limit for tick distribution in Norway (Hvidsten et al., 2015; Larsson, Hvidsten, Stuen, Henningsson, & Wilhelmsson, 2018; Paulsen et al., 2015; Soleng et al., 2018). Roe deer is a browser, meaning that it eats leaves, soft shoots, or fruits of tall, generally woody plants such as shrubs in the lowlands, with preferences for forest clearings and being territorial in the main tick season (Hofmann, 1989). Red deer is an intermediate, opportunistic, mixed feeder, meaning that it would eat both leaves and grass in the lowland, and mainly, in the western part of the country, often in areas of dense forest (Hofmann, 1989). Moose is a browser which preferences dense forests and is often feeding on water plants in lakes and wet areas (Apollonio et al., 2010; Franzmann & Schwartz, 2007), with a wide distribution in Norway both inland and in coastal areas (Solberg et al., 2010).

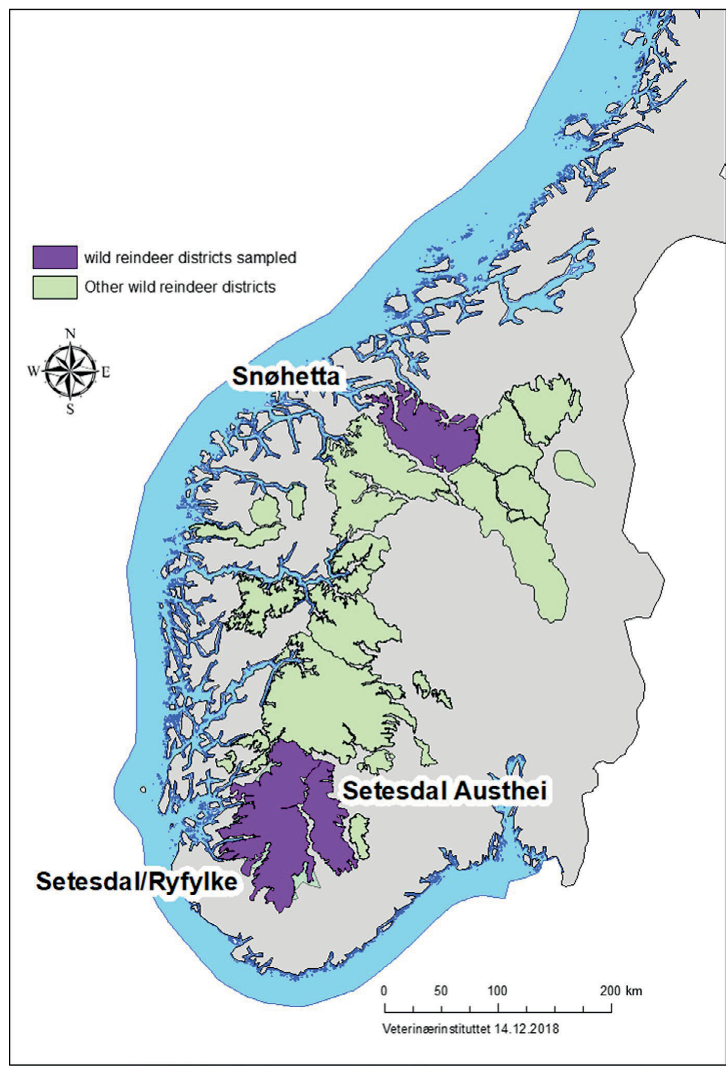

FIGURE 1 Geographical locations of the sampling sites of wild reindeer sera included in the study. The coloured areas in the map indicates the Norwegian wild reindeer management districts (in light green), with those in pink depicting districts with samples included in this study

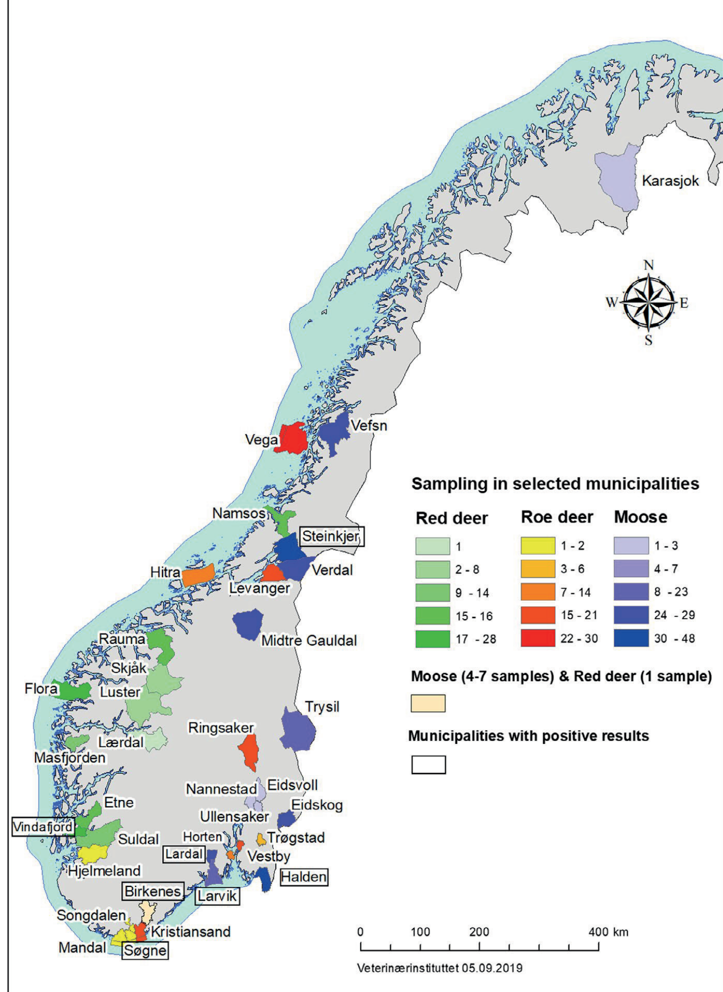

FIGURE 2 Geographical locations of the sampling sites of moose, red deer and roe deer sera included in the study. The coloured areas in the map indicates sampling in a municipality, and municipalities labelled with a square represents municipalities with seropositive samples

The aim of the study was to investigate the seroprevalence of TBEV-specific antibodies in cervids in Norway and the possible emergence of new foci. Furthermore, a second aim was to evaluate if cervids in Norway can function as possible sentinel animals for the distribution of TBEV as a supplement to surveillance of TBEV in ticks. This is based on the assumption that cervids as sentinels have: (a) measurable antibody response after infection with TBEV, (b) territory or home range that overlaps the area where ticks are present, (c) sufficient population size and can be easily captured and sampled.

Based on the current geographic distribution of ticks and cervid species, we hypothesize that red deer, roe deer and moose may function as sentinel species, especially along the coastline. We also hypothesize that wild reindeer can function as a relevant sentinel species and as an early-warning system for spread of ticks to higher altitudes.

2 | MATERIALS AND METHODS

2.1 | Sample collection and selection criteria

The Norwegian health monitoring program for deer and muskox (HOP) has been ongoing since 1998 and provides an overview and knowledge of the state of health of Norwegian populations of deer and muskox. In 2013, a broad national sampling was organized and approximately 700 animals were sampled. Criteria for sample selection were: (a) collection in areas with known abundance of cervids, (b) collection during summer months, which coincides with the highest period of tick activity (between April and November), (c) collection of samples of each cervid species in areas with and without reported tick presence. Hunters were asked to collect blood from the thoracic cavity with a plastic Pasteur pipette and transfer it to full blood tubes. The blood samples were sent at ambient

TABLE 1 Seroprevalence of tick-borne encephalitis virus in different cervid species hunted in Norway in 2013

	Total analysed sera	TBEV positive by ELISA (borderline)	Confirmed TBEV positive by serum neutralisation test	Number of municipalities/herding districts with positive animals[a]	Seroprevalence %
Moose	286	29 (3)	27	5/15	9.4
Roe deer	148	0 (1)	1	1/12	0.7
Red deer	140	2 (0)	2	2/11	1.4
Reindeer	83	1 (2)	0	0/3	0
Total	657	32 (6)	30		4.6

[a]The number/y depicts the total number of municipalities or herding districts from which samples were obtained.

temperature to the Norwegian Veterinary Institute (approximately within 1 to 3 days after collection). Upon arrival they were centrifuged at 685 *g* for 10 min. Serum was transferred to 5-mL tubes, and the samples stored at −40°C until use. Due to the blood sampling from the hunted animals, some samples were haemolysed (<10% of total samples), especially for the roe deer samples.

2.2 | Serological methods

Serum samples from 286 moose, 148 roe deer, 140 red deer, and 83 reindeer (Figure 1, Figure 2, and Tables S1 and S2) were screened for TBE immunoglobulin G (IgG) antibodies with a modified commercial enzyme-linked immunosorbent assay (ELISA, Enzygnost® Anti-TBE virus IgG, Siemens) according to the manufacturer's protocol, as described previously (Ytrehus et al., 2013). The ELISA was modified using peroxidase-labelled affinity purified antibody to deer IgG (H + L) produced in rabbit (TriChem ApS-interkemi). The conjugate was diluted 1:10,000 in IgG Conjugate Buffer Microbiol (Enzygnost® Anti-Rubella Virus IgG, Siemens). Previously confirmed TBE IgG positive and negative roe deer and moose sera by serum neutralisation test (SNT) were used as internal controls.

To confirm the TBE ELISA results, all positive and borderline serum samples were re-tested by a TBEV-specific SNT at the Center for Virology of the Medical University of Vienna, as described previously (Stiasny, Holzmann, & Heinz, 2009). Briefly, serial dilutions of heat-inactivated samples were incubated with TBEV (strain Neudoerfl) for 1h at 37°C. Baby hamster kidney (BHK-21) cells were added and incubated for three days. The presence of virus in the cell culture supernatant was assessed by ELISA. The virus neutralisation titre was defined as the reciprocal of the sample dilution that showed a 90% reduction in the absorbance readout compared to the control without antibody. Samples with titres equal to ten and higher were defined as TBE seropositive.

All positive and borderline serum samples by TBE ELISA were also analysed for IgG antibodies to LIV using haemagglutination inhibition test (HI) and SNT at Moredun Research Institute in Scotland as described previously (Clarke & Casals, 1958; Grist, 1966). The HI test for antibody to LIV was performed using gander erythrocytes, as described by Clarke and Casals (1958), and modified to use tissue culture–grown virus and a microtiter plate. This assay format is validated at Moredun Research Institute in routine-diagnostic use for many species, including deer (Ytrehus et al., 2013). Nonspecific inhibitors and goose erythrocyte agglutinins were removed by kaolin and goose erythrocyte absorption. Positive and negative controls (ovine sera) were included in each test batch to confirm assay performance. Samples giving a result of HI at a titre of greater than 20 were considered positive. Samples with a titre of 10 were considered inconclusive, and titres of <10 were considered negative.

For confirmation of the LIV HI results and for comparison to TBEV titres, all positive and borderline samples from the TBE ELISA screening test were re-tested by LIV SNT using the constant virus varying serum method (Grist, 1966). The test was modified to be performed in 96-well plates using BHK-21 cells with the LIV strain L31 using 30–300 median tissue culture infective dose (TCID50) per well. Virus controls, known positive and negative serum controls, toxicity controls, and uninfected control wells were run in each test. Serum samples with a titre higher or equal to 4 were interpreted as IgG positives against LIV by SNT.

The combination of the four serological tests was used to determine if a sample contained antibodies homologue to the TBEV-complex antigens. Specifically, the TBE ELISA was performed to screen the serum samples followed by validation by TBEV SNT. Due to the history of LIV in Norway the samples were also analysed by LIV HI and LIV SNT to assess possible cross-reactions between viruses within the complex. The titres of TBEV SNT and LIV SNT were compared and evaluated.

2.3 | Statistics

Statistical analysis was carried out using Stata/SE 14 for Windows (Stata Corp.). We used the Spearman correlation (ρ) to assess the relationships between SNTs. The squared value ρ^2 can be interpreted in terms of predictive power (explained variability) of one SNT ranks by the other SNT ranks. *p*-value was considered significant if below .05 (Thrusfield, 2007).

3 | RESULTS

A total of 657 cervid specimens from Norway were analysed for the presence of IgG antibodies against TBEV. The collection sites for

serum from wild reindeer, red deer, roe deer and moose are shown in Figures 1 and 2.

In total, 38 samples were positive by TBEV ELISA. The overall seroprevalence of antibodies against the TBEV complex in the cervid specimens from Norway confirmed by TBEV SNT was 4.6% (30/657 TBEV seropositive cervids). The highest number of TBEV seropositive cervids was detected in the county of Vestfold (Larvik and Lardal municipalities) in south-eastern Norway. Seropositive cervids were also detected in the counties of Aust-Agder (Birkenes municipality) and Vest-Agder (Søgne municipality) located in southern Norway, Østfold (Halden municipality) in south-eastern Norway, Rogaland (Vindafjord municipality) in western Norway and Trøndelag (Steinkjer municipality) in central Norway (Figure 2 and Tables S1 and S2). No antibodies against TBEV were confirmed in any of the wild reindeer samples (0/83). Antibodies against TBEV detected by SNT were present in 9.4% (27/286) of the analysed moose sera, 1.4% (2/140) in red deer and 0.7% (1/148) in roe deer. The majority (27/30) of the positive serum samples originated from moose (Table 1).

All TBE IgG positive and borderline samples from the ELISA were also examined for the presence of antibodies to LIV by HI and SNT. Seroreactivity to LIV was detected in 30 of the 38 ELISA positive TBEV samples by LIV HI-test, and in 32 of the same 38 samples by LIV SNT.

A strong correlation was found between TBEV and LIV SNTs using the Spearman correlation (ρ = .75) (p-value > .001). Detailed information on individual results are summarized in Tables S1 and S2.

4 | DISCUSSION

The present study represents the first comprehensive screening of cervid species in Norway for viruses in the TBEV complex. We identified TBEV complex neutralizing antibodies in moose and in small numbers in roe deer and red deer. The majority of the positive serum samples from cervids included in this study originated from south-eastern Norway. This is in the area where human TBE cases have been reported in Norway according to the Norwegian Surveillance System for Communicable Diseases (MSIS). TBE positive samples were furthermore detected in the counties of Østfold, Rogaland and Trøndelag, which is located outside the area of reported human cases. This supports previous findings of TBE antibodies in blood donors, and in TBEV in ticks and unpasteurized cow milk (Larsen et al., 2014; Paulsen et al., 2015, 2019; Soleng et al., 2018).

The presence of TBE antibodies in moose has only been studied in Sweden in the early 1960s (Svedmyr, Zeipel, Borg, & Hansen, 1965) and more recently in Norway (Ytrehus et al., 2013) and Finland (Tonteri, Jokelainen, Matala, Pusenius, & Vapalahti, 2016). Given that the distribution of moose is mostly restricted to north-eastern Europe (Scandinavia, Finland Latvia, Estonia and Poland) with some additional animals in the Czech Republic, Ukraine and Belarus, it is not surprising that the number of studies in this species is limited (Imhoff et al., 2015). It is often difficult to compare studies using different methodologies and sampling techniques. The previous Swedish and Norwegian studies seem to be based on animals taken almost exclusively from endemic areas, which might help explain the high prevalences found in those studies (Svedmyr et al., 1965; Ytrehus et al., 2013). We therefore believe the best source for comparison comes from the Finnish study. Tonteri and colleagues tested animals from both endemic and non-endemic areas, and found a low prevalence of 0.74%, whereas our results reveal a prevalence of 9.4%.

The positive moose sample from the municipality of Steinkjer in central Norway represents the northernmost detection of a large TBEV seropositive animal in Norway. No human cases have been reported in this area. Moreover, Steinkjer is located too far away from TBEV endemic areas to attribute migration of mammals from endemic areas (Norwegian Surveillance System for Communicable Diseases (MSIS), 2019). This, in accordance with previous findings in ticks and cow's milk in non-endemic areas (Paulsen et al., 2015, 2019; Soleng et al., 2018), seems to indicate that TBEV is spreading northwards, which may be of relevance for public health considerations.. One must also take into consideration the role of migrating birds in the distribution TBEV in Norway (Hasle, 2013; Hasle et al., 2009; Waldenstrom et al., 2007). Moose preference for foraging in wet/lake areas may also contribute to the higher prevalence observed, as several studies (including in Scandinavia) have clearly identified waterbodies and well-connected forests of oak, birch or pine, as relevant factors for tick abundance (Zeimes, Olsson, Hjertqvist, & Vanwambeke, 2014). Since moose is more sparsely distributed along the western Norwegian coast than in inland areas, it would be interesting to obtain samples in the western parts of the country in the future.

We found TBEV complex neutralizing antibodies in two red deer and one roe deer. One red deer and one roe deer that were positive originated from endemic areas with well-documented human TBE cases (Norwegian Surveillance System for Communicable Diseases (MSIS), 2019). This study identified one seropositive red deer along the western coast of Norway, an area where TBEV has been documented in ticks (Paulsen et al., 2015). There are, however, few studies of TBEV complex in red deer, making it difficult to conclude if these results result from an “off-target” sampling or if red deer are in fact not as susceptible as other cervids to TBEV.

The TBE seropositive red der from the western coast of Norway, had a high LIV SNT titre (20 for TBEV and 128 for LIV). Interestingly, this red deer was hunted in Vindafjord, which is located in western Norway, close to the area with reported LIV infections in sheep in the 1980s and early 90s (Norwegian Veterinary Institute., 2019; Ulvund, Vik, & Krogsrud, 1983). This could indicate that LIV might circulate in western Norway. Ytrehus et al. (2013) found antibodies against TBEV and LIV in Farsund in southern Norway, supporting the conclusion of a possible co-circulation, which has also been demonstrated in Bornholm in Denmark (Jensen, Skarphedinsson, & Semenov, 2004; Ytrehus et al., 2013). There have been no reports of clinical LIV cases among sheep in Norway since it was last diagnosed in 1991 (Gao et al., 1993; Norwegian Veterinary Institute., 2019;

Ulvund et al., 1983). In our opinion, it would seem implausible that a virus known to cause neurological disease in sheep could be circulating in one of the highest sheep density areas in Norway without any clinical reports for more than twenty years. In addition, 7,615 *I. ricinus* ticks have been analysed for LIV in Norway, and all were found to be negative (Paulsen et al., 2017). It is recommended to confirm the ELISA results by SNT, since TBEV and LIV are genetically closely related and antibodies to either virus may cross-react in the test, as seem to be the case in our study (Calisher et al., 1989; Klaus, Ziegler, Kalthoff, Hoffmann, & Beer, 2014).

Roe deer is one of the most surveyed species of cervids for TBEV in Europe. In many countries across Europe, roe deer is a key host for ticks, and due to the high animal densities and broad geographic spread, a good indicator for the occurrence of human TBEV infections. A recent study on roe deer in Denmark revealed an overall seropositivity against TBEV complex viruses of approximately 7% (Andersen et al., 2019). This study found positive animals in known endemic areas but also helped to map new risk areas for TBE. Other recent studies in roe deer have revealed varying prevalences: in Germany, 10% (Balling et al., 2014), in the Netherlands, 2% (Jahfari et al., 2017), in Austria, 2.4% (Duscher, Wetscher, Baumgartner, & Walder, 2015) and in Belgium 5.1% (Tavernier et al., 2015). In our study, one sample (0.7%) was TBE positive. Observations from other countries reveal relatively higher prevalences in roe deer. However, in our study, only 32 of 148 samples were collected in areas with reported human TBE cases. Further studies with a greater sampling size in endemic areas should be conducted to clarify to what extent roe deer can function as a sentinel species in Norway.

All wild reindeer tested in our survey were found to be negative to both TBEV and LIV by neutralizing assays. The likely absence of TBEV in these animals may be of special relevance in understanding the epidemiology of tick-borne diseases in a climate change perspective. Wild reindeer in southern and central Norway tend to range at higher altitudes, away from the coastline. Several studies have shown the negative effect of increasing altitude on all tick stages due to the effect of temperature, which limits questing periods and development rates in ticks (Jouda, Perret, & Gern, 2004a, 2004b; Perret, Guigoz, Rais, & Gern, 2000; Randolph, 2004). A shift in the altitudinal distribution of *I. ricinus* has been documented in Scotland, suggesting that the abundance of ticks at higher altitudes will increase as a response to climate change (Gilbert, 2010). In this perspective, wild reindeer can represent a unique sentinel species to understand the changes in tick distribution and abundance at high altitudes.

5 | CONCLUSION

The present study represents the first comprehensive screening of cervid species in Norway for TBE antibodies and provides updated information on the distribution of TBEV and indicates that TBEV is spreading northwards in Norway. In many ways similar to other screenings across Europe, our results indicate that cervids are useful as sentinel animals for distribution of TBEV, in addition to studies in ticks.

This study supports previous findings of TBEV in ticks, which indicates that TBEV is distributed in Norway more widely than suggested by human TBE cases. There is a growing interest in the use of wild animals as sentinel species for understanding the epidemiology of emerging diseases and detecting them as early as possible. This approach, in line with the ONE HEALTH concept, has clear benefits in terms of both public and animal health, and warrants further studies on wildlife sentinels and reservoirs. Moose because of their wide distribution in Norway, habitat and foraging preferences, may constitute an important "candidate" for sentinel species. Wild reindeer ranging at high altitudes in southern Norway may have an important function as an early-warning system for spread of ticks in altitude as a result, among other factors, of climatic changes. Finally, the possibility of other flaviviruses closely related to TBEV circulating in Norway should also be further investigated. The information from this study is highly relevant for public health considerations.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ETHICAL APPROVAL

The work presented in this manuscript required no specific ethical approvals.

ORCID

Katrine M. Paulsen https://orcid.org/0000-0001-5680-7426
Carlos G. das Neves https://orcid.org/0000-0003-0348-4808
Erik G. Granquist https://orcid.org/0000-0002-2740-8562
Knut Madslien https://orcid.org/0000-0002-2886-4949
Benedikte N. Pedersen https://orcid.org/0000-0002-6154-9668
Rose Vikse https://orcid.org/0000-0002-3706-1603
Åshild K. Andreassen https://orcid.org/0000-0002-1614-6747

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

TableS1-S2

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Table S1 Samples collected by species and municipalities (for wild-reindeer by management areas). Municipalities in bold represent those were positive TBE animals were identified.

Moose		**Red deer**		**Roe deer**		**Wild Reindeer**	
Municipality	*Number of animals*	*Municipality*	*Number of animals*	*Municipality*	*Number of animals*	*Reindeer district*	*Number of animals*
Birkenes	7	**Birkenes**	1	Hitra	14	Setesdal Austhei	26
Eidskog	27	Etne	15	Hjelmeland	2	Setesdal Ryfylke	27
Eidsvoll	2	Flora	21	Horten	12	Snøhetta	30
Eidsvoll	1	Luster	8	Kristiansand	18		
Halden	43	Lærdal	1	Levanger	21		
Karasjok komm	1	Masfjorden	14	Mandal	1		
Lardal	29	Namsos	16	Ringsaker	21		
Larvik	23	Rauma	15	Songdalen	1		
Midtre Gauldal	28	Skjåk	7	**Søgne**	1		
Nannestad	1	Suldal	14	Trøgstad	6		
Nannestad	1	**Vindafjord**	28	Vega	30		
Steinkjer	48			Vestby	21		
Trysil	20						
Ullensaker	3						
Vefsn	27						
Verdal	25						
Total	***286***		***140***		***148***		***83***
						Grand total	**657**

Table S2 Results for tick-borne encephalitis virus by enzyme linked immunosorbent assay and serum neutralisation test, and for louping ill virus by haemagglutination inhibition test and serum neutralisation test. All cervid samples included in this study where obtained during the hunting period in Norway in 2013.

Species	Municipality	TBEV ELISA IgG	TBEV IgG titre by serum neutralisation test*	LIV IgG titre by haemagglutination inhibition test**	LIV IgG titre by serum neutralisation test ***
roe deer	Søgne	Borderline	15	<10	8
red deer	Vindafjord	Positive	20	80	128
red deer	Birkenes	Positive	640	320	256
moose	Birkenes	Positive	120	40	64
moose	Halden	Borderline	<10	<10	<4
moose	Halden	Positive	20	10	8
moose	Lardal	Positive	60	20	16
moose	Lardal	Borderline	30	10	<4
moose	Lardal	Positive	2560	≥ 320	8
moose	Lardal	Positive	60	<10	16
moose	Lardal	Positive	80	40	64
moose	Lardal	Positive	160	40	45
moose	Lardal	Positive	60	20	22
moose	Lardal	Positive	160	10	16
moose	Lardal	Positive	640	80	90
moose	Lardal	Positive	640	40	64
moose	Larvik	Positive	160	80	180
moose	Larvik	Positive	1280	320	256
moose	Larvik	Positive	640	80	90
moose	Larvik	Positive	120	10	8
moose	Larvik	Positive	160	40	16
moose	Larvik	Positive	40	<10	11
moose	Larvik	Positive	320	80	32
moose	Larvik	Positive	unknown	80	90
moose	Larvik	Positive	80	<10	11
moose	Larvik	Positive	320	80	64
moose	Larvik	Positive	480	320	180
moose	Larvik	Positive	80	40	128
moose	Larvik	Positive	60	20	22
moose	Larvik	Positive	40	10	16
moose	Larvik	Positive	480	80	64
moose	Steinkjer	Positive	160	40	45
moose	Steinkjer	Borderline	<10	<10	11
moose	Vefsn	Positive	<10	<10	unknown
moose	Vefsn	Positive	<10	<10	4
reindeer	Setesdal Ryfylke	Borderline	< 10	10	unknown
reindeer	Setesdal Austhei	Positive	<10	<10	<4
reindeer	Setesdal Austhei	Borderline	<10	<10	<4

* Titre equal to 10 or higher was evaluated as positive by TBEV serum neutralisation test
** Titres greater than 20 were considered positive by LIV haemagglutination inhibition test, and samples with titre 10 were considered inconclusive.
***Titres equal to 4 or higher were considered as positive by LIV serum neutralisation test

PAPER 2

Title

Tick-borne encephalitis virus in cows and unpasteurised cow milk from Norway

Short running title

Tick-borne encephalitis virus in milk and cows

Authors

Katrine M. Paulsen[1,2], Snorre Stuen[3], Carlos G. das Neves[4], Faisal Suhel[4], Deepa Gurung[1], Arnulf Soleng[5], Karin Stiasny[6], Rose Vikse[1], Åshild K. Andreassen*[1] and Erik G. Granquist*[2]

*Both authors contributed equally to this work

Corresponding author: Katrine M. Paulsen. Katrine.Mork.Paulsen@nmbu.no

1: Norwegian Institute of Public Health, Division for Infection Control and Environmental Health, Department of Virology, PO-Box 222 Skøyen, NO-0213 Oslo, Norway

2: Norwegian University of Life Sciences, Faculty of Veterinary Medicine, Department of Production Animal Clinical Sciences, Ullevålsveien 72, NO-0454 Oslo, Norway.

3: Norwegian University of Life Sciences, Department of Production Animal Clinical Sciences, Section of Small Ruminant Research and Herd Health, Kyrkjeveien 332/334, NO-4325 Sandnes, Norway

4: Norwegian Veterinary Institute, PO Box 750 Sentrum, N0106 Oslo, Norway

5: Norwegian Institute of Public Health, Division for Infection Control and Environmental Health, Department of Pest Control, PO-Box 222 Skøyen, NO-0213 Oslo, Norway

6: Medical University of Vienna, Center for Virology, Kinderspitalgasse 15, 1090 Vienna, Austria

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Summary

Tick-borne encephalitis virus (TBEV) is recognised as the most important zoonotic tick-transmitted virus in Europe. TBEV is mainly transmitted to humans through bites from TBEV-infected ticks (*Ixodes ricinus* and *Ixodes persulcatus*). However, alimentary infection after consumption of unpasteurised milk and cheese from domestic ruminants have been reported. There is little information about TBEV in ruminants in Norway. The objectives of this study were to analyse unpasteurised cow milk for TBEV RNA, and to study the presence of IgG antibodies to TBEV in the same animals. A total of 112 milk and blood samples were collected from cows from five different farms spread from southern to northern Norway. The milk samples were analysed by an in-house reverse transcription (RT) real-time polymerase chain reaction (PCR) and confirmed by pyrosequencing. Serum samples were screened by a commercial enzyme linked immunosorbent assay (ELISA) and verified by a TBEV-specific serum neutralisation test (SNT). We found TBEV RNA in unpasteurised milk collected from farms in the municipalities of Mandal, Skedsmo and Brønnøy in 5.4% of the tested animals. Specific antibodies to TBEV were only detected in Arendal, where 88.2% of the tested animals were positive. Further studies on milk containing TBEV RNA should be performed to conclude if TBEV found in unpasteurised milk in Norway is infectious, which could be of great importance in a One Health perspective.

Keywords

Tick-borne encephalitis virus (TBEV), unpasteurised milk, domestic ruminants, real-time PCR, serum neutralisation test.

Impacts

- This is the first report of tick-borne encephalitis virus in unpasteurised milk in Norway.
- The study provides updated information on TBEV distribution in Norway.
- Norwegian authorities are currently considering to allow the sale of unpasteurised dairy products. Our study indicates that a risk assessment may be suitable to evaluate the consequences of consuming unpasteurised dairy products from domestic ruminants grazing in areas of Norway where TBEV is detected.

60 ## Introduction

61 Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis (TBE)
62 in humans, and it is the most important zoonotic tick-transmitted virus in Europe from a medical
63 perspective (Suss, 2011). TBEV is a positive-sense, single-stranded RNA virus in the
64 *Flaviviridae* family, within the *Flavivirus* genus. TBEV is occasionally transmitted to humans
65 through bites from TBEV-infected *Ixodes ricinus* or *Ixodes persulcatus* ticks, which are
66 recognised as both vectors and reservoirs (Lindquist & Vapalahti, 2008). TBEV infection in
67 humans often result in unspecific transient febrile symptoms, but the clinical outcome ranges
68 from asymptomatic to severe infection in the central nervous system (Kaiser, 2012). The virus
69 is traditionally divided into three main subtypes, the European, the Siberian and the Far Eastern
70 (Ecker *et al.*, 1999). At least two additional subtypes are known at present, the Baikalian and
71 the Himalayan subtypes (Kovalev & Mukhacheva, 2017, Dai *et al.*, 2018). In Norway, only the
72 European subtype is found (Andreassen *et al.*, 2012, Paulsen *et al.*, 2015, Soleng *et al.*, 2018).
73 The closely related Louping ill virus (LIV) has been detected in Norway. However, no
74 outbreaks have been reported since the last outbreak in 1991, reported by the Norwegian
75 Veterinary Institute (Gao *et al.*, 1993, Ulvund *et al.*, 1983). The disease caused by the different
76 subtypes of TBEV varies in severity and mortality; the European and Siberian subtypes
77 generally have fatality rates of approximately 1 to 3%, while the Far Eastern subtype fatality
78 rate might be as high as 20 to 40% (Dorrbecker *et al.*, 2010, Gritsun *et al.*, 2003a).

79 Consumption of raw milk and other dairy product seems to be an increasing trend both in
80 Norway as well as in other European countries due to the alleged health benefits and better taste
81 of natural products. Outbreaks of *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*,
82 *Campylobacter* spp. and TBEV have been reported after consumption of unpasteurised milk in
83 Europe (Bogovic & Strle, 2015, Costard *et al.*, 2017, Willis *et al.*, 2018). Alimentary TBEV
84 infection following consumption of unpasteurised milk and cheese from domestic ruminants
85 have been reported in approximately 1% of all TBE-cases (Kriz *et al.*, 2009). However, this
86 number may differ significantly. The highest occurrence of alimentary TBE is known from
87 Slovakia, where up to 17% of TBE-cases are caused by consumption of infected milk (Kerlik
88 *et al.*, 2018). For other countries, the available information is limited. The largest reported
89 outbreak of alimentary TBE occurred in Slovakia in 1951-52, where at least 660 people became
90 infected (Kerlik *et al.*, 2018).

91 In most of the alimentary TBE-cases, the virus is transmitted through unpasteurised milk and
92 cheese from goat, but TBEV infection through consumption of unpasteurised milk from cow

93 and sheep has also been reported (Balogh *et al.*, 2010, Caini *et al.*, 2012, Gresikova *et al.,* 1975,
94 Holzmann *et al.*, 2009, Hudopisk *et al.*, 2013, Markovinovic *et al.*, 2016). Infected domestic
95 ruminants do not display clinical symptoms, but they may develop a viremia with a duration of
96 approximately one week (Baloh *et al*., 2012, Van Tongeren, 1955). In milk samples from
97 infected ruminants, TBEV has been detected for up to 19 days post infection (Baloh *et al*.,
98 2012). Detectable antibodies in ruminants have been found for at least 28 months after infection
99 (Klaus *et al*., 2014). The clinical manifestation of alimentary TBE in humans may differ from
100 TBE after tick-bites. The alimentary TBE is biphasic, similar to infection caused by the
101 European subtype of TBEV, with some observed differences. Alimentary-transmitted TBE has
102 a shorter incubation period compared to TBEV infection through tick-bite (3 to 4 days and 7 to
103 14 days, respectively). While the biphasic form is dominant for alimentary TBE, the biphasic
104 form represents about 20 to 30% of all TBEV infections after tick-bite. Non-severe
105 meningoencephalitis is observed for alimentary TBE, while clinical manifestations of tick-
106 associated TBE may be more severe with "aseptic" meningitis, meningoencephalitis, and
107 meningomyeloencephalitis (Gritsun *et al.*, 2003b, Ruzek *et al.*, 2010).

108 There is limited knowledge of the presence of TBEV in domestic ruminants in Norway. A study
109 from 1973 detected a seroprevalence of 17.7% in bovine sera in western Norway (Traavik,
110 1973). According to the Norwegian Surveillance System of Communicable Diseases (MSIS),
111 the incidence of human TBE is low in Norway, with a total of 143 reported cases since 1997,
112 of which 16 were reported in 2017. The human cases are limited to southern Norway
113 (Norwegian Institute of Public Health, MSIS, 2018). However, *I. ricinus* ticks carrying TBEV
114 have been detected in coastal areas from the county of Østfold in the southeast up to Brønnøy
115 located in the county of Nordland in northern Norway. This indicates that the virus is more
116 widespread in Norway than the reported human cases may suggest (Andreassen *et al*., 2012,
117 Larsen *et al.*, 2014, Paulsen *et al.,* 2015, Soleng *et al*., 2018). Outbreaks of alimentary TBEV
118 infections can be prevented by pasteurising milk before consumption or by vaccination against
119 TBEV (Hudopisk *et al*., 2013, Offerdahl *et al.*, 2016). For instance, immunisation of goats has
120 been demonstrated as an effective method of preventing TBEV infection from unpasteurised
121 milk (Balogh *et al*., 2012).

122 The objectives of this study were to analyse unpasteurised milk samples from cows for TBEV
123 RNA, and to study the presence of antibodies to TBEV in the same animals.

124 # Materials and methods

125 **Collection of milk and blood samples**

126 A total of 112 milk samples were collected from grazing dairy cows between June 2014 and
127 September 2017 on farms located in the municipalities of Skedsmo, Arendal, Mandal, Finnøy
128 and Brønnøy in Norway. The Skedsmo samples were collected in June, the Brønnøy and Finnøy
129 samples were collected in September, and the Mandal and Arendal samples were collected in
130 October (Figure 1, Table 1). All cows included in the study have been grazing close to their
131 respective farms during daytime for more than one year. Blood samples were taken from the
132 same animals and separated to serum. In addition to the milk samples from individual cows, a
133 total of five samples from bulk milk tanks at the farms, were tested from Skedsmo, Mandal
134 (two samples), Finnøy and Brønnøy. The samples were stored in sterile tubes at -80°C until
135 further processing. All farms, except the farm in Skedsmo, are situated in areas where ticks are
136 known to be abundant. Of the five municipalities in this study, Mandal and Arendal are the only
137 two where human cases of TBE have been reported in Norway (Norwegian Institute of Public
138 Health, MSIS, 2018).

139 **Detection of tick-borne encephalitis virus in raw milk from cows**

140 Fat from all milk samples was removed by centrifuging at 6,000 x g for 10 minutes according
141 to Cisak *et al.* (2010). Viral RNA from skimmed milk was then extracted using QIAamp® Viral
142 RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's
143 instructions. The elution volume was 60 µL. Directly after extraction, the RNA was reversely
144 transcribed to cDNA with random primers (High-Capacity cDNA Reverse Transcription Kit,
145 Applied Biosystems, Foster city, CA, USA), followed by detection of TBEV RNA with in-
146 house RT real-time PCR and pyrosequencing assays according to Andreassen *et al*. (2012). The
147 real-time PCR amplifies a 54 base pair fragment located on the envelope gene of TBEV,
148 specific to the Norwegian TBEV-strain (Andreassen *et al*. 2012, Skarpaas *et al.,* 2006). All real-
149 time PCR positive samples were further analysed by pyrosequencing according to the
150 manufacturer's manual for sequence analysis (SQA) on the BioTage system (Pyromark ID,
151 QIAGEN, GmbH, Hilden, Germany). A positive control ("Soukup") was used in the real-time
152 PCR, and further in the pyrosequencing analysis, to compare and confirm the sequences
153 revealed from the positive samples (Andreassen *et al*. 2012).

154 **Detection of IgG antibodies against tick-borne encephalitis virus in cow serum**

155 Serum samples from cows were screened for IgG antibodies against TBEV by a commercial
156 enzyme linked immunosorbent assay (ELISA, Enzygnost® Anti-TBE virus IgG, Siemens

157 Healthcare, GmbH, Marburg, Germany) according to the manufacturer's protocol, with one
158 modification: the conjugate was changed to peroxidase-labelled antibody to bovine IgG diluted
159 1: 30 000 (KLP, Gaithersburg, USA). The conjugate was diluted in IgG Conjugate Buffer
160 Microbiol (Enzygnost® Anti-Rubella Virus IgG, Siemens Healthcare, GmbH, Marburg,
161 Germany). Serum from calves vaccinated against TBE with the TicoVac-vaccine (Pfizer Ltd,
162 Ramsgate Road, Sandwich, Kent, CT13 9NJ, UK), were used as positive controls and serum
163 from calves, which never had been exposed to ticks as negative controls.

164 Positive samples from the ELISA were re-tested in a TBEV-specific serum neutralisation test
165 (SNT) at the Center for Virology of the Medical University of Vienna, as described by Stiasny
166 *et al.* (2009). Briefly, serial dilutions of heat-inactivated samples were incubated with TBEV
167 (strain Neudoerfl) for 1h at 37°C. Baby hamster kidney (BHK-21) cells were added and
168 incubation was continued for three days. The presence of virus in the cell culture supernatant
169 was assessed by ELISA. The virus neutralisation titre was defined as the reciprocal of the
170 sample dilution that showed a 90% reduction in the absorbance readout compared to the control
171 without antibody. Samples with titres equal to 10 and higher were defined as TBE seropositive.

172 Results

173 Tick-borne encephalitis virus in raw milk from cows

174 TBEV RNA was detected by RT real-time PCR and pyrosequencing in six of the 112 (5.4%)
175 analysed raw milk samples from cows. The positive samples originated from Mandal, Skedsmo
176 and Brønnøy, in 28.6%, 13.6%, and 2.1% of the animals, respectively. In Mandal, two of the
177 three real-time PCR positive samples were confirmed by pyrosequencing. Five positive samples
178 from Skedsmo were detected by real-time PCR, three of them were confirmed by
179 pyrosequencing. At Brønnøy, one sample was positive by both real-time PCR and
180 pyrosequencing (Table 1).

181 Both bulk tank milk samples from Mandal were positive by real-time PCR and confirmed by
182 pyrosequencing, while the remaining tank samples were negative (data not shown).

183 Antibodies against tick-borne encephalitis virus in cow serum

184 IgG antibodies against TBEV were detected by ELISA in 16 of the 112 tested animals.
185 However, positive samples by SNT were only detected in Arendal, where 15 out of 17 (88.2%)
186 samples were positive. The titres in the SNT ranged from 15 to 1280. ELISA-positive samples
187 from Skedsmo and Finnøy were negative by SNT (Table 2, Supplementary material Table 1).

188 None of the cows with TBEV-positive milk by RT real-time PCR and pyrosequencing had
189 detectable TBE-antibodies in serum by SNT (Table 1 and 2).

190 Discussion

191 This is the first study to report tick-borne encephalitis virus (TBEV) in unpasteurised cow milk
192 in Norway. TBEV RNA was detected in raw milk collected from areas both with and without
193 reported human TBE-cases.

194 A study from TBE-endemic areas in Poland found, by RT-PCR, an overall TBEV-prevalence
195 of 11.1% in unpasteurised milk from cows (Cisak *et al.*, 2010). The overall prevalence was
196 lower in the current study (5.4%). Regions with no previously reported TBE-cases were
197 included in our study, which is in contrast to the Polish study, where only high-endemic areas
198 were included (Cisak *et al*., 2010). In Norway, TBEV has a wider geographic distribution than
199 the human cases reported by MSIS, as recently documented by studies on ticks (*Ixodes ricinus*)
200 and healthy blood donors (Larsen *et al*., 2014, Paulsen *et al.*, 2015, Soleng et *al.*, 2018). In
201 Brønnøy in Nordland county, Soleng *et al*. (2018) detected a TBEV prevalence of up to 3% and
202 9% in *I. ricinus* nymphs and adults, respectively. Our data from one positive milk sample in
203 Brønnøy supports the suspicion that TBEV is circulating close to the northern border of *I.*
204 *ricinus* ticks' geographical distribution in Norway (Soleng *et al*., 2018).

205 The TBEV RNA positive milk samples from Skedsmo were somewhat unexpected, because
206 ticks only sporadically are found on livestock in this area (personal communication by the
207 farmer). However, *I. ricinus* ticks may appear outside their normal range via transportation by
208 mammals and migratory birds, which also may play an important role in transmission and
209 distribution of TBEV (Labuda & Nuttall, 2004). Large mammals may facilitate short to
210 medium-range transportation of ticks, while birds may transport ticks over long distances and
211 across geographical barriers (Hasle *et al*., 2009).

212 Seroprevalence studies on TBE in domestic animals in Europe have demonstrated that animals
213 may serve as useful sentinels for detection of TBEV risk areas (Klaus *et al.*, 2012, Klaus *et al.*,
214 2010, Rieille *et al.*, 2017, Salat *et al.*, 2017). In the present study, a total of 112 serum samples
215 from five farms were analysed. TBEV-specific antibodies were detected in cattle in Arendal
216 only. The fact that TBE-antibodies were detected in cows from Arendal is in accordance with
217 the reported human TBE-cases, as this region has the highest number of reported TBE-cases
218 annually (Norwegian Institute of Public Health, MSIS, 2018).

A previous study has shown that goats and sheep have a measurable antibody response for at least 28 months after primary infection (Balogh *et al.*, 2012). The antibody response seems to vary between species, and it is unclear if all animals exposed to the virus develop an immune response (Klaus *et al*., 2012, Klaus *et al*., 2010). This might explain why the samples taken from Skedsmo, Mandal and Brønnøy had detectable TBEV RNA in the milk, but all animals tested negative for neutralizing antibodies to TBEV in the serum. Balogh *et al.* (2012) showed that infected goats had measurable virus in the milk for up to 19 days post infection, but immunised goats did not shed TBEV through the milk. The positive serum samples from Arendal were sampled in October. If these cows were infected, and thereby immunised in the spring or summer, there will be no virus left in the milk samples taken in the fall, but the IgG will remain detectable for several months. This might be the reason why we did not detect TBEV RNA in the cow milk from this area, where 15 out of 17 animals had TBEV-specific antibodies. The sampling from Skedsmo occurred in June, and three milk samples were found to be positive for TBEV RNA. It is not known if these animals developed neutralising antibodies to TBEV post infection. While the age of the cows and introduction of animals to new areas may affect the results, all animals in the present study were adults, and had been grazing in the same area for more than one season.

Sera testing positive for TBEV-specific IgG antibodies by ELISA may cross-react to the closely related Louping ill virus (LIV). For this reason, a neutralisation test is recommended to confirm the ELISA results (Rieille *et al*., 2017). However, TBEV and LIV are so closely related that antibodies to either virus may also react in a neutralisation test. On the other hand, LIV has not been detected in Arendal previously, and Louping ill cases have not been reported in Norway since the last report from the Norwegian Veterinary Institute in 1991 (Gao *et al.*, 1993, Ulvund *et al.*, 1983, Ytrehus *et al.*, 2013). Furthermore, 7,615 ticks collected from all over Norway have been analysed for LIV, all were found to be LIV negative (Paulsen *et al.*, 2017).

A study by Cisak *et al*. (2010) found no correlation between milk samples tested by ELISA for presence of specific antibodies against TBEV with those obtained by RT-PCR, and they suggested that the ELISA is less appropriate for detecting the presence of TBEV-specific antibodies in milk. For this reason, ELISA on our collected milk samples was not performed.

Detection of TBEV RNA in unpasteurised milk alone cannot prove that TBEV is endemic in an area, but detection of TBEV RNA in ticks and results from serological assays may indicate that the virus is circulating in an area. The presence of TBEV may therefore pose a direct risk for the human population living in, or visiting these areas, even without any reported TBE-

252 cases. In this study, we found no relationship between TBEV RNA in milk and TBE IgG in
253 serum. However, TBEV RNA in ticks has been detected in all areas, except for Skedsmo,
254 where, to our knowledge, no ticks have been examined for TBEV.

255 Further studies on TBEV in milk should be performed to conclude if TBEV found in
256 unpasteurised milk in Norway is infectious. These results, as others reported in Europe, may
257 point to the importance of considering a precautionary principle when consuming unpasteurised
258 milk products in areas where TBEV is distributed (Balogh *et al*., 2012 Offerdahl *et al*., 2013).
259 Previous studies have demonstrated that pasteurisation of milk is an effective method to prevent
260 TBEV infections from dairy products (Hudopisk *et al*., 2013, Offerdahl *et al*., 2016). Further
261 seroprevalence studies in domestic animal populations with broader geographical coverage and
262 greater sample size should be carried out, accompanied by risk assessments to evaluate both the
263 prevalence of TBEV RNA in milk and the consequences of consuming unpasteurised milk from
264 ruminants in Norway. Furthermore, a seroprevalence study on people working in close contact
265 with these animals could provide important epidemiological data for risk evaluation, as they
266 could have been exposed to ticks or may be infected via unpasteurised milk.

267 Conflict of Interest Statement

268 The authors have no conflict of interest to declare.

269 Ethical considerations

270 All blood samples and milk samples were considered diagnostic and were collected by trained
271 veterinarians. Vaccination of calves, to serve as positive controls, was authorised by the
272 Norwegian Food Safety Authority (FOTS ID 8135).

