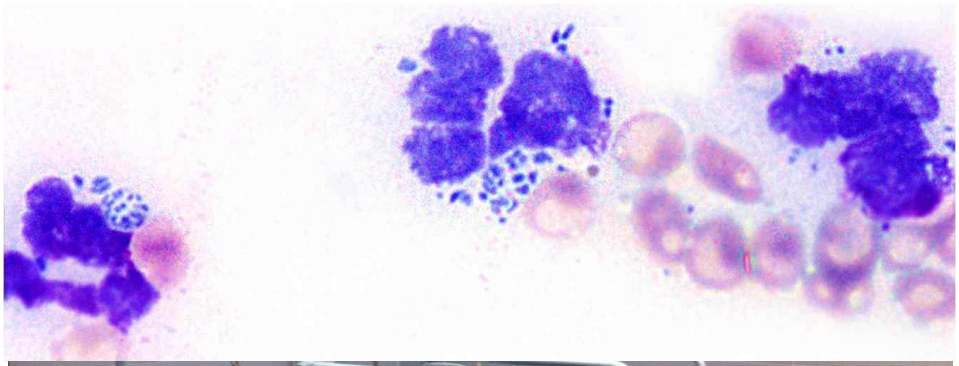
Experimental tick transmission studies in sheep with *Anaplasma phagocytophilum*



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Abstract

Anaplasma phagocytophilum, an intracellular, gram-negative bacteria, survives and replicates in neutrophilic granulocytes (Stuen et al. 2013). The disease it causes is called granulocytic anaplasmosis which can affect many vertebrate animals, including sheep, horses and also humans (Goldman & Green 2009). *I. ricinus,* which is widespread in Europe, is the most common tick known as a vector for this disease (Stuen et al. 2013). *A. phagocytophilum* mainly cause fever which lasts for 1-2 weeks (Stuen & Longbottom 2011). Other clinical signs are mild thrombocytopenia (Carrade et al.), reduced weight gain (Stuen et al. 2002), rigors, headache, myalgia and malaise (Goldman & Green 2009).
This study was designed to achieve two goals. At first, the aim was to investigate if *Dermacentor reticulatus* and *Amblyomma hebraeum* were, besides *I. ricinus* possible vectors for the transmission of *A. phagocytophilum*. *D. reticulatus* is the main vector of *Babesia canis* and other piroplasm species(Karbowiak 2014) and is distributed in many parts of Europe (from Atlantic ocean to Kazakhstan), particularly in wooded areas (Wall & Shearer 1997; Taylor et al. 2007). *Amblyomma hebraeum* is distributed in the rural areas of southern Africa (including South-Africa, Zimbabwe and Mozambique) and is known as the vector of *Rickettsia africae* which is known as ''African tick bite fever'' (Mabey et al. 2013). If the transmission of *A. phagocytophilum* by *A. hebraeum* and *D. reticulatus* can succeed, there is a significant possibility for the occurrence of *A. phagocytophilum* in *A. hebraeum* and *D. reticulatus* in areas of the world where these ticks are common (southern Africa and Western-Europe).
The second goal was to determine if blood samples from different animals coming from different areas in the Netherlands or faeces (tested positive for *A. phagocytophilum*) from infected ticks will cause the same symptoms in sheep and contain the same strain of *Anaplasma phagocytophilum*. These products were injected in individual sheep.

In this study, three experimental rounds were designed to investigate the two main goals. Each experimental round (which lasted 14 days) contained four sheep which were each exposed to *A. phagocytophilum* differently under laboratory conditions. The sheep were monitored by daily temperature measurement and if fever was measured, blood was collected and examined by making blood smear (to see if inclusion bodies/morulae were present in the neutrophil granulocytes) and by performing a PCR/RLB. Ticks were collected when they were engorged or at the end of the experimental round. When they were fed on a sheep which had a febrile period, they were tested to determine their infection rate.

Transmission of *A. phagocytophilum* did not succeed in *D. reticulatus* and *A. hebraeum*. The sheep did not get any symptoms, so blood collection was not implemented. *I. ricinus* did successfully transmit *A. phagocytophilum*, which proves that the experimental model was accurate. Why transmission did not occur, is not clear yet, so further research is necessary.
The experimental round in which the second hypotheses was tested, showed that all the sheep injected with the different blood samples did get symptoms which were all similar to each other. After PCR/RLB, different strains of *A. phagocytophilum* were found in the blood. The sheep who was injected with the infected tick faeces did not develop any symptoms, so no blood was collected.

Keywords: *Anaplasma phagocytophilum, I. ricinus, D. reticulatus, A. hebraeum,* sheep, zoonosis, transmission, different *A. phagocytophilum* strains, tick faeces.

Introduction

Background information

Anaplasma phagocytophilum

Anaplasma phagocytophilum is an obligate intracellular, gram-negative bacterium, which has a tropism for phagocytes. It survives and replicates in neutrophilic granulocytes (Stuen et al. 2013). The disease it causes is called granulocytic anaplasmosis (Goldman & Green 2009). *Anaplasma* is known (sometimes even resulting in death) in several hosts: domestic ruminants (sheep and cattle), but also in horses, dogs, cats, deer, wild rodents and even in humans (Stuen et al. 2013).

The genus *Anaplasma* belongs to the family of Anaplasmataceae and is one of the four genera together with *Ehrlichia*, *Neorickettsia* and *Wolbachia*. Because of a nucleotide similarity of 99,1% between the three species *Ehrlichia equi*, *Ehrlichia phagocytophila* and human granulocytic agent, Dumler and colleagues have combined these three and regrouped them under the name *Anaplasma phagocytophilum* (Goldman & Green 2009; Woldehiwet 2010).
After regrouping of the Family Anaplasmataceae by Dumler et al., *A. phagocytophilum* was found as the cause of many diseases including tick-borne fever (TBF) in ruminants, equine granulocytic anaplasmosis(EGA) in horses, canine granulocytic anaplasmosis (CGA) in dogs and human granulocytic anaplasmosis (HGA) (Reppert et al. 2014).

A. phagocytophilum transmission will occur by *Ixodes* tick species worldwide, especially on the northern hemisphere. In Europe, the average *A. phagocytophilum* prevalence in *I. ricinus* (the most common tick in The Netherlands) ranges between 1% and approximately 20% (Stuen et al. 2013). This makes *I. ricinus* the main vector for the transmission of *A. phagocytophilum*. The disease can be transmitted when an *A. phagocytophilum*-infected *I. ricinus* is attached to the host and has a blood meal (Goldman & Green 2009).

Clinical signs consist mainly of high fever. Within 14 days after exposure of *A. phagocytophilum*, sheep develop clinical signs from which fever will last for 1-2 weeks. Tick-borne fever (a term which is exclusively used to describe an infection with *A. phagocytophilum*) is seldom fatal, unless it is complicated by secondary infections as a result of immunosuppