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Intrauterine and tick-transmission of Anaplasma phagocytophilum in persistently infected lambs

Master of science in Biological Chemistry

By
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Universitetet i Stavanger

Norges miljø- og biovitenskapelige universitet

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Anne Mette Cruys Sagen

Abstract

The most important tick borne pathogen in animals is *Anaplasma phagocytophilum* (*A. phagocytophilum* and the main vector transmitting this pathogen is the tick *Ixodes ricinus (I. ricinus).* Tick-borne fever and anaplasmosis caused by this bacterium is one of the most common vector borne pathogens in Norway and Europe, *A. phagocytophilum* is widespread on south western coast areas in Norway. It has been estimated that more than 300. 000 lambs are infected with *A. phagocytophilum* each year and that it contribute to severe welfare challenges and economic losses for the Norwegian sheep industry

A. phagocytophilum causes a persistent infection in several animal species, which contributes to the transmission and spread of the bacterium. The objectives of this study were to investigate if persistently infected sheep could transmit the bacterium *A. phagocytophilum* via intrauterine transmittance pathway to its newborn lambs, and in addition to determine if persistently infected sheep could transmit the bacterium to *I. ricinus* ticks around one year after the primary infection. The study included twelve persistently infected lambs previously inoculated with *A. phagocytophilum* (M73220) and one uninfected control. The lambs were followed up with measurement of rectal temperature and blood sampling every day the first month after primary infection, thereafter blood were collected monthly. The lambs were mated and nine were pregnant. Blood samples were collected from newborn lambs and from tissue samples if the lamb died. Eleven to twelve months after primary infection the ewes were grafted with nymphs, there were taken blood samples of the ewes and engorged nymphs were collected within 7 days. Blood samples, tissue samples and nymphs were analyzed with real-time PCR (qPCR), if PCR positive the samples were further analyzed by semi-nested PCR. The PCR positive samples from semi-nested PCR were further investigated by sequencing. Thirty percent of newborn lambs were PCR positive (qPCR) including dead lambs. One sample from one newborn lamb was verified for *A. phagocytophilum* by sequencing. In addition nymphs (33 %) from altogether four lambs were PCR positive. The present study indicates tick transmission and intrauterine transmission of *A. phagocytophilum* in persistently infected sheep. The persistently infected sheep can carry the bacterium from one grazing pasture to another and to ticks around one year after primary infection. Offspring of persistently infected sheep may also be infected. The importance of these findings for the epidemiology of *A. phagocytophilum* is unknown and needs to be further investigated.

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Abbreviations

BLAST	Basic Local Alignment Tool
Bp	Base pairs
Cp	Crossing Point
DNA	Deoxyribonucleotide Acid
dNTP	Deoxynucleotide
ddNTP	Dideoxynucleotide
ELISA	Enzyme Linked Immunosorbent Assay
HGA	Human Granulocytic Anaplasmosis
IFA	Immunofluorescent Antibody Test
MGPs	Magnetic Particle Suspension
MSIS	Register for communicable diseases in Norway (Meldingssystem for smittsomme sykdommer)
MSP	Major Surface Protein
NCBI	National Center for Biotechnology Information
NMBU	Norwegian University of Life Sciences
NKS	Norwegian White Breed
PCR	Polymerase Chain Reaction
p.i.	Post Infection
qPCR	Real-Time Polymerase Chain Reaction
SUS	Stavanger University Hospital
TBE	Tick-borne Encephalitis
TBF	Tick-borne Fever
T_m	Melting temperature
UIS	University of Stavanger

1.0 Introduction

1.1 Aims of the study

A. phagocytophilum which is the causative agent of tick-borne fever is widespread on pastures in Norway and can cause severe economic losses and contribute to welfare challenges in sheep flocks. It has been estimated that more than 300. 000 lambs are infected each year (Stuen et al. 2002a). The infection causes immunosuppression in the affected animals which may result in reduced productivity, secondary infections and crippling conditions (Foggie 1951). In addition, *A. phagocytophilum* establishes a persistent infection in affected animals, mainly due to antigenic variation in the major surface proteins (MSP) (Brayton et al. 2001). Earlier study indicated that the bacterium can persist for 25 months in infected sheep (Foggie 1951), this allows the bacterium to be carried from one pasture season to another. The bacterium can also cause human granulocytic anaplasmosis and is an emerging concern for human health especially in the US (Dumler et al. 2001, Thomas et al. 2009)

The objectives of this study were to investigate if the persistently infected sheep could transmit the bacterium *A. phagocytophilum* via intrauterine transmittance pathway to its newborn lambs, and in addition determine if persistently infected sheep could transmit the bacterium to *I. ricinus* ticks approximately one year after initial infection.

1.2 History

The disease tick-borne fever (TBF) was first described in Scotland in 1932 where sheep in a flock got a fever reaction by grazing on tick-infested pastures. The disease could also be transmitted by blood inoculation to other sheep (Stockman 1918, Gordon 1932). In 1933, the tick *I. ricinus* was found to be the main vector of transmittance (MacLeod 1933). The causative agent of TBF was first described in 1940 as *Rickettsia phagocytophila* due to its morphological similarity to rickettsias (Foggie 1951). The microorganism was named *Ehrlichia phagocytophila* (*E. phagocytophila*) in 1974 in Bergey`s manual of determinative bacteriology (Phillip 1974) and has recently been named *Anaplasma phagocytophilum* (Dumler et al. 2001). In Norway, a serious disease in ruminants named sjodogg was described already in 1780 and in 1939 sjodogg was confirmed to be the same as TBF (Thorshaug 1940).

1.3 *Ixodes ricinus*

1.3.1 Classification

The main vector of *A. phagocytophilum,* the tick *I. ricinus,* is an arthropod (Table 1.3.1) which is an external parasite that feeds exclusively on blood from different hosts (Mehl 1987).

Table 1.3.1 Classification of *Ixodes ricinus* (Sonenshine and Roe 2014).

Kingdom	*Animalia*
Phylum	*Arthropoda*
Class	*Arachnida*
Subclass	*Acari*
Order	*Ixodida*
Superorder	*Parasitiformes*
Family	*Ixodidae*
Genus	*Ixodes*
Species	*Ixodes ricinus*

1.3.2 Distribution

Twelve different tick species have earlier been detected in Norway (Mehl 1987). In recent years, due to migrating birds, new tick species have been reported such as *Hyalomma rufipes* and *Dermacentor spp* (Hasle et al. 2009). However, the most important tick species is *I. ricinus.* This tick has been known for centuries in Norway and has several local geographical names, including flått, fløtt, skautroll, lyngbobb, skinnflått, einebærlus, hantikk, påte, rauarev, kuflått, skoglus and several more (Tambs-Lyche 1990). *I. ricinus* is widely distributed in Norway along the coast of southern Norway (Figure 1.3.1), it prefers humid woodland areas (70-80 % humidity) and shelter in the ground vegetation (Sonenshine and Roe 2014).

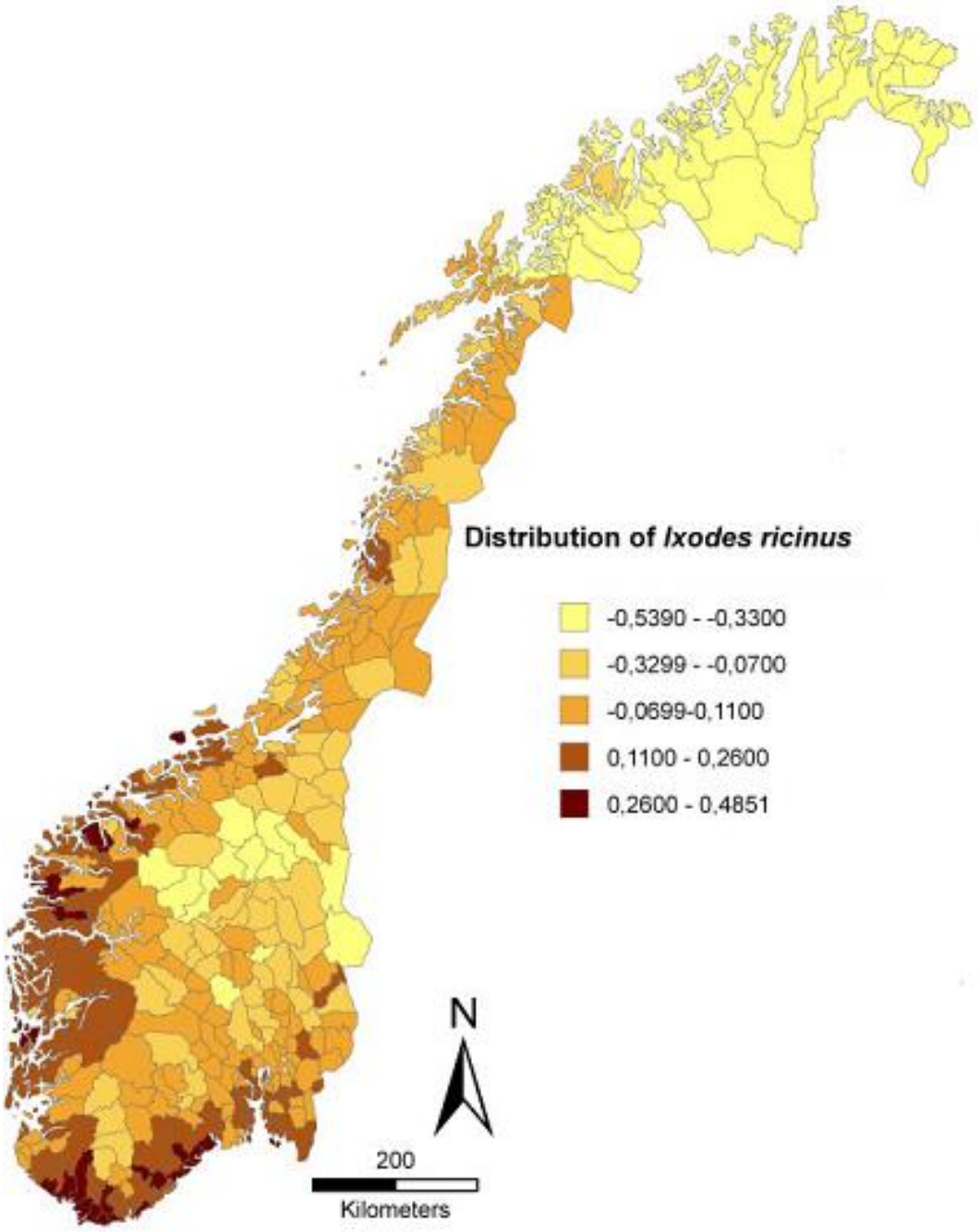

Figure 1.3.1 *Ixodes ricinus* distribution in Norway. The distribution map displays tick detection within municipalities, abundance with increasing darkness of color (Jore et al. 2011).

1.3.3 Lifecycle

The lifecycle of *I. ricinus* consists of four phases' egg, larva, nymph and adult. The larvae have three pairs of legs and are less than 1 mm before the blood meal. The nymphs and adults have four pairs of legs and are approximately 1.5 mm and 3 mm, respectively, before sucking blood. During a blood meal the female tick can expand up to 150 times its original size (Figure 1.3.2) (Mehl 1987, Sonenshine and Roe 2014).

Figure 1.3.2 *I. ricinus* engorged female (Zimmermann 2010).

The tick has to feed and digest a blood meal in order to enter the next stage of development (Figure 1.3.3). *I. ricinus* is a three host tick meaning that the tick feeds of three different hosts during its development cycle. The larvae feed on rodents such as mice and rats, and also small birds. The nymph feeds on birds and small mammals, detaches and molts into a full grown female or male tick. The full-grown female tick feeds on large mammals such as deer, badger and fox and also on domestic animals for instance sheep, cattle, cats, dogs and horses, however the full-grown male tick does seldom feed. The full engorged female tick lays upto 3000 eggs and then dies (Mehl 1987, Talleklint and Jaenson 1996, Sonenshine and Roe 2014). It can be mentioned, that *I. ricinus* can absorb water directly from sub-saturated atmospheres on its mouth parts. In addition, the tick may be host seeking at 4-5 °C and can survive for several weeks down to -7 °C (Sonenshine and Roe 2014).

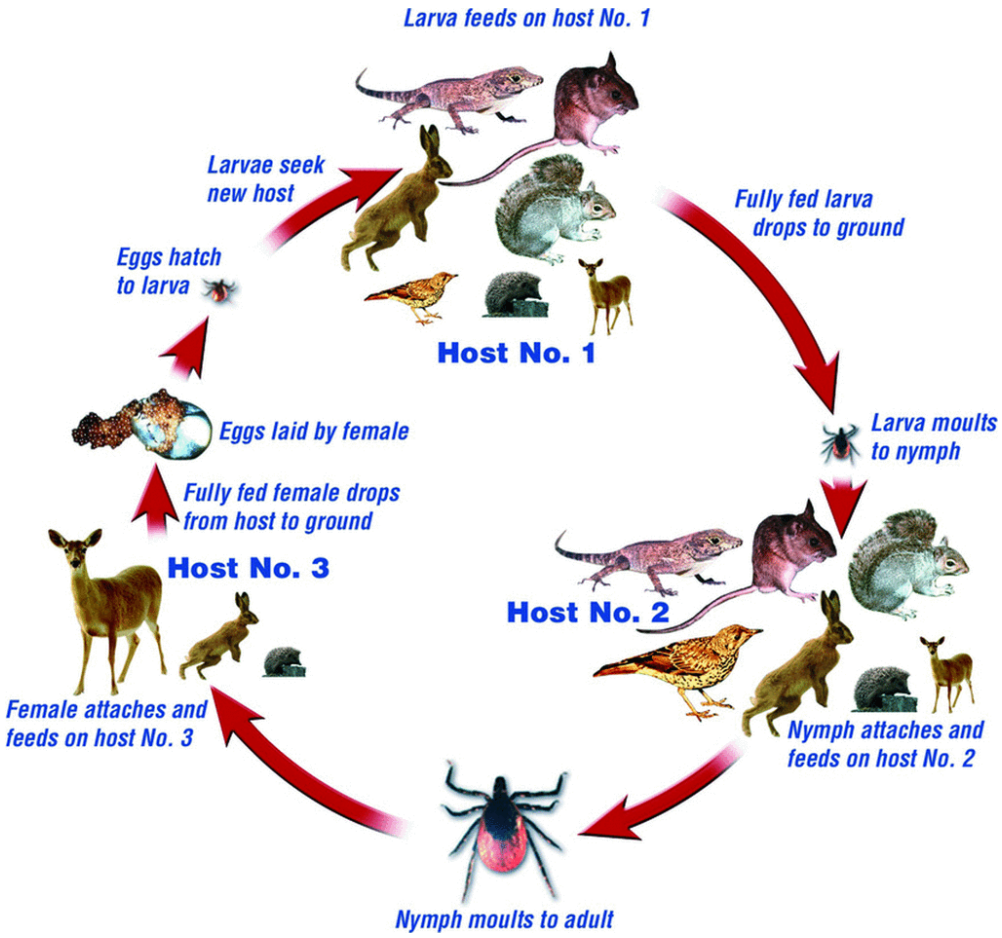

Figure 1.3.3 Life cycle of *Ixodes ricinus*. Each stage takes approximately a year to complete. The relative size of the animals approximates their significance as hosts for the different life-cycle stages in a typical woodland habitat (Mannelli 2012).

1.3.4 Tick – borne pathogens

Ticks in general are the most important disease transmitting vectors due to its transmittance of a great variety of pathogenic organisms, whereas *I. ricinus* is the most important vector of tick-borne diseases of humans and animals in Western Europe (Sonenshine and Roe 2014). In Norway, the main tick-borne pathogens are *Borrelia burgdorferi* sensu lato (*B. burgdorferi s. l.)*, *Babesia divergens (B. divergens), A. phagocytophilum* and Tick-borne encephalitis (TBE) virus (Table 1.3.2). The most important disease in humans (Lyme borreliosis) is caused by *B. burgdorferi s. l.,* while the most important tick borne pathogen in animals is *A. phagocytophilum* (Eldøen et al. 2001, Stuen 2007).

The number of tick-borne diseases in humans in Norway varies. There are approximately 300 annual incidents of disseminated Lyme-borreliosis, approximately 13 incidents a year of TBE, but only one case of human babesiosis has so far been reported (MSIS 2015). Since anaplasmosis is not notifiable, the numbers of identified human cases are unknown, but approximately 20-30 cases have been verified (Stuen, personal information).

Table 1.3.2 The main tick-borne pathogens detected in Norway.

Agents	**Clinical symptoms in humans**	**Cases in Norway**
Bacteria		
Anaplasma phagocytophilum	Fever, headache, myalgia and malaise, Incubation period: 5-10 days Treatment with tetracyclines (Dumler et al. 2007)	Approximately 20-30 incidents (Stuen S, personal information)
Borrelia burgdorferi* sensu lato *(B. afzelii, B. garnii) ***Borrelia burgdorferi* sensu stricto**	Variable, may include fever, rash, facial paralysis and arthritis. (Depending on stage of infection) Incubation period: 3-30 days (Dattwyler and Luft 1991).	Approximately 300 incidents per year (MSIS 2015)
Virus		
Tick-borne encephalitis virus **(Louping-ill)**	Fever, fatigue, malaise headache and neurological symptoms Incubation period: 4-28 days (Mickiene et al. 2002).	Approximately 13 incidents per year (MSIS 2015)
Protozoa		
Babesia divergens	Fever, flu like symptoms, fatigue, body pain and anemia. Incubation period: 1-6 weeks (Wormser et al. 2006)	1 case (2007) (MSIS 2015).