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426

427 **Tables**

428 **Table 1** Prevalence of tick-borne encephalitis virus in 112 unpasteurised milk samples
429 collected from cows at five farms in Norway

Location (municipality, county, date)	No. of milk samples	Positive by real-time PCR	Confirmed by pyrosequencing (prevalence)
Skedsmo, Akershus, 02.06.2014	22	5	3 (13.6%)
Arendal, Aust-Agder, 05.10.2015	17	0	0 (0%)
Mandal, Vest-Agder, 01.10.2014	7	3	2 (28.6%)
Finnøy, Rogaland, 11.09.2017	19	0	0 (0%)
Brønnøy, Nordland, 02.09.2015	47	1	1 (2.1%)
Total	112	9	6 (5.4%)

430

431 **Table 2** Seroprevalence of IgG antibodies against tick-borne encephalitis virus in 112 serum
432 samples collected from cows at five farms in Norway

Location (municipality, county, date)	No. of serum samples	Positive by ELISA	Confirmed by SNT (seroprevalence)
Skedsmo, Akershus, 02.06.2014	22	1	0
Arendal, Aust-Agder, 05.10.2015	17	14	15 (88.2%)
Mandal, Vest-Agder, 01.10.2014	7	0	0
Finnøy, Rogaland, 11.09.2017	19	1	0
Brønnøy, Nordland, 02.09.2015	47	0	0
Total	112	21	15 (13.4%)

433

434 **Figures**
435
436 **Figure 1** Geographical locations of the five farms included in the study. A total of 112 cow
437 milk and serum samples were collected from Skedsmo (Akershus county), Arendal (Aust-
438 Agder county), Mandal (Vest-Agder county) and Finnøy (Rogaland county). Map from
439 Kartverket (Creative Commons Attribution ShareAlike 3.0)

Brønnøy
Finnøy
Skedsmo
Arendal
Mandal

Supplementary material:

Table 1 ELISA results and neutralisation test titre values from serum samples collected from cows from five farms in Norway

Sample site	Sample ID	corrected absorbance value*	ELISA result	Neutralisation test titer
Skedsmo	1	0.214	borderline	not tested
Skedsmo	2	0.164	negative	not tested
Skedsmo	3	0.151	negative	not tested
Skedsmo	4	0.189	negative	not tested
Skedsmo	5	0.223	borderline	not tested
Skedsmo	6	0.236	borderline	<10
Skedsmo	7	0.144	negative	not tested
Skedsmo	8	0.151	negative	not tested
Skedsmo	9	0.738	positive	<10
Skedsmo	10	0.193	negative	not tested
Skedsmo	11	0.171	negative	not tested
Skedsmo	12	0.297	borderline	<10
Skedsmo	13	0.110	negative	not tested
Skedsmo	14	0.130	negative	not tested
Skedsmo	15	0.196	negative	not tested
Skedsmo	16	0.166	negative	not tested
Skedsmo	17	0.137	negative	not tested
Skedsmo	18	0.124	negative	not tested
Skedsmo	19	0.173	negative	not tested
Skedsmo	20	0.205	negative	not tested
Skedsmo	21	0.162	negative	not tested
Skedsmo	22	0.162	negative	not tested
Positive control	P/P	0.683	positive	not tested
Negative control	N/N	0.024	negative	not tested
Vaccinated control		2.169	positive	not tested
Unvaccinated control		0.167	negative	not tested
Arendal	1	0.421	positive	40
Arendal	2	2.540	positive	480
Arendal	3	0.841	positive	60
Arendal	4	0.247	borderline	15
Arendal	5	1.740	positive	160
Arendal	6	1.180	positive	120
Arendal	7	1.060	negative	60
Arendal	8	0.170	negative	not tested
Arendal	9	2.360	positive	320
Arendal	10	0.646	positive	80
Arendal	11	0.638	positive	40
Arendal	12	0.592	positive	30
Arendal	13	0.190	negative	not tested

Arendal	14	0.811	positive	15
Arendal	15	3.90	positive	1280
Arendal	16	1.900	positive	120
Arendal	17	0.621	positive	60
Positive Control	P/P	0.728	positive	not tested
Negative Control	N/N	0.011	positive	not tested
Vaccinated control		1.560	positive	not tested
Unvaccinated control		0.134	negative	not tested
Mandal	1	0.090	negative	not tested
Mandal	2	0.076	negative	not tested
Mandal	3	0.072	negative	not tested
Mandal	4	0.094	negative	not tested
Mandal	5	0.181	negative	not tested
Mandal	6	0.124	negative	not tested
Mandal	7	0.097	negative	not tested
Positive control	P/P	0.683	negative	not tested
Negative control	N/N	0.024	negative	not tested
Vaccinated control		1.558	positive	not tested
Unvaccinated control		0.197	negative	not tested
Finnøy	1	0.543	positive	<10
Finnøy	2	0.131	negative	not tested
Finnøy	3	0.156	negative	not tested
Finnøy	4	0.128	negative	not tested
Finnøy	5	0.154	negative	not tested
Finnøy	6	0.123	negative	not tested
Finnøy	7	0.154	negative	not tested
Finnøy	8	0.114	negative	not tested
Finnøy	9	0.114	negative	not tested
Finnøy	10	0.052	negative	not tested
Finnøy	11	0.109	negative	not tested
Finnøy	12	0.138	negative	not tested
Finnøy	13	0.147	negative	not tested
Finnøy	14	0.180	negative	not tested
Finnøy	15	0.202	negative	not tested
Finnøy	16	0.112	negative	not tested
Finnøy	17	0.151	negative	not tested
Finnøy	18	0.121	negative	not tested
Finnøy	19	0.131	negative	not tested
Positive control	P/P	0.544	positive	not tested
Negative control	N/N	0.015	negative	not tested
Vaccinated control		1.227	positive	not tested
Unvaccinated control		0.166	negative	not tested
Brønnøy	1	0.130	negative	not tested
Brønnøy	2	0.090	negative	not tested
Brønnøy	3	0.124	negative	not tested

Brønnøy	4	0.139	negative	not tested
Brønnøy	5	0.010	negative	not tested
Brønnøy	6	0.093	negative	not tested
Brønnøy	7	0.098	negative	not tested
Brønnøy	8	0.086	negative	not tested
Brønnøy	9	0.123	negative	not tested
Brønnøy	10	0.094	negative	not tested
Brønnøy	11	0.100	negative	not tested
Brønnøy	12	0.114	negative	not tested
Brønnøy	13	0.107	negative	not tested
Brønnøy	14	0.111	negative	not tested
Brønnøy	15	0.009	negative	not tested
Brønnøy	16	0.009	negative	not tested
Brønnøy	17	0.155	negative	not tested
Brønnøy	18	0.102	negative	not tested
Brønnøy	19	0.107	negative	not tested
Brønnøy	20	0.102	negative	not tested
Brønnøy	21	0.085	negative	not tested
Brønnøy	22	0.084	negative	not tested
Brønnøy	23	0.125	negative	not tested
Brønnøy	24	0.103	negative	not tested
Brønnøy	25	0.109	negative	not tested
Brønnøy	26	0.118	negative	not tested
Brønnøy	27	0.089	negative	not tested
Brønnøy	28	0.111	negative	not tested
Brønnøy	29	0.107	negative	not tested
Brønnøy	30	0.107	negative	not tested
Brønnøy	31	0.081	negative	not tested
Brønnøy	32	0.092	negative	not tested
Brønnøy	33	0.087	negative	not tested
Brønnøy	34	0.117	negative	not tested
Brønnøy	35	0.108	negative	not tested
Brønnøy	36	0.105	negative	not tested
Brønnøy	37	0.116	negative	not tested
Brønnøy	38	0.086	negative	not tested
Brønnøy	39	0.090	negative	not tested
Brønnøy	40	0.096	negative	not tested
Brønnøy	41	0.086	negative	not tested
Brønnøy	42	0.051	negative	not tested
Brønnøy	43	0.093	negative	not tested
Brønnøy	44	0.116	negative	not tested
Brønnøy	45	0.128	negative	not tested
Brønnøy	46	0.113	negative	not tested
Brønnøy	47	0.117	negative	not tested
Positive control	P/P	0.764	positive	not tested

Negative control	N/N	0.025	negative	not tested
Vaccinated control		1.150	positive	not tested
Unvaccinated control		0.159	negative	not tested

* Skedsmo: Positive > 0.309. Borderline 0.209-0.309. Negative < 0.209
Arendal: Positive > 0.311. Borderline 0.211-0.311. Negative < 0.211
Mandal: Positive > 0.324. Borderline 0.224-0.324. Negative < 0.224
Finnøy: Positive > 0.315. Borderline 0.215-0.315. Negative < 0.215
Brønnøy: Positive > 0.325. Borderline 0.225-0.325. Negative < 0.225

Measurement correction

The absorbance values (read on an ELISA reader at 450 nm) are corrected by multiplying with a correction factor. The correction factor is found by dividing the nominal value (given by the kit) by the mean absorbance of the positive control samples.

Qualitative evaluation

To calculate the cut-off value for the IgG test, use the mean absorbance value of Anti-TBE Virus Reference N/N and add 0.200. Positive samples are samples with a corrected absorbance value greater than the cut-of value plus 0.100. All samples with a value between the cut-off value and the positive value are considered as borderline samples.

PAPER 3

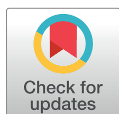
RESEARCH ARTICLE

Experimental infection of lambs with tick-borne encephalitis virus and co-infection with *Anaplasma phagocytophilum*

Katrine M. Paulsen[1,2]*, **Erik G. Granquist**[2], **Wenche Okstad**[3], **Rose Vikse**[1], **Karin Stiasny**[4], **Åshild K. Andreassen**[1]☯, **Snorre Stuen**[3]☯

1 Department of Virology, Division for Infection Control and Environmental Health, Norwegian Institute of Public Health, Oslo, Norway, **2** Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway, **3** Section of Small Ruminant Research and Herd Health, Department of Production Animal Clinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Sandnes, Norway, **4** Center for Virology, Medical University of Vienna, Vienna, Austria

☯ These authors contributed equally to this work.
* katrine.mork.paulsen@nmbu.no, katrine-mork.paulsen@fhi.no



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Abstract

Tick-borne encephalitis virus (TBEV) is a zoonotic pathogen which may cause tick-borne encephalitis (TBE) in humans and animals. More than 10,000 cases of TBE are reported annually in Europe and Asia. However, the knowledge on TBE in animals is limited. Co-infection with *Anaplasma phagocytophilum* and louping ill virus (LIV), a close relative to TBEV, in sheep has been found to cause more severe disease than single LIV or *A. phagocytophilum* infection. The aim of this study was to investigate TBEV infection and co-infection of TBEV and *A. phagocytophilum* in lambs. A total of 30 lambs, aged five to six months, were used. The experiment was divided into two. In part one, pre- and post-infection of TBEV and *A. phagocytophilum* was investigated (group 1 to 4), while in part two, co-infection of TBEV and *A. phagocytophilum* was investigated (group 5 and 6). Blood samples were drawn, and rectal temperature was measured daily. Lambs inoculated with TBEV displayed no clinical symptoms, but had a short or non-detectable viremia by reverse transcription real-time PCR. All lambs inoculated with TBEV developed neutralizing TBEV antibodies. Our study is in accordance with previous studies, and indicates that TBEV rarely causes symptomatic disease in ruminants. All lambs inoculated with *A. phagocytophilum* developed fever and clinical symptoms of tick-borne fever, and *A. phagocytophilum* was present in the blood samples of all infected lambs, shown by qPCR. Significantly higher mean TBEV titer was detected in the group co-infected with TBEV and *A. phagocytophilum*, compared to the groups pre- or post-infected with *A. phagocytophilum*. These results indicate that co-infection with TBEV and *A. phagocytophilum* in sheep stimulates an increased TBEV antibody response.

Introduction

The disease tick-borne encephalitis (TBE) in humans and animals is caused by tick-borne encephalitis virus (TBEV). TBEV is a member of the genus *flavivirus* within the family *flaviviridae*, and it is mainly transmitted to humans and animals through bites by TBEV-infected *Ixodes ricinus* or *Ixodes persulcatus* ticks [1]. In addition, TBEV has been detected in unpasteurized milk from domestic ruminants and there are reported human cases of alimentary TBE from consumption of unpasteurized milk and other dairy products [2–9].

In humans, TBE may vary from asymptomatic to severe infection in the central nervous system, and the number of annually reported human TBE cases is increasing in Europe and Asia [10, 11]. Most animals do not develop symptomatic disease when infected with TBEV. However, the knowledge on TBE in animals is limited. TBE has been described with neurological symptoms in dogs, horses, and, in one case, monkey (*Macaca sylvanus*) [12–16]. TBE in small ruminants is presumably rare, with only a few reported cases [17, 18]. Large and small mammals along with migratory birds are known to be important for the distribution and transmission of the virus [19–26].

Anaplasma phagocytophilum is the causative agent of tick-borne fever in ruminants and is transmitted by the same tick species as TBEV in Europe, namely *I*. *ricinus* [27]. The intracellular bacterium is known to affect domestic ruminants, humans and wild animals [27, 28]. *A*. *phagocytophilum* has a great negative impact on the sheep farming and it has been estimated that more than 300,000 lambs are infected by *A*. *phagocytophilum* annually in Norway [29]. Infection with *A*. *phagocytophilum* results in immune suppression and the most typical symptoms in domestic ruminants include high fever, depression, reduced appetite, and sudden drop in milk yield [30, 31]. Reduced weight gain in infected lambs has also been observed [32, 33].

Because several tick-borne pathogens often circulate in the same area, humans and animals may be infected with multiple pathogens from tick-bites [34]. A recent study in Norway by Kjelland et al. (2018), reported co-infected ticks with *Borrelia afzelii* and *Neoehrlichia mikurensis*. The same study found several tick-borne pathogens, including TBEV and *A*. *phagocytophilum*, in the same locations [35]. Co-infection with *A*. *phagocytophilum* and other pathogens in sheep has been found to cause more severe disease compared to infection with a single pathogen [36, 37]. Previous studies have shown that co-infection with *A*. *phagocytophilum* and louping ill virus (LIV) in sheep may give fatal clinical outcomes [36, 38]. TBEV and LIV are closely related, and it has been speculated whether similar clinical outcomes could occur from co-infection with *A*. *phagocytophilum* and TBEV. A recently published experimental study on the immune responses to TBEV and LIV in sheep, showed that the infected sheep developed neutralizing antibodies for both viruses, which seemed to limit the infection caused by TBEV, but not the infection caused by LIV [39]. Furthermore, prior inoculation with TBEV appeared to reduce the disease severity and viremia caused by LIV, but it did not prevent LIV infection [39]. The objective of this study was to study the effect of TBEV infection and co-infection of TBEV and *A*. *phagocytophilum* in lambs.

Materials and methods

Ethics statement

The study was authorized by the Norwegian Animal Research Authority (Norwegian Food Safety Authority, FOTS ID 8632, FOTS ID 8135). Blood samples were collected by trained veterinarians, and all lambs were observed daily.

Table 1. Overview of the study groups and the experimental design of part one and part two of the experimental study with infection of tick-borne encephalitis virus (TBEV) and *Anaplasma phagocytophilum* in lambs.

	Part one: Pre- and post- infection of TBEV and *A. phagocytophilum*				Part two: Co-infection of TBEV and *A. phagocytophilum*	
Day	Group 1_a	Group 2_a	Group 3_a	Group 4_a	Group 5_a	Group 6_a
0	Inoculated with $TBEV_b$	Inoculated with *A. phagocytophilum*$_c$	Inoculated with *A. phagocytophilum*$_c$	Negative controls. Inoculated with uninfected cell medium$_d$	Inoculated with TBEV and *A. phagocytophilum*$_{b,c}$	Negative controls. Inoculated with physiological saline solution$_d$
21	Inoculated with *A. phagocytophilum*$_c$	Inoculated with uninfected cell medium$_c$	Inoculated with $TBEV_b$	Negative controls. Inoculated with uninfected cell medium$_d$	End of experiment	
42	End of experiment				-	

[a] Each group consisted of five lambs.

[b]TBEV was inoculated subcutaneously (1 ml of the strain Hohosterwitz, approximately $6.5x10^6$ focus forming units per ml (FFU/ml).

[c]*A. phagocytophilum* was inoculated intravenously (0.4 ml of heparinised sheep blood stabilized with 10% demethyl sulphoxide (DMSO), approximately $1x10^6$ infected cells, GenBank accession number M73220).

[d]1 ml negative control medium and saline were inoculated subcutaneously.

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Experimental design and blood sampling

This study was conducted at the Norwegian University of Life Sciences (NMBU) in Sandnes, Norway. The study was divided in two parts. A total of 30 lambs, at the age of five to six months of the breed "Norwegian white sheep", were used. Part one included only rams, and was performed in the autumn of 2017. Part two consisted entirely of ewes, and was carried out in the autumn of 2018 (Table 1).

The main reason for the difference in gender between part one and part two was the limited number of animals available. No differences between genders have been observed previously in experimental infection with *A. phagocytophilum* in sheep [40]. The main reason to split male and female lambs in two separate groups was to avoid disturbances due to rutting behavior of young males. The lambs were used to handling before the start of the experiment. Sedatives were not used.

In part one, the animals were divided into four groups of five ram lambs (group 1–4, Table 1). On day 0, lambs in group 1 were inoculated with 1 ml of the TBEV-strain Hochosterwitz (European subtype, approximately $6.5x10^6$ focus forming units per ml (FFU/ml)), and lambs in group 2 and 3 were inoculated with 1 ml *A. phagocytophilum* (0.4 ml of heparinised sheep blood stabilized with 10% demethyl sulphoxide (DMSO), approximately 10^6 infected cells, GenBank accession number M73220). The lambs in group 4 were negative controls, and were inoculated with uninfected cell medium from the virus cultivation. On day 21, lambs in group 1 were inoculated with the same strain of *A. phagocytophilum*, and lambs in group 3 with the same strain of TBEV as described above. Lambs in group 2 served as *A. phagocytophilum* controls.

TBEV and the negative control medium were inoculated subcutaneously and *A. phagocytophilum* intravenously. The experimental infection model with intravenous inoculation of *A. phagocytophilum* has been used for several years at NMBU in Sandnes [40]. In addition, no difference in clinical manifestation has previously been observed after subcutaneous, intradermal or intravenous inoculation, except for a delay in incubation period after subcutaneous/intradermal inoculation. TBEV was inoculated subcutaneously to mimic tick bites, and because TBEV has been inoculated subcutaneously in mouse models and in studies in sheep

previously. For practical reasons and to avoid any mixture with the subcutaneous TBEV inoculation, *Anaplasma phagocytophilum* was inoculated intravenously.

Blood samples were drawn from *Vena jugularis* using vacuette tubes from all lambs on day 0, 2, 4, 6, 8, 10, 14, 18, 21, 23, 25, 27, 29, 31, 35, 39 and 42 (two EDTA tubes of 2 ml and one serum-tube with clot activator of 9 ml, Vacuette® Greiner Bio-One GmbH, Kremsmünster, Austria). The experimental period in part one ended on day 42 (Table 1). All lambs from part one of the study were euthanized, and brain samples were obtained for PCR analysis. The animals were euthanized by intravenous injection of pentobarbital sodium 400 mg/ml (Euthasol vet, Le Vet B.V., Oudewater, The Netherlands) at 140 mg/kg).

Study part two was designed similarly with two groups of five ewe lambs each (group 5 and 6 Table 1). The experimental period in part two ended on day 21. The same strains and batches of TBEV and *A*. *phagocytophilum* as above were inoculated to group 5 on day 0, while physiological saline solution was used as negative control and inoculated to group 6 on the same day. Blood samples were drawn from *Vena jugularis* using vacuette tubes all animals on day 0, 2, 4, 6, 8, 10, 14, 18 and 21 (two 2 ml EDTA-tubes, and one 4 ml serum-tube with clot activator, Vacuette® Greiner Bio-One GmbH, Kremsmünster, Austria).

All serum tubes were separated by centrifugation within two hours post sampling, and stored at -80 °C until analysis. One EDTA tube was stored at -20 °C for *A*. *phagocytophilum* PCR, while the second tube was used for hematology.

Hematology

Hematological analyses were performed on the ADVIA 120 instrument (Siemens healthcare, Erlangen Germany) with veterinary software for sheep blood.

Detection of tick-borne encephalitis virus

RNA from the serum samples was extracted on QIAcube with QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. RNA from the brain samples was extracted by RNeasy mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. Immediately after the extraction process, the RNA was reversely transcribed to cDNA with random primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster city, CA, USA). To detect TBEV RNA, an in-house reverse transcriptase (RT) real-time PCR was performed according to Andreassen et al. (2012). The real-time PCR amplifies a 54 base pair (bp) fragment located on the envelope gene of TBEV. A positive RNA control ("Soukup") was used in the real-time PCR [41]. Nuclease free water was used as negative control.

Detection of antibodies to tick-borne encephalitis virus

Serum samples from lambs were analyzed for TBEV IgG by a commercial enzyme linked immunosorbent assay (ELISA, Enzygnost® Anti-TBE virus IgG, Siemens Healthcare, GmbH, Marburg, Germany) according to the manufacturer's protocol, with one modification: the conjugate was changed to Peroxidase-Labeled Anti-Sheep IgG antibody (KPL, Gaithersburg, USA). The IgG conjugate was diluted 1:50,000. Serum from sheep vaccinated against TBEV with the TicoVac-vaccine (Pfizer Ltd, Ramsgate Road, Sandwich, Kent, CT13 9NJ, UK) was used as positive control, and serum from sheep which had never been exposed to ticks was used as negative control [8]. All positive and borderline samples from the ELISA were further tested in a TBEV-specific serum neutralization test (SNT) at the Center for Virology of the Medical University of Vienna, as described previously [42].

Detection of *Anaplasma phagocytophilum*

DNA from the EDTA blood samples were extracted on MagNA Pure 96 with MagNA Pure 96 DNA and viral NA large volume kit (Roche Molecular Systems, Inc. Basel, Switzerland) according to the manufacturer's recommendations. To detect *A. phagocytophilum* DNA, a quantitative real-time PCR method was performed according to Henningsson et al. 2015. This method amplifies a 64 bp fragment of the gltA gene of the bacterium [43]. A positive *A. phagocytophilum* control and a synthetic plasmid (pAP-GltA cloned in pUC57, GenScript Cooperation, Scotch plains, NJ) were used in the qPCR. Nuclease free water was used as negative control.

Statistics

All clinical and laboratory data were collected in Microsoft Excel (2016) spreadsheets and transferred to Stata 14.2 for Windows (StataCorp, 4905 Lakeway Drive. College Station, Texas 77845) for statistical analysis. The quality of data and distributions were analyzed using tabulations and histograms. Initial analyses included multilevel linear regression modelling of each of the continuous outcome variables; rectal temperature, neutrophil counts, lymphocyte counts, monocyte counts, quantitative PCR of *A. phagocytophilum* and TBEV titer. Predictors were "Group" (exposure) and "Day" of infection and the random effects variable was "The individual lambs". The statistical analyses were performed on day 0 to day 21 post inoculation with TBEV and *A. phagocytophilum*. Residuals were estimated and visualized in quantile plots. $p < 0.05$ was considered significant. Additional, descriptive statistical analyses were performed in Excel and GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA).

Results

Part one: pre- and post-infection of TBEV and *A. phagocytophilum*

The lambs in group 1 and 3, which had been inoculated with TBEV, displayed no clinical TBE symptoms or fever, and had a short or non-detectable viremia by RT real-time PCR on serum samples. On day two post TBEV infection, four of five lambs in group 1 tested positive for TBEV in the serum, while in group 3 two of five lambs were positive. One of five lambs in group 3 tested positive for TBEV on day four post TBEV infection. All samples were negative on day six and throughout the experiment (S1 Table). The brain samples collected from the lambs at the end of the experiment (day 42) were all found to be TBEV negative by RT real-time PCR (data not shown).

The results from serum neutralization test showed that the lambs inoculated with TBEV (group 1 and 3) developed neutralizing antibodies to the virus (Fig 1, S1 Table). The lambs had detectable neutralizing antibodies in the serum from day six post TBEV infection, and throughout the experiment. No significant difference in the mean TBEV titer between group 1 and 3 was found ($p>0.05$).

All lambs inoculated with *A. phagocytophilum* (group 1, 2 and 3) developed fever and clinical symptoms of *A. phagocytophilum* infection (Fig 2, S1 Table). One of the lambs was diagnosed with pneumonia and was euthanized before the end of the study according to animal welfare standards. *A. phagocytophilum* was detected by qPCR in all blood samples from day 2 post infection and throughout the experiment (Fig 2, S1 Table). No significant difference in the mean *A. phagocytophilum* concentration between group 1, 2 and 3 was found ($p>0.05$). Furthermore, no significant difference in the mean rectal temperature related to the *A. phagocytophilum* infection between group 1, 2 and 3 was found ($p>0.05$).

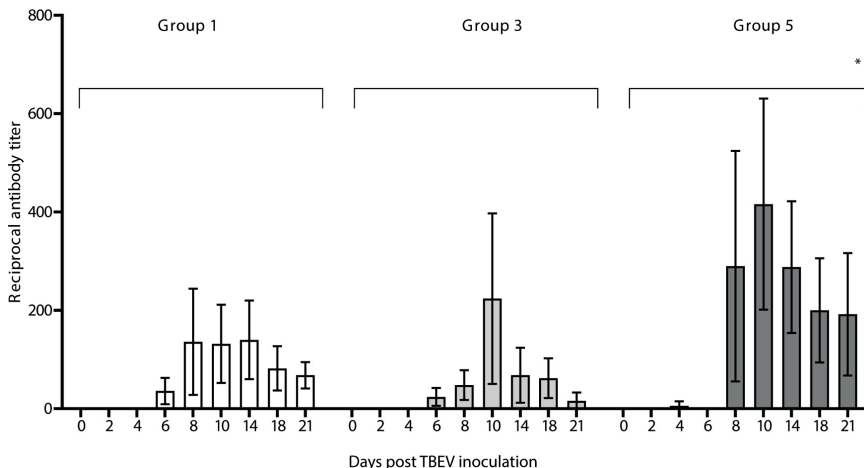

Fig 1. Mean reciprocal TBEV antibody titer in lambs post TBEV infection. TBEV titers (Y axis) were measured by serum neutralization test in group 1, 3 and 5 on day 0 to day 21 post inoculation with TBEV (X axis). Group 5 had significantly higher mean TBEV titer values than group 1 and 3, indicated with*. Standard deviations (SD) are illustrated with error bars. The groups which were not inoculated with TBEV are not included in the figure, and did not develop neutralizing antibodies to the virus.

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For the hematological analysis, group 2 had a significantly higher mean monocyte count compared to group 1 and 3 ($p < 0.05$). No significant difference in the mean neutrophil and lymphocyte counts was found between group 1, 2 and 3 ($p > 0.05$, Fig 3, S1 Table).

Part two: Co-infection of TBEV and *A. phagocytophilum*

The lambs in group 5, which were co-infected with TBEV and *A. phagocytophilum*, displayed no clinical TBE symptoms, and the viremia was either not detectable or short-lived. Two of five lambs had detectable TBEV RNA in serum on day two, and one of five on day four and six. All serum samples tested negative for TBEV RNA on day eight and throughout the experiment. Similarly to part one in the present study, all lambs inoculated with TBEV developed neutralizing TBEV antibodies from day 4 and 8 post inoculation (Fig 1, S1 Table).

The lambs in group 5 developed fever and clinical signs of tick-borne fever, and the bacterium was detected by qPCR from day 2 post infection and throughout the experiment (Fig 2, S1 Table).

Statistical comparison of study part one and two

A significantly higher mean TBEV titer was found in group 5 where the lambs were co-infected with TBEV and *A. phagocytophilum*, compared to group 1 which received an infection of TBEV on day 0 and *A. phagocytophilum* on day 21 ($p < 0.05$). Similarly, group 5 had a significantly higher mean TBEV titer compared to group 3 which had been infected with *A. phagocytophilum* on day 0 and TBEV on day 21 ($p < 0.05$). No differences in terms of viremia between pre-, post- and co-infection of TBEV and *A. phagocytophilum* was found.

No significant difference was found in the mean rectal temperature related to the *A. phagocytophilum* infection or the mean concentration of *A. phagocytophilum* in the blood samples between group 1, 2, 3 and 5 ($p > 0.05$).

Fig 2. Mean rectal temperature and mean concentration of *Anaplasma phagocytophilum* in the lambs. The mean rectal temperature (Y axis) and mean concentration of *A. phagocytophilum* in the blood measured by qPCR (copies per µL (logarithmic), Y axis) of each group on day 0 to day 42 post inoculation (X axis). The dotted line at 40 degrees Celsius indicates fever. The vertical dashed line in graph A to D indicates the second challenge day on day 21. Part two of the experiment (graph E and F) concluded on day 21. Standard deviations (SD) are illustrated by the error bars.

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Fig 3. Mean counts of neutrophils and monocytes in the lambs in the experimental study. Mean counts (Y axis) of neutrophils (circular dots) and monocytes (squares) in the experiment. Normal counts in sheep are 0.8–5.0 (10^9 cells per liter) for neutrophils and <0.75 (10^9 cells per liter) for monocytes. The vertical dashed line in graph A to D indicates the second challenge on day 21. Standard deviations (SD) are illustrated by the error bars. The lack of endpoints in some error bars in graph A are due to outliers.

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For the hematological analysis, a significantly higher mean count of monocytes was found after inoculation with *A*. *phagocytophilum* in group 1 (day 21–42) and group 3 (day 0–21) compared to group 5 (day 0–21). Similarly, post TBEV inoculation, a significantly higher mean number of monocytes was found in group 1 (day 0–21) than in group 5 (day 0–21). No significant difference in the mean count of neutrophils and lymphocytes was found between the *A*. *phagocytophilum* infected groups in part 1 and part 2, however, a significantly higher mean neutrophil count was found in group 1 compared to group 5 on day 0 to 21 post TBEV-inoculation ($p<0.05$, S1 Table).

Discussion

There is a lack of information on the veterinary aspects of TBEV. This study aimed to investigate infection of TBEV and co-infection of TBEV and *A*. *phagocytophilum* in lambs. All TBEV infected lambs developed neutralizing TBEV antibodies, without displaying any clinical symptoms of TBE, and had a very short viremia. A significantly higher mean TBEV titer was found in the group co-infected with TBEV and *A*. *phagocytophilum* compared to the other groups. These results indicate that co-infection of TBEV and *A*. *phagocytophilum* in lambs may stimulate a higher TBEV antibody response compared to a single infection of TBEV, or a prior infection with *A*. *phagocytophilum*. The reason for this is, however, unknown.

The significant difference in the TBEV antibody titer could have been affected by the difference of the gender of the lambs in part one (rams) and part two (ewes). A previous study on *A*. *phagocytophilum* infection in laboratory mice found that infected male mice had increased *A*. *phagocytophilum* DNA load and number of infected neutrophils [44]. In the present study, no significant difference was found in the mean *A*. *phagocytophilum* DNA load, but a significantly higher mean neutrophil count was found in group 1 compared to group 5 post TBEV infection. Although TBEV viremia was low or non-detectable, the differences in gender could have affected the TBEV titers and the neutrophil counts. TBEV infection of lambs from different genders and ages have, however, not shown any differences in the clinical symptoms (unpublished data).

In our study, the mean number of monocytes was found to be significantly higher in group 2 than in all the other groups infected with *A*. *phagocytophilum*. Furthermore, groups 1 and 3 had a significantly higher mean monocyte count compared to group 5. Monocytes have been found to be important in combating *A*. *phagocytophilum* infection [45], and also to contribute to the cell-mediated immune response to TBEV [46, 47]. A significantly higher mean monocyte count was found in group 1 (day 0 to 21) than in group 5 (day 0 to 21) post TBEV infection. These results may indicate that when a single infection of *A*. *phagocytophilum* or TBEV occur (group 1 and 3), a higher cell-mediated immune response is developed, compared to co-infection with TBEV and *A*. *phagocytophilum* (group 5). However, no significant differences were found in the mean bacterial load of *A*. *phagocytophilum*, nor the clinical symptoms of the lambs.

The results from our study are in accordance with previous studies and together they indicate that TBEV rarely leads to symptomatic disease in sheep [17, 18, 39]. Co-infection with LIV and *A*. *phagocytophilum* is known to cause severe disease in sheep [36]. In our study, co-infection with *A*. *phagocytophilum* and TBEV did not seem to impact the clinical symptoms in lambs, even though LIV is genetically closely related to TBEV [48]. The absence of clinical TBE cases in sheep may be due to poor replication of the virus in sheep cells [39].

A recent experimental study on TBEV and LIV in sheep by Mansfield et al. (2016), found no clinical symptoms following TBEV infection, although a neutralizing antibody response was established [39]. Similar results were found in the current study. The study by Mansfield

et al. (2016), found that the low antibody titer post TBEV infection was likely a reflection of the low viral load within the sheep infected with TBEV. Comparable results were found in this study, where a low viremia was detected in some of the lambs a few days post TBEV inoculation. Although a low and short-lived viremia was found, there is a known possibility of alimentary transmitted TBEV, which shows that ruminants develop a viremia post TBEV infection [2–5, 49–52]. Furthermore, an experimental study in goats detected TBEV viremia with a duration of up to 19 days [53]. The reason for the prolonged viremic period detected in goats compared to sheep is unknown, but it might indicate that goats are more susceptible to TBEV infection than sheep, or that there are differences in the pathogenicity of the viral strains.

In summary, the present study shows that all TBEV-infected lambs developed neutralizing TBEV antibodies without displaying any clinical symptoms of TBE. A significantly higher mean TBEV titer was found in the group co-infected with TBEV and *A. phagocytophilum* compared to the other groups. For future experimental studies in domestic ruminants other and possibly more virulent TBEV-strains should be considered to confirm the effects of co-infection using animals of the same gender.

Supporting information

S1 Table. Data on the rectal temperature, hematological variables, PCR results of tick-borne encephalitis virus and *Anaplasma phagocytophilum*, enzyme-linked immunosorbent assay and TBE titers. Abbreviations in S1 Table:
TBEV PCR: Tick-borne encephalitis virus real-time polymerase chain reaction (0 = negative, 1 = positive)
TBEV ELISA: Tick-borne encephalitis enzyme-linked immunosorbent assay (0 = negative, 1 = positive)
TBEV titer: Tick-borne encephalitis titer by serum neutralization test
Wbc: White blood cells
Rbs: Red blood cells
Hgb: Hemoglobin
Hct: Hematocrit
MCV: Mean cell volume
MCH: Mean cellular hemoglobin
RDV: Red cell distribution width
HDW: Hemoglobin distribution width
Neut: Neutrophils
Lymp: Lymphocytes
Mono: Monocytes
Eos: Eosinophils
Baso: Basophils
LUC: Large unstained cells.
(XLSX)

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Author Contributions

Conceptualization: Katrine M. Paulsen, Erik G. Granquist, Rose Vikse, Åshild K. Andreassen, Snorre Stuen.

Data curation: Katrine M. Paulsen, Erik G. Granquist, Snorre Stuen.

Formal analysis: Katrine M. Paulsen, Erik G. Granquist.

Funding acquisition: Erik G. Granquist, Åshild K. Andreassen.

Investigation: Katrine M. Paulsen, Erik G. Granquist, Wenche Okstad, Karin Stiasny, Snorre Stuen.

Methodology: Katrine M. Paulsen, Erik G. Granquist, Åshild K. Andreassen, Snorre Stuen.

Project administration: Katrine M. Paulsen, Erik G. Granquist, Åshild K. Andreassen, Snorre Stuen.

Supervision: Erik G. Granquist, Åshild K. Andreassen, Snorre Stuen.

Validation: Katrine M. Paulsen, Erik G. Granquist, Snorre Stuen.

Visualization: Katrine M. Paulsen, Erik G. Granquist.

Writing – original draft: Katrine M. Paulsen.

Writing – review & editing: Katrine M. Paulsen, Erik G. Granquist, Wenche Okstad, Rose Vikse, Karin Stiasny, Åshild K. Andreassen, Snorre Stuen.

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Supplementary Table 1

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	0	8	1	39,5	0,00	0	0	0	0	11,31	11,98	114	32,10	26,80	9,60	18,20	25,80	4,00	6,31	0,51	0,39	0,08	0,01
2017	0	36	1	39,7	0,00	0	0	0	0	9,34	10,82	103	28,30	26,20	9,50	17,10	24,90	4,54	3,89	0,38	0,36	0,10	0,08
2017	0	78	1	39,5	0,00	0	0	0	0	12,75	11,73	117	34,00	29,00	10,00	18,40	24,20	3,34	8,75	0,36	0,12	0,08	0,10
2017	0	119	1	39,6	0,00	0	0	0	0	15,05	10,85	114	32,10	30,30	10,80	18,30	27,20	3,90	9,66	0,80	0,44	0,12	0,14
2017	0	264	1	39,7	0,00	0	0	0	0	13,11	10,54	105	31,30	29,70	10,00	18,80	24,90	7,02	4,14	1,29	0,37	0,12	0,17
2017	1	8	1	39,3																			
2017	1	36	1	40,7																			
2017	1	78	1	39,5																			
2017	1	119	1	39,2																			
2017	1	264	1	39,6																			
2017	2	8	1	38,8	32,55	0	0	0	0	8,10	11,10	108	30,50	27,40	9,70	18,50	26,30	3,33	3,94	0,48	0,22	0,08	0,07
2017	2	36	1	39,3	0,00	0	0	0	0	8,56	10,88	107	28,70	26,40	9,90	17,20	24,90	4,03	3,17	0,94	0,18	0,09	0,15
2017	2	78	1	39,3	30,40	0	0	0	0	11,28	11,39	114	33,60	29,50	10,00	18,60	24,60	2,54	7,82	0,71	0,11	0,09	0,01
2017	2	119	1	39,2	31,01	0	0	0	0	9,68	9,74	105	29,70	30,50	10,80	18,10	27,10	2,69	6,29	0,40	0,19	0,08	0,03
2017	2	264	1	39,4	28,30	0	0	0	0	12,97	10,41	105	30,90	29,60	10,10	18,70	25,00	7,54	4,16	0,81	0,27	0,08	0,11
2017	3	8	1	39,1																			
2017	3	36	1	39,3																			
2017	3	78	1	39,3																			
2017	3	119	1	39,6																			
2017	3	264	1	40,1																			
2017	4	8	1	38,7	0,00	0	0	0	0	9,78	13,03	130	37,70	28,90	10,00	18,30	23,90	4,60	3,85	0,96	0,26	0,08	0,03
2017	4	36	1	39,0	0,00	0	0	0	0	7,29	11,39	111	31,30	27,50	9,80	17,00	23,60	2,49	4,22	0,15	0,26	0,06	0,12
2017	4	78	1	39,3	0,00	0	0	0	0	12,63	11,81	119	36,40	30,80	10,10	18,10	22,20	3,87	8,23	0,32	0,09	0,09	0,03
2017	4	119	1	39,4	0,00	0	0	0	0	12,76	10,55	116	33,30	31,60	11,00	18,10	25,80	4,27	7,42	0,64	0,29	0,10	0,03
2017	4	264	1	39,6	0,00	0	0	0	0	13,88	10,93	113	33,50	30,70	10,40	18,70	24,30	7,93	5,10	0,44	0,32	0,07	0,02
2017	5	8	1	39,2																			
2017	5	36	1	39,4																			
2017	5	78	1	39,4																			
2017	5	119	1	39,4																			
2017	5	264	1	39,8																			
2017	6	8	1	39,1	0,00	1	80	0	0	9,52	11,37	108	30,70	27,00	9,50	18,70	26,50	4,00	4,25	0,90	0,14	0,07	0,16
2017	6	36	1	39,3	0,00	1	15	0	0	7,74	10,64	102	27,70	26,00	9,60	17,10	25,30	2,89	3,37	1,05	0,27	0,06	0,10
2017	6	78	1	39,4	0,00	1	40	0	0	12,46	11,06	110	32,10	29,00	10,00	18,80	24,90	3,41	7,07	1,72	0,08	0,08	0,11
2017	6	119	1	39,6	0,00	1	30	0	0	13,49	9,30	101	28,00	30,10	10,90	18,70	27,50	4,45	7,86	0,83	0,20	0,09	0,05
2017	6	264	1	39,7	0,00	0	15	0	0	13,19	10,35	106	30,30	29,30	10,20	18,60	25,20	6,59	5,35	0,70	0,34	0,10	0,11
2017	7	8	1	39,0																			
2017	7	36	1	39,5																			
2017	7	78	1	39,9																			
2017	7	119	1	39,6																			
2017	7	264	1	39,6																			
2017	8	8	1	39,1	0,00	1	320	0	0	9,02	11,38	111	31,00	27,30	9,80	18,70	26,70	3,72	4,52	0,51	0,19	0,06	0,02
2017	8	36	1	39,5	0,00	0	40	0	0	10,04	10,77	105	28,50	26,40	9,70	17,30	25,60	5,88	3,42	0,43	0,25	0,05	0,01
2017	8	78	1	39,7	0,00	1	120	0	0	11,73	11,04	112	32,70	29,60	10,10	18,80	24,90	4,24	6,84	0,36	0,11	0,08	0,10
2017	8	119	1	39,7	0,00	1	80	0	0	11,90	9,42	103	29,00	30,70	10,90	19,20	28,10	4,36	6,68	0,57	0,20	0,08	0,02
2017	8	264	1	39,8	0,00	1	120	0	0	12,99	10,43	107	31,20	29,90	10,20	18,60	24,80	7,29	4,22	0,93	0,39	0,09	0,07
2017	9	8	1	39,4																			
2017	9	36	1	39,7																			
2017	9	78	1	39,7																			
2017	9	119	1	39,7																			
2017	9	264	1	39,8																			
2017	10	8	1	39,4	0,00	1	240	0	0	7,22	11,24	109	30,60	27,20	9,70	18,90	26,80	3,58	2,98	0,35	0,24	0,06	0,01
2017	10	36	1	39,5	0,00	1	20	0	0	9,63	9,90	96	26,10	26,40	9,70	17,70	26,10	5,36	3,37	0,48	0,33	0,08	0,01