1.4 *Anaplasma phagocytophilum*

1.4.1 Classification

Before reclassification, *A. phagocytophilum* was formerly known as *Ehrlichia phagocytophila, Ehrlichia equi* and human granulocytic ehrlichiosis agent (Dumler et al. 2001). *A. phagocytophilum* belongs to the family *Anaplasmataceae* (Table 1.4.1, Figure 1.4.1) and includes five well-studied genera *Anaplasma, Ehrlichia, Neorickettsia, Aegyptianella* and *Wolbachia,* and two less-studied genera *Candidatus* Neoehrlichia and *Candidatus* Xenohaliotis (Dumler et al. 2001, Rikihisa 2011).

A. phagocytophilum is the causative agent of granulocytic anaplasmosis, also known as TBF, sjodogg or pasture fever in ruminants. The agent has been detected in several other animals, such as dogs and cats (Lewis et al. 1975), goats (MacLeod 1933), cattle (Hudson 1950), horses (Gribble 1969) and roe deer (Stuen 1996). In humans, the disease is now called human granulocytic anaplasmosis (HGA)

Table 1.4.1 Classification of the *Anaplasmataceae* family (Dumler et al. 2001, Rymaszewska and Grenda 2008, Rikihisa 2011, Li et al. 2015)

Family						
Anaplasmataceae						
Order						
Rickettsiales						
Class						
Alphaproteobacteria						
Genus						
Anaplasma	*Ehrlichia*	*Neorickettsia*	*Aegyptianella*	*Wolbachia*	*Candidatus* Neoehrlichia	*Candidatus* Xenohaliotis
Species						
A. marginale *A. platys* *A. phagocytophilum* *A. bovis* *A. ovis* *A. centrale* *A. capra*	*E. ruminantum* *E. muris* *E. chaffeensis* *E. ewingii* *E. canis*	*N. helminthoeca* *N. sennetsu* *N. risticcii*	*A. pallorum*	*W. pipentis*	*Ca.* Neoehrlichia mikurensis *Ca.* Neoehrlichia lotoris	*Ca.* Xenohaliotis *californiensis*

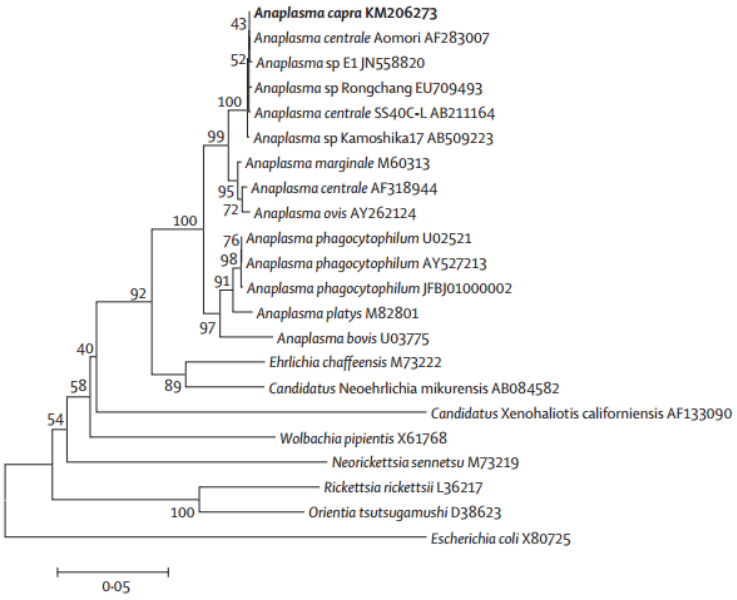

Figure 1.4.1 Phylogenetic members of the genus *Anaplasma* in the family *Anaplasmataceae*. Constructed from analysis of 1250 bp (base pair) of the 16S rRNA gene sequences, the scale bar indicates estimated evolutionary distance. GenBank accession numbers are included (Li et al. 2015).

1.4.2 Morphology

A. phagocytophilum is a pleomorphic gram-negative bacterium (rickettsia), generally 0.4 µm to 2 µm long. The bacterium is obligate intracellular and cannot survive for a long time extracellular (Rikihisa 1991). *A. phagocytophilum* lacks all genes for synthesis of lipid A and most genes for the biosynthesis of peptidoglycan, which is important for structural strength in gram-negative bacteria. The bacteria therefore incorporates cholesterol from the host membrane to ensure stability (Lin and Rikihisa 2003a). *A. phagocytophilum* invades white blood-cells, monocytes and granulocytes by caveolae-mediated endocytosis (Lin and Rikihisa 2003b), whereas the main host cell is the neutrophil. In the neutrophils, the bacterium induces the formation of a membrane-bound niche, a parasitophorus vacuole, which does not fuse with the lysozymes and therefore prohibit the lysosomal destruction. The organism is internalized by the host in separate phagosomes in the cytoplasm (Mott et al. 1999). These data and several microarray studies suggest that *A. phagocytophilum* survives by modulating critical cell signaling mechanisms involved in phagocytic activation and the differentiation of infected neutrophils (Yoshiie et al. 2000, Rikihisa 2011). The bacterium can be visualized as membrane bound inclusions (Figure 1.4.2) (Popov et al. 1998).

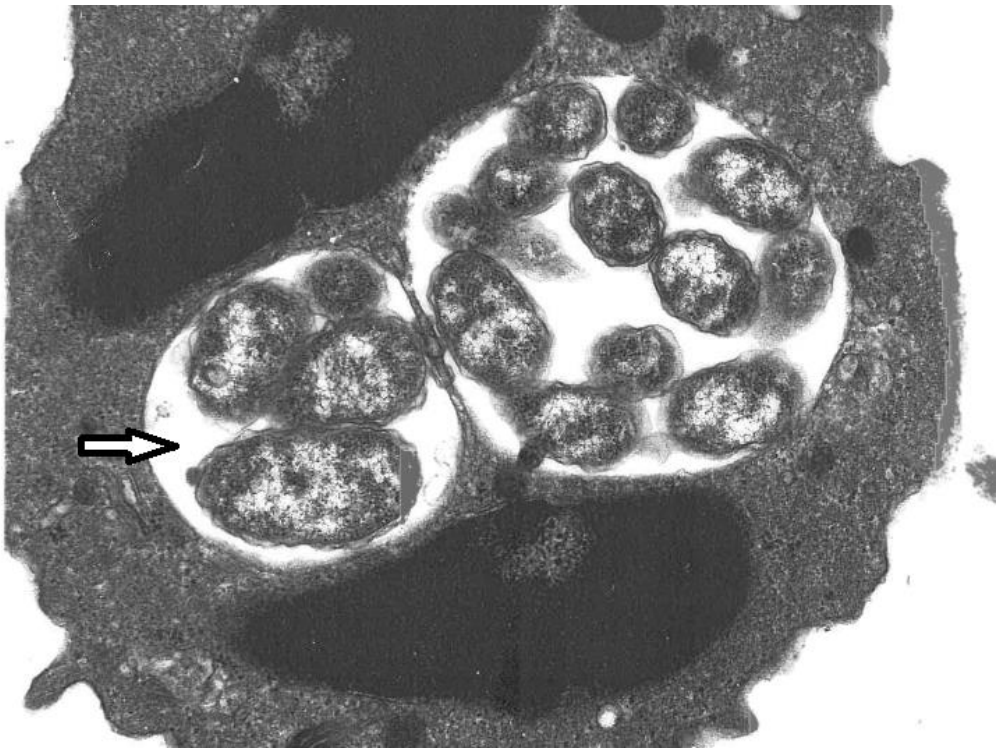

Figure 1.4.2 Electron microphotograph of an *A. phagocytophilum* infected neutrophil granulocyte with cytoplasmic inclusions (morulae) (arrow). The intracellular vacuoles protect the bacterium from phagocytosis (Photo: V. Popov).

1.4.3 Strain variants

Different strain variants of *A. phagocytophilum* is based on sequencing of several genes such as *ankA, groESL, msp4* and 16S rRNA. Variations in the 16S rRNA nucleotide sequence are shown in Table 1.4.2 (Stuen et al. 2002b).

Table 1.4.2 Overview of the differences in the 5`end of the 16S rRNA gene sequence (bp 81-186) of *Anaplasma* strains (Stuen et al. 2002b)

Sequence[a]	Accession no.	Species	Nucleotide change
TTATTCTTT**A**TAGCTTGCTAT**A**AAGAATA**G**TTAGTGGCAGACGGGTGAGTAATGCA	U02521	*A. phagocytophila* prototype	Prototype
.....**A**.....	M73220	*A. phagocytophila* variant 1	100^{A}
.....**G**.....**A**.....	AF336220	*A. phagocytophila* variant 2	80^{G}, 100^{A}
.....**G**.....**A**.....	AJ242784	*A. phagocytophila* variant 3	92^{G}, 100^{A}
.....**G**.....	AJ242783	*A. phagocytophila* variant 4	92^{G}
.....G.....	AY035312	*A. phagocytophila* variant 5	93^{G}
.....**A**.....G.....	AF227954	*E. phagocytophila*	100^{A}, 121^{G}
.....G.....	AF036647	*E. equi*	90^{G}
.....G.....	AF036646	*E. equi*	94^{G}
.....**GG**..G.....	AF205140	HGE agent	92^{G} 93^{G} 96^{G}

[a] Nucleotides at positions 80, 92, and 100, respectively, are indicated in boldface for each sequence.

Anaplasma strains have been found to differ in host specificity and can give different clinical manifestations in infection experiments (Stuen et al. 2003). For example, a Californian strain that was infectious to equines was not infectious to ruminants (Stannard et al. 1969). It has also been shown that animals can be infected by different strains at the same time and that different strains may

interfere with each other in the host (Stuen et al. 2005b). Different species of *Anaplasma* genus with their disease characteristics are shown in Table 1.4.3.

Table 1.4.3 Characteristics of the genus *Anaplasma* (Rymaszewska and Grenda 2008, Li et al. 2015).

Species	Diseases	Hosts	Host cells	Vectors
A. phagocytophilum	Human granulocytic anaplasmosis, TBF, equine ehrlichiosis	Human, horse, small ruminants, rodents, dog, cat, several species	Mainly granulocytes	*Ixodes sp,* occationally other tick species
A. marginale ***A. centrale***	Bovine anaplasmosis	Bovine, deer, rabbit	Erythrocytes, monocytes	*Haemaphysalis sp, Rhipicephalus sp, Amblyomma sp, Ixodes sp*
A. platys	Canine cyclic thrombocytopenia	Dog	Platelets, monocytes	*Rhipicephalus sanguineus*
A. ovis	Ovine anaplasmosis	Ruminants	Erythrocytes	*Dermacentor sp Rhipicephalus sp*
A. bovis	Bovine anaplasmosis	Ruminants	Erythrocytes	*Ixodes sp, Haemaphysalis sp.*
A. capra	Anaplasmosis	Human, ruminants	Erythrocytes	*I. persulcatus*

1.4.4 Clinical characteristics

TBF in ruminants is normally a benign rickettsiosis usually without complications. The most common characteristics of TBF in sheep are high fever, severe neutropenia and inclusions in neutrophils. The incubation period is around 3-14 days by intravenous inoculation or experimental exposal to infected ticks (Gordon 1932, Macleod 1936, Foggie 1951). Fever, often over 41 °C, is the main clinical symptom in the acute phase, where the maximum temperature usually occurs the second day of the bacteremia and can last for one to two weeks (Foggie 1951, Stuen 1993). Neutropenia, abnormal low concentration of neutrophils in the blood, last for approximately 1-2 weeks (Foggie 1951, Campbell et al. 1994). The fever reaction of the sheep may differ according to host species, immunologic response and age of animal involved (Foggie 1951, Woldehiwet and Scott 1982, Stuen 1993, Stuen et al. 1998a).

Different genotypes of *A. phagocytophilum* can give different clinical symptoms (Stuen et al. 1998a). Other clinical signs such as reduced appetite, dullness and coughing have been reported, but are often mild or absent. However death or other clinical symptoms is usually due to secondary infections, because *A. phagocytophilum* causes immunosuppression that could last for at least 6 weeks (see also complications 1.4.6) (Campbell et al. 1994, Larsen et al. 1994, Stuen 1996, Granquist et al. 2008).

In humans, the most frequent clinical symptoms are fever, headache, myalgia and malaise (Aguero-Rosenfeld et al. 1996, Dumler et al. 2005, Bakken and Dumler 2008). More rear symptoms are nausea, vomiting, respiratory distress and neurological involvement (Bakken et al. 1996a, Walker and Dumler 1996, Dumler et al. 2005). Recently studies suggest that HGA is a mild infection that often remains undiagnosed in humans and thereby the infection is resolved without antibiotic therapy. However, severe cases may occur and fatal infection are reported (Bakken et al. 1998, Bakken and Dumler 2015). Severe complications may also occur, such as meningoencephalitis, acute respiratory distress syndrome, acute renal failure, rhabdomyolysis, cranial nerve palsies, coagulopathy, myocarditis and brachial plexopathy (Dumler et al. 2005).

1.4.5 Diagnosis

The diagnosis in sheep is based on clinical manifestations, such as onset of high fever (≥41 °C), hematological changes and presence of cytoplasmic inclusions. Blood smear analysis (Figure 1.4.3) by light-field microscopy in the fever period is usually enough to confirm the diagnosis (Tuomi 1966, Popov et al. 1998, Stuen et al. 2003). Also electron microscopy can be used to verify an acute infection with visualization of cytoplasmic vacuoles (Tuomi 1966, Woldehiwet and Scott 1982, Rikihisa 1991, Popov et al. 1998). Beyond the acute phase of the infection, determination of microbe DNA in blood and tissue is based on polymerase chain reaction (PCR) (Bakken et al. 1994, Stuen et al. 1999). Determination of antibodies against *A. phagocytophilum* can support the diagnosis and is determined either by an indirect immunofluorescent antibody test (IFA), a complement fixation test or a counter-current immunoelectrophoresis test (Webster and Mitchell 1988, Paxton and Scott 1989). An enzyme linked immunosorbent assay (ELISA) method has been reported, but is not yet commercial available (Stuen S, personal information). Antibodies against *A. phagocytophilum* may remain for several years after infection (Paxton and Scott 1989, Stuen et al. 2003). At post mortem, an enlargement of the spleen (splenomegalia), upto 4-5 times its original size, is the main pathological change described (1.4.4) (Gordon 1932, Overas et al. 1993).

In humans, the diagnosis is based on clinical manifestations, blood smear investigation, PCR analysis and serological tests (Bakken et al. 1994, Hardalo et al. 1995, Bakken et al. 1996a).

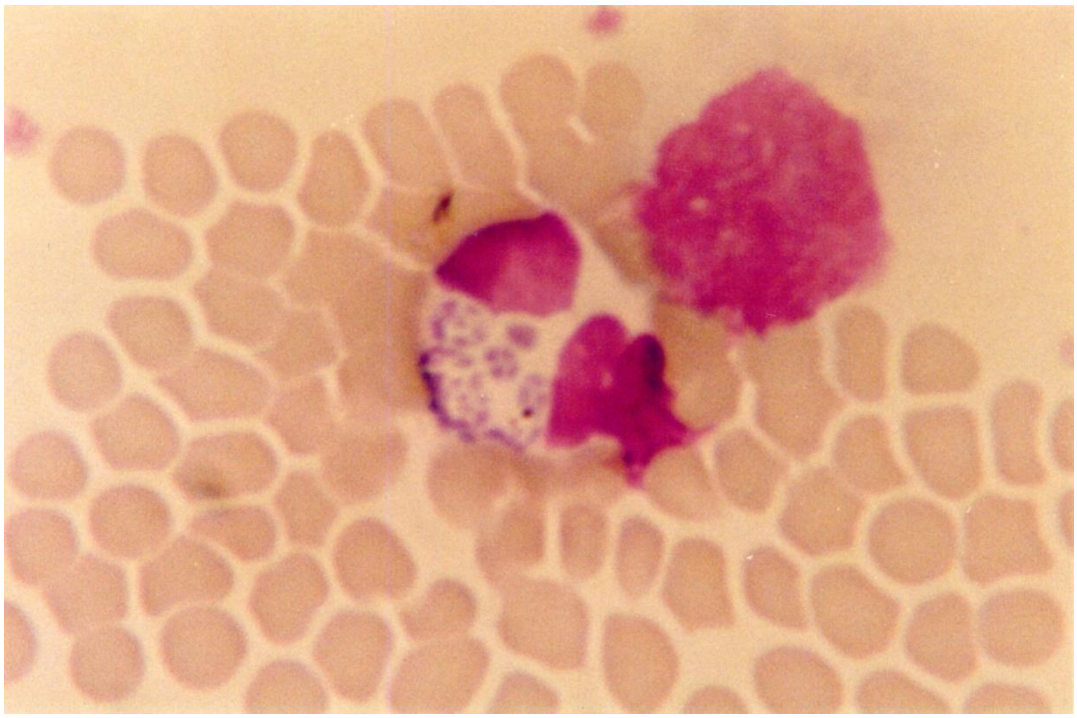

Figure 1.4.3 Inclusions (morulae) in a neutrophil, blood smear stained by May-Grünwald Giemsa (Photo: S. Stuen).