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	10	78	1	39,7	0,00	1	120	0	0	8,66	11,28	114	33,50	29,70	10,10	19,00	25,50	3,64	3,30	1,46	0,11	0,04	0,11
2017	10	119	1	39,6	0,00	1	160	0	0	8,44	9,17	101	28,20	30,80	11,00	19,60	28,20	3,04	4,72	0,30	0,25	0,08	0,05
2017	10	264	1	40,7	0,00	1	120	0	0	29,25	10,59	110	31,40	29,60	10,40	18,80	25,70	23,53	4,73	0,78	0,11	0,08	0,02
2017	11	8	1	39,0																			
2017	11	36	1	39,6																			
2017	11	78	1	39,5																			
2017	11	119	1	39,3																			
2017	11	264	1	39,5																			
2017	12	8	1	39,4																			
2017	12	36	1	39,5																			
2017	12	78	1	39,5																			
2017	12	119	1	39,0																			
2017	12	264	1	39,5																			
2017	13	8	1	39,3																			
2017	13	36	1	39,2																			
2017	13	78	1	39,8																			
2017	13	119	1	39,5																			
2017	13	264	1	39,5																			
2017	14	8	1	39,4	0,00	1	240	0	0	11,92	11,45	112	31,50	27,50	9,80	18,80	26,10	5,46	5,30	0,76	0,21	0,06	0,12
2017	14	36	1	39,4	0,00	1	20	0	0	8,56	10,74	104	28,60	26,60	9,70	17,50	25,20	3,52	4,01	0,60	0,36	0,06	0,01
2017	14	78	1	39,5	0,00	1	120	0	0	11,11	10,97	113	32,70	29,80	10,30	18,70	25,40	3,07	7,52	0,33	0,08	0,08	0,03
2017	14	119	1	39,8	0,00	1	160	0	0	10,95	9,66	107	29,90	31,00	11,10	19,40	28,70	4,00	6,12	0,60	0,11	0,09	0,02
2017	14	264	1	39,3	0,00	1	160	0	0	10,52	10,19	105	30,40	29,80	10,30	18,50	25,90	4,60	4,49	0,46	0,87	0,09	0,01
2017	15	8	1	39,2																			
2017	15	36	1	39,5																			
2017	15	78	1	39,4																			
2017	15	119	1	39,3																			
2017	15	264	1	40,6																			
2017	16	8	1	39,2																			
2017	16	36	1	39,0																			
2017	16	78	1	39,2																			
2017	16	119	1	39,4																			
2017	16	264	1	39,5																			
2017	17	8	1	39,4																			
2017	17	36	1	39,5																			
2017	17	78	1	39,4																			
2017	17	119	1	39,7																			
2017	17	264	1	39,6																			
2017	18	8	1	39,6	0,00	1	120	0	0	10,31	10,90	107	31,10	28,60	9,80	18,20	23,90	4,73	4,86	0,17	0,35	0,07	0,13
2017	18	36	1	39,7	0,00	1	10	0	0	7,86	10,76	105	30,00	27,90	9,70	17,30	24,30	3,16	4,09	0,06	0,41	0,05	0,08
2017	18	78	1	39,5	0,00	1	80	0	0	10,64	10,08	104	31,40	31,10	10,40	17,80	22,50	2,47	7,51	0,46	0,10	0,10	0,00
2017	18	119	1	39,4	0,00	1	80	0	0	9,04	9,51	107	30,80	32,40	11,20	18,90	26,00	1,85	6,64	0,33	0,13	0,07	0,02
2017	18	264	1	40,1	0,00	1	120	0	0	23,38	9,94	104	30,10	30,30	10,50	18,20	25,50	15,87	6,08	0,77	0,55	0,10	0,02
2017	19	8	1	39,3																			
2017	19	36	1	39,4																			
2017	19	78	1	39,3																			
2017	19	119	1	39,2																			
2017	19	264	1	39,8																			
2017	20	8	1	39,1																			
2017	20	36	1	39,3																			
2017	20	78	1	38,9																			
2017	20	119	1	39,4																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc 10^9/l 4,5-12,0	Rbc 10^{12}/l 9,0-14,0	Hgb g/l 90-140	Hct % 27,0-40,0	MCV fl 25,0-35,0	MCH pg 8,0-12,0	RDW % 15,0-30,0	HDW g/l 17,0-25,0	Neut 10^9/l 0,8-5,0	Lymp 10^9/l 1,2-6,0	Mono 10^9/l <0,75	Eos 10^9/l <1,0	Baso 10^9/l <0,3	LUC 10^9/l <0,25
2017	20	264	1	39,9																			
2017	21	8	1	39,2	0,00	1	80	0	0	8,62	11,70	113	32,80	28,00	9,70	18,40	25,50	3,28	4,50	0,55	0,20	0,06	0,03
2017	21	36	1	39,5	0,00	1	20	0	0	7,97	10,92	107	29,60	27,10	9,80	17,80	25,80	3,28	3,90	0,45	0,23	0,10	0,01
2017	21	78	1	39,3	0,00	1	80	0	0	11,39	11,19	115	34,50	30,90	10,20	18,20	24,80	2,71	6,69	1,76	0,05	0,09	0,10
2017	21	119	1	39,5	0,00	1	80	0	0	8,37	9,85	110	31,80	32,30	11,20	19,30	27,30	1,55	6,02	0,63	0,07	0,08	0,03
2017	21	264	1	39,7	0,00	1	80	0	0	16,01	10,56	109	32,20	30,40	10,30	18,40	26,40	10,13	4,56	0,88	0,22	0,06	0,17
2017	22	8	1	39,2																			
2017	22	36	1	39,2																			
2017	22	78	1	39,1																			
2017	22	119	1	39,2																			
2017	22	264	1	39,1																			
2017	23	8	1	39,1				131	2,12	9,31	11,32	110	31,70	28,00	9,80	18,40	25,00	3,96	4,33	0,73	0,19	0,07	0,03
2017	23	36	1	39,2				120	2,08	7,39	10,25	101	27,80	27,20	9,80	18,10	25,50	3,07	3,53	0,39	0,25	0,09	0,06
2017	23	78	1	39,3				0	0,00	10,94	10,82	110	33,20	30,70	10,20	18,40	24,50	3,08	5,90	1,71	0,05	0,09	0,11
2017	23	119	1	39,2				141	2,15	9,44	9,63	108	30,70	31,90	11,20	19,40	27,50	1,94	6,79	0,54	0,07	0,09	0,00
2017	23	264	1	39,5				109	2,04	16,02	10,23	106	31,00	30,30	10,40	18,20	25,80	9,82	4,68	1,03	0,34	0,08	0,07
2017	24	8	1	39,3																			
2017	24	36	1	40,6																			
2017	24	78	1	39,2																			
2017	24	119	1	40,6																			
2017	24	264	1	40,3																			
2017	25	8	1	41,7				127284	5,10	2,63	10,67	109	29,90	28,00	10,20	18,20	23,40	1,28	1,06	0,03	0,10	0,01	0,14
2017	25	36	1	41,8				351824	5,55	4,46	10,12	103	27,60	27,30	10,20	17,70	23,90	2,69	1,23	0,25	0,09	0,01	0,19
2017	25	78	1	40,6				34516	4,54	8,98	10,49	112	32,90	31,40	10,60	17,90	22,30	4,53	4,11	0,14	0,06	0,07	0,07
2017	25	119	1	42,2				247343	5,39	5,35	9,68	113	30,60	31,60	11,70	18,90	26,80	2,88	2,09	0,11	0,11	0,02	0,14
2017	25	264	1	41,4				1089917	6,04	17,20	9,88	106	29,30	29,60	10,70	18,00	25,40	12,18	2,21	0,70	1,90	0,05	0,16
2017	26	8	1	41,7																			
2017	26	36	1	42,1																			
2017	26	78	1	41,9																			
2017	26	119	1	41,9																			
2017	26	264	1	41,4																			
2017	27	8	1	40,6				375213	5,57	5,62	12,22	121	33,20	27,10	9,90	18,70	26,60	3,63	1,47	0,35	0,06	0,01	0,11
2017	27	36	1	41,0				280237	5,45	6,14	11,72	114	31,00	26,40	9,70	18,40	27,00	4,05	1,31	0,56	0,07	0,02	0,13
2017	27	78	1	41,3				381880	5,58	7,10	10,90	114	32,30	29,70	10,50	18,40	24,90	4,81	1,68	0,50	0,04	0,00	0,07
2017	27	119	1	40,9				236952	5,37	6,47	10,11	116	31,20	30,90	11,50	19,30	29,90	3,68	2,08	0,25	0,05	0,02	0,38
2017	27	264	1	41,4				108554	5,04	14,21	10,44	109	30,40	29,10	10,40	18,00	26,50	10,25	1,73	1,69	0,23	0,03	0,29
2017	28	8	1	40,2																			
2017	28	36	1	40,5																			
2017	28	78	1	41,1																			
2017	28	119	1	40,2																			
2017	28	264	1	41,4																			
2017	29	8	1	40,8				82410	4,92	4,49	10,70	105	29,00	27,10	9,80	18,60	26,00	1,90	1,77	0,41	0,10	0,02	0,28
2017	29	36	1	40,5				170267	5,23	4,51	10,50	103	28,00	26,70	9,90	18,80	28,20	2,00	1,58	0,54	0,06	0,04	0,30
2017	29	78	1	40,3				249117	5,40	5,61	10,46	109	31,00	29,60	10,40	18,30	25,40	3,16	1,91	0,16	0,07	0,03	0,28
2017	29	119	1	41,1				115685	5,06	5,65	8,98	100	28,20	31,40	11,20	19,30	29,30	1,87	2,83	0,59	0,09	0,03	0,24
2017	29	264	1	40,9				0	0,00	14,51	9,53	100	27,80	29,10	10,50	17,80	24,70	10,19	2,18	1,10	0,18	0,05	0,81
2017	30	8	1	40,1																			
2017	30	36	1	39,7																			
2017	30	78	1	40,3																			
2017	30	119	1	40,3																			
2017	30	264	1	40,8																			
2017	31	8	1	40,5				3691	3,57	4,65	10,84	106	29,30	27,10	9,80	18,50	25,80	0,89	2,92	0,50	0,07	0,04	0,24

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	31	36	1	40,7				56197	4,75	4,15	10,76	104	28,60	26,60	9,70	18,70	27,30	0,63	2,57	0,59	0,05	0,04	0,28
2017	31	78	1	40,4				3262	3,51	6,72	9,82	104	29,40	29,90	10,60	18,30	24,90	1,56	4,12	0,80	0,09	0,08	0,06
2017	31	119	1	40,2				6334	3,80	7,38	9,16	102	28,70	31,30	11,20	19,20	29,40	0,43	5,54	1,12	0,04	0,08	0,17
2017	31	264	1	41,2				564736	5,75	14,51	9,63	98	28,10	29,20	10,20	18,10	24,50	8,09	4,70	1,04	0,10	0,12	0,46
2017	32	8	1	40,1																			
2017	32	36	1	39,7																			
2017	32	78	1	40,5																			
2017	32	119	1	40,2																			
2017	32	264	1	40,6																			
2017	33	8	1	39,9																			
2017	33	36	1	39,8																			
2017	33	78	1	40,0																			
2017	33	119	1	39,9																			
2017	33	264	1	40,0																			
2017	34	8	1	40,3																			
2017	34	36	1	39,7																			
2017	34	78	1	39,8																			
2017	34	119	1	39,7																			
2017	34	264	1	39,5																			
2017	35	8	1	40,2				1077	3,03	4,80	10,77	106	29,10	27,00	9,80	18,90	25,90	0,88	3,01	0,57	0,08	0,04	0,22
2017	35	36	1	39,5				358	2,56	3,82	10,57	103	28,30	26,80	9,70	18,80	27,30	0,70	2,14	0,73	0,07	0,01	0,17
2017	35	78	1	39,6				666	2,82	6,92	9,55	100	29,10	30,50	10,50	18,80	24,50	1,33	4,01	1,31	0,12	0,03	0,12
2017	35	119	1	39,9				7497	3,87	6,30	9,13	102	28,60	31,30	11,20	18,80	28,20	0,96	3,20	1,95	0,05	0,04	0,09
2017	35	264	1	39,4				0	0,00	9,92	9,85	101	29,40	29,90	10,30	20,50	25,60	1,86	4,30	3,40	0,12	0,08	0,16
2017	36	8	1	39,4																			
2017	36	36	1	39,4																			
2017	36	78	1	39,6																			
2017	36	119	1	39,8																			
2017	36	264	1	39,8																			
2017	37	8	1	39,1																			
2017	37	36	1	39,7																			
2017	37	78	1	40,6																			
2017	37	119	1	39,5																			
2017	37	264	1	39,5																			
2017	38	8	1	40,6																			
2017	38	36	1	39,3																			
2017	38	78	1	39,4																			
2017	38	119	1	39,3																			
2017	38	264	1	39,4																			
2017	39	8	1	40,2				31854	4,50	8,40	10,92	105	30,00	27,50	9,60	18,70	25,20	5,94	1,57	0,69	0,08	0,03	0,09
2017	39	36	1	39,2				62	1,80	3,36	10,50	100	28,50	27,10	9,60	18,80	26,60	0,47	1,92	0,76	0,08	0,02	0,11
2017	39	78	1	39,3				320	2,51	7,47	10,07	105	30,70	30,50	10,40	19,30	24,50	0,56	4,89	1,37	0,07	0,04	0,55
2017	39	119	1	39,7				10	1,02	6,64	9,31	102	29,80	32,00	11,00	18,80	27,90	1,02	3,45	1,96	0,08	0,02	0,11
2017	39	264	1	39,4				1456	3,16	12,09	10,12	108	30,90	30,50	10,70	22,90	26,90	2,58	7,20	2,08	0,10	0,09	0,04
2017	40	8	1	38,8																			
2017	40	36	1	39,1																			
2017	40	78	1	38,8																			
2017	40	119	1	39,0																			
2017	40	264	1	40,1																			
2017	41	8	1	39,1																			
2017	41	36	1	39,1																			
2017	41	78	1	39,2																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	41	119	1	38,7																			
2017	41	264	1	39,4																			
2017	42	8	1	39,1				0	0,00	4,75	10,64	103	29,10	27,40	9,70	18,80	25,80	1,32	2,56	0,64	0,12	0,03	0,09
2017	42	36	1	38,7				0	0,00	6,64	10,23	100	28,20	27,60	9,80	18,50	25,30	2,81	3,30	0,16	0,20	0,05	0,11
2017	42	78	1	39,1				0	0,00	8,87	10,20	107	31,40	30,80	10,50	19,00	24,00	2,79	5,24	0,72	0,07	0,03	0,02
2017	42	119	1	39,3				46	1,67	9,65	9,09	101	29,20	32,10	11,20	18,50	27,20	2,62	6,02	0,61	0,28	0,10	0,01
2017	42	264	1	39,8				352	2,55	17,31	10,98	115	34,40	31,40	10,50	23,00	26,60	5,92	9,86	1,21	0,11	0,09	0,12
2017	0	11	2	39,4				0	0,00	13,31	10,55	107	30,80	29,20	10,20	15,20	23,10	4,07	8,34	0,45	0,22	0,10	0,12
2017	0	48	2	39,3				0	0,00	8,97	11,73	122	35,00	29,80	10,40	17,10	25,60	2,75	4,92	0,76	0,33	0,09	0,12
2017	0	93	2	39,7				0	0,00	15,70	10,62	111	32,40	30,50	10,50	18,30	23,00	4,34	9,49	0,74	0,93	0,14	0,05
2017	0	149	2	39,3				0	0,00	13,98	10,20	105	30,40	29,80	10,30	17,20	21,20	5,41	7,91	0,36	0,18	0,09	0,04
2017	0	263	2	39,4				0	0,00	5,78	10,90	113	33,10	30,40	10,40	18,20	22,10	1,94	3,47	0,13	0,17	0,04	0,04
2017	1	11	2	39,2																			
2017	1	48	2	39,6																			
2017	1	93	2	38,7																			
2017	1	149	2	39,1																			
2017	1	263	2	39,3																			
2017	2	11	2	39,1				55	1,75	10,13	10,61	109	31,30	29,50	10,30	15,10	22,70	2,36	7,26	0,11	0,19	0,09	0,11
2017	2	48	2	39,7				238	2,38	9,32	11,94	125	35,60	29,80	10,50	17,00	26,10	3,03	5,11	0,81	0,26	0,07	0,04
2017	2	93	2	39,0				0	0,00	15,69	11,47	121	35,10	30,60	10,50	18,40	23,90	3,20	10,45	0,82	0,99	0,17	0,05
2017	2	149	2	39,1				1	0,29	13,22	10,84	113	32,50	30,00	10,40	17,40	21,80	3,45	8,94	0,29	0,18	0,16	0,20
2017	2	263	2	39,5				57	1,77	4,01	10,58	112	32,20	30,50	10,60	18,30	22,80	1,37	2,36	0,12	0,12	0,02	0,03
2017	3	11	2	40,6																			
2017	3	48	2	40,5																			
2017	3	93	2	39,4																			
2017	3	149	2	40,2																			
2017	3	263	2	39,9																			
2017	4	11	2	42,0				9479	3,98	7,83	11,31	117	33,20	29,40	10,40	15,10	22,90	4,01	3,31	0,08	0,29	0,02	0,13
2017	4	48	2	42,1				532546	5,73	5,14	10,41	114	31,40	30,10	11,00	16,60	25,00	3,32	1,46	0,19	0,07	0,01	0,08
2017	4	93	2	40,4				0	0,00	10,53	10,88	118	32,50	29,90	10,90	18,20	23,90	3,86	6,03	0,27	0,19	0,09	0,09
2017	4	149	2	41,8				259486	5,41	5,81	10,15	107	31,00	30,50	10,50	17,20	20,50	2,75	2,60	0,27	0,11	0,01	0,07
2017	4	263	2	41,8				352523	5,55	4,67	10,89	117	33,30	30,60	10,80	17,80	21,70	2,94	1,45	0,06	0,11	0,02	0,10
2017	5	11	2	41,4																			
2017	5	48	2	41,6																			
2017	5	93	2	41,6																			
2017	5	149	2	41,5																			
2017	5	263	2	42,0																			
2017	6	11	2	41,2				258685	5,41	5,16	10,73	113	30,00	28,00	10,50	15,20	24,10	2,70	1,92	0,20	0,09	0,02	0,23
2017	6	48	2	41,7				711134	5,85	5,02	11,94	129	34,50	28,90	10,80	16,90	29,20	3,37	1,12	0,09	0,08	0,00	0,35
2017	6	93	2	41,7				572353	5,76	6,43	11,00	119	32,50	29,50	10,90	17,90	23,30	3,61	2,27	0,10	0,09	0,01	0,35
2017	6	149	2	40,9				420646	5,62	6,61	11,28	119	32,60	28,90	10,50	17,40	23,30	3,55	2,30	0,41	0,11	0,01	0,23
2017	6	263	2	41,4				385851	5,59	3,82	10,62	114	31,30	29,40	10,80	17,80	23,60	1,97	1,09	0,47	0,09	0,02	0,17
2017	7	11	2	41,1																			
2017	7	48	2	41,5																			
2017	7	93	2	40,7																			
2017	7	149	2	40,2																			
2017	7	263	2	41,5																			
2017	8	11	2	41,2				122342	5,09	6,41	11,02	113	31,90	28,90	10,30	15,50	24,70	2,08	3,59	0,21	0,19	0,05	0,30
2017	8	48	2	41,1				339527	5,53	8,80	11,75	123	34,60	29,50	10,50	16,90	30,60	5,87	1,96	0,43	0,08	0,03	0,44
2017	8	93	2	40,7				201708	5,30	6,98	11,16	119	33,50	30,00	10,60	17,40	22,90	3,29	2,83	0,30	0,19	0,04	0,33
2017	8	149	2	39,3				130317	5,12	6,27	9,84	102	29,00	29,40	10,40	17,60	23,30	1,98	3,30	0,66	0,10	0,05	0,19
2017	8	263	2	40,5				87660	4,94	4,08	9,84	105	29,70	30,10	10,70	17,40	23,40	1,83	1,74	0,33	0,09	0,02	0,07

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	9	11	2	40,3																			
2017	9	48	2	41,7																			
2017	9	93	2	41,3																			
2017	9	149	2	41,0																			
2017	9	263	2	40,6																			
2017	10	11	2	40,9				16797	4,23	5,39	10,67	108	30,60	28,70	10,10	15,70	24,90	0,88	3,56	0,70	0,09	0,03	0,12
2017	10	48	2	40,8				709350	5,85	10,60	11,74	126	34,80	29,60	10,70	16,70	30,20	7,28	2,82	0,33	0,07	0,03	0,07
2017	10	93	2	41,8				92948	4,97	7,44	10,67	115	32,00	30,00	10,80	17,00	22,10	2,46	4,29	0,38	0,12	0,04	0,15
2017	10	149	2	39,9				6817	3,83	7,80	9,90	100	28,80	29,10	10,10	17,60	23,40	0,96	5,88	0,74	0,05	0,09	0,08
2017	10	263	2	40,3				5431	3,73	4,70	10,20	109	30,60	30,00	10,70	17,20	23,30	0,69	3,40	0,43	0,04	0,06	0,07
2017	11	11	2	40,0																			
2017	11	48	2	40,6																			
2017	11	93	2	40,8																			
2017	11	149	2	40,1																			
2017	11	263	2	39,7																			
2017	12	11	2	40,2																			
2017	12	48	2	40,4																			
2017	12	93	2	40,4																			
2017	12	149	2	39,7																			
2017	12	263	2	39,7																			
2017	13	11	2	40,4																			
2017	13	48	2	40,3																			
2017	13	93	2	40,9																			
2017	13	149	2	40,1																			
2017	13	263	2	39,8																			
2017	14	11	2	40,7				19161	4,28	5,35	10,92	113	31,50	28,90	10,30	15,60	23,30	1,56	2,90	0,65	0,07	0,05	0,12
2017	14	48	2	40,5				5305	3,72	13,73	12,94	143	39,60	30,60	11,10	17,30	29,70	8,33	3,36	1,45	0,08	0,07	0,45
2017	14	93	2	40,5				2122	3,33	4,79	10,52	113	32,00	30,40	10,70	16,90	21,90	1,01	3,45	0,23	0,05	0,01	0,02
2017	14	149	2	39,3				143	2,16	6,80	10,38	106	30,30	29,20	10,30	17,60	22,60	0,63	3,94	1,85	0,03	0,04	0,31
2017	14	263	2	39,9				5497	3,74	3,97	10,46	111	32,30	30,90	10,60	17,70	22,70	0,87	2,53	0,49	0,04	0,02	0,02
2017	15	11	2	41,6																			
2017	15	48	2	41,2																			
2017	15	93	2	40,3																			
2017	15	149	2	39,3																			
2017	15	263	2	39,7																			
2017	16	11	2	40,3																			
2017	16	48	2	41,0																			
2017	16	93	2	39,9																			
2017	16	149	2	38,9																			
2017	16	263	2	39,5																			
2017	17	11	2	39,3																			
2017	17	48	2	40,8																			
2017	17	93	2	39,8																			
2017	17	149	2	39,2																			
2017	17	263	2	39,5																			
2017	18	11	2	39,7				18	1,29	4,96	10,14	104	29,40	29,00	10,30	15,50	23,10	0,44	3,91	0,45	0,10	0,03	0,02
2017	18	48	2						0,00														
2017	18	93	2	39,6				11149	4,05	7,67	10,29	111	31,30	30,40	10,80	17,20	24,40	1,89	4,71	0,79	0,19	0,04	0,05
2017	18	149	2	38,8				2024	3,31	8,34	9,49	97	28,90	30,50	10,30	17,60	21,10	2,09	5,58	0,51	0,11	0,04	0,02
2017	18	263	2	39,0				0	0,16	5,35	9,90	108	31,40	31,70	10,90	17,60	22,60	1,49	3,43	0,32	0,08	0,02	0,01
2017	19	11	2	39,6																			
2017	19	48	2																				

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	19	93	2	40,0																			
2017	19	149	2	39,2																			
2017	19	263	2	39,1																			
2017	20	11	2	39,6																			
2017	20	48	2																				
2017	20	93	2	39,2																			
2017	20	149	2	39,1																			
2017	20	263	2	39,1																			
2017	21	11	2	39,4				146	2,17	6,05	9,81	99	28,90	29,50	10,10	15,90	23,10	1,08	4,18	0,62	0,05	0,09	0,02
2017	21	48	2						0,00														
2017	21	93	2	39,0				1629	3,21	6,64	10,47	113	32,50	31,10	10,80	16,80	21,70	0,94	4,54	0,94	0,09	0,03	0,10
2017	21	149	2	39,3				1	0,23	9,29	9,93	101	30,10	30,30	10,20	19,40	23,30	2,46	4,09	2,45	0,09	0,05	0,16
2017	21	263	2	39,2				0	0,11	7,91	10,16	108	32,50	32,00	10,70	18,00	23,10	2,62	2,92	2,14	0,11	0,03	0,09
2017	22	11	2	39,2																			
2017	22	48	2																				
2017	22	93	2	39,3																			
2017	22	149	2	38,8																			
2017	22	263	2	39,3																			
2017	23	11	2	39,4						5,85	9,93	101	29,00	29,20	10,10	18,10	24,30	1,44	3,86	0,33	0,06	0,09	0,08
2017	23	48	2																				
2017	23	93	2	39,2						8,55	10,38	112	32,30	31,10	10,70	16,90	21,80	1,84	6,14	0,27	0,09	0,06	0,14
2017	23	149	2	39,2						9,75	10,50	109	32,00	30,50	10,40	20,10	23,50	2,76	6,53	0,17	0,08	0,05	0,16
2017	23	263	2	39,5						9,53	10,90	117	34,90	32,00	10,80	18,30	22,80	4,36	4,69	0,31	0,12	0,03	0,02
2017	24	11	2	39,7																			
2017	24	48	2																				
2017	24	93	2	39,4																			
2017	24	149	2	39,0																			
2017	24	263	2	39,4																			
2017	25	11	2	39,2						4,95	9,58	101	28,20	29,40	10,50	18,80	24,60	0,70	3,80	0,29	0,11	0,03	0,02
2017	25	48	2																				
2017	25	93	2	39,3						10,72	9,79	108	30,00	30,60	11,10	17,40	23,70	3,14	6,93	0,08	0,41	0,06	0,10
2017	25	149	2	39,1						11,36	9,65	105	30,40	31,60	10,90	19,50	21,00	3,74	7,01	0,39	0,13	0,07	0,02
2017	25	263	2	39,1						7,13	9,42	105	30,30	32,20	11,20	18,20	22,20	2,29	4,59	0,02	0,15	0,02	0,05
2017	26	11	2	39,2																			
2017	26	48	2																				
2017	26	93	2	39,2																			
2017	26	149	2	39,0																			
2017	26	263	2	39,1																			
2017	27	11	2	39,7						6,98	10,13	104	29,40	29,10	10,30	19,10	26,30	2,39	3,97	0,45	0,13	0,02	0,01
2017	27	48	2																				
2017	27	93	2	39,6						7,56	10,74	114	33,10	30,80	10,60	17,10	22,70	1,91	4,81	0,36	0,41	0,05	0,01
2017	27	149	2	39,0						12,28	10,88	110	32,80	30,10	10,10	20,00	23,70	3,31	8,44	0,31	0,09	0,07	0,05
2017	27	263	2	38,9						7,07	10,43	114	32,90	31,60	10,90	18,40	23,40	2,39	2,87	1,51	0,20	0,03	0,08
2017	28	11	2	39,3																			
2017	28	48	2																				
2017	28	93	2	39,2																			
2017	28	149	2	39,1																			
2017	28	263	2	39,4																			
2017	29	11	2	39,4						6,88	9,17	93	26,60	29,00	10,20	18,70	25,50	2,42	3,95	0,32	0,14	0,04	0,01
2017	29	48	2																				
2017	29	93	2	39,5						9,49	10,43	111	32,20	30,90	10,46	16,90	21,90	3,63	5,14	0,23	0,32	0,08	0,09
2017	29	149	2	39,1						10,57	10,26	105	31,10	30,30	10,30	20,00	23,30	2,47	7,60	0,16	0,11	0,06	0,18

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	29	263	2	39,4						7,48	9,89	103	31,40	31,70	10,50	18,20	22,70	2,77	3,06	1,46	0,09	0,04	0,06
2017	30	11	2	39,8																			
2017	30	48	2																				
2017	30	93	2	40,3																			
2017	30	149	2	39,3																			
2017	30	263	2	39,9																			
2017	31	11	2	39,5						6,80	9,14	94	26,70	29,30	10,20	19,80	25,90	1,92	4,41	0,20	0,11	0,08	0,08
2017	31	48	2																				
2017	31	93	2	39,5						9,66	10,44	109	32,30	30,90	10,50	17,00	21,40	2,24	6,50	0,54	0,18	0,12	0,08
2017	31	149	2	39,0						9,32	10,21	105	31,10	30,40	10,30	19,90	23,50	2,13	6,54	0,51	0,10	0,04	0,01
2017	31	263	2	40,1						8,78	9,42	100	29,80	31,70	10,60	18,40	22,70	4,22	2,88	1,45	0,11	0,02	0,10
2017	32	11	2	39,5																			
2017	32	48	2																				
2017	32	93	2	39,4																			
2017	32	149	2	38,8																			
2017	32	263	2	39,8																			
2017	33	11	2	39,5																			
2017	33	48	2																				
2017	33	93	2	39,4																			
2017	33	149	2	39,1																			
2017	33	263	2	39,9																			
2017	34	11	2	39,5																			
2017	34	48	2																				
2017	34	93	2	39,0																			
2017	34	149	2	39,3																			
2017	34	263	2	39,6																			
2017	35	11	2	39,6						6,66	9,33	98	28,50	30,50	10,50	22,40	28,20	1,58	3,35	1,39	0,13	0,08	0,13
2017	35	48	2																				
2017	35	93	2	39,3						9,11	10,16	106	31,40	30,90	10,50	17,10	22,80	2,74	5,39	0,18	0,62	0,06	0,13
2017	35	149	2	39,1						11,10	10,33	106	31,60	30,60	10,20	19,80	23,70	2,69	7,41	0,73	0,13	0,11	0,03
2017	35	263	2	39,6						6,16	9,28	99	29,60	32,00	10,70	18,00	23,10	2,08	3,59	0,30	0,10	0,02	0,07
2017	36	11	2	39,6																			
2017	36	48	2																				
2017	36	93	2	39,4																			
2017	36	149	2	39,1																			
2017	36	263	2	39,6																			
2017	37	11	2	39,6																			
2017	37	48	2																				
2017	37	93	2	39,2																			
2017	37	149	2	39,0																			
2017	37	263	2	39,6																			
2017	38	11	2	40,4																			
2017	38	48	2																				
2017	38	93	2	39,2																			
2017	38	149	2	39,0																			
2017	38	263	2	39,5																			
2017	39	11	2	39,3						7,60	9,29	96	28,00	30,10	10,40	22,50	29,30	3,69	3,53	0,17	0,11	0,06	0,05
2017	39	48	2																				
2017	39	93	2	39,2						10,11	9,74	102	30,10	30,90	10,40	17,30	23,10	2,92	5,94	0,34	0,74	0,09	0,08
2017	39	149	2	39,1						11,04	10,18	104	31,10	30,50	10,20	19,80	24,00	3,24	7,29	0,15	0,09	0,09	0,17
2017	39	263	2	39,4						5,60	9,47	101	30,60	32,30	10,70	18,10	23,50	2,14	3,20	0,08	0,07	0,02	0,09
2017	40	11	2	39,5																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	40	48	2																				
2017	40	93	2	39,1																			
2017	40	149	2	39,0																			
2017	40	263	2	39,2																			
2017	41	11	2	38,7																			
2017	41	48	2																				
2017	41	93	2	39,4																			
2017	41	149	2	38,9																			
2017	41	263	2	39,1																			
2017	42	11	2	39,7						7,68	10,06	106	31,00	30,80	10,60	22,00	27,90	2,89	4,22	0,26	0,16	0,07	0,07
2017	42	48	2																				
2017	42	93	2	39,2						13,08	9,67	102	29,60	30,70	10,60	17,70	23,50	4,88	7,13	0,41	0,47	0,16	0,04
2017	42	149	2	38,9						12,79	10,98	112	33,40	30,40	10,20	19,30	23,30	4,69	7,05	0,83	0,12	0,07	0,04
2017	42	263	2	39,7						8,43	9,80	106	31,30	32,00	10,80	17,90	23,20	4,67	3,40	0,14	0,09	0,03	0,08
2017	0	24	3	39,4				0	0	13,56	11,27	118	33,00	29,30	10,50	17,10	22,70	4,80	7,57	0,82	0,26	0,09	0,01
2017	0	55	3	39,7				0	0	10,65	10,57	109	29,70	28,10	10,30	17,30	23,70	3,91	5,81	0,61	0,25	0,05	0,03
2017	0	108	3	39,4				0	0	9,00	10,68	110	31,00	29,00	10,30	17,20	23,80	1,96	6,20	0,57	0,13	0,07	0,07
2017	0	176	3	39,3				0	0	5,76	10,30	104	28,40	27,60	10,10	18,80	27,70	1,42	4,03	0,18	0,10	0,02	0,00
2017	0	504	3	39,4				0	0	9,60	10,16	109	30,90	30,40	10,70	17,10	22,20	2,11	5,55	1,62	0,20	0,04	0,07
2017	1	24	3	39,3																			
2017	1	55	3	39,4																			
2017	1	108	3	39,2																			
2017	1	176	3	39,2																			
2017	1	504	3	39,4																			
2017	2	24	3	39,3				117	2,07	13,40	10,41	108	30,50	29,30	10,40	17,20	23,20	5,04	7,30	0,46	0,25	0,11	0,24
2017	2	55	3	39,2				60	1,79	11,21	10,88	110	30,80	28,30	10,10	17,20	24,30	4,46	5,88	0,47	0,25	0,05	0,09
2017	2	108	3	38,9				124	2,10	9,23	10,58	107	30,70	29,00	10,10	17,20	24,60	2,50	5,99	0,47	0,12	0,08	0,07
2017	2	176	3	39,2				81	1,91	6,63	10,57	107	29,40	27,80	10,20	18,70	28,00	2,36	3,91	0,15	0,12	0,03	0,04
2017	2	504	3	39,5				112	2,05	9,44	10,01	107	30,40	30,40	10,70	17,10	22,30	2,95	5,88	0,20	0,24	0,04	0,14
2017	3	24	3	40,2																			
2017	3	55	3	40,6																			
2017	3	108	3	40,9																			
2017	3	176	3	40,8																			
2017	3	504	3	41,3																			
2017	4	24	3	41,8				388781	5,59	8,00	10,23	107	30,10	29,40	10,50	16,80	21,20	4,45	3,02	0,11	0,20	0,02	0,21
2017	4	55	3	41,8				243415	5,39	3,72	10,15	105	29,40	29,00	10,40	17,20	22,80	1,96	1,37	0,20	0,13	0,01	0,05
2017	4	108	3	42,1				179505	5,25	4,13	10,63	110	31,20	29,40	10,40	17,10	23,80	2,11	1,67	0,19	0,09	0,01	0,05
2017	4	176	3	41,9				135807	5,13	6,59	10,07	101	27,60	27,40	10,00	18,10	25,80	2,33	1,05	0,11	0,07	0,01	0,02
2017	4	504	3	42,4				287052	5,46	5,05	9,80	105	30,60	31,20	10,80	16,70	19,30	2,83	1,71	0,06	0,26	0,01	0,18
2017	5	24	3	41,4																			
2017	5	55	3	41,3																			
2017	5	108	3	41,6																			
2017	5	176	3	41,7																			
2017	5	504	3	41,5																			
2017	6	24	3	40,8				264964	5,42	9,75	10,72	112	30,30	28,30	10,40	17,80	25,00	4,74	3,83	0,66	0,13	0,02	0,37
2017	6	55	3	41,0				187807	5,27	5,90	11,81	122	32,40	27,40	10,30	18,10	29,30	3,74	1,27	0,40	0,08	0,00	0,40
2017	6	108	3	41,4				280370	5,45	4,68	11,66	120	32,70	28,00	10,30	17,70	28,60	2,96	0,81	0,75	0,07	0,00	0,08
2017	6	176	3	41,2				172047	5,24	3,96	11,09	113	29,70	26,80	10,20	18,40	30,10	2,49	1,03	0,29	0,07	0,00	0,08
2017	6	504	3	41,5				366978	5,56	6,09	10,05	108	29,70	29,60	10,70	17,20	23,00	3,46	1,20	0,74	0,16	0,02	0,51
2017	7	24	3	40,8																			
2017	7	55	3	40,8																			
2017	7	108	3	40,5																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc 10^9/l	Rbc 10^{12}/l	Hgb g/l	Hct %	MCV fl	MCH pg	RDW %	HDW g/l	Neut 10^9/l	Lymp 10^9/l	Mono 10^9/l	Eos 10^9/l	Baso 10^9/l	LUC 10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	7	176	3	41,0																			
2017	7	504	3	40,9																			
2017	8	24	3	40,4				203989	5,31	7,30	9,99	105	28,90	28,90	10,50	17,50	24,30	3,16	2,71	1,17	0,12	0,04	0,10
2017	8	55	3	41,3				154458	5,19	4,33	9,73	99	27,30	28,10	10,20	18,30	29,50	2,41	1,18	0,58	0,08	0,02	0,08
2017	8	108	3	40,4				101495	5,01	5,47	10,43	108	30,10	28,90	10,40	17,80	28,50	1,99	2,29	0,69	0,06	0,04	0,40
2017	8	176	3	41,4				90546	4,96	3,68	9,19	93	25,20	27,40	10,10	18,20	28,70	1,79	1,39	0,38	0,07	0,02	0,03
2017	8	504	3	41,0				82024	4,91	4,43	9,57	103	28,80	30,10	10,70	17,20	22,00	1,01	2,31	0,54	0,10	0,02	0,44
2017	9	24	3	40,1																			
2017	9	55	3	40,9																			
2017	9	108	3	40,7																			
2017	9	176	3	40,4																			
2017	9	504	3	40,9																			
2017	10	24	3	40,3				2756	3,44	9,02	10,84	113	31,40	29,90	10,40	17,40	24,30	0,83	6,99	0,86	0,04	0,09	0,22
2017	10	55	3	40,9				41473	4,62	5,25	9,84	100	27,80	28,20	10,20	18,40	29,30	1,30	2,98	0,64	0,05	0,06	0,23
2017	10	108	3	40,0				5036	3,70	6,17	10,27	106	29,80	29,00	10,30	17,90	28,60	0,50	4,97	0,30	0,03	0,07	0,30
2017	10	176	3	40,5				6190	3,79	3,53	9,24	93	25,20	27,30	10,10	18,10	28,50	0,36	2,31	0,65	0,03	0,03	0,16
2017	10	504	3	40,6				8503	3,93	6,00	10,11	106	30,20	29,80	10,50	17,30	23,30	0,43	4,05	1,00	0,06	0,08	0,38
2017	11	24	3	40,4																			
2017	11	55	3	40,1																			
2017	11	108	3	39,9																			
2017	11	176	3	41,1																			
2017	11	504	3	40,0																			
2017	12	24	3	40,1																			
2017	12	55	3	41,0																			
2017	12	108	3	39,6																			
2017	12	176	3	39,9																			
2017	12	504	3	39,4																			
2017	13	24	3	40,9																			
2017	13	55	3	40,2																			
2017	13	108	3	39,5																			
2017	13	176	3	40,3																			
2017	13	504	3	39,3																			
2017	14	24	3	39,9				130	2,12	7,14	10,95	114	31,80	29,10	10,40	17,50	23,70	1,39	4,19	1,16	0,06	0,04	0,30
2017	14	55	3	40,3				677	2,83	4,61	9,01	93	25,90	28,80	10,30	18,80	28,10	1,37	2,04	0,94	0,05	0,01	0,20
2017	14	108	3	39,6				1426	3,15	5,29	10,10	106	29,70	29,40	10,50	18,50	27,30	0,37	2,61	1,97	0,08	0,02	0,25
2017	14	176	3	39,8				257	2,41	4,80	10,06	103	28,20	28,00	10,20	18,90	28,10	0,48	2,77	1,29	0,05	0,03	0,18
2017	14	504	3	39,9				10004	4,00	6,31	10,33	110	31,80	30,80	10,70	17,80	22,30	1,92	2,64	1,53	0,09	0,01	0,12
2017	15	24	3	39,6																			
2017	15	55	3	39,9																			
2017	15	108	3	39,4																			
2017	15	176	3	39,7																			
2017	15	504	3	39,5																			
2017	16	24	3	40,2																			
2017	16	55	3	40,7																			
2017	16	108	3	39,1																			
2017	16	176	3	40,5																			
2017	16	504	3	39,2																			
2017	17	24	3	39,2																			
2017	17	55	3	39,4																			
2017	17	108	3	39,2																			
2017	17	176	3	39,3																			
2017	17	504	3	39,3																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	18	24	3	39,5				78	1,90	8,25	9,83	102	29,50	30,00	10,40	17,20	21,60	2,23	4,85	1,00	0,10	0,05	0,03
2017	18	55	3	39,5				210	2,32	4,80	9,30	97	27,60	29,70	10,40	18,20	26,10	1,23	2,91	0,52	0,10	0,02	0,01
2017	18	108	3	38,9				3187	3,50	7,27	9,79	101	29,40	30,10	10,30	18,40	26,10	1,58	5,01	0,49	0,15	0,04	0,01
2017	18	176	3	38,6				2	0,50	3,72	9,49	97	27,50	29,00	10,20	18,50	26,60	0,39	2,29	0,88	0,06	0,03	0,07
2017	18	504	3	39,0				0	0,00	7,42	9,06	97	28,80	31,80	10,70	17,90	20,60	2,35	3,04	1,70	0,20	0,02	0,11
2017	19	24	3	39,0																			
2017	19	55	3	39,4																			
2017	19	108	3	38,7																			
2017	19	176	3	38,9																			
2017	19	504	3	39,0																			
2017	20	24	3	39,4																			
2017	20	55	3	39,6																			
2017	20	108	3	39,1																			
2017	20	176	3	39,0																			
2017	20	504	3	38,9																			
2017	21	24	3	39,2	0,00	0	0	3	0,60	9,34	9,88	101	29,40	29,80	10,20	17,70	23,20	3,23	4,87	1,06	0,11	0,04	0,04
2017	21	55	3	39,2	0,00	0	0	141	2,15	5,36	9,82	101	28,70	29,20	10,30	18,70	27,30	0,90	3,83	0,30	0,09	0,03	0,20
2017	21	108	3	38,9	0,00	0	0	0	0,00	6,26	10,17	105	30,40	29,90	10,30	18,70	26,90	0,90	4,71	0,55	0,05	0,03	0,02
2017	21	176	3	39,1	0,00	0	0	3	0,56	5,25	9,97	101	28,90	29,00	10,10	18,90	28,00	1,61	3,41	0,05	0,08	0,02	0,09
2017	21	504	3	39,1	0,00	0	0	0	0	9,51	9,19	96	28,60	31,10	10,50	19,10	23,20	2,57	5,96	0,62	0,31	0,04	0,01
2017	22	24	3	39,4																			
2017	22	55	3	39,0																			
2017	22	108	3	39,0																			
2017	22	176	3	39,1																			
2017	22	504	3	39,4																			
2017	23	24	3	39,0	0,00	0	0			8,98	9,93	104	29,50	29,70	10,50	18,10	23,30	2,90	3,72	2,05	0,10	0,05	0,17
2017	23	55	3	39,1	31,01	0	0			4,58	9,71	100	28,50	29,30	10,30	18,90	27,10	1,35	2,03	1,01	0,07	0,02	0,11
2017	23	108	3	39,8	31,89	0	0			7,96	10,30	107	30,60	29,70	10,40	18,70	27,10	2,21	5,34	0,26	0,08	0,04	0,02
2017	23	176	3	39,2	0,00	0	0			5,23	10,32	107	29,80	28,90	10,30	19,10	28,30	1,42	3,57	0,06	0,05	0,03	0,10
2017	23	504	3	39,3	0,00	0	0			10,63	9,44	101	29,70	31,50	10,70	19,90	24,60	3,54	6,31	0,19	0,36	0,06	0,17
2017	24	24	3	39,3																			
2017	24	55	3	39,5																			
2017	24	108	3	39,2																			
2017	24	176	3	39,4																			
2017	24	504	3	39,2																			
2017	25	24	3	39,4	0,00	0	0			11,21	9,66	102	29,80	30,90	10,50	18,20	21,20	4,67	3,80	2,29	0,23	0,04	0,18
2017	25	55	3	39,3	0,00	0	0			6,15	9,28	98	28,20	30,40	10,60	18,20	23,20	2,71	2,97	0,31	0,14	0,02	0,01
2017	25	108	3	39,2	35,16	0	0			8,05	9,58	102	29,50	30,80	10,70	18,50	25,10	2,41	5,10	0,31	0,18	0,04	0,01
2017	25	176	3	39,0	0,00	0	0			5,81	9,51	100	27,90	29,30	10,60	19,00	26,30	1,79	3,70	0,08	0,09	0,04	0,10
2017	25	504	3	39,5	0,00	0	0			12,36	8,72	97	28,50	32,70	11,10	19,30	21,20	4,79	6,73	0,19	0,50	0,05	0,11
2017	26	24	3	40,1																			
2017	26	55	3	39,5																			
2017	26	108	3	39,2																			
2017	26	176	3	39,5																			
2017	26	504	3	39,5																			
2017	27	24	3	39,6	0,00	0	30			10,43	10,35	106	30,50	29,50	10,20	18,70	24,50	3,73	4,13	2,11	0,22	0,05	0,19
2017	27	55	3	39,3	0,00	0	0			7,09	9,64	100	27,80	28,80	10,30	18,60	26,50	2,99	3,55	0,19	0,14	0,03	0,20
2017	27	108	3	38,8	0,00	1	10			8,79	9,77	101	28,80	29,50	10,30	18,70	26,40	2,84	5,32	0,48	0,09	0,04	0,02
2017	27	176	3	39,5	0,00	1	40			6,87	10,34	107	29,70	28,70	10,30	19,50	28,60	2,66	3,84	0,12	0,06	0,04	0,14
2017	27	504	3	39,2	0,00	1	40			11,57	9,77	105	30,70	31,40	10,80	20,00	24,20	3,68	7,08	0,35	0,35	0,08	0,04
2017	28	24	3	39,3																			
2017	28	55	3	39,4																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc 10^9/ l 4,5-12,0	Rbc 10^{12}/l 9,0-14,0	Hgb g/l 90-140	Hct % 27,0-40,0	MCV fl 25,0-35,0	MCH pg 8,0-12,0	RDW % 15,0-30,0	HDW g/l 17,0-25,0	Neut 10^9/ l 0,8-5,0	Lymp 10^9/ l 1,2-6,0	Mono 10^9/ l <0,75	Eos 10^9/ l <1,0	Baso 10^9/ l <0,3	LUC 10^9/ l <0,25
2017	28	108	3	39,2																			
2017	28	176	3	39,8																			
2017	28	504	3	39,3																			
2017	29	24	3	39,3	0,00	1	80			9,87	9,58	99	28,30	29,60	10,30	18,60	23,90	3,51	5,34	0,81	0,15	0,05	0,01
2017	29	55	3	39,1	0,00	1	0			8,49	9,39	94	27,00	28,80	10,00	18,40	26,00	3,47	3,43	1,23	0,17	0,03	0,16
2017	29	108	3	39,1	0,00	1	40			8,73	9,54	96	28,40	29,70	10,10	18,60	26,50	2,13	6,16	0,21	0,06	0,06	0,11
2017	29	176	3	39,6	0,00	1	60			6,12	9,47	95	27,30	28,80	10,10	19,40	28,50	1,70	3,08	1,11	0,08	0,03	0,13
2017	29	504	3	39,1	0,00	1	60			9,45	9,53	101	30,00	31,50	10,60	19,80	24,60	2,59	6,22	0,19	0,27	0,05	0,13
2017	30	24	3	39,3																			
2017	30	55	3	39,3																			
2017	30	108	3	38,9																			
2017	30	176	3	39,2																			
2017	30	504	3	39,2																			
2017	31	24	3	39,5	0,00	1	480			8,04	9,53	99	28,60	30,00	10,40	18,70	23,60	2,38	4,85	0,60	0,14	0,05	0,02
2017	31	55	3	39,4	0,00	1	320			9,26	9,59	96	27,80	29,00	10,00	18,20	25,50	3,80	4,83	0,21	0,21	0,04	0,16
2017	31	108	3	39,1	0,00	1	80			7,95	9,70	98	28,70	29,60	10,20	18,40	26,30	1,69	5,74	0,26	0,13	0,04	0,09
2017	31	176	3	39,7	0,00	1	160			6,78	9,46	96	27,20	28,70	10,10	19,10	27,60	2,42	3,83	0,36	0,12	0,02	0,02
2017	31	504	3	39,4	0,00	1	80			9,09	9,67	105	30,30	31,30	10,90	19,70	24,90	3,03	5,19	0,52	0,29	0,05	0,02
2017	32	24	3	39,8																			
2017	32	55	3	39,3																			
2017	32	108	3	39,2																			
2017	32	176	3	39,5																			
2017	32	504	3	39,4																			
2017	33	24	3	39,4																			
2017	33	55	3	39,3																			
2017	33	108	3	39,4																			
2017	33	176	3	39,4																			
2017	33	504	3	39,4																			
2017	34	24	3	39,6																			
2017	34	55	3	39,4																			
2017	34	108	3	39,4																			
2017	34	176	3	39,9																			
2017	34	504	3	39,2																			
2017	35	24	3	39,6	0,00	1	80			9,10	10,10	105	30,80	30,50	10,40	19,30	24,30	3,44	4,67	0,79	0,13	0,04	0,03
2017	35	55	3	39,4	0,00	1	160			7,72	9,85	101	28,90	29,40	10,20	18,40	25,60	3,08	3,40	0,88	0,20	0,04	0,12
2017	35	108	3	39,6	0,00	1	40			7,24	9,69	100	29,40	30,30	10,30	19,20	26,80	2,37	4,28	0,24	0,09	0,05	0,19
2017	35	176	3	39,5	0,00	1	40			5,26	9,54	96	27,50	28,90	10,10	18,90	27,20	1,56	3,41	0,08	0,09	0,03	0,09
2017	35	504	3	39,2	0,00	1	20			9,94	9,81	105	31,10	31,70	10,70	19,60	24,00	2,95	6,10	0,20	0,43	0,05	0,20
2017	36	24	3	39,4																			
2017	36	55	3	39,9																			
2017	36	108	3	39,5																			
2017	36	176	3	39,8																			
2017	36	504	3	39,4																			
2017	37	24	3	39,3																			
2017	37	55	3	39,6																			
2017	37	108	3	39,1																			
2017	37	176	3	39,8																			
2017	37	504	3	39,4																			
2017	38	24	3	39,2																			
2017	38	55	3	39,6																			
2017	38	108	3	39,4																			
2017	38	176	3	39,5																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	38	504	3	39,7																			
2017	39	24	3	39,5	0,00	1	80			9,06	10,10	105	30,70	30,40	10,40	19,40	24,50	3,43	3,78	1,52	0,12	0,06	0,15
2017	39	55	3	39,7	0,00	1	120			4,98	10,54	110	31,20	29,60	10,50	18,50	25,20	2,10	2,33	0,40	0,09	0,01	0,04
2017	39	108	3	39,1	0,00	1	30			6,52	9,66	101	29,40	30,40	10,50	19,20	27,10	1,46	4,50	0,44	0,08	0,03	0,01
2017	39	176	3	39,8	0,00	1	60			6,17	9,55	95	27,70	29,10	10,00	18,70	27,20	2,18	3,66	0,13	0,07	0,04	0,08
2017	39	504	3	39,7	0,00	1	20			9,27	9,73	105	31,00	31,90	10,80	19,40	24,10	3,40	5,27	0,22	0,20	0,04	0,13
2017	40	24	3	39,2																			
2017	40	55	3	39,2																			
2017	40	108	3	39,2																			
2017	40	176	3	39,3																			
2017	40	504	3	39,3																			
2017	41	24	3	39,4																			
2017	41	55	3	39,1																			
2017	41	108	3	38,9																			
2017	41	176	3	39,6																			
2017	41	504	3	39,3																			
2017	42	24	3	39,3	0,00	1	20			8,21	10,35	109	31,80	30,70	10,60	19,40	24,10	2,76	4,55	0,57	0,12	0,05	0,16
2017	42	55	3	39,3	0,00	1	0			6,77	9,88	103	28,90	29,20	10,40	18,10	24,60	2,69	2,79	0,99	0,14	0,04	0,13
2017	42	108	3	39,3	0,00	1	20			6,99	9,97	105	30,10	30,20	10,50	18,70	26,00	2,62	3,97	0,18	0,07	0,04	0,10
2017	42	176	3	39,5	0,00	0	0			6,97	9,68	100	27,80	28,70	10,30	18,40	27,10	3,17	3,43	0,14	0,09	0,03	0,10
2017	42	504	3	39,3	0,00	1	40			7,99	10,49	117	33,50	32,00	11,10	19,20	23,70	2,06	4,91	0,63	0,31	0,04	0,03
2017	0	15	4	39,3	0,00	0	0	0	0	13,42	11,23	112	31,90	28,40	9,90	17,20	23,70	3,47	9,01	0,59	0,14	0,09	0,11
2017	0	61	4	39,5	0,00	0	0	0	0	10,01	11,32	118	35,90	31,50	10,30	17,90	21,20	3,08	6,09	0,45	0,18	0,09	0,12
2017	0	118	4	39,4	0,00	0	0	0	0	15,46	11,23	112	32,80	29,20	10,00	17,50	22,80	4,12	8,50	1,99	0,60	0,17	0,09
2017	0	252	4	39,1	0,00	0	0	0	0	9,74	13,48	128	37,00	27,50	9,50	18,60	24,90	4,15	4,72	0,43	0,19	0,09	0,16
2017	0	510	4	39,1	0,00	0	0	0	0	7,86	10,25	101	27,70	27,00	9,90	18,90	28,60	2,41	4,52	0,53	0,26	0,06	0,07
2017	1	15	4	39,2																			
2017	1	61	4	39,2																			
2017	1	118	4	38,8																			
2017	1	252	4	39,4																			
2017	1	510	4	39,3																			
2017	2	15	4	39,1	0,00	0	0	0	0	12,71	11,22	111	32,10	28,60	9,90	17,20	23,90	3,58	8,53	0,20	0,14	0,10	0,17
2017	2	61	4	39,2	0,00	0	0	0	0	9,64	10,90	114	34,30	31,40	10,40	17,90	21,40	3,14	5,77	0,32	0,23	0,09	0,10
2017	2	118	4	38,5	0,00	0	0	0	0	14,34	11,56	116	33,90	29,30	10,00	17,90	24,00	3,11	9,01	1,32	0,56	0,19	0,16
2017	2	252	4	39,4	0,00	0	0	0	0	13,85	13,83	132	37,90	27,40	9,50	18,30	25,20	4,99	7,79	0,78	0,15	0,10	0,05
2017	2	510	4	39,5	0,00	0	0	0	0	7,51	10,56	106	28,80	27,30	10,00	18,60	28,60	2,73	4,18	0,36	0,19	0,04	0,01
2017	3	15	4	38,6																			
2017	3	61	4	38,3																			
2017	3	118	4	38,7																			
2017	3	252	4	38,2																			
2017	3	510	4	39,1																			
2017	4	15	4	38,8	0,00	0	0	0	0	12,68	10,66	108	31,70	29,70	10,10	16,90	20,30	3,72	8,38	0,19	0,16	0,07	0,15
2017	4	61	4	39,0	0,00	0	0	0	0	9,00	10,71	114	34,50	32,20	10,70	17,50	19,50	2,68	5,76	0,27	0,16	0,06	0,07
2017	4	118	4	38,6	0,00	0	0	0	0	13,60	10,19	106	30,10	29,60	10,40	17,40	22,00	2,77	9,94	0,18	0,58	0,10	0,03
2017	4	252	4	39,4	0,00	0	0	0	0	16,32	12,60	123	36,60	29,00	9,70	17,50	20,80	8,73	6,45	0,86	0,16	0,10	0,03
2017	4	510	4	39,1	0,00	0	0	0	0	7,23	10,27	105	27,80	27,10	10,20	17,80	23,60	3,24	3,34	0,36	0,21	0,05	0,03
2017	5	15	4	38,9																			
2017	5	61	4	39,3																			
2017	5	118	4	39,1																			
2017	5	252	4	39,4																			
2017	5	510	4	39,3																			
2017	6	15	4	38,9	0,00	0	0	0	0	12,93	10,15	102	28,60	28,20	10,10	17,70	24,50	4,05	8,23	0,23	0,13	0,11	0,19