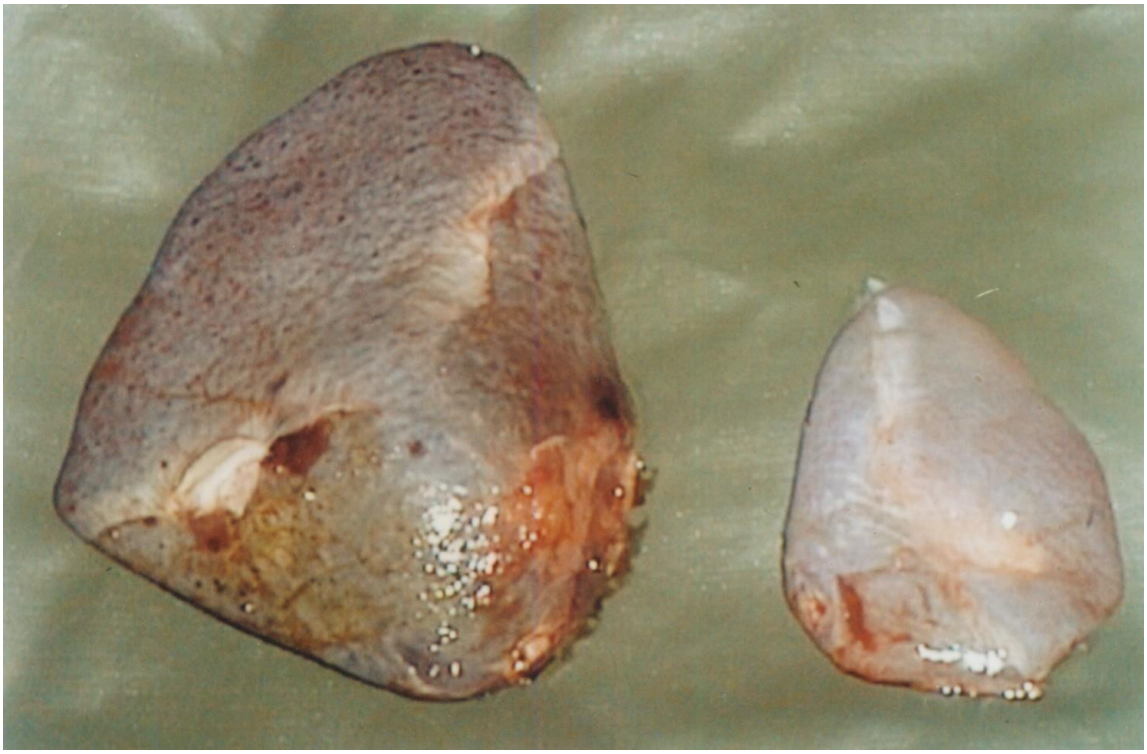

Figure 1.4.4 Splenomegalia in a 4 week old lamb infected with *A. phagocytophilum* (left) compared to an uninfected lamb (right) (Photo: S. Stuen).

1.4.6 Complications

As mentioned earlier, TBF is normally a mild rickettsiosis, but serious complications can occur and may even be fatal. Abortion (Jamieson 1950, Stamp and Watt 1950, Overas 1959, Wilson et al. 1964, Jones and Davies 1995) and infertility due to impaired spermatogenesis in rams and bulls have been reported (Watson 1964, Retief et al. 1971). However the most serious complication is immunosuppression, which causes the animal to be more predisposed for secondary infections (Foggie 1951, Campbell et al. 1994, Granquist et al. 2008) such as louping-ill (MacLeod 1933), "*Pasteurella" septicaemia* and tick pyaemia, the last condition is caused by a *Staphylococcus aureus* infection, which is characterized by paralysis and crippling lameness (Foggie 1956, Webster and Mitchell 1989). Other secondary infections, often identified as septicaemia, can be caused by microorganisms such as *Escherichia coli, Klebsiella pneumonia, Mannheima haemolytica* and *Streptococcus* spp (Brodie et al. 1986, Overas et al. 1993, Stuen and Kjolleberg 2000, Jenkins et al. 2001).

1.4.7 Hematology

Hematology in *A. phagocytophilum* infected sheep is characterized by severe leucopenia, which includes lymphocytopenia, thrombocytopenia and prolonged neutropenia (Taylor et al. 1941). The neutropenia may last for 1-2 weeks and the neutrophil count may be decreased for several months (Foggie 1951, Campbell et al. 1994, Stuen 2003). The lymphocytopenia is caused by a significant decrease in cells bearing surface immunoglobulins, B-lymphocytes (Batungbacal et al. 1982). The T-lymphocytes are also reduced, where both helper (CD4) and cytotoxic/suppressor (CD8) T-cells are significantly lowered. In addition, cells not carrying either B-epitopes or T-epitopes are usually significantly increased (Woldehiwet 1991).

1.4.8 Transmission and persistence

Transmission of *A. phagocytophilum* from tick to host usually occurs within a day or approximately 24 hours after attachment (Foggie 1951, des Vignes et al. 2001). In *I. ricinus* ticks, there is no evidence of transovarial transmittance of the bacterium (MacLeod 1933, Webster and Mitchell 1989). The eggs of *I. ricinus* are not infected and thereby not the unfed larvae. The larvae become infected by ingesting blood from an infectious host or by cofeeding transmission from an infected tick on the same host. Maintenance in nature is dependent on infected hosts (Long et al. 2003).

A. phagocytophilum may cause a persistent infection in different hosts, meaning it can circulate and cause infection over a long period of time. Several micro-organisms have evolved effective ways to escape the host immunity system and can in this way persist in the host (Barbour and Restrepo 2000). The host is not able to clear the infectious agent and it was shown that sheep could still be

infective 25 months after the initial infection (Foggie 1951, Boldogh et al. 1996). It may be difficult to verify a persistent infection, especially since an antibody test cannot be used; the host animal can be seropositive for several months after treatment (Paxton and Scott 1989, Stuen et al. 2003).

The mechanism that causes *A. phagocytophilum* infection to persist is not known, but it is suggested that antigenic variation in the MSP's is one major reason (Brayton et al. 2001). The antigenic variation is caused by a change in the polypeptide or polysaccharide antigens of the surface proteins and these changes are often reversible. Due to this feature, the microorganism can change the exposed surface antigens and thereby avoid the immune system because of the constantly changing antigens (Barbour and Restrepo 2000, Granquist et al. 2008, Granquist et al. 2010c). The persistency of *A. phagocytophilum* shows also a cyclic pattern, a cyclic bacteraemia characterized by varying numbers of organisms in the blood (Granquist et al. 2010b).

A. phagocytophilum has been found to persist not only in sheep (Foggie 1951, Paxton and Scott 1989, Stuen et al. 1998b), but also in other mammals such as cattle (Pusterla et al. 1998, Larson et al. 2006), dogs (Ewing and Buckner 1965, Egenvall et al. 2000), horses (Nyindo et al. 1978, Madigan 1993) and red deer (Stuen et al. 2001). In contrast, no evidence of persistent *A. phagocytophilum* infection in humans has been described (Stuen, personal information).

1.4.9 Treatment and prophylaxis

It is difficult to control ticks and tick-borne infections in sheep. The best option is to avoid tick infested pastures, but this management is normally not feasible. The most common tick control on domestic animals is regularly application of pour-on treatment with pyrethroids (Brodie et al. 1986). The drug of choice against *A. phagocytophilum* infection is tetracyclines in both human and animals (Woldehiwet and Scott 1993, Dumler et al. 2007). However, an earlier study indicated that a 5 day long treatment with oxytetracycline was not enough to clear the *A. phagocytophilum* infection from experimentally infected lambs (Stuen and Bergstrom 2001). Other antibiotics such as ampicillin, penicillin and streptomycin have not been shown to be effective (Synge 1976, Anika et al. 1986, Woldehiwet and Scott 1993). No vaccine against ticks has been developed, with the exception of TickGuard^{PLUS} a vaccine against the tick *Riphicephalus* (formerly *Boophilus*) *microplus* (Willadsen 2004). In addition, no effective vaccine against *A. phagocytophilum* is yet available, although several vaccine candidates have been suggested (Ijdo et al. 1998, Herron et al. 2000, Ge and Rikihisa 2006).

2.0 Materials and methods

The study is divided in two different experiments; intrauterine transmission of *A. phagocytophilum* from infected sheep to offspring, and tick transmission of *A. phagocytophilum* from persistently infected sheep.

2.1 The intrauterine transmission experiment

Thirteen lambs, 5-6 months old, of the Norwegian white breed (NKS) were used. Twelve lambs were inoculated intravenously with 1 ml of a whole blood dimethyl sulphoxide stabilate of an *A. phagocytophilum* strain (GenBank accession number M73220) originally isolated from a sheep in a local sheep flock (Stuen et al. 1992). One lamb was left as uninfected control. The lambs had not previously been on tick infected pasture and were housed indoors during the whole experimental period. Rectal temperature was measured daily and blood samples (EDTA) were collected regularly the first month after inoculation. Thereafter, blood samples including serum samples were obtained monthly for 11-12 months. The lambs were mated around 4-6 weeks post infection (p.i.) with *A. phagocytophilum*. After delivery the following spring, blood samples (EDTA) and serum samples from newborn lambs were obtained from the day of birth (day 0) and thereafter at day 3, 7, 14, 28 and day 42. If the lamb died, tissue samples were collected, such as brain, lung, heart, spleen, liver and heart blood. In addition, birth fluids and colostrum were collected at birth. The newborn lambs from the uninfected ewe were used as controls.

2.2 The tick transmission experiment

The same infected lambs as in the transovarial transmission experiment were used 11-12 months after primary infection. The wool in a chosen area of the lamb was sheared and the skin was disinfected by 70 % ethanol. A cap was glued to the skin surface using Copydex (Henkel, Winsford, UK). Every lamb was grafted with approximately 50 nymphs of the tick species *I. ricinus* inside the cap (Figure 2.1). The nymphs were breed in a controlled climate chamber (Figure 2.2) by IS Insect Services GmbH, Germany (http://www.insectservices.de/), the nymphs were not exposed to any known infections prior to this experiment. Blood samples (EDTA) were collected from the lamb at the same day as grafting (day 0) and thereafter on days 2, 3, 4, 5 and day 6. At day 7, the nymphs and caps were removed. Half of the engorged nymphs were incubated in small tubes, in a refrigerated exicator with saturated solution of $MgSO_4$ to molt into adult ticks (to be used in another experiment). The rest of the nymphs were frozen in -75 °C.

All blood samples and tissue samples were extracted for deoxyribonucleotide acid (DNA) and investigated by real-time polymerase chain reaction analysis (qPCR). All PCR positive samples from qPCR were verified by melting point (T_m) analysis and thereafter investigated by semi-nested PCR. All blood samples, serum samples and nymphs from the animals were stored at -20 °C.

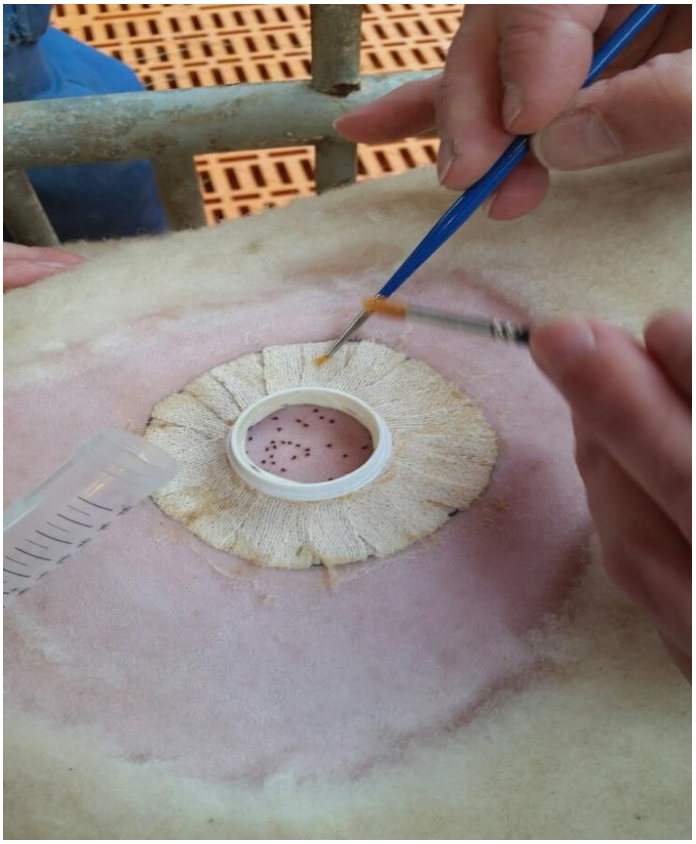

Figure 2.1 Grafting of nymphs, (Photo: Sagen AM).

Figure 2.2 The climate chamber with breeding cages from IS Insect Services, Germany (Photo: IS Insect Services GmbH, Germany).

2.3 DNA extraction from blood

DNA from the blood samples was extracted with the use of MagNA Pure LC Instrument (Roche Diagnostics), DNA Isolation Kit I according to manufacturers and instrument protocol. The DNA I Blood Cells High Performance program was used. The isolation procedure uses magnetic-bead technology. The samples are lysed by incubation with the use of a special buffer containing a chaotropic salt and proteinase K solution. Magnetic glass particles are added and DNA binds to their surfaces. Unbound substances are removed by several washing steps and purified DNA is eluted by using a low salt buffer (Figure 2.3). The kit included wash buffer I, wash buffer II, lysis/binding buffer, proteinase K, magnetic particle suspension (MGPs) and elution buffer. The blood samples were thawed in room temperature and 200 µl was used for extraction. The eluted DNA samples were stored in -20 °C.

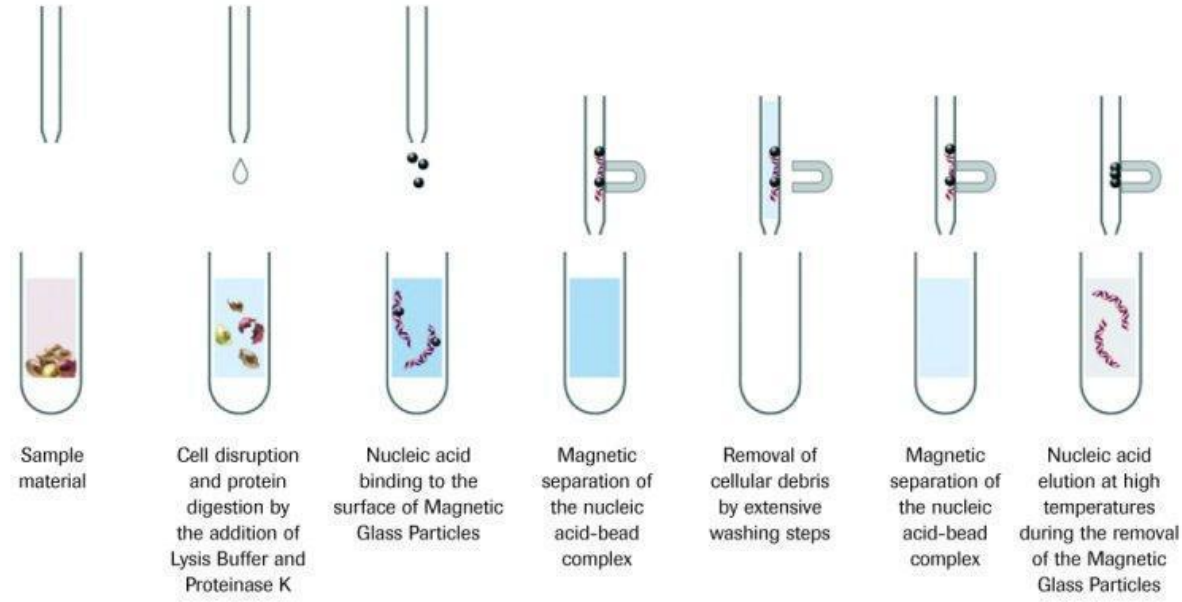

Figure 2.3: Principle of nucleic acid isolation performed automatically by the MagNA Pure Compact Instrument (Roche Lifescience 2003).

2.4 DNA extraction from tissue

DNA from the tissue samples was extracted with the use of MagNA Pure LC Instrument (Roche Diagnostics), DNA Isolation Kit II according to manufacturers and the machines protocol. The DNA II Tissue External Proteinase K program was used. The isolation process uses the same technology as for DNA extraction from blood (Figure 2.3). The kit included wash buffer I, wash buffer II, wash buffer III, lysis/binding buffer, proteinase K, MGPs, elution buffer and tissue lysis buffer. 25 mg tissue was weighed and homogenized in 160 µl Tissue lysis buffer and 40 µl Proteinase K, using a mortar. The samples were incubated in 55 °C in continuous shaking overnight. 100 µl of the homogenized tissue samples were used for extraction and the eluted DNA samples were stored at -20 °C.

2.5 DNA extraction from ticks

DNA from engorged nymphs was extracted with the use of DNeasy® Blood & Tissue kit (cat. nos. 69504) provided from Qiagen (Qiagen, Venlo, Netherlands). The extraction was performed according to manufacturer's supplementary protocol: Purification of total DNA from ticks using the DNeasy® Blood & Tissue kit (Appendix A.1). The protocol was performed without carrier RNA and two spin columns were used per sample. The collected nymphs were divided in 4 – 6 tubes of 5 nymphs each. The nymphs were washed with 70 % ethanol and sterile water, and then air dried before any analysis. The nymphs were homogenized by using two methods; one method using scalpel to cut the nymphs and one method using Lysis Matrix tubes.

Scalpel: Tubes were thawed, 180 µl ATL buffer was added, and then each of the nymphs were drawn to the upper rim of the tube and cut once longitudinally and diagonally, thereafter 20 µl proteinase K was added.

Lysis matrix tubes: Nymphs were homogenized with Lysis Matrix tubes provided by MP Biomedical (MP Biomedical, Santa Ana, California), with 1.4 mm ceramic spheres. The nymphs were thawed and 360 µl ATL buffer were added. The nymphs were bead-beated in the Fastprep 6.5 m/s for 25 seconds; this was repeated until tissue was homogenized. Thereafter 40 µl proteinase K was added.

All homogenized samples were incubated in 56 °C overnight after adding proteinase K. The eluted DNA samples were stored at -20 °C.

2.6 Real-time PCR for identification of positive samples, targeting the *msp2 (p44)* gene

Conventional Polymerase chain reaction (PCR) (Figure 2.4) includes denaturing, annealing and extension, and is an enzymatic DNA amplification process to mass-duplicate DNA where the results are copies of the targeted sequence (Roche Lifescience 2009).

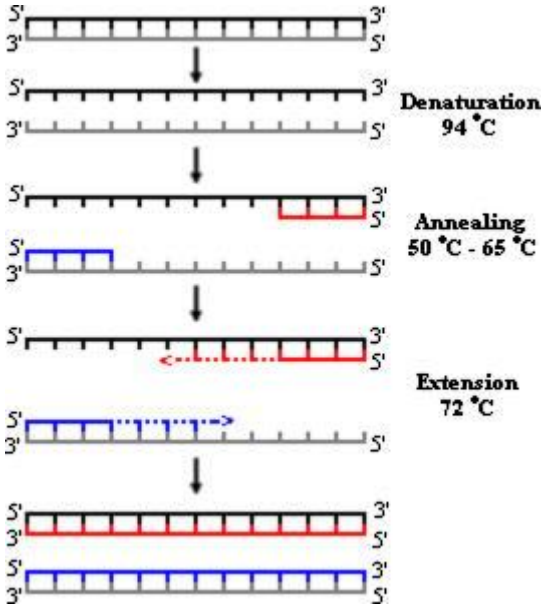

Figure 2.4 The PCR principle, denaturation; the double stranded DNA is denatured by heating to approximately 95 °C, annealing; temperature is lowered to approximately 60 °C and primers attach to the targeted sequence, extension; polymerase attach to the single stranded DNA sequence and extends to double stranded DNA. The process is repeated 35-45 cycles to obtain a large amount of amplicon (Lee et al. 2007).

Quantitative PCR (qPCR) also called kinetic PCR or real-time PCR is an advanced version of the conventional PCR technique, where qualitative and quantitative DNA analysis is possible. In qPCR, the detection and amplification process is monitored as it is occurring. The PCR product is measured by using a fluorescent dye which binds to the double-stranded DNA and the measurements are plotted on a curve. The amplification process doubles the amount of target DNA for every cycle until its fluorescence exceeds background. The cycle when the products fluorescence becomes visible and the intensity rises, is called the crossing point (Cp). The curve can be divided into three phases; the early background phase, the middle exponential phase and the late plateau phase (Figure 2.5). The LightCycler software uses the second derivate maximum method to determine the Cp (Espy et al. 2006, Roche Lifescience 2009). The samples with a Cp were validated by T_m analysis (Panjkovich and Melo 2005).

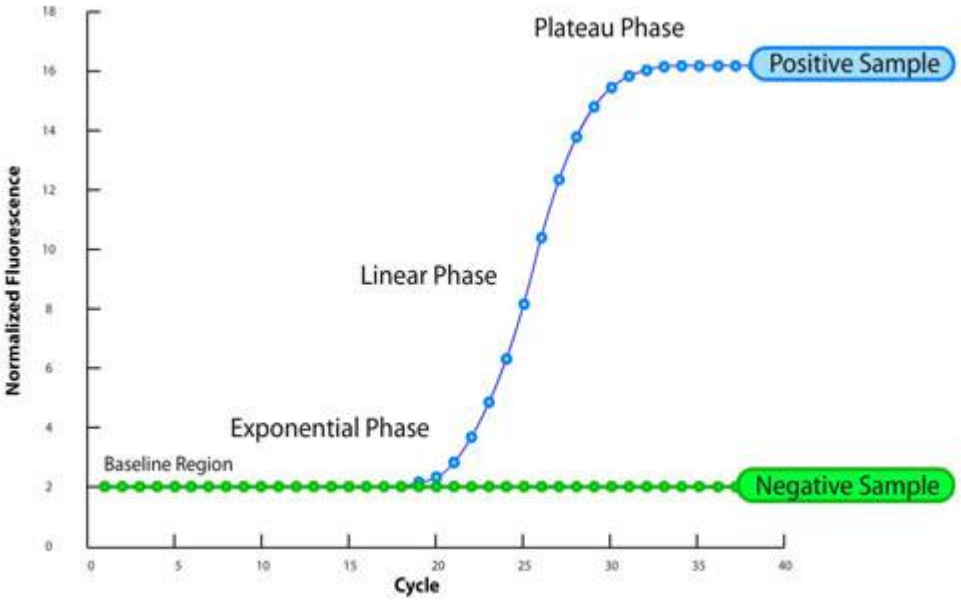

Figure 2.5 PCR kinetic profile (Roche Lifescience 2009).

The qPCR analysis was performed in a Light Cycler 480 Instrument (Roche Diagnostics) as described in previously studies (Granquist et al. 2010c). The reaction mix per sample consisted of forward primer ApMSP2252 (ACAGTCCAGCGTTTAGCAAGA), reverse primer ApMSP2459 (GCACCACCAATACCATAACCA), RNAse free H_2O, Light Cycler 480 DNA SYBR Green I Master mix and template. The extracted DNA samples and reaction mix were loaded onto a 96 well white plate. The plates were sealed with sealing foil and centrifuged for 2-3 minutes at 1200 rpm. The primers (TIB MOLBIOL Syntheselabor GmbH, Germany) amplifies a product of 208 bp covering the conserved N-terminal region of the expression site for the *msp2 (p44)* gene (Granquist et al. 2010b). The parameters and reaction mix for the absolute quantification (used for all samples) are listed in Appendix A.2. In every run, a negative and a positive control were included with the samples. The positive control was obtained from an inoculated animal (Id: 40104) during the acute course of infection, whereas the negative control was reaction mix without any added substance. The samples with a Cp indicate that *A. phagocytophilum* DNA are present. All samples with a Cp were validated by T_m analysis with a range of 82 – 83 °C. All samples with indication of *A. phagocytophilum* DNA were further analyzed by semi-nested PCR and sequenced to verify if *A. phagocytophilum* was present.

2.7 Semi-nested PCR

Semi-nested PCR is a method to be used to detect a small specific region of a DNA molecule. In this technique the PCR is used in series, in this case two reactions with one forward primer and two reverse primers. The first reaction was performed with an outer set of primers using the forward primer16S-F5 (5`AGTTTGATCATGGTTCAGA) and the reverse primer ANA-R4B (5`CGAACAACGCTTGC) for the amplification of a 507 bp fragment *rrs (*16S rRNA gene) in *A. phagocytophilum*, which includes both target sequence and non-specific DNA fragments. Thereafter the products are used in a second reaction with the same forward primer (16S-F5) and a new reverse primer ANA-R5 (5`TCCTCTAGACCAGCTATA); to amplify a more delimited amplicon of the target sequence; a 282 bp fragment. This method is often used where very low amount of target DNA are present or with problems such as contamination DNA or background disturbance (Wilson 1987, Chelly et al. 1989, Mulhardt 2010).

Semi-nested PCR analysis were carried out on a PTC-200 instrument (MJ Research, St Bruno, Canada) on samples with indication of *A. phagocytophilum* DNA present as described in previously studies (Stuen et al. 2005a, Stuen et al. 2005b). The parameters and reaction mix used in semi-nested PCR are listed in Appendix A.3.

2.8 Gel-electrophoresis

The amplicons from the semi-nested PCR were validated by gel electrophoresis on a 2 % agarose gel with GelRed, a fluorescent nucleicacid dye. A DNA marker (Appendix A.3) and a mixture of loading buffer and PCR product (1:6) were then added to the wells, according to manufacturer's recommendations (Sigma-Aldrich). The gel electrophoresis were run with parameters; 90 V, 400 mA for 40-45 minutes. The finished gel was photographed in a Kodak Image Station 4000R (Carestream Health 2002). Positive amplicons appears on the image with a 282 bp gel-band.

2.9 Sequencing

2.9.1 PCR product purification

The products were cleansed with Exostar to remove leftover primers and remaining deoxynucleotides (dNTPs) (Illustra ExoProStar 1-step enzymatic PCR and seq. cleanup kit) (GE Healthcare 2013). Five µl PCR products and two µl ExoProStar was added to a PCR tube and the tubes were placed in the PCR machine with parameters 37 °C for 15 minutes and 80 °C for 15 minutes. Clean PCR products were stored in -20 ° until used for BigDye sequencing.

2.9.2 Sequencing of the 16S rRNA-gene

Fluorescently labeled dyes are incorporated into PCR products by using either 5`-dye labeled primers or 3`-dye labeled dideoxynucleotide terminators (ddNTPs) to determine the order of the bases in a DNA sample. AmpliTaq® polymerases was used for primer extension and dyed ddNTPs were used in this experiment (Figure 2.6) (Lee et al. 1992, AppliedBiosystems 2013).

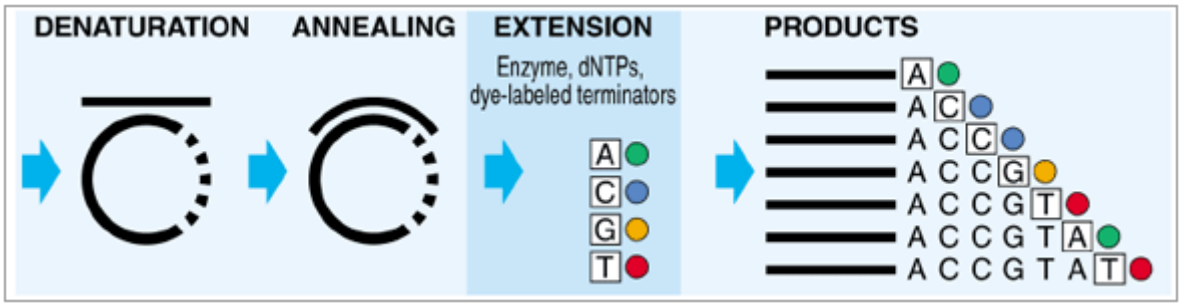

Figure 2.6 One cycle of dye terminator cycle sequencing (AppliedBiosystems 2013).

The sequencing reaction for this project was capillary electrophoresis; where the sequencing products (negatively charged DNA) enter a capillary via electro kinetic injection and the current is applied to continue the electrophoresis. The fluorescent labeled DNA fragments travel towards the positive electrode, moves across a laser beam which causes the dyes to fluorescence and are detected by a CCD camera on the genetic analyzer. The signal is converted to digital data by base calling the fluorescence intensity from each data point. Base calling is the process of assigning bases to chromatogram peaks. The different dyes emit a different wavelength when excited and therefore all colors and all bases can be detected in one capillary injection (Figure 2.7) (Woolley and Mathies 1995, AppliedBiosystems 2010).

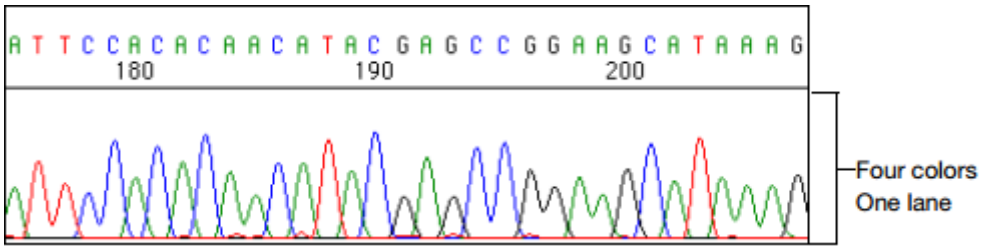

Figure 2.7 Fluorescent sequencing (AppliedBiosystems 2010).

For sequencing, the BigDye Terminator v1.1 kit were used as recommended from manufacturer, the reaction mix and parameters used for BigDye are listed in Appendix A.4 (AppliedBiosystems 2002). Sequencing of the BigDye products were performed in an ABI PRISM 310 Genetic Analyzer (AppliedBiosystems 2010). The finished sequences were compared with databases by BLAST (Basic Local Alignment Tool) search in NCBI`s webpage (http://blast.ncbi.nlm.nih.gov/Blast/) and Table 1.4.2 for determination if *A. phagocytophilum* DNA was present and which variant. Only the products with positive gelbands from the gel electrophoresis were prepared for sequencing. The ABI PRISM 310 Genetic Analyzer was prepared and calibrated according to application manual. POP-6™ denaturing polymer was used with 1 × Genetic Analyzer Buffer with EDTA. The samples were heated in 95 °C for 5 minutes; thereafter the samples were vortexed and spun quickly before placing in the genetic analyzer. The prepared samples were sequenced in one direction with the reverse primer (ANA-R5) as start point, the analysis of sequences were performed with reverse complement sequences.

2.10 Serological tests

Sera were analyzed using an IFA test to determine the antibody titer to an equine variant of *A. phagocytophilum* (Artursson et al. 1999, Stuen and Bergström 2001). The IFA test uses the method of fluorescent antibody staining (Weller and Coons 1954). The method is divided into two steps, where the first step uses an unlabeled antibody which binds to the antigenic substrate forming an antigen-antibody complex. If there are no antibodies in the serum, no complex will be formed. The second step uses an antibody carrying fluorophore which binds to the antigen-antibody complex formed in step one. Positive serum samples will fluorescence when observed under a fluorescence microscope.

Briefly, two-fold dilutions of sera were added to slides precoated with *A. phagocytophilum* antigen (Protatec, St. Paul, Minnesota). Bound antibodies were visualized by fluorescein-isothiocyanate (FITC) –conjugated rabbit-anti-sheep immunoglobulin (Cappel, Organon Teknika West Chester, PA). Sera were screened for antibodies at dilution 1:40. If positive, the serum was further diluted and retested. A titer of 1.6 ($\log_{10}$ reciprocal of 1:40) or more was regarded positive.

3.0 Results

Twelve lambs were infected in the autumn (day 0) and the fever reaction (≥40 °C) started on day 5 p.i. The rectal temperature fluctuated from the first fever reaction until the measurement period was finished (Figure 3.1). Other clinical observations were dullness and reduced appetite for one or two days.

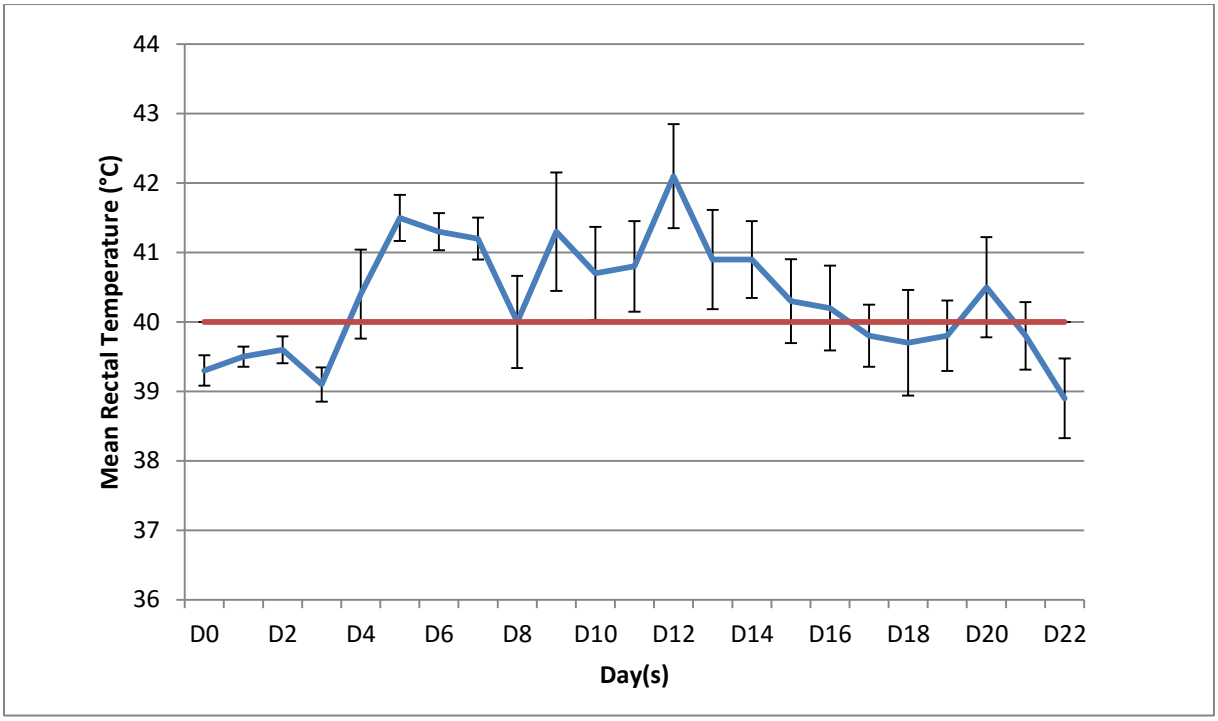

Figure 3.1 Mean rectal temperature (± SD) of 12 lambs 5-6 months old, infected with *A. phagocytophilum* (D0 – day of inoculation). Rectal temperature ≥40 °C (red line) was considered as fever.

The lambs were mated 4-6 weeks p.i. and 9 became pregnant. In the following spring, a total of 20 lambs were born, whereas 6 died within 2 days. Three of the lambs died due to caesarean on the ewe, 14 days before estimated delivery. These lambs were weakborn and died within 10 – 15 minutes. One of the other dead lambs was stillborn. The last two lambs were weakborn, and died without any specific findings at post mortem.

3.1 Intrauterine transmission experiment

3.1.1 Real-time PCR analysis

3.1.1.1 DNA extraction

The eluted DNA samples were all measured for DNA content. The DNA were extracted from blood samples, and the DNA content were measured with a DeNovix DS-11 Spectrophotometer according to manufacturer's recommendations (DeNovix 2013). In order to observe the reproducibility all eluted DNA samples from infected animals were measured in triplets, chosen samples are shown in Table 3.1.

Table 3.1 Mean DNA content (± SD) in triplets from 4 infected lambs at different days p.i.

ID	Date	DNA content (µg/ml)
S1	22.03.15	10.93 ± 0.37
S4	22.03.15	25.31 ± 0.32
S9	06.04.15	17.16 ± 0.22
S10	25.04.15	28.56 ± 0.46

3.1.1.2 Optimizing qPCR

DNA sample from lamb S4 at day 5 p.i. were analyzed in a dilution series (10^{-1} to 10^{-5}) to find the best suited dilution to use for the rest of the samples (Figure 3.2). All dilutions gave a sigmoidal curve with a Cp under 35 (Cp: 17.74 (10^{-1}), 21.78 (10^{-2}), 25.65 (10^{-3}), 27.65 (10^{-4}) and 33.08 (10^{-5})); the 10^{-5} dilution shows that the qPCR may detect samples with a very low amount of DNA. The dilution of 10^{-2} was thought to be the best suited, thereafter all samples were diluted 1:100 with sterile water before qPCR analysis. The 10^{-2} dilution had also previously been used in a study based on quantification of *A. phagocytophilum* (Granquist et al. 2010c).