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	6	61	4	39,3	0,00	0	0	0	0	8,64	10,00	105	30,90	30,90	10,50	18,10	22,10	2,23	5,57	0,61	0,13	0,08	0,02
2017	6	118	4	39,2	0,00	0	0	0	0	13,97	11,11	112	31,70	28,50	10,10	17,80	24,60	3,86	8,48	1,00	0,34	0,11	0,17
2017	6	252	4	39,5	0,00	0	0	0	0	15,10	13,07	126	35,30	27,00	9,60	18,10	26,20	7,06	6,52	1,24	0,11	0,11	0,05
2017	6	510	4	40,3	0,00	0	0	0	0	7,84	10,15	101	27,00	26,60	10,00	18,60	28,90	3,45	3,90	0,15	0,16	0,05	0,12
2017	7	15	4	38,8																			
2017	7	61	4	39,0																			
2017	7	118	4	38,7																			
2017	7	252	4	39,7																			
2017	7	510	4	39,4																			
2017	8	15	4	39,4	0,00	0	0	0	0	10,23	10,39	106	29,70	28,50	10,20	18,00	24,40	4,24	5,34	0,37	0,14	0,06	0,07
2017	8	61	4	39,4	0,00	0	0	0	0	9,88	10,30	108	32,50	31,50	10,50	17,80	21,50	3,58	5,52	0,43	0,18	0,06	0,11
2017	8	118	4	39,2	0,00	0	0	0	0	14,30	10,50	107	30,30	28,90	10,20	18,00	24,40	5,06	8,06	0,67	0,36	0,12	0,03
2017	8	252	4	39,8	0,00	0	0	0	0	17,55	12,92	125	35,10	27,10	9,70	17,90	25,90	11,41	4,67	1,23	0,08	0,10	0,06
2017	8	510	4	39,8	0,00	0	0	0	0	7,13	9,82	100	26,70	27,20	10,20	18,50	28,60	2,81	3,44	0,60	0,13	0,03	0,12
2017	9	15	4	38,9																			
2017	9	61	4	39,1																			
2017	9	118	4	38,6																			
2017	9	252	4	39,9																			
2017	9	510	4	39,4																			
2017	10	15	4	39,2	0,00	0	0	0	0	12,97	11,04	113	31,90	28,90	10,30	18,80	25,00	3,63	8,32	0,81	0,10	0,09	0,01
2017	10	61	4	39,0	0,00	0	0	0	0	9,38	10,80	108	31,90	31,70	10,70	18,10	21,80	3,30	5,56	0,16	0,16	0,06	0,15
2017	10	118	4	39,4	0,00	0	0	0	0	14,61	10,86	111	31,90	29,30	10,20	18,00	23,90	4,43	8,53	0,78	0,66	0,18	0,02
2017	10	252	4	39,8	0,00	0	0	0	0	20,97	11,94	117	32,50	27,20	9,80	17,70	26,00	11,32	7,54	1,34	0,56	0,14	0,07
2017	10	510	4	39,5	0,00	0	0	0	0	7,00	9,91	100	27,10	27,40	10,10	18,40	28,40	2,88	3,26	0,61	0,18	0,03	0,04
2017	11	15	4	39,4																			
2017	11	61	4	39,0																			
2017	11	118	4	39,2																			
2017	11	252	4	39,8																			
2017	11	510	4	39,4																			
2017	12	15	4	39,1																			
2017	12	61	4	39,0																			
2017	12	118	4	39,4																			
2017	12	252	4	39,6																			
2017	12	510	4	39,4																			
2017	13	15	4	38,7																			
2017	13	61	4	38,9																			
2017	13	118	4	39,0																			
2017	13	252	4	39,5																			
2017	13	510	4	39,6																			
2017	14	15	4	39,1	0,00	0	0	0	0	11,32	10,88	111	32,10	29,50	10,20	18,80	24,60	3,59	5,87	1,53	0,13	0,07	0,14
2017	14	61	4	39,0	0,00	0	0	0	0	9,68	10,57	113	33,80	32,00	10,70	18,00	21,50	3,90	5,34	0,15	0,14	0,05	0,11
2017	14	118	4	39,1	0,00	0	0	0	0	15,03	11,07	115	32,90	29,70	10,40	18,20	24,10	4,36	9,41	0,72	0,37	0,15	0,02
2017	14	252	4	39,3	0,00	0	0	0	0	21,38	12,16	117	33,60	27,60	9,60	17,30	25,30	13,31	6,65	0,91	0,21	0,15	0,14
2017	14	510	4	39,5	0,00	0	0	0	0	7,78	10,65	110	29,50	27,70	10,30	18,40	28,40	3,72	3,57	0,31	0,12	0,06	0,00
2017	15	15	4	39,3																			
2017	15	61	4	39,1																			
2017	15	118	4	39,6																			
2017	15	252	4	39,3																			
2017	15	510	4	39,3																			
2017	16	15	4	38,9																			
2017	16	61	4	39,1																			
2017	16	118	4	40,2																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	16	252	4	39,1																			
2017	16	510	4	39,3																			
2017	17	15	4	39,3																			
2017	17	61	4	39,6																			
2017	17	118	4	39,5																			
2017	17	252	4	39,1																			
2017	17	510	4	39,4																			
2017	18	15	4	38,9	0,00	0	0	0	0	13,64	9,59	101	29,40	30,70	10,50	17,80	20,70	5,45	7,48	0,46	0,13	0,09	0,03
2017	18	61	4	38,5	0,00	0	0	0	0	8,23	9,82	108	32,30	32,90	11,00	17,60	19,70	2,88	5,00	0,08	0,11	0,05	0,11
2017	18	118	4	39,2	0,00	0	0	0	0	12,59	10,52	109	32,00	30,40	10,40	17,70	22,10	3,44	8,13	0,64	0,22	0,13	0,03
2017	18	252	4	38,9	0,00	0	0	0	0	16,26	11,50	114	33,70	29,30	9,90	16,60	21,00	7,41	7,24	1,08	0,25	0,16	0,13
2017	18	510	4	39,4	0,00	0	0	0	0	6,69	9,78	99	26,90	27,50	10,10	17,90	24,20	2,32	3,78	0,34	0,18	0,04	0,04
2017	19	15	4	38,7																			
2017	19	61	4	39,0																			
2017	19	118	4	38,9																			
2017	19	252	4	39,1																			
2017	19	510	4	39,1																			
2017	20	15	4	39,1																			
2017	20	61	4	38,6																			
2017	20	118	4	38,9																			
2017	20	252	4	39,3																			
2017	20	510	4	39,2																			
2017	21	15	4	39,5	0,00	0	0	0	0	11,68	10,22	102	30,50	29,90	10,00	18,60	23,60	2,99	6,61	1,66	0,09	0,10	0,23
2017	21	61	4	39,2	0,00	0	0	0	0	8,69	9,86	104	32,00	32,40	10,60	17,90	21,00	2,55	5,34	0,63	0,09	0,06	0,01
2017	21	118	4	39,1	0,00	0	0	0	0	13,72	9,88	101	29,60	29,90	10,20	18,00	23,70	3,15	8,08	1,97	0,27	0,16	0,10
2017	21	252	4	39,4	0,00	0	0	0	0	16,92	12,13	118	34,00	28,00	9,70	17,20	24,40	8,78	6,80	0,87	0,26	0,17	0,04
2017	21	510	4	39,4	0,00	0	0	0	0	7,35	10,07	101	28,10	27,90	10,00	18,70	28,30	2,95	3,93	0,23	0,09	0,06	0,09
2017	22	15	4	38,6																			
2017	22	61	4	38,8																			
2017	22	118	4	38,6																			
2017	22	252	4	39,1																			
2017	22	510	4	39,0																			
2017	23	15	4	39,1	0,00	0	0	0	0	11,55	10,82	111	32,30	29,90	10,30	18,70	24,60	3,22	6,60	1,49	0,07	0,10	0,07
2017	23	61	4	39,0	0,00	0	0	0	0	7,54	10,08	108	32,70	32,50	10,70	18,00	21,40	2,39	4,46	0,54	0,07	0,06	0,03
2017	23	118	4	38,7	0,00	0	0	0	0	12,71	10,99	115	33,30	30,30	10,40	18,30	24,40	3,19	7,12	1,82	0,28	0,14	0,16
2017	23	252	4	39,0	0,00	0	0	0	0	11,98	12,49	125	34,80	27,90	10,00	17,40	25,30	6,58	4,51	0,56	0,20	0,11	0,03
2017	23	510	4	39,1	0,00	0	0	0	0	6,68	10,93	110	30,60	28,00	10,10	18,80	29,00	2,65	3,49	0,35	0,11	0,04	0,04
2017	24	15	4	39,2																			
2017	24	61	4	39,2																			
2017	24	118	4	39,1																			
2017	24	252	4	39,2																			
2017	24	510	4	39,4																			
2017	25	15	4	39,1	0,00	0	0	0	0	11,01	10,12	108	31,80	31,40	10,70	18,10	20,70	3,56	6,45	0,71	0,16	0,08	0,06
2017	25	61	4	39,8	0,00	0	0	0	0	5,10	9,35	103	31,10	33,30	11,00	17,70	18,90	2,69	2,15	0,12	0,04	0,04	0,07
2017	25	118	4	39,4	0,00	0	0	0	0	14,58	10,16	108	31,00	30,50	10,60	17,90	22,70	5,68	7,96	0,49	0,34	0,10	0,01
2017	25	252	4	39,3	0,00	0	0	0	0	15,43	11,17	113	33,60	30,10	10,20	16,70	20,30	7,62	6,59	0,79	0,29	0,13	0,01
2017	25	510	4	39,3	0,00	0	0	0	0	6,80	10,03	103	28,00	27,90	10,30	17,60	23,10	3,34	2,96	0,33	0,12	0,05	0,01
2017	26	15	4	39,3																			
2017	26	61	4	41,0																			
2017	26	118	4	39,4																			
2017	26	252	4	39,3																			
2017	26	510	4	39,4																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc 10^9/l	Rbc 10^{12}/l	Hgb g/l	Hct %	MCV fl	MCH pg	RDW %	HDW g/l	Neut 10^9/l	Lymp 10^9/l	Mono 10^9/l	Eos 10^9/l	Baso 10^9/l	LUC 10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	27	15	4	39,3	0,00	0	0	0	0	13,72	10,59	110	31,30	29,60	10,30	18,60	24,10	5,14	7,45	0,78	0,10	0,11	0,15
2017	27	61	4	39,7	0,00	0	0	0	0	7,75	10,39	114	33,00	31,80	11,00	18,00	21,20	3,21	3,87	0,49	0,06	0,06	0,06
2017	27	118	4	39,1	0,00	0	0	0	0	14,04	10,26	109	30,40	29,60	10,60	18,20	23,80	5,14	7,78	0,67	0,28	0,13	0,04
2017	27	252	4	38,7	0,00	0	0	0	0	12,66	12,18	120	33,70	27,70	9,90	17,10	24,80	4,28	7,55	0,24	0,19	0,15	0,26
2017	27	510	4	39,2	0,00	0	0	0	0	6,36	10,27	105	28,60	27,90	10,30	18,80	28,50	2,33	3,54	0,35	0,08	0,05	0,02
2017	28	15	4	39,2																			
2017	28	61	4	39,2																			
2017	28	118	4	39,0																			
2017	28	252	4	39,2																			
2017	28	510	4	39,3																			
2017	29	15	4	39,3	0,00	0	0	0	0	11,72	10,06	102	30,00	29,90	10,20	18,50	24,10	3,57	7,29	0,63	0,14	0,09	0,01
2017	29	61	4	39,1	0,00	0	0	0	0	10,83	10,35	113	33,50	32,40	10,90	17,90	21,40	3,93	5,84	0,83	0,08	0,10	0,05
2017	29	118	4	39,1	0,00	0	0	0	0	13,79	9,77	100	29,10	29,80	10,30	18,10	23,80	4,44	8,14	0,78	0,23	0,15	0,05
2017	29	252	4	39,3	0,00	0	0	0	0	12,44	12,09	118	33,90	28,00	9,70	17,20	24,80	4,90	6,41	0,76	0,14	0,20	0,03
2017	29	510	4	39,3	0,00	0	0	0	0	7,24	10,10	102	28,30	28,00	10,10	18,90	29,50	2,98	3,53	0,51	0,10	0,06	0,05
2017	30	15	4	39,0																			
2017	30	61	4	38,9																			
2017	30	118	4	38,9																			
2017	30	252	4	39,0																			
2017	30	510	4	39,6																			
2017	31	15	4	39,2	0,00	0	0	0	0	12,00	10,62	110	31,90	30,00	10,30	18,50	23,90	3,66	6,67	1,31	0,11	0,08	0,17
2017	31	61	4	39,0	0,00	0	0	0	0	7,93	10,35	110	33,20	32,10	10,70	17,60	20,70	2,61	4,82	0,12	0,09	0,09	0,19
2017	31	118	4	39,2	0,00	0	0	0	0	13,60	9,81	102	29,40	29,90	10,40	18,00	23,50	4,00	8,58	0,57	0,23	0,17	0,05
2017	31	252	4	39,3	0,00	0	0	0	0	12,73	11,97	117	33,50	28,00	9,80	17,00	24,70	4,37	7,28	0,81	0,11	0,12	0,05
2017	31	510	4	39,6	0,00	0	0	0	0	7,76	10,10	103	28,60	28,30	10,20	18,90	28,80	3,16	3,76	0,59	0,09	0,05	0,10
2017	32	15	4	39,0																			
2017	32	61	4	38,9																			
2017	32	118	4	39,0																			
2017	32	252	4	39,3																			
2017	32	510	4	39,3																			
2017	33	15	4	39,2																			
2017	33	61	4	38,8																			
2017	33	118	4	38,8																			
2017	33	252	4	39,1																			
2017	33	510	4	39,3																			
2017	34	15	4	39,6																			
2017	34	61	4	38,8																			
2017	34	118	4	39,2																			
2017	34	252	4	39,5																			
2017	34	510	4	39,5																			
2017	35	15	4	39,3	0,00	0	0	0	0	11,86	10,85	109	32,60	30,00	10,00	18,30	22,90	4,27	5,68	1,42	0,09	0,06	0,33
2017	35	61	4	39,2	0,00	0	0	0	0	9,40	10,40	110	33,60	32,30	10,60	17,40	19,60	3,56	4,97	0,60	0,14	0,06	0,07
2017	35	118	4	39,0	0,00	0	0	0	0	12,69	10,08	103	30,70	30,50	10,20	18,20	23,10	3,94	6,63	1,68	0,22	0,13	0,08
2017	35	252	4	39,2	0,00	0	0	0	0	11,59	12,38	121	35,20	28,40	9,80	17,30	24,40	4,24	6,54	0,43	0,11	0,11	0,17
2017	35	510	4	39,6	0,00	0	0	0	0	7,31	10,33	104	29,30	28,40	10,10	18,70	28,00	2,98	3,59	0,54	0,10	0,05	0,04
2017	36	15	4	38,9																			
2017	36	61	4	38,7																			
2017	36	118	4	39,2																			
2017	36	252	4	39,6																			
2017	36	510	4	39,2																			
2017	37	15	4	38,8																			
2017	37	61	4	38,9																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2017	37	118	4	39,1																			
2017	37	252	4	39,1																			
2017	37	510	4	39,4																			
2017	38	15	4	39,0																			
2017	38	61	4	39,0																			
2017	38	118	4	38,9																			
2017	38	252	4	39,2																			
2017	38	510	4	39,5																			
2017	39	15	4	39,2	0,00	0	0	0	0	13,08	10,53	107	31,80	30,20	10,10	18,10	22,80	5,24	5,55	1,90	0,09	0,05	0,25
2017	39	61	4	39,2	0,00	0	0	0	0	9,55	10,26	109	33,30	32,40	10,60	17,50	20,00	3,85	4,29	1,05	0,17	0,08	0,11
2017	39	118	4	39,2	0,00	0	0	0	0	13,41	10,17	107	31,00	30,50	10,60	18,60	23,80	4,44	8,25	0,33	0,22	0,14	0,03
2017	39	252	4	39,4	0,00	0	0	0	0	14,73	12,58	124	35,80	28,40	9,90	17,30	24,70	7,07	5,57	1,66	0,14	0,13	0,15
2017	39	510	4	39,6	0,00	0	0	0	0	9,09	10,24	105	28,90	28,20	10,20	18,70	28,40	4,78	3,74	0,35	0,11	0,06	0,06
2017	40	15	4	39,4																			
2017	40	61	4	39,8																			
2017	40	118	4	39,0																			
2017	40	252	4	39,0																			
2017	40	510	4	39,2																			
2017	41	15	4	39,3																			
2017	41	61	4	38,9																			
2017	41	118	4	39,0																			
2017	41	252	4	39,1																			
2017	41	510	4	39,3																			
2017	42	15	4	39,5	0,00	0	0	0	0	17,28	10,57	110	31,60	29,90	10,40	18,10	23,30	8,85	7,17	1,07	0,08	0,09	0,02
2017	42	61	4	38,9	0,00	0	0	0	0	8,85	10,19	110	32,90	32,30	10,80	17,60	20,30	3,18	4,15	1,19	0,19	0,05	0,10
2017	42	118	4	39,0	0,00	0	0	0	0	14,99	10,34	107	31,30	30,30	10,30	18,50	23,70	5,31	8,35	0,87	0,30	0,14	0,02
2017	42	252	4	39,2	0,00	0	0	0	0	12,85	11,90	118	33,70	28,30	9,90	17,10	24,80	5,69	4,97	1,83	0,12	0,10	0,14
2017	42	510	4	39,2	0,00	0	0	0	0	7,39	10,26	105	29,10	28,30	10,20	18,80	28,50	2,80	3,77	0,57	0,11	0,05	0,09
2018	0	26	5	39,0	0,00	0	0	0	0	12,71	11,40	119	34,80	30,60	10,40	17,50	20,10	3,82	8,07	0,27	0,37	0,08	0,09
2018	0	38	5	38,8	0,00	0	0	0	0	12,26	10,67	116	32,10	30,00	10,90	17,20	22,50	4,91	6,72	0,43	0,09	0,07	0,03
2018	0	92	5	39,4	0,00	0	0	0	0	11,03	10,51	114	33,70	32,00	10,90	18,10	21,30	3,47	7,24	0,14	0,07	0,07	0,02
2018	0	143	5	39,8	0,00	0	0	0	0	12,35	12,34	119	34,10	27,60	9,70	17,30	23,00	5,33	6,55	0,25	0,10	0,06	0,06
2018	0	150	5	39,7	0,00	0	0	0	0	7,36	10,97	119	33,80	30,80	10,90	17,90	23,00	2,28	4,62	0,37	0,05	0,03	0,01
2018	1	26	5	38,8																			
2018	1	38	5	39,0																			
2018	1	92	5	38,7																			
2018	1	143	5	39,5																			
2018	1	150	5	39,2																			
2018	2	26	5	39,0	34,60	0	0	44	1,7	10,59	11,64	121	35,50	30,50	10,40	17,50	19,90	3,16	6,90	0,16	0,14	0,08	0,15
2018	2	38	5	40,0	35,00	0	0	20	1,3	6,97	10,84	116	32,20	29,80	10,70	17,20	22,70	2,50	3,98	0,42	0,03	0,03	0,01
2018	2	92	5	40,1	0,00	0	0	102	2,0	13,92	10,13	111	31,80	31,40	10,90	18,00	21,60	7,04	6,30	0,22	0,07	0,04	0,25
2018	2	143	5	39,5	0,00	0	0	145	2,2	9,18	11,92	117	32,90	27,60	9,80	17,10	22,10	3,46	5,30	0,30	0,06	0,03	0,02
2018	2	150	5	39,3	0,00	0	0	33	1,5	4,42	11,05	121	33,90	30,70	10,90	17,90	22,40	1,58	2,54	0,15	0,06	0,01	0,08
2018	3	26	5	39,5																			
2018	3	38	5	39,6																			
2018	3	92	5	40,1																			
2018	3	143	5	40,0																			
2018	3	150	5	41,3																			
2018	4	26	5	41,7	0,00	0	10	175115	5,2	5,93	10,11	110	31,00	30,70	10,90	17,20	19,70	3,06	2,50	0,19	0,06	0,02	0,11
2018	4	38	5	41,5	0,00	0	20	106817	5,0	6,94	9,94	107	29,60	29,80	10,80	16,90	22,60	3,30	3,17	0,14	0,03	0,02	0,28
2018	4	92	5	41,8	34,50	0	0	315074	5,5	6,91	9,01	102	29,20	32,40	11,30	17,20	19,10	3,47	3,10	0,09	0,06	0,02	0,16
2018	4	143	5	41,6	0,00	0	0	174751	5,2	5,19	10,04	98	27,90	27,80	9,80	16,80	23,80	2,36	2,49	0,18	0,04	0,01	0,12