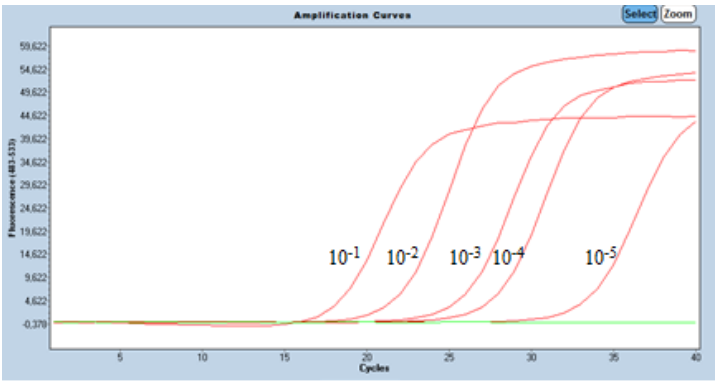

Figure 3.2 Dilution series (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of positive control S4 at day 5 after inoculation, negative controls (reaction mix without DNA) are shown in green.

3.1.1.3 Reproducibility of qPCR

In order to investigate the reproducibility of the qPCR, 24 of the DNA samples from persistently infected lambs were run in triplets, to confirm the LC-machines sensitivity and if there was any significant deviation. The triplet run (± SD) of lamb S4 at day 5 p.i. gave a mean of 22.13 ± 0.3 (Figure 3.3). The amplification curves show little difference for the Cp. The negative control (mix without DNA) was negative.

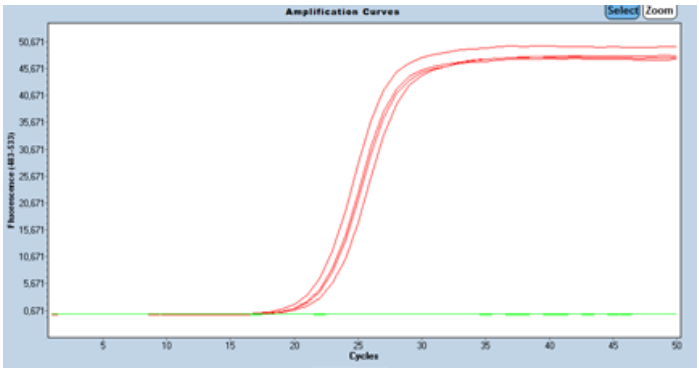

Figure 3.3 Triplet runs with one positive control S4 at day 6 after inoculation (Cp`s of 22.14, 21.98 and 22.57) and negative control (mix without DNA) (green).

3.1.1.4 qPCR analysis of persistently infected lambs and newborn lambs

Persistently infected lambs

The samples used in the first qPCR analysis were obtained from inoculated lambs with fever (≥40 °C) to confirm an *A. phagocytophilum* infection. All except the control animal (S2) had a fever reaction at day 5 p.i. and a positive PCR product was detected in all of these samples (Figure 3.4). The samples from this date were analyzed with a positive (*A. phagocytophilum* infected lamb - previously sequenced) and a negative control (mix without DNA).

Blood obtained before and after birth were first chosen to be analyzed. All pregnant lambs except one had an indication of infection before giving birth, while three lambs had an indication of infection after delivery. Three lambs S3, S5 and S10 had an indication of infection both right before and after giving birth, while S10 had an indication of infection at the same day as delivery (Table 3.2.). Thereafter, all of the blood samples obtained from year 2015 were analyzed. All samples with sigmoidal curve profiles were further validated by T_m analysis with a range of 82° - 83 °C (Table 3.2).

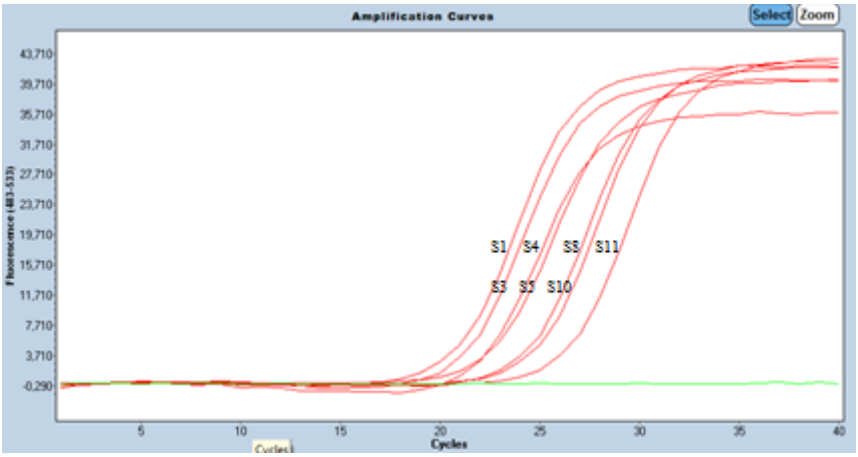

Figure 3.4 DNA samples from lambs (S1, S3, S4, S5, S8, S10 and S11) 5 days p.i. with fever reaction (≥40 °C), negative control in green (mix without DNA).

Table 3.2 Overview qPCR results of blood samples from infected lambs, (Cp values – in brackets). (na = not analyzed, + = positive, - = negative, C = control). Lambing period (blue): 22.03.15 – 25.04.15.

ID	13.10.14	19.1.15	22.3.15	6.4.15	25.4.15	18.5.15	6.7.15	20.8.15	23.9.15
S1	+ (19.86)	-	-	-	+ (32.46)	-	-	-	-
S2 (C)	na	na	-	-	-	-	-	-	-
S3	+ (25.79)	-	-	+ (32.38)	+ (29.92)	-	-	-	-
S4	+ (19.69)	-	+ (37.8)	+ (36.51)	-	-	-	+ (36.5)	-
S5	+ (23.61)	+ (36.7)	-	+ (36.50)	-	+ (37.1)	-	-	-
S6	+ (27.60)	-	na	+ (38.0)	+ (33.87)	-	-	-	-
S7	+ (23.13)	-	-	+ (31.96)	+ (30.11)	-	-	-	-
S8	+ (20.58)	-	-	+ (37.0)	+ (31.76)	-	-	-	-
S9	+ (24.09)	-	-	-	+ (30.94)	-	-	-	-
S10	+ (19.82)	-	+ (39.0)	+ (32.18)	+ (38.5)	-	-	-	-
S11	+ (26.17)	+ (30.69)	-	+ (37.0)	-	+ (24.88)	-	-	-
S12	+ (17.36)	-	-	+ (38.0)	-	-	-	-	-
S13	+ (24.62)	-	-	-	-	+ (36.7)	-	-	-

Newborn lambs

Positive products were obtained from both blood and tissue samples of newborn lambs. In total, 84 blood samples from newborn lambs were investigated, whereas indication of an *A. phagocytophilum* infection was suspected in two lambs, belonging to the same ewe (Table 3.3).

Table 3.3 Overview of qPCR results of living newborn lambs (na = not analyzed, + = positive, - = negative, C = control)

ID	D0	D3	D7	D14	D28	D42
S10B	-	-	-	-	-	-
S10C	-	-	-	-	-	-
S3A	-	-	-	-	-	-
S3B	-	-	+ (36.0)	-	+ (37.0)	-
S3C	-	-	+ (34.75)	-	-	-
S2A (C)	-	-	-	-	-	-
S2B (C)	-	-	-	-	-	-
S13A	-	-	-	-	-	-
S12A	-	-	-	-	-	-
S12B	-	-	-	-	-	-
S7A	-	-	-	-	-	-
S7B	-	-	-	-	-	-
S9A	-	-	-	-	-	-
S8A	-	-	-	-	-	-

A total of 65 tissue samples from 6 dead lambs were analyzed and *A. phagocytophilum* infection were suspected in different tissues from four lambs, were three of these lambs belonged to the same ewe (Table 3.4).

Table 3.4 Overview of tissue sample qPCR results from dead lambs (na = not analyzed, + = positive, - = negative)

Tissue	S10A	S9B	S5A	S5B	S5C	S12C
Brain	+ (36.54)	-	-	-	-	-
Heart	+ (35.5)	-	-	-	+ (38.83)	-
Lung	-	-	+ (36.4)	-	-	-
Liver	-	-	-	+ (40.59)	-	-
Kidney	-	-	+ (36.3)	+ (37.8)	+ (37.6)	-
Spleen	+ (36.5)	-	+ (38.0)	+ (36.0)	+ (40.0)	-
Navel	-	-	-	-	-	-
Blood	-	-	-	-	-	-
Urine	-	-	-	-	-	na
Abomasum content	na	-	-	-	-	-
Bladder	na	-	na	-	na	-
Skin	na	-	na	na	na	na
Uracus	na	-	na	na	na	na
Testicle	na	-	na	na	na	na
Abomasum	na	-	na	na	na	na

3.1.2 Semi-nested PCR targeting 16S rRNA gene and sequencing analysis of infected lambs

PCR products from the qPCR analysis with indication of *A. phagocytophilum* infection were analyzed by semi-nested PCR and gel electrophoresis in a 2 % agarose gel, with added GelRed for visualization. The gels were run for 45 minutes. The samples with an indication or a trace of infection were all further investigated by sequencing.

Figure 3.5 shows the PCR results from two selected infected lambs with a fever reaction (≥40 °C). The PCR products indicated an *A. phagocytophilum* infection, except the negative controls (mix without DNA). The DNA sample from animal S4 were used further as positive control.

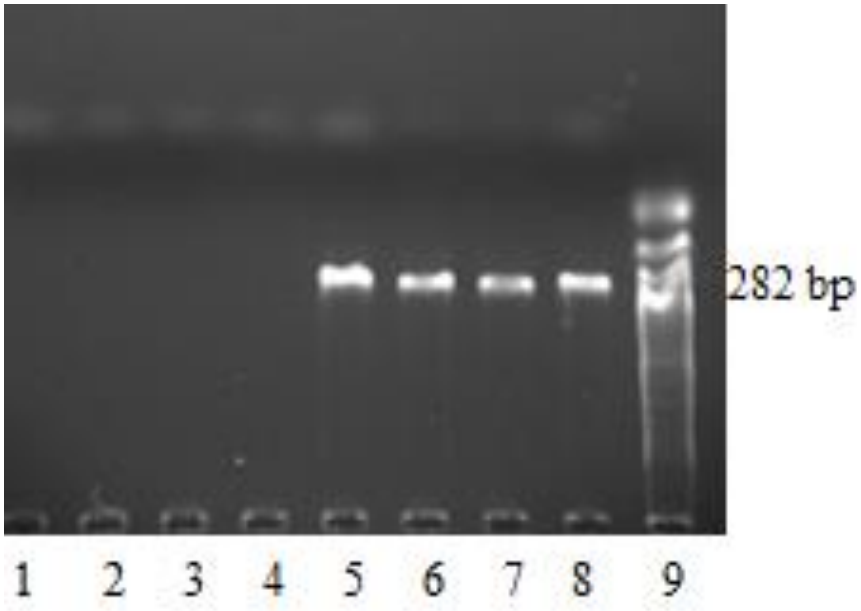

Figure 3.5 Gel electrophoresis results of DNA samples from two animals, S4 and S11 infected with *A. phagocytophilum* with a fever reaction. (1 – 4) negative controls (mix without DNA), (5) S11, (6 – 7) S4 (undiluted), (8) S4 10^{-2} dilution, (9) DNA marker.

There were 24 blood samples from inoculated animals from year 2015, investigated with semi-nested PCR. Seven of these samples were confirmed positive for *A. phagocytophilum* variant 1 confirmed by using the BLAST program from NCBI and Table 1.4.2 (NCBI 2016). Variant 1 *A. phagocytophilum* is marked yellow in the sequences (Table 3.5). Colostrum and fetal fluids from pregnant ewes were collected and analyzed with PCR. None of these samples were PCR positive (data not shown).

Table 3.5 Sequencing results of infected lambs, the sequence for *A. phagocytophilum* variant 1 (acc. No M73220, Table 1.3.2) is marked in yellow.

ID	Sample date	Sequence	Cp values (qPCR)
S3	25.04.15	TTTAGTTTGATCATGGTTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTCAAGGCCTACCTTCCCTTTCTCGGGGCGGGT	29.92
S4	06.04.15	ATCGTAGTTTGATCATGGTTCAGAACGAACGCCGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCGTGCGGGGGAAAGATATTATAACTATTAGATGAGCCTATGTTAGATTAGTTTTTTTGCCCAGGGGGGGGCCTTTTTTTTTTTTTCTTGCGGGTT	36.51
S5	19.01.15	TAGTTTGATATGGTTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCAATTGCGTTCTTCCCGC	36.7
S7	25.04.15	TAGTTTGAATCATGGTTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCATTTGCCCTCTGCGGTT	30.11
S9	25.04.15	TAGTTTGATCATGGTTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGCGTGGCCTACTTATCTTGTTCTCGCGCT	30.94
S11	19.01.15	TAGTTTGATCATGGTTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGCCTACCTTTTCGTTCTTTCGCC	30.69
S11	18.05.15	TAGTTTGATCATGGTTCAGAACGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTATAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTTAGATTAGCTATTTGGTAGGGCCTTGTCCTCTCTTTTTTTTTCTTCGGCGC	24.88

3.1.2 Semi-nested PCR targeting 16SrRNA gene and sequencing analysis of newborn lambs

In total, there were 3 blood samples and 12 tissue samples from newborn lambs with indication of infection from the qPCR analysis. One tissue sample (S10B brain) was PCR positive from the semi-nested PCR results (Figure 3.6). This sample was further investigated by sequencing.

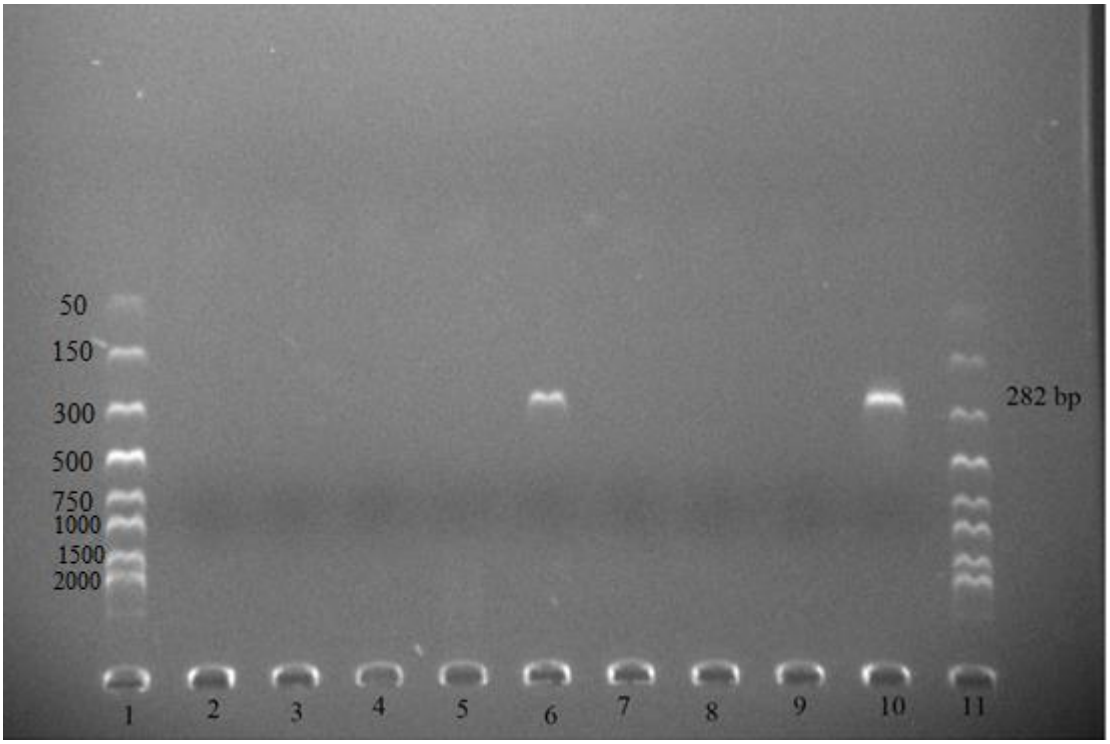

Figure 3.6 Gel electrophoresis results of DNA samples from 4 newborn lambs. (1) DNA marker, (2 – 3) S5A; kidney and lung, (4 – 6) S10B; heart, spleen and brain, (7) S3B; D7, (8 – 9) S3C; D7 and D28, (10) Positive control (infected animal S4), (11) DNA marker.

The product from the newborn lamb (S10B) was confirmed positive for *A. phagocytophilum* variant 1 obtained by sequencing results, by using the BLAST program and Table 1.3.2 (NCBI 2016). The sequencing products were blasted against *Anaplasma phagocytophilum* (taxid: 948).

Table 3.6 Sequencing results from one infected newborn lamb (S10B), the sequence for *A. phagocytophilum* variant 1 (acc. no M73220, Table 1.3.2) is marked in yellow.

ID	Sample	Sequence	BLAST results
S10B	Brain	TTAGTTTGTACATGGTTCAGAACGAACGCTGGCGGCAAGCT TAACACATGCAAGTCGAACGGATTATTCTTTATAGCTTGCTA TAAAGAATAATTAGTGGCAGACGGGTGAGTAATGCATAGGA ATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGT AATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTA GATGAGCCTATGTTAGATTAGCTAGTTGGTAGGGTAAAGGC CTACCAAGGCGCAGTCTTTG	100 % identical to acc. No M73220.1 *Ehrlichia phagocytophila* 16S ribosomal RNA gene, partial sequence. Total score: 449, Query cover: 90 %. 99 % identical to acc. No AF336220.2 *Ehrlichia phagocytophila* 16S ribosomal RNA gene, partial sequence. Total score: 459, Query cover: 95 %.

3.1.4 Serological tests

The IFA test performed shows that all infected lambs were seropositive the whole experimental period, except one (Table 3.7). A titer less than 40 was considered as negative. The serological results indicate that sheep can be seropositive for one year after the primary infection.

Table 3.7 Reciprocal antibody titers to *A. phagocytophilum* in lambs inoculated on day 0. A titer less than 40 was considered negative. – titer < 40

ID	Day 0	Day 21	Day 56	Day 98	Day 273	Day 350
S1	-	> 1280	> 1280	> 1280	160	80
S2 (C)	-	-	-	-	-	-
S3	-	> 1280	> 1280	320	1280	80
S4	-	> 1280	320	320	160	640
S5	-	> 1280	> 1280	> 1280	1280	320
S6	-	> 1280	320	320	80	40
S7	-	> 1280	> 1280	320	640	160
S8	-	> 1280	320	> 1280	320	80
S9	-	> 1280	> 1280	320	160	160
S10	-	> 1280	320	320	1280	80
S11	-	> 1280	> 1280	320	320	160
S12	-	> 1280	> 1280	320	80	-
S13	-	> 1280	> 1280	320	160	160

Serum obtained from precolostral newborn lambs was negative. However maternal *A. phagocytophilum* antibodies were transmitted to all lambs that received colostrum (data not shown).

3.2 Tick transmission experiment

Around eleven months after primary infection the ewes were used for grafting of nymphs. A total of 650 nymphs were used in this experiment and approximately 50 nymphs were grafted on each ewe. Seven days after grafting a mean of 39 (79 %) living engorged nymphs from each lamb and 9 (21 %) dead/unengorged (Table 3.8) were collected. The collected nymphs were divided in 4 – 6 tubes of 5 nymphs each.

Table 3.8 Overview of results (percentage in brackets) of nymphs grafted on persistently infected lambs

ID	Engorged nymphs (%)	Dead/ unengorged (%)	Total
S1	40 (75)	14 (25)	54
S2	31 (57)	23 (43)	54
S3	40 (93)	3 (7)	43
S4	34 (76)	11 (24)	45
S5	43 (86)	7 (14)	50
S6	43 (88)	6 (12)	49
S7	33 (63)	19 (37)	52
S8	44 (79)	12 (21)	56
S9	40 (83)	8 (17)	48
S10	39 (78)	9 (22)	50
S11	40 (82)	17 (18)	49
S12	31 (66)	11 (34)	47
S13	48 (100)	0 (0)	48
Mean	**39 (79)**	**9 (21)**	**48**

3.2.1 DNA extraction from *I. ricinus* nymphs

DNA was extracted from nymphs using two homogenization methods; one where the nymphs were cut with a scalpel and another method by using Lysis Matrix tubes with ceramic beads. There were 4 tubes from each ewe, with 5 nymphs each; two tubes were homogenized by using scalpel and the other two by using Lysis Matrix tubes. By using scalpel, the nymphs were parted into 4 pieces, and after an overnight lyzation, only small parts of the exoskeleton were still intact. In contrast, the exoskeleton was not completely ruptured, by using the lysis matrix tubes and after a day of lyzation the exoskeletons were unchanged. Eleven of the lambs had one or two extra pooled tubes of ticks. These were extracted for DNA by using the scalpel method. The eluted DNA was measured with a DeNovix DS-11 (DeNovix 2013). DNA measured from homogenized nymphs is listed in Table 3.9. The method by using scalpel gave a higher yield of DNA, compared to the two batches where using the lysis matrix tubes (marked in *italic*).

Table 3.9 DNA of samples from pooled nymphs, batches 1 – 4 (B1 – B4) selected from four animals (S1, S5, S9 and S10). The nymphs from B1/B2 were homogenized with scalpel and B3/B4 (*italic)* was homogenized by using Lysis Matrix tubes.

ID	Sample	DNA content (µg/ml)
S1	B1	91.0 ± 0.62
	B2	137.3 ± 0.4
	B3	*62.31 ± 1.05*
	B4	*48.78 ± 0.9*
S5	B1	74.51 ± 0.44
	B2	91.1 ± 0.26
	B3	*26.75 ± 0.73*
	B4	*21.81 ± 0.68*
S9	B1	109.95 ± 0.68
	B2	101.81 ± 0.32
	B3	*41.98 ± 0.71*
	B4	*29.71 ± 1.1*
S10	B1	95.1 ± 0.9
	B2	81.68 ± 0.33
	B3	*32.92 ± 1.3*
	B4	*25.21 ± 0.1*

3.2.2 Real-time PCR analysis of infected animals and nymphs

Blood samples (EDTA) were collected from the lamb at the same day as grafting (day 0) and thereafter on days 2, 3, 4, 5 and day 6. All of the 78 blood samples were PCR negative by qPCR for *A. phagocytophilum* DNA (data not shown).

By qPCR, 6 nymph samples from 4 different animals were found PCR positive and verified by T_m analysis. Only samples homogenized by using the scalpel method were positive (Table 3.10).

Table 3.10 qPCR results from nymphs grafted on persistently infected lambs (na = not analyzed, + = positive, - = not proven, C = control Cp value in brackets). Batch 1 – 6 (B1 – B6)

ID	B1	B2	*B3*	*B4*	B5	B6
S1	-	-	-	-	-	-
S2 (C)	-	-	-	-	-	na
S3	+ (37.0)	+ (36.0)	-	-	+ (37)	na
S4	-	-	-	-	-	na
S5	+ (36.0)	-	-	-	-	-
S6	-	-	-	-	-	na
S7	-	-	-	-	+ (38)	na
S8	-	-	-	-	-	-
S9	-	-	-	-	-	-
S10	-	+ (37.0)	-	-	-	na
S11	-	-	-	-	na	na
S12	-	-	-	-	na	na
S13	-	-	-	-	-	na

3.2.3 Semi-nested PCR and sequencing of nymphs

Nymph tissue samples found positive by qPCR were analyzed with semi-nested PCR and 4 samples indicated an *A. phagocytophilum* from the gel electrophoresis results. Three of the nymph samples were grafted on the same ewe, S3, and one from S7 (data not shown). However none of the samples gave a positive sequence for *A. phagocytophilum.* In Figure 3.7 two batches from S3 are shown, batch 1 (line 5) and batch 2 (line 9) which shows a very weak gelband.

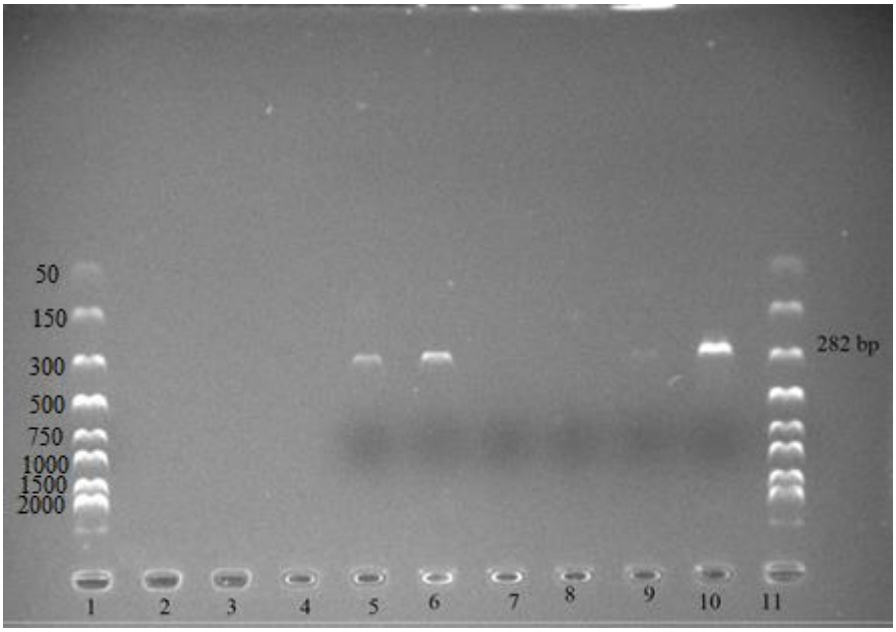

Figure 3.7 Gel electrophoresis results of DNA samples from 3 pooled nymph samples. (1) DNA marker, (2 – 4) empty, (5) S3 Batch 1, (6) positive control (S10B Brain), (7) negative control (mix without DNA), (8) S10 Batch 2, (9) S3 Batch 2, (10) positive control (S4 day 5 p.i), (11) DNA marker.

4.0 Discussion

4.1 Methods

4.1.1 DNA Extraction

DNA extraction is a critical point in the confirmation of *A. phagocytophilum* and there are many different methods and commercial kits available. Separation of the host and the bacteria DNA is not yet possible with current technology. *A. phagocytophilum* is an intracellular bacterium and the isolation method used should give as high possible yield of total DNA, which includes DNA from both sheep and *A. phagocytophilum.*

DNA extraction from blood and tissue

MagNA Pure LC Instrument (Roche Diagnostics) and DNA Isolation Kit I (blood) and II (tissue) were used according to manufacturers and instrument protocol. The isolation method used had successfully been performed in earlier studies (Stuen et al. 2011). The MagNA Pure extraction method is based on in-housed automated nucleic acid extraction (Roche Lifescience 2003) and due to full automation, contamination in the instrument should be very low. The high yield of total DNA (30-60 ng), indicates this as a reliable method for DNA isolation. False-positive results are most likely to occur due to hands-on manipulation of samples before operating the instrument or by cross-contamination of the samples (Kwok and Higuchi 1989). To prevent contamination, the experiment was performed under strict safety precautions. For instance the plastic containers and pipet tips for the instrument were stored in clean plastic bags and all handling of samples and equipment were performed with gloves.

DNA extraction of Nymphs

For DNA isolation of engorged nymphs, chloroform and phenol DNA extraction were considered to be used (Halos et al. 2004), because of availability and the low costs. However this method was eliminated due to its potential health risks. Therefore DNA extraction of nymphs was performed using DNeasy® Blood & Tissue kit (cat. nos. 69504) provided from Qiagen. This method was chosen due to its availability and because the manufacturers had created a supplementary protocol for purification of total DNA from engorged ticks. In addition, the method had successfully been used in previously studies (Wilhelmsson et al. 2010). The most important routine precautions to avoid contamination were to use new and clean pipet tips for every sample. The microcentrifuge tubes and Eppendorf tubes were sterile and carefully marked and kept in a well ordered system to avoid cross-contamination of samples. All handling of samples and equipment were performed with gloves.

Two different methods were used for the pre-treatment of the nymphs; one where the nymphs were cut by scalpel as recommended in the supplementary protocol (Qiagen 2008) and one with the use of lysis matrix tubes containing ceramic beads (Feria-Arroyo et al. 2014, MP Biomedicals 2016). The final yield of DNA, using the scalpel method was higher. By using the ceramic bead, the exoskeleton or capitulum were not completely ruptured. *A. phagocytophilum* DNA of the salivary glands may therefore not have been isolated (Hodzic et al. 1998, Liu et al. 2011). In the present study, *A. phagocytophilum* DNA were only detected from nymphs cut with the scalpel method, indicating that this pre-treatment method is a more reliable method, compared to the ceramic beads, for DNA extraction of nymphs and the detection of *A. phagocytophilum*. However, more samples are needed to be analyzed in order to verify this assumption.

4.1.2 Real-time PCR targeting the *msp2 (p44)* gene

Real-time qPCR used in this thesis was based on SYBRgreen I master mix assay by targeting the major outer membrane protein gene, *msp2 (p44),* as performed in previously studies (Granquist et al. 2008, Granquist et al. 2010c). Samples from 5-6 months old lambs, 5 days p.i. were analyzed first to verify that the method worked. All samples except control lamb were positive and the negative control was negative which indicates that the PCR-assay was not contaminated and that both primers and parameters worked as they should.

The qPCR used is a sensitive method to detect DNA from the *msp2* region of *A. phagocytophilum* (Courtney et al. 2004), and are highly dependent on accuracy which in turn depends on several critical factors; specificity, sensitivity, quality of template DNA and effectivity (Espy et al. 2006). In order to investigate the reproducibility and sensitivity of the method, one sample (lamb S4) at day 5 p.i. were analyzed with triplet and dilution measurements. The triplet measurements showed little deviation and indicates that the reproducibility is reliable (Figure 3.3). The dilution series confirms that the qPCR is a sensitive method for detecting low amounts of target *A. phagocytophilum* DNA (Figure 3.2). One blood sample from an infected sheep with a high Cp of 36.7 was successfully sequenced with a positive match for *A. phagocytophilum* DNA. This indicates that the template parameters are reliable for the detection of the *msp2 (p44)* gene in *A. phagocytophilum* DNA (Roche Lifescience 2009, Granquist et al. 2010a). There are also possibilities for instrument failure, but no error codes or messages were received when operating the instrument.

In qPCR, the PCR reaction is monitored in real-time and the detection is by fluorescence, by using fluorescent SYBR Green which binds to the double-stranded DNA. The time/cycles it takes to detect a specific DNA sequence depends on the initial concentration in the sample. The Cp alone is therefore not enough to verify an *A. phagocytophilum* infection. A T_m analysis is therefore necessary for

verification of *A. phagocytophilum*; the SYBR Green cannot separate between specific and non-specific PCR products (Teo et al. 2002, Roche Lifescience 2009). The combination of Cp analysis and T_m analysis has proven to be sufficient, since PCR products with the same size and with the same primer set should get the same melting temperature (Panjkovich and Melo 2005). If the primers create primer dimers or cross-reactions of other DNA areas than the target area, a T_m analysis will separate the false-positive and the real-positive samples (Bustin 2002). A positive and a negative sample were included in all analyses with all samples. The primers ApMSP2252 and ApMSP2459 used in this experiment have also been used in previously studies without complications (Granquist et al. 2010b). Another sensitive PCR method to detect *A. phagocytophilum* is to use probe assays, which is a specific sequence technique relying on oligonucleotide probes that hybridize to their complementary sequence in the target PCR product and thus only detect this specific product (Roche Lifescience 2009). Using a probe to detect *A. phagocytophilum* by targeting the *msp2* gene, has successfully been performed in earlier studies (Courtney et al. 2004, Hulinska et al. 2004, Drazenovich et al. 2006). This was not performed in the present study.

In the acute phase of infection all inoculated animals had all a Cp under 30, which indicates a high amount of the targeted *A. phagocytophilum* DNA (Table 3.2). Samples after this date (13.10.14) had all Cp over 30 except one. The low amount of *A. phagocytophilum* DNA may be due to cyclic pattern (cyclic bacteraemia) characterized by varying and diminishing numbers of organisms in the blood during the persistent period of the infection (Granquist et al. 2010).

4.1.3 Semi-nested PCR targeting 16S rRNA gene and sequencing

After targeting the *msp2 (p44)* gene with qPCR, the samples with an indication of infection were further investigated by using semi-nested PCR, based on the method described in previously studies (Stuen et al. 2005a). The primers used 16S F5, ANA-R4B and ANA-R5, had successfully given specific sequencing results of gene variants of *A. phagocytophilum* in earlier studies and no other primers were therefore considered for this thesis (Stuen et al. 2005a). Semi-nested PCR gives room for many error sources, because of the hands-on manipulation of samples, including pipetting of samples, cross-contamination of samples, contamination of agarose gel and PCR-assay. Another weakness of the method is the two PCR steps; contamination in the first PCR will affect the results in the next PCR. Variation of template content can also affect the results: decreasing the template amount may cause too little 16S rRNA product from *A. phagocytophilum* for any amplification; however by increasing the amount of template there could be too much background noise from sheep DNA disturbing the amplification process (Kramer and Coen 2001). Another factor is the Mg^{2+} content; in general can too much Mg^{2+} contribute to the creation of unspecific amplicons and too little Mg^{2+}

could contribute to yield reduction due to reduced primer binding (Saiki 1989). However, the method used has earlier been optimized (Stuen et al. 2005a).

The products from semi-nested PCR were further investigated with an agarose gel-electrophoresis with 2 % agarose gel (Southern 1975). All samples analyzes were managed with the same parameters and the gel bands were verified with a known positive control (*Anaplasma phagocytophilum,* GenBank accession number M73220) and a DNA marker. In order to verify the method, the first samples investigated were the samples from inoculated lambs 5 days p.i. When performing the gel electrophoresis all 12 samples from infected lambs 5 days p.i. gave strong gel-bands at 282 bp, which show that the method worked as it should. The negative control was also negative which show that the PCR-assay and primers were not contaminated by any hands-on manipulation.

Of a total of 44 samples analyzed with semi-nested PCR, only 13 had strong gel-bands of 282 bp. These were further analyzed by sequencing. By using the BLAST program and by visual inspection, eight samples were confirmed to obtain *A. phagocytophilum* DNA by sequencing. Other sequences detected, were from an uncultured bacteria clone of the 16S rRNA gene (BLAST results). Sequencing results are highly dependent on PCR conditions, primer design and purification methods (AppliedBiosystems 2010). Also low amount of *A. phagocytophilum* DNA in the PCR products can give inconclusive results or no sequences at all. However, all detected sequences in this thesis were readable.

Three blood samples and twelve tissue samples altogether from six newborn lambs were PCR positive for *A. phagocytophilum* by qPCR, while there was only one of these samples recorded positive by the semi-nested PCR. These analysis results give an indication that the qPCR may be more sensitive than the semi-nested PCR in the detection of *A. phagocytophilum,* as earlier reported (Drazenovich et al. 2006).