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2018	4	150	5	42,1	0,00	1	0	154958	5,2	3,86	9,82	107	29,80	30,40	10,90	17,60	22,40	2,13	1,32	0,28	0,06	0,01	0,06
2018	5	26	5	41,9																			
2018	5	38	5	41,8																			
2018	5	92	5	41,5																			
2018	5	143	5	42,0																			
2018	5	150	5	41,7																			
2018	6	26	5	40,9	0,00	1	0	219661	5,3	5,32	10,58	112	31,10	29,40	10,50	17,50	21,20	2,46	2,32	0,41	0,04	0,02	0,07
2018	6	38	5	41,2	0,00	1	0	94427	5,0	3,82	10,25	112	29,90	29,20	10,90	17,40	24,90	1,70	1,64	0,38	0,02	0,00	0,07
2018	6	92	5	41,3	0,00	1	0	823909	5,9	7,71	9,66	108	30,00	31,00	11,20	18,00	22,50	4,42	2,67	0,19	0,09	0,01	0,33
2018	6	143	5	41,5	34,66	1	0	194630	5,3	4,29	10,54	105	29,20	27,70	10,00	17,20	23,10	2,33	1,64	0,04	0,02	0,01	0,24
2018	6	150	5	41,2	0,00	1	0	148282	5,2	3,40	9,55	107	28,20	29,50	11,20	18,20	25,70	2,20	0,86	0,07	0,04	0,00	0,23
2018	7	26	5	41,0																			
2018	7	38	5	41,1																			
2018	7	92	5	41,3																			
2018	7	143	5	41,4																			
2018	7	150	5	41,4																			
2018	8	26	5	40,8	0,00	1	640	140926	5,1	4,56	9,70	103	29,10	30,00	10,60	17,40	20,40	1,67	2,30	0,37	0,05	0,02	0,15
2018	8	38	5	40,5	0,00	1	10	85317	4,9	3,17	9,29	99	27,60	29,70	10,60	17,10	23,30	0,86	1,80	0,17	0,02	0,02	0,30
2018	8	92	5	40,9	0,00	1	160	302937	5,5	5,18	8,77	98	27,40	31,20	11,20	18,20	22,20	2,68	2,21	0,16	0,05	0,01	0,06
2018	8	143	5	41,5	0,00	1	320	232546	5,4	4,59	9,84	97	27,50	27,90	9,90	17,10	22,90	2,51	1,76	0,19	0,03	0,01	0,10
2018	8	150	5	41,0	0,00	1	320	66876	4,8	1,87	8,12	90	24,20	30,40	11,00	18,30	24,80	1,02	0,68	0,05	0,02	0,00	0,10
2018	9	26	5	40,5																			
2018	9	38	5	40,3																			
2018	9	92	5	40,1																			
2018	9	143	5	41,2																			
2018	9	150	5	40,3																			
2018	10	26	5	40,5	0,00	1	640	26880	4,43	5,50	10,11	104	30,40	30,10	10,30	17,40	20,60	0,98	3,79	0,54	0,03	0,05	0,13
2018	10	38	5	39,2	0,00	1	320	705	2,85	4,28	9,20	101	28,10	30,50	11,00	17,40	23,20	0,23	3,18	0,64	0,03	0,06	0,14
2018	10	92	5	40,4	0,00	1	160	52362	4,72	5,07	8,92	97	28,00	31,30	10,80	18,20	22,00	1,23	3,48	0,07	0,03	0,06	0,20
2018	10	143	5	40,7	0,00	1	640	112314	5,05	3,47	8,64	85	24,10	27,90	9,90	16,90	23,30	1,26	1,90	0,21	0,04	0,03	0,02
2018	10	150	5	40,0	0,00	1	320	3148	3,50	2,63	8,82	97	27,00	30,60	11,00	18,00	24,20	0,31	1,92	0,31	0,02	0,04	0,03
2018	11	26	5	40,0																			
2018	11	38	5	39,5																			
2018	11	92	5	40,0																			
2018	11	143	5	40,4																			
2018	11	150	5	39,9																			
2018	12	26	5	39,6																			
2018	12	38	5	39,5																			
2018	12	92	5	39,7																			
2018	12	143	5	40,0																			
2018	12	150	5	39,8																			
2018	13	26	5	39,1																			
2018	13	38	5	39,2																			
2018	13	92	5	39,3																			
2018	13	143	5	40,2																			
2018	13	150	5	40,8																			
2018	14	26	5	39,4	0,00	1	480	8466	3,93	5,79	10,73	111	32,80	30,60	10,30	17,40	20,60	1,31	3,47	0,73	0,03	0,03	0,21
2018	14	38	5	39,1	0,00	1	320	43	1,65	6,85	9,85	109	30,30	30,80	11,00	18,60	24,50	0,19	5,89	0,35	0,01	0,07	0,34
2018	14	92	5	39,4	0,00	1	160	1050	3,02	4,17	9,43	105	30,20	32,00	11,10	20,10	23,00	0,36	2,75	0,93	0,02	0,02	0,09
2018	14	143	5	40,1	0,00	1	320	133	2,13	2,88	9,69	101	28,60	29,50	10,40	20,40	25,70	0,30	1,91	0,55	0,02	0,01	0,08
2018	14	150	5	40,2	0,00	1	160	243	2,39	2,97	8,97	99	27,90	31,10	11,10	18,60	24,50	0,54	1,91	0,46	0,03	0,01	0,03
2018	15	26	5	40,1																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc 10^9/l 4,5-12,0	Rbc 10^{12}/l 9,0-14,0	Hgb g/l 90-140	Hct % 27,0-40,0	MCV fl 25,0-35,0	MCH pg 8,0-12,0	RDW % 15,0-30,0	HDW g/l 17,0-25,0	Neut 10^9/l 0,8-5,0	Lymp 10^9/l 1,2-6,0	Mono 10^9/l <0,75	Eos 10^9/l <1,0	Baso 10^9/l <0,3	LUC 10^9/l <0,25
2018	15	38	5	39,3																			
2018	15	92	5	39,3																			
2018	15	143	5	39,8																			
2018	15	150	5	39,9																			
2018	16	26	5	38,5																			
2018	16	38	5	38,8																			
2018	16	92	5	39,2																			
2018	16	143	5	39,9																			
2018	16	150	5	39,6																			
2018	17	26	5	38,8																			
2018	17	38	5	39,1																			
2018	17	92	5	39,0																			
2018	17	143	5	39,6																			
2018	17	150	5	39,2																			
2018	18	26	5	39,1	0,00	1	240	8119	3,91	7,84	10,05	109	31,00	30,80	10,80	17,40	20,00	2,47	4,77	0,43	0,07	0,05	0,04
2018	18	38	5	39,1	0,00	1	320	39	1,60	8,15	9,50	106	29,40	31,00	11,10	18,50	24,50	1,95	5,18	0,93	0,05	0,04	0,01
2018	18	92	5	39,5	0,00	1	40	3152	3,50	5,66	9,15	103	30,00	32,70	11,20	19,40	21,60	0,97	3,89	0,73	0,05	0,02	0,01
2018	18	143	5	39,5	0,00	1	240	1994	3,30	6,19	9,52	101	28,70	30,20	10,60	21,30	26,60	1,26	4,29	0,46	0,09	0,06	0,02
2018	18	150	5	39,1	0,00	1	160	760	2,88	3,55	9,02	101	28,20	31,20	11,20	18,20	24,10	0,83	2,12	0,18	0,20	0,01	0,21
2018	19	26	5	39,1																			
2018	19	38	5	38,9																			
2018	19	92	5	39,2																			
2018	19	143	5	39,8																			
2018	19	150	5	39,2																			
2018	20	26	5	38,7																			
2018	20	38	5	39,8																			
2018	20	92	5	39,0																			
2018	20	143	5	39,6																			
2018	20	150	5	38,8																			
2018	21	26	5	38,7	0,00	1	320	0	0,00	9,63	10,72	113	32,50	30,30	10,60	17,60	21,40	2,55	6,67	0,11	0,11	0,05	0,15
2018	21	38	5	40,7	0,00	1	120	172	2,24	5,55	9,94	108	30,10	30,30	10,90	18,40	25,20	0,41	4,76	0,13	0,03	0,03	0,19
2018	21	92	5	38,9	0,00	1	40	0	0	9,06	9,69	105	30,70	31,60	10,80	19,20	22,80	2,71	5,83	0,40	0,05	0,04	0,02
2018	21	143	5	39,5	0,00	1	320	0	0	7,88	10,30	106	30,50	29,60	10,30	20,30	25,40	2,21	5,07	0,42	0,13	0,04	0,02
2018	21	150	5	38,9	0,00	1	160	0	0	5,17	10,07	106	30,60	30,40	10,60	18,20	25,70	1,36	3,52	0,11	0,04	0,03	0,11
2018	0	13	6	39,6	0,00	0	0	0	0	11,26	11,17	117	33,30	29,80	10,50	17,70	23,70	3,51	7,23	0,28	0,06	0,09	0,09
2018	0	15	6	39,0	0,00	0	0	0	0	10,25	11,13	117	32,40	29,10	10,50	17,40	22,20	1,93	7,78	0,23	0,07	0,05	0,18
2018	0	41	6	38,8	0,00	0	0	0	0	10,56	12,00	119	32,40	27,00	9,90	19,30	25,40	2,76	6,92	0,63	0,10	0,05	0,09
2018	0	99	6	39,2	0,00	0	0	0	0	7,19	10,64	111	32,40	30,50	10,40	16,10	22,10	2,33	4,50	0,18	0,06	0,04	0,08
2018	0	220	6	39,0	0,00	0	0	0	0	8,85	12,49	130	35,20	28,20	10,40	18,10	26,50	2,05	5,91	0,69	0,06	0,06	0,08
2018	1	13	6	39,3																			
2018	1	15	6	38,8																			
2018	1	41	6	38,7																			
2018	1	99	6	39,0																			
2018	1	220	6	38,9																			
2018	2	13	6	39,1	0,00	0	0	0	0	10,31	11,89	125	35,80	30,10	10,50	17,70	23,20	2,71	7,00	0,27	0,04	0,05	0,23
2018	2	15	6	39,2	0,00	0	0	0	0	9,22	11,13	115	32,40	29,10	10,40	17,50	21,60	1,88	6,40	0,75	0,04	0,04	0,11
2018	2	41	6	38,9	0,00	0	0	0	0	10,12	12,16	118	32,90	27,10	9,70	19,40	25,30	3,18	6,46	0,31	0,09	0,07	0,01
2018	2	99	6	39,6	0,00	0	0	0	0	8,39	11,19	115	34,00	30,40	10,30	16,20	21,70	3,60	4,39	0,30	0,04	0,03	0,02
2018	2	220	6	40,1	0,00	0	0	0	0	9,15	11,85	121	33,10	27,90	10,20	18,00	25,70	3,53	5,28	0,17	0,05	0,04	0,09
2018	3	13	6	40,0																			
2018	3	15	6	39,1																			
2018	3	41	6	38,6																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l	10^9/l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2018	3	99	6	39,2																			
2018	3	220	6	39,1																			
2018	4	13	6	39,1	0,00	0	0	0	0	10,72	12,19	130	35,80	29,40	10,60	17,70	22,90	2,32	7,76	0,08	0,13	0,05	0,37
2018	4	15	6	39,2	0,00	0	0	0	0	9,46	11,41	121	32,90	28,80	10,60	17,80	22,90	1,82	6,87	0,61	0,08	0,06	0,02
2018	4	41	6	39,2	0,00	0	0	0	0	10,48	11,73	117	33,00	28,10	10,00	18,80	21,90	2,92	6,79	0,51	0,12	0,06	0,07
2018	4	99	6	39,2	0,00	0	0	0	0	6,87	11,00	116	34,20	31,10	10,50	15,90	22,30	1,77	4,70	0,15	0,11	0,05	0,08
2018	4	220	6	40,1	0,00	0	0	0	0	8,99	12,84	132	35,50	27,70	10,30	18,00	24,50	2,53	5,60	0,53	0,07	0,05	0,20
2018	5	13	6	39,7																			
2018	5	15	6	39,3																			
2018	5	41	6	39,0																			
2018	5	99	6	39,5																			
2018	5	220	6	39,8																			
2018	6	13	6	39,0	0,00	0	0	0	0	11,13	12,10	128	36,50	30,20	10,60	17,70	23,50	2,17	7,93	0,49	0,04	0,09	0,41
2018	6	15	6	38,9	0,00	0	0	0	0	9,74	10,76	113	31,20	29,00	10,50	17,50	22,30	2,03	6,52	0,92	0,07	0,04	0,16
2018	6	41	6	38,9	0,00	0	0	0	0	9,49	11,04	111	29,90	27,00	10,10	19,50	25,60	2,54	6,19	0,52	0,07	0,06	0,12
2018	6	99	6	39,4	0,00	0	0	0	0	6,98	10,70	114	32,70	30,60	10,60	16,30	22,90	2,02	4,54	0,10	0,02	0,04	0,25
2018	6	220	6	40,1	0,00	0	0	0	0	8,42	11,73	121	32,40	27,60	10,40	17,90	26,40	2,26	5,60	0,44	0,04	0,04	0,04
2018	7	13	6	39,2																			
2018	7	15	6	39,2																			
2018	7	41	6	39,0																			
2018	7	99	6	39,3																			
2018	7	220	6	39,7																			
2018	8	13	6	39,1	0,00	0	0	0	0	9,35	11,46	120	35,20	30,70	10,50	17,50	22,40	1,75	6,71	0,46	0,04	0,07	0,31
2018	8	15	6	39,0	0,00	0	0	0	0	9,50	10,57	113	31,10	29,40	10,70	17,30	21,20	3,17	5,74	0,33	0,06	0,03	0,17
2018	8	41	6	39,4	0,00	0	0	0	0	11,73	10,58	106	28,60	27,00	10,00	19,50	24,90	5,27	5,58	0,48	0,08	0,04	0,28
2018	8	99	6	39,3	0,00	0	0	0	0	6,29	10,36	108	31,70	30,60	10,50	16,00	21,60	2,10	3,76	0,24	0,05	0,04	0,10
2018	8	220	6	39,0	0,00	0	0	0	0	8,48	11,70	122	32,60	27,90	10,40	18,00	25,90	1,99	5,98	0,20	0,06	0,06	0,20
2018	9	13	6	39,0																			
2018	9	15	6	38,7																			
2018	9	41	6	38,8																			
2018	9	99	6	38,9																			
2018	9	220	6	38,8																			
2018	10	13	6	39,1	0,00	0	0	0	0	9,52	11,55	123	35,00	30,30	10,60	17,70	23,90	2,32	6,60	0,47	0,04	0,06	0,04
2018	10	15	6	39,0	0,00	0	0	0	0	10,53	10,38	110	30,10	29,00	10,60	17,40	22,10	3,17	6,32	0,89	0,07	0,03	0,05
2018	10	41	6	39,1	0,00	0	0	0	0	9,10	10,71	105	29,00	27,10	9,80	19,70	25,70	2,57	5,91	0,45	0,10	0,05	0,02
2018	10	99	6	39,0	0,00	0	0	0	0	5,79	10,24	107	31,60	30,80	10,40	16,20	22,30	1,67	3,74	0,27	0,05	0,05	0,02
2018	10	220	6	38,8	0,00	0	0	0	0	7,58	11,62	121	33,00	28,40	10,40	18,20	26,20	2,08	5,04	0,26	0,06	0,05	0,08
2018	11	13	6	39,2																			
2018	11	15	6	39,1																			
2018	11	41	6	38,6																			
2018	11	99	6	38,9																			
2018	11	220	6	38,8																			
2018	12	13	6	39,5																			
2018	12	15	6	39,2																			
2018	12	41	6	38,2																			
2018	12	99	6	39,2																			
2018	12	220	6	39,1																			
2018	13	13	6	39,1																			
2018	13	15	6	38,9																			
2018	13	41	6	38,7																			
2018	13	99	6	38,9																			
2018	13	220	6	38,6																			

Year	Day of experiment	Animal no	Group	Rectal temperature °C	TBEV PCR Ct.	TBE ELISA	TBE titer	*A. phagocytophilum* PCR concentration copies/µL	*A. phagocytophilum* PCR concentration LOG10 copies/µL	Wbc	Rbc	Hgb	Hct	MCV	MCH	RDW	HDW	Neut	Lymp	Mono	Eos	Baso	LUC
										10^9/ l	10^{12}/l	g/l	%	fl	pg	%	g/l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l	10^9/ l
										4,5-12,0	9,0-14,0	90-140	27,0-40,0	25,0-35,0	8,0-12,0	15,0-30,0	17,0-25,0	0,8-5,0	1,2-6,0	<0,75	<1,0	<0,3	<0,25
2018	14	13	6	39,2	0,00	0	0	0	0	11,29	11,37	120	34,60	30,50	10,60	17,60	23,40	2,80	7,70	0,53	0,06	0,07	0,13
2018	14	15	6	38,9	0,00	0	0	0	0	9,00	10,85	114	31,90	29,40	10,50	17,50	22,50	2,52	5,59	0,70	0,05	0,03	0,12
2018	14	41	6	38,8	0,00	0	0	0	0	9,01	10,69	107	29,10	27,30	10,00	19,80	26,20	2,52	5,97	0,39	0,06	0,05	0,01
2018	14	99	6	39,1	0,00	0	0	0	0	6,77	10,19	108	31,60	31,10	10,60	16,10	22,30	2,63	3,86	0,11	0,03	0,04	0,09
2018	14	220	6	38,9	0,00	0	0	0	0	8,86	11,49	120	32,70	28,50	10,40	18,20	26,20	2,51	5,98	0,10	0,05	0,06	0,15
2018	15	13	6	39,7																			
2018	15	15	6	39,1																			
2018	15	41	6	38,9																			
2018	15	99	6	39,3																			
2018	15	220	6	38,6																			
2018	16	13	6	39,6																			
2018	16	15	6	39,3																			
2018	16	41	6	38,7																			
2018	16	99	6	39,2																			
2018	16	220	6	38,8																			
2018	17	13	6	38,5																			
2018	17	15	6	39,0																			
2018	17	41	6	38,7																			
2018	17	99	6	38,8																			
2018	17	220	6	38,2																			
2018	18	13	6	39,6	0,00	0	0	0	0	10,82	10,74	113	31,90	29,70	10,50	17,60	24,10	3,27	6,65	0,71	0,11	0,06	0,03
2018	18	15	6	39,1	0,00	0	0	0	0	9,03	10,10	108	29,20	28,90	10,60	17,60	22,80	2,34	5,84	0,62	0,09	0,04	0,10
2018	18	41	6	39,2	0,00	0	0	0	0	10,94	11,15	113	31,30	28,10	10,10	19,20	23,10	3,44	6,77	0,51	0,10	0,05	0,08
2018	18	99	6	39,0	0,00	0	0	0	0	6,86	9,82	110	30,50	31,10	11,20	15,90	22,50	2,77	3,61	0,37	0,06	0,04	0,01
2018	18	220	6	38,9	0,00	0	0	0	0	9,00	11,62	120	32,60	28,10	10,30	18,10	25,30	2,79	5,61	0,44	0,08	0,06	0,02
2018	19	13	6	39,8																			
2018	19	15	6	39,4																			
2018	19	41	6	39,5																			
2018	19	99	6	39,6																			
2018	19	220	6	39,0																			
2018	20	13	6	39,1																			
2018	20	15	6	38,9																			
2018	20	41	6	38,8																			
2018	20	99	6	39,0																			
2018	20	220	6	38,9																			
2018	21	13	6	38,9	0,00	0	0	0	0	11,08	11,12	115	32,90	29,60	10,30	17,60	25,00	2,92	7,36	0,62	0,07	0,07	0,03
2018	21	15	6	39,1	0,00	0	0	0	0	4,53	10,34	107	29,40	28,40	10,40	17,50	23,80	1,79	2,28	0,41	0,03	0,01	0,00
2018	21	41	6	38,8	0,00	0	0	0	0	8,98	11,22	110	30,10	26,80	9,80	19,50	26,90	2,17	5,95	0,65	0,05	0,04	0,12
2018	21	99	6	38,9	0,00	0	0	0	0	6,23	10,97	114	33,70	30,70	10,40	16,30	22,80	1,84	3,83	0,44	0,06	0,05	0,01
2018	21	220	6	38,9	0,00	0	0	0	0	8,97	11,35	117	31,40	27,70	10,30	18,40	27,60	2,30	6,29	0,14	0,06	0,04	0,13

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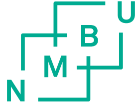

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

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