4.2 Transmission of *A. phagocytophilum*

All inoculated lambs had a fever reaction 5 days p.i and were all confirmed positive for *A. phagocytophilum* infection by PCR and gene sequencing (Figure 3.1). The highest rectal temperature recorded was 42.1 °C and the longest fever period recorded was 18 days. Other clinical observations were one or two days of dullness and reduced appetite. High fever (≥41 °C) is one of the most common clinical characteristics in lambs infected with *A. phagocytophilum,* together with the other clinical manifestations mentioned (Woldehiwet and Scott 1993). The mechanism which causes the fever reaction is not known, but it is suggested that it is due to bacterial lipopolysaccharides or by the production of endogenous pyrogens by neutrophils and monocytes (Stuen 2003).

4.2.1 Intrauterine transmission

As mentioned earlier, in the lambing period (Table 3.2) all pregnant lambs except one had an indication of infection right before delivery, while three lambs had an indication of infection right after giving birth. Three lambs S3, S5 and S10 had an indication of infection both right before and after delivery; the newborn lambs from these ewes were recorded as PCR positive for *A. phagocytophilum*. In the present study, more animals were found infected before than after lambing. There were no records of problems with pregnancy or abortion during this study, however only eight of the twelve persistently infected lambs were found pregnant. If these barren ewes are due to an *A. phagocytophilum* infection has to be further investigated.

The usual transmittance pathway for *A. phagocytophilum* is by tick transmission, but there are also other transmission pathways recorded. For instance, in humans transmittance has been recorded by blood transfusion (Jereb et al. 2012), and after butchering of infected animals (via skin lesions) (Bakken et al. 1996b). Nosocomial transmission has also been suggested (Zhang et al. 2008). There have also been recorded incidents via perinatal transmission in humans, but the route of infection was difficult to determine (Horowitz et al. 1998, Dhand et al. 2007). In addition incidents of intrauterine transmission of *A. phagocytophilum* have been recorded in cattle during the acute phase of the infection (Pusterla et al. 1997, Henniger et al. 2013). It may be mentioned that the closely related bacterium *A. marginale,* have been reported to be transmitted to calves via placenta in chronically infected cows (Grau et al. 2013).

In sheep, a human isolate (NY-18) of *A. phagocytophilum* was transmitted to a newborn lamb during pregnancy in the acute stage of infection (Reppert et al. 2013), however there is no evidence yet for intrauterine transmission in persistently infected sheep. In the present study, six newborn lambs were PCR positive (33 %) and one newborn lamb was confirmed with *A. phagocytophilum* DNA by gene sequencing.

Of the six PCR positive newborn lambs, all except one lamb died after birth (Table 3.3 and 3.4). If an *A. phagocytophilum* infection causes weakborn lambs is an open question, however there were no specific pathological changes at necropsy of these dead newborn lambs. Two and three of the PCR positive newborn lambs were also siblings, respectively. The mothers of these lambs (S3 and S5) were all PCR positive both right before and right after delivery.

In sheep, maternal immunoglobulin is not normally transferred to the fetus; newborn lamb will therefore obtain antibodies by ingesting colostrum during the first 48 hours (Brambell 1970). Fetal lambs can synthesize immunoglobulin after 70 days of gestation and therefore be immunological competent during the last period of pregnancy (Fahey and Brandon 1978). Infection before 70 days of pregnancy may lead to tolerance of the infection (Tizard 1992). In the present study, serological tests of the precolostral blood were negative; indicating that *A. phagocytophilum* positive lambs may have been infected before day 70. However, this has to be confirmed in further studies with a focus on the first period of pregnancy. Newborn lambs could have been infected by *A. phagocytophilum* by ingestion of fetal fluid and colostrum, but both fluids were PCR negative in all pregnant ewes. In addition, three of the positive newborn lambs died before any colostral intake.

These results indicate that an intrauterine transmission of *A. phagocytophilum* may be possible in the persistent phase of the infection. However, only *A. phagocytophilum* DNA was detected in this present study and there is no evidence of an active infection of the newborn lambs. In order to confirm an *A. phagocytophilum* infection in newborn lambs, the newborn lambs should have been followed for a longer period and blood may have been inoculated into susceptible lambs or tick transmission studies should have been used in order to verify a viable infection (Stuen 2003).

4.2.2 Persistently infected lambs – transmission to ticks

A. phagocytophilum has been shown to be persistent in several different animals such as sheep and cattle (Foggie 1951, Paxton and Scott 1989, Pusterla et al. 1998, Stuen et al. 1998b, Larson et al. 2006). The bacterial persistence is a trait in which the bacterium is able to resist and adapt to several environmental changes. The mechanism for *A. phagocytophilum* to persist is not known, but it is suggested that the bacterium is able to escape the host immune response by changing its MSP's (Brayton et al. 2001). By constant changing of exposed surface antigens, the bacterium avoids the host immune system (Barbour and Restrepo 2000, Granquist et al. 2008, Granquist et al. 2010b). *A. phagocytophilum* infection is maintained as an persistent subclinical infection (Stuen et al. 1998b). The persistency of the closely related bacterium *A. marginale* shows a cyclic pattern of 6-8 week intervals (Kieser et al. 1990). The cycling of *A. phagocytophilum* has shown to be less consistent and the cycles are not clearly defined (Stuen et al. 1998b, Granquist et al. 2010b). In the present study, every lamb was confirmed positive for *A. phagocytophilum* at several occasions in the persistent period (Table 3.2). However, these lambs also tested negative on several occasions, whereas number of organisms may be beyond the detection limit by PCR (Stuen et al. 2008). In order to observe a cyclic pattern in the blood, more samples should have been collected and investigated.

A persistently infected host may be a reservoir host with the ability to infect vectors. In the present study, 50 nymphs were grafted on each of the 13 ewes. On each ewe, a mean of 79 % living and 21 % dead nymphs were collected. The reason for the number of dead nymphs is unknown. However, nymphs need to be host seeking for attachment and a similar number of dead or unengorged nymphs has been seen in earlier tick studies (Stuen personal information).

None of the blood samples from persistently infected ewes were found PCR positive 11-12 months after the primary infection. However nymphs from four different ewes (33 %) were PCR positive for *A. phagocytophilum* DNA after feeding blood on the same infected animals. A PCR negative animal does not necessarily exclude that the animals are free of *A. phagocytophilum.* In order to verify both negative and positive animals, more samples should have been collected and more sensitive methods could have been used. The present study indicates that using blood sucking ticks is a more sensitive method than PCR for detecting *A. phagocytophilum* in persistently infected sheep. An earlier study, has also found *A. phagocytophilum* infection in engorged ticks collected on sheep, although the infection was not detected by PCR in peripheral blood from same the sheep (Ogden et al. 2003).

5.0 Conclusion

The present study indicated that the bacterium can be transmitted from mother to offspring during pregnancy. The present study also indicates that a persistent infection in sheep can infect ticks one year after the primary infection. The lambs were infected experimentally in controlled environment, but these infection routes may also occur in the field.

These findings are important for further epidemiological studies. Intrauterine transmission and tick-transmission can be an important role in the spread of *A. phagocytophilum*. A persistent infection of *A. phagocytophilum* in sheep means the bacterium can be carried from pasture to pasture, from year to year and ticks may acquire the infection from these persistently infected animals. This has to be considered when purchasing animals from farms that earlier have been on tick infested pastures. For further investigation on intrauterine transmission of *A. phagocytophilum* it would be beneficial to use more animals, and perform more investigation on newborn lambs. In order to verify a viable infection, newborn lambs should have been followed for a longer period and blood from these lambs should have been used to inoculate susceptible lambs or to infect blood sucking ticks.

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Appendix

A.1 Purification of total DNA from ticks using two methods

The nymphs were pooled, 4-6 nymphs in 4-6 tubes per sheep.

DNeasy® Blood & Tissue kit

1. The nymphs were thawed and placed in an Eppendorf tube with 180 µl buffer ATL and vortexed.
2. Each of the moistened ticks were drawn up to the edge of the tube and cut once diagonally and once longitudinally. In the mixture, 20 µl proteinase K was added and vortexed, and then incubated overnight at 56 °C.
3. After incubation the mixture was vortexed and 200 µl AL buffer was added, then vortexed again and incubated for 10 minutes at 70 °C.
4. The mixture pipetted and divided into two spin columns and centrifuged at 8000 rpm for 1 minute.
5. The spin column was placed in a new collection tube and 500 µl AW1 buffer was added, the column was centrifuged at 8000 rpm for 1 minute.
6. The spin column was placed in a new collection tube and 500 µl AW2 buffer was added, the column was centrifuged at 14,000 rpm for 3 minutes.
7. The spin column was placed in a new clean Eppendorf tube, and 35 µl AE buffer was added directly onto the membrane. The column was incubated in room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute to elute DNA.
8. Another 30 µl AE buffer was added directly onto the membrane. The column was incubated in room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute to elute DNA. The two spin columns from each sample eluted a total of 130 µl DNA and were collected in one Eppendorf tube at the end.

Lysis Matrix Tubes

1. The nymphs were thawed and placed in a Lysis Matrix tube with ceramic beads and added 360 µl buffer ATL and vortexed.
2. The tubes were placed in the Fastprep 12 and the parameters were 6.5 m/s for 25 seconds. After the run, the samples had to cool down. This step was repeated 6 times (until completely homogenization).

3. The mixtures were pipet to a clean Eppendorf tube and were added 40 µl proteinase K. The tubes were incubated overnight at 56 °C.
4. Step 3-8 from DNeasy® Blood & Tissue kit were then followed (see above).

A.2 Reaction mix and parameters for qPCR

The amplification was performed on a LightCycler instrument (Roche Diagnostics) and the enzyme used was SYBRgreen (Table A.1). Primer descriptions and information are shown in Figure A.1 and A.2.

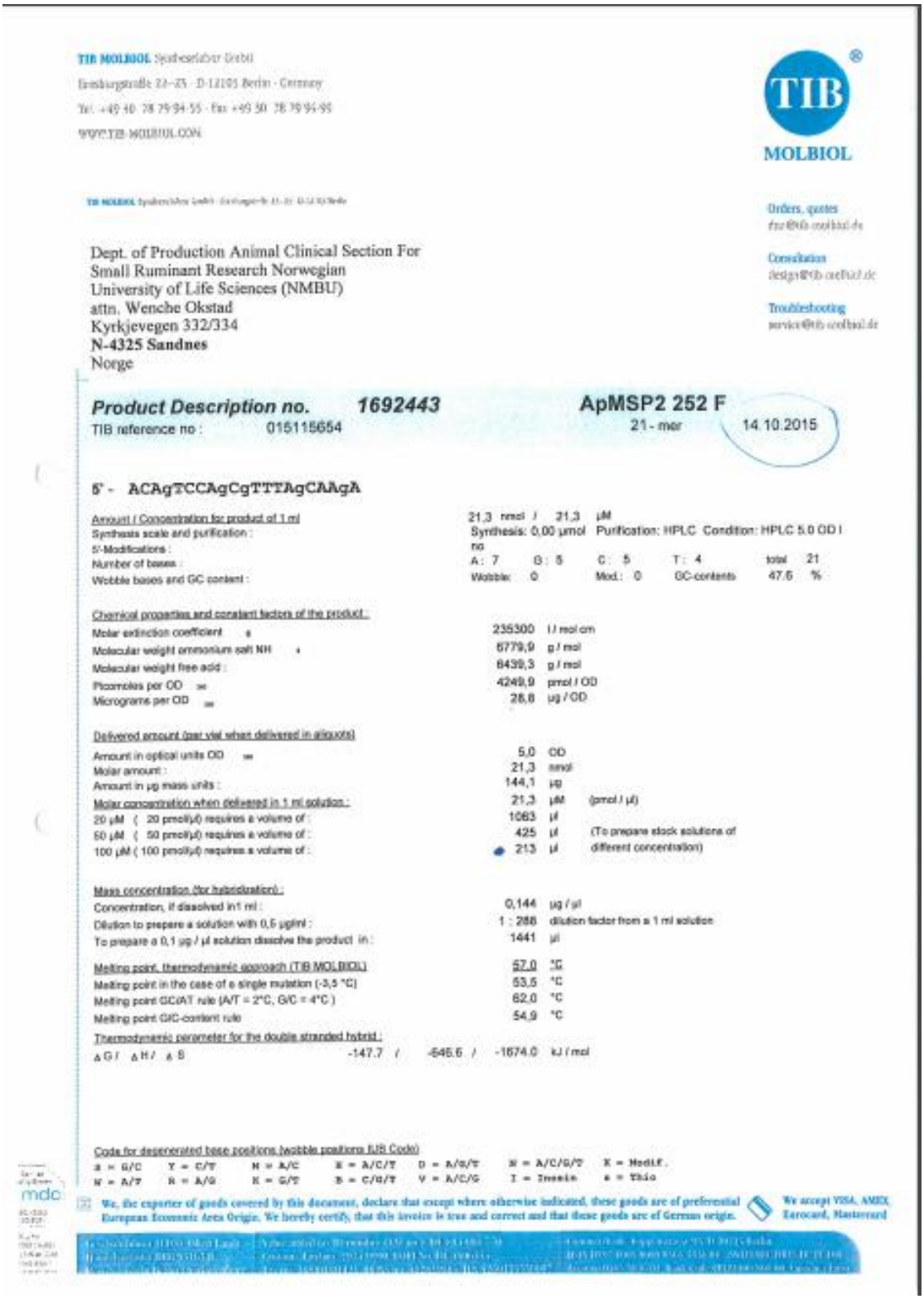
Table A.1 qPCR reaction mix per sample

Forward primer ApMSP2252 (10pmol/ µl)	1.0 µl
Revers primer ApMSP2459 (10pmol/ µl)	1.0 µl
SYBRgreen I Master	10.0 µl
Rnase free H_2O	3.0 µl
Template	5.0 µl
Total	20 µl

All of the samples were analyzed with the same parameters in the LightCycler instrument (Table A.2).

Table A.2 qPCR parameters

Step	**Temperature**	**Time**
1 pre-incubation	95 °C	5 minutes
2 amplification	95 °C	10 seconds
3	56 °C	10 seconds
4	72 °C	10 Seconds
5	Repeat step 2 – 4	40 times
6 melting curve	95 °C	5 seconds
7	65 °C	1 minute
8 cooling	40 °C	30 seconds



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Dept. of Production Animal Clinical Section For
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Product Description no. ***1692443*** **ApMSP2 252 F**
TIB reference no : 015115654 21 - mer 14.10.2015

5' - ACAgTCCAgCgTTTAgCAAgA

Amount / Concentration for product of 1 ml					21,3 nmol / 21,3 µM		
Synthesis scale and purification :					Synthesis: 0,00 µmol Purification: HPLC Condition: HPLC 5.0 OD I no		
5'-Modifications :							
Number of bases :	A: 7	G: 5	C: 5	T: 4	total 21		
Wobble bases and GC content :	Wobble: 0		Mod.: 0	GC-contents	47.6 %		
Chemical properties and constant factors of the product :							
Molar extinction coefficient ε					235300 l / mol cm		
Molecular weight ammonium salt NH4 +					6779,9 g / mol		
Molecular weight free acid :					6439,3 g / mol		
Picomoles per OD 260					4249,9 pmol / OD		
Micrograms per OD 260					28,8 µg / OD		
Delivered amount (per vial when delivered in aliquots)							
Amount in optical units OD 260					5,0 OD		
Molar amount :					21,3 nmol		
Amount in µg mass units :					144,1 µg		
Molar concentration when delivered in 1 ml solution :					21,3 µM	(pmol / µl)	
20 µM (20 pmol/µl) requires a volume of :					1063 µl		
50 µM (50 pmol/µl) requires a volume of :					425 µl	(To prepare stock solutions of	
100 µM (100 pmol/µl) requires a volume of :					213 µl	different concentration)	
Mass concentration (for hybridization) :							
Concentration, if dissolved in1 ml :					0,144 µg / µl		
Dilution to prepare a solution with 0,5 µg/ml :					1 : 288 dilution factor from a 1 ml solution		
To prepare a 0,1 µg / µl solution dissolve the product in :					1441 µl		
Melting point, thermodynamic approach (TIB MOLBIOL)					57,0 °C		
Melting point in the case of a single mutation (-3,5 °C)					53,5 °C		
Melting point GC/AT rule (A/T = 2°C, G/C = 4°C)					62,0 °C		
Melting point G/C-content rule					54,9 °C		
Thermodynamic parameter for the double stranded hybrid :							
Δ G / Δ H / Δ S	-147.7	/	-646.6	/	-1674.0 kJ / mol		

Code for degenerated base positions (wobble positions IUB Code)

S = G/C	Y = C/T	M = A/C	K = A/C/T	D = A/G/T	N = A/C/G/T	X = Modif.	
W = A/T	R = A/G	H = G/T	B = C/G/T	V = A/C/G	I = Inosin	e = Thio	

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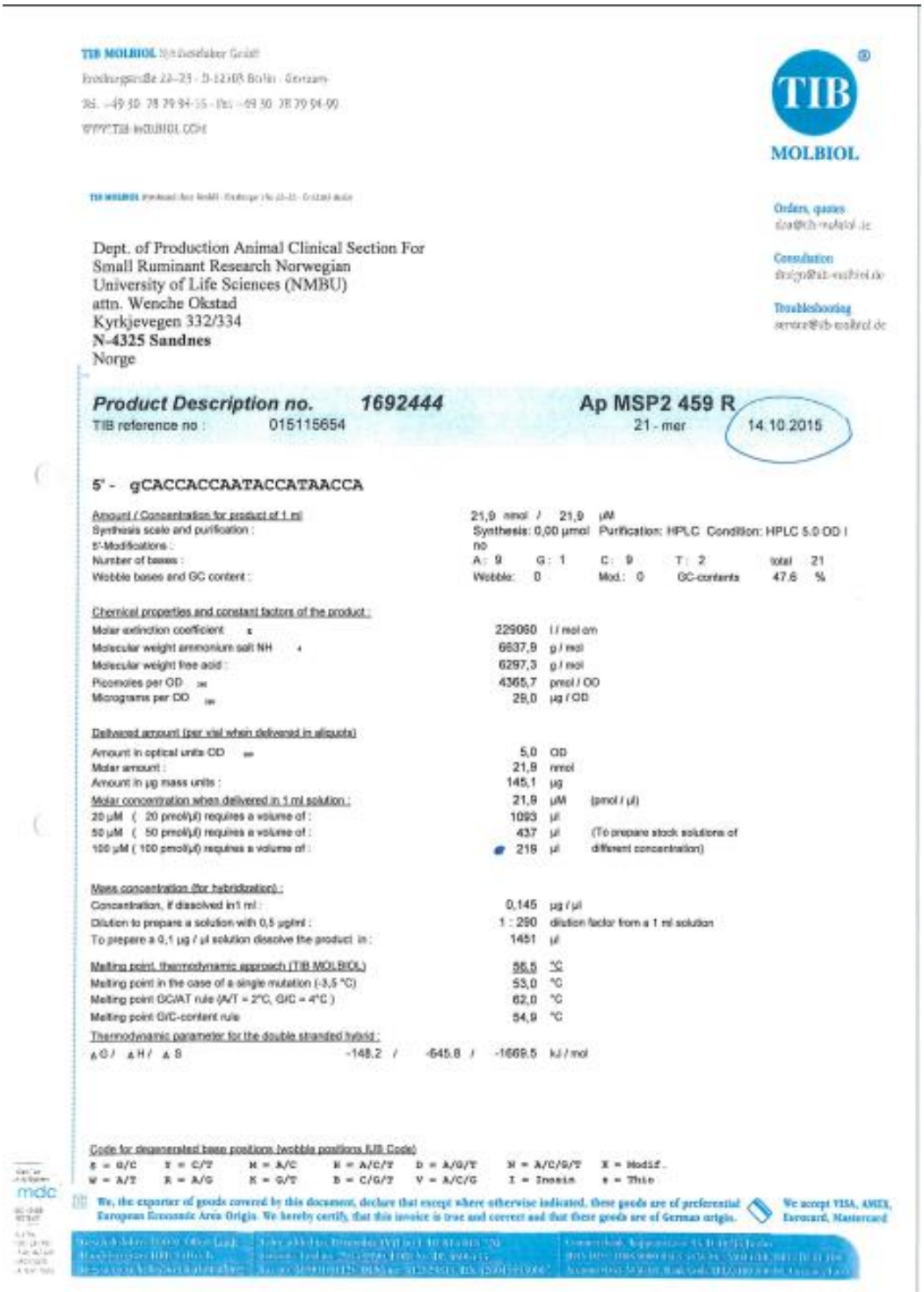
Figure A.1 Forward primer ApMSP2 252 F for qPCR.



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Dept. of Production Animal Clinical Section For
Small Ruminant Research Norwegian
University of Life Sciences (NMBU)
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Norge

Product Description no. ***1692444*** **Ap MSP2 459 R**

TIB reference no : 015115654 21 - mer 14.10.2015

5' - gCACCACCAATACCATAACCA

Amount / Concentration for product of 1 ml	21,9 nmol / 21,9 µM						
Synthesis scale and purification :	Synthesis: 0,00 µmol	Purification: HPLC	Condition: HPLC 5.0 OD I no				
5'-Modifications :							
Number of bases :	A: 9	G: 1	C: 9	T: 2	total	21	
Wobble bases and GC content :	Wobble: 0		Mod.: 0	GC-contents	47.6	%	

Chemical properties and constant factors of the product :		
Molar extinction coefficient ε	229080	l / mol cm
Molecular weight ammonium salt NH 4	6637,9	g / mol
Molecular weight free acid :	6297,3	g / mol
Picomoles per OD 260	4365,7	pmol / OD
Micrograms per OD 260	28,0	µg / OD

Delivered amount (per vial when delivered in aliquots)			
Amount in optical units OD 260	5,0	OD	
Molar amount :	21,9	nmol	
Amount in µg mass units :	145,1	µg	
Molar concentration when delivered in 1 ml solution :	21,9	µM	(pmol / µl)
20 µM (20 pmol/µl) requires a volume of :	1093	µl	
50 µM (50 pmol/µl) requires a volume of :	437	µl	(To prepare stock solutions of
100 µM (100 pmol/µl) requires a volume of :	219	µl	different concentration)

Mass concentration (for hybridization) :		
Concentration, if dissolved in1 ml :	0,145	µg / µl
Dilution to prepare a solution with 0,5 µg/ml :	1 : 290	dilution factor from a 1 ml solution
To prepare a 0,1 µg / µl solution dissolve the product in :	1451	µl

Melting point, thermodynamic approach (TIB MOLBIOL)	56,5	°C
Melting point in the case of a single mutation (-3,5 °C)	53,0	°C
Melting point GC/AT rule (A/T = 2°C, G/C = 4°C)	62,0	°C
Melting point G/C-content rule	54,9	°C

Thermodynamic parameter for the double stranded hybrid :

ΔG / ΔH / Δ S	-148.2 /	-645.8 /	-1669.5	kJ / mol

Code for degenerated base positions (wobble positions IUB Code)

S = G/C	Y = C/T	M = A/C	R = A/G/T	D = A/G/T	N = A/C/G/T	X = Modif.
W = A/T	R = A/G	K = G/T	B = C/G/T	V = A/C/G	I = Inosin	e = Thio

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Figure A.2 Reverse primer ApMSP2 459 R for qPCR.

A.3 Reaction mix and Parameters used in Semi-nested PCR

The amplification of *rrs* (16S rRNA gene) was performed on PTC-2000 instrument (MJ Research). The enzyme used was Amplitaq Gold (Table A.3 and A.4). Primer descriptions and information are shown in Figure A.4 – A.6.

Table A.3 Semi-nested PCR reaction mix I per sample

Forward Primer 16sF5 (10 pmol/µl)	1.0 µl
Reverse Primer AnaR4b (10 pmol/µl)	1.0 µl
10 × PCR Gold Buffer	2.0 µl
MgCl (25 mM)	1.2 µl
dNTP (2 mM)	2.0 µl
Rnase free H_2O	7.6 µl
Amplitaq Gold (5 unit/ µl)	0.2 µl
Template	5.0 µl
Total	20 µl

Table A.4 Semi-nested PCR reaction mix II per sample

Forward Primer 16sF5 (10 pmol/µl)	1.0 µl
Reverse Primer AnaR5 (10 pmol/µl)	1.0 µl
10 × PCR Gold Buffer	2.0 µl
MgCl (25 mM)	1.2 µl
dNTP (2 mM)	2.0 µl
Rnase free H_2O	11.6 µl
Amplitaq Gold (5 unit/ µl)	0.2 µl
Template	1.0 µl
Total	20 µl

All of the samples were analyzed with the same parameters in the PTC-2000 instrument (Table A.5).

Table A.5 Semi-nested PCR parameters

Step	**Temperature**	**Time**
1	95 °C	5 minutes
2	94 °C	30 seconds
3	60 °C	30 seconds
4	-1 °C/cycle	
5	72 °C	30 seconds
6	Repeat step 2 – 5	7 times
7	94 °C	30 seconds
8	52 °C	30 seconds
9	72 °C	30 seconds
10	Repeat step 7 – 9	30 times (20 for PCRII)
11	72 °C	5 minutes
12	4 °C	forever
13	End	

Data sheet on the DNA marker used for the gel electrophoresis are shown in Figure A.3.

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

SIGMA

ProductInformation

PCR Marker, 50-2,000 bp

Catalog Number **P9577**
Storage Temperature –20 °C

Product Description
This PCR Marker has been especially designed for size determination of PCR generated DNA fragments. The recommended agarose gel concentration is 2.0%. The marker is composed of 8 bands, 50 - 2,000 bp in predictably spaced (ladder) double stranded DNA recombinant fragments. The PCR Marker is supplied in 1× PCR Loading Buffer and is ready to use. It is recommended to use 5 µl of the PCR Marker per lane and one vial is sufficient for 50 applications.

Reagents
One vial of the PCR Marker (Catalog Number P2993) containing 250 µl.

One vial containing 0.5 ml of 6× PCR Loading Buffer (Catalog Number P7206) is supplied for preparation of PCR generated DNA fragment samples by the researcher.

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability
Store the PCR Marker (Catalog Number P2993) at –20 °C. The 6× PCR Loading Buffer (Catalog Number P7206) should be stored at 2–8 °C after receipt. It may require gentle heating (25-37 °C) to completely go into solution after freezing. Do not use if precipitates are present as this will affect the current flow in electrophoresis.

Procedure
The 2.0% (w/v) agarose gel is prepared with 1× TAE (40 mM Tris acetate, pH 8.3, with 1 mM EDTA) running buffer. Load 5 µl of the PCR Marker and the PCR generated DNA fragment samples on the agarose gel. The PCR Marker is ready to use and the PCR fragments may be prepared using the diluted 6× PCR Loading Buffer (Catalog Number P7206). The gel was run ~30 minutes in 1× TAE. After ethidium bromide staining, 8 bands (50-2,000 bp) are observed and the pattern is consistent with the expected fragment sizes.

Fragment Sizes: base pairs

50
150
300
500
750
1,000
1,500
2,000

The ethidium bromide background can be reduced by destaining 30–45 minutes in 1× electrophoresis buffer.

JLL,JWM,KS,MAM 11/06-1

Figure A.3 PCR marker data sheet.

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Product Description no. ***1709928*** **16S-F5**

TIB reference no : 016100037 19 - mer 07.01.2016

5' - AgTTTgATCATggTTCAgA

Amount / Concentration for product of 1 ml	5,0 nmol / 5,0 µM
Synthesis scale and purification :	Synthesis: 0,01 µmol Purification: GSF Condition: 5 nmol lyo
5'-Modifications :	no
Number of bases :	A: 5 G: 5 C: 2 T: 7 total 19
Wobble bases and GC content :	Wobble: 0 Mod.: 0 GC-contents 36.8 %

Chemical properties and constant factors of the product :

Molar extinction coefficient ε	208960 l / mol cm
Molecular weight ammonium salt NH	6164,4 g / mol
Molecular weight free acid :	5857,9 g / mol
Picomoles per OD	4785,8 pmol / OD
Micrograms per OD	29,5 µg / OD

Delivered amount (per vial when delivered in aliquots)

Amount in optical units OD	1,0 OD	5 OD
Molar amount :	5,0 nmol	21,3 nmol
Amount in µg mass units :	30,9 µg	
Molar concentration when delivered in 1 ml solution :	5,0 µM	(pmol / µl)
20 µM (20 pmol/µl) requires a volume of :	250 µl	
50 µM (50 pmol/µl) requires a volume of :	100 µl	(To prepare stock solutions of
100 µM (100 pmol/µl) requires a volume of :	50 µl	different concentration)

Mass concentration (for hybridization) :

Concentration, if dissolved in 1 ml :	0,031 µg / µl
Dilution to prepare a solution with 0,5 µg/ml :	1 : 62 dilution factor from a 1 ml solution
To prepare a 0,1 µg / µl solution dissolve the product in :	309 µl

Melting point, thermodynamic approach (TIB MOLBIOL)	46,5 °C
Melting point in the case of a single mutation (-3,5 °C)	43,0 °C
Melting point GC/AT rule (A/T = 2°C, G/C = 4°C)	52,0 °C
Melting point G/C-content rule	47,1 °C

Thermodynamic parameter for the double stranded hybrid :

ΔG / ΔH / ΔS -117.6 / -531.8 / -1389.9 kJ / mol

Code for degenerated base positions (wobble positions IUB Code)

S = G/C	Y = C/T	M = A/C	H = A/C/T	D = A/G/T	N = A/C/G/T	X = Modif.
W = A/T	R = A/G	K = G/T	B = C/G/T	V = A/C/G	I = Inosin	* = Thio

Figure A.4 Forward primer 16S – F5, for semi-nested PCR.

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Product Description no. ***1709929*** **ANA-R4B**

TIB reference no : 016100037 14 - mer 07.01.2016

5' - CgAACAACgCTTgC

Amount / Concentration for product of 1 ml	5,0 nmol / 5,0 µM
Synthesis scale and purification :	Synthesis: 0,01 µmol Purification: GSF Condition: 5 nmol lyo
5'-Modifications :	no
Number of bases :	A: 4 G: 3 C: 5 T: 2 total 14
Wobble bases and GC content :	Wobble: 0 Mod.: 0 GC-contents 57.1 %

Chemical properties and constant factors of the product :	
Molar extinction coefficient	148880 l / mol cm
Molecular weight ammonium salt NH	4454,3 g / mol
Molecular weight free acid :	4232,9 g / mol
Picomoles per OD	6716,8 pmol / OD
Micrograms per OD	29,9 µg / OD
Delivered amount (per vial when delivered in aliquots)	
Amount in optical units OD	0,7 OD
Molar amount :	5,0 nmol
Amount in µg mass units :	22,2 µg
Molar concentration when delivered in 1 ml solution :	5,0 µM (pmol / µl)
20 µM (20 pmol/µl) requires a volume of :	250 µl
50 µM (50 pmol/µl) requires a volume of :	100 µl (To prepare stock solutions of
100 µM (100 pmol/µl) requires a volume of :	50 µl different concentration)
Mass concentration (for hybridization) :	
Concentration, if dissolved in 1 ml :	0,022 µg / µl
Dilution to prepare a solution with 0,5 µg/ml :	1 : 44 dilution factor from a 1 ml solution
To prepare a 0,1 µg / µl solution dissolve the product in :	222 µl
Melting point, thermodynamic approach (TIB MOLBIOL)	47,6 °C
Melting point in the case of a single mutation (-3,5 °C)	44,1 °C
Melting point GC/AT rule (A/T = 2°C, G/C = 4°C)	44,0 °C
Melting point G/C-content rule	42,7 °C
Thermodynamic parameter for the double stranded hybrid :	
ΔG / ΔH / ΔS	-106.3 / -456.3 / -1174.7 kJ / mol

Code for degenerated base positions (wobble positions IUB Code)

S = G/C	Y = C/T	M = A/C	H = A/C/T	D = A/G/T	N = A/C/G/T	X = Modif.
W = A/T	R = A/G	K = G/T	B = C/G/T	V = A/C/G	I = Inosin	s = Thio

Figure A.5 Reverse primer ANA – R4B for first semi-nested PCR reaction.

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Product Description no. ***1709930*** **ANA-R5**

TIB reference no : 016100037 19 - mer 07.01.2016

5' - TCCTCTCAgACCAgCTATA

Amount / Concentration for product of 1 ml			5,0 nmol / 5,0 µM				
Synthesis scale and purification :			Synthesis: 0,01 µmol Purification: GSF Condition: 5 nmol lyo no				
5'-Modifications :							
Number of bases :	A : 5	G : 2	C : 7	T : 5		total	19
Wobble bases and GC content :	Wobble: 0		Mod : 0	GC-contents		47.4	%

Chemical properties and constant factors of the product :

Molar extinction coefficient ε	191370	l / mol cm
Molecular weight ammonium salt NH 4	6014,4	g / mol
Molecular weight free acid :	5707,8	g / mol
Picomoles per OD 260	5225,5	pmol / OD
Micrograms per OD 260	31,4	µg / OD

Delivered amount (per vial when delivered in aliquots)

Amount in optical units OD 260	1,0	OD
Molar amount :	5,0	nmol
Amount in µg mass units :	30,1	µg
Molar concentration when delivered in 1 ml solution :	5,0	µM (pmol / µl)
20 µM (20 pmol/µl) requires a volume of :	250	µl
50 µM (50 pmol/µl) requires a volume of :	100	µl (To prepare stock solutions of
100 µM (100 pmol/µl) requires a volume of :	50	µl different concentration)

Mass concentration (for hybridization) :

Concentration, if dissolved in1 ml :	0,030	µg / µl
Dilution to prepare a solution with 0,5 µg/ml :	1 : 60	dilution factor from a 1 ml solution
To prepare a 0,1 µg / µl solution dissolve the product in :	301	µl
Melting point, thermodynamic approach (TIB MOLBIOL)	46,3	°C
Melting point in the case of a single mutation (-3,5 °C)	42,8	°C
Melting point GC/AT rule (A/T = 2°C, G/C = 4°C)	56,0	°C
Melting point G/C-content rule	51,4	°C

Thermodynamic parameter for the double stranded hybrid :

Δ G / Δ H / Δ S	-119.1 /	-546.1 /	-1433.0	kJ / mol

Code for degenerated base positions (wobble positions IUB Code)

S = G/C	Y = C/T	M = A/C	K = A/C/T	D = A/G/T	H = A/C/G/T	X = Nodle,
W = A/T	R = A/G	B = G/T	N = C/G/T	V = A/C/G	I = Inosin	e = Thio

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Figure A.6 Reverse primer ANA – R5 for second semi-nested PCR reaction.

A.4 Reaction mix and Parameters used in Big Dye and Sequencing

BigDye Terminator v1.1 All of the samples were analyzed with the same parameters in the PTC-2000 instrument) (MJ Research), reaction mix and parameters are listed in Table A.6 and A.7.

Table A.6 BigDye Terminator v1.1. Reaction mix per sample

BigDye Terminator v1.1	4.0 µl
Buffer × 5	2.0 µl
Reverse Primer AnaR5 (10pmol/ µl)	0.2 µl
Rnase free H_2O	13.3 µl
Template (PCRII products)	0.5 µl
Total	20 µl

Table A.7 BigDye Terminator v1.1 Parameters

Step	**Temperature**	**Time**
1	96 °C	1 minute
2	96 °C	10 seconds
3	50 °C	5 seconds
4	60 °C	4 minutes
5	Repeat Step 2 – 4	24 times
6	4 °C	Forever
7	End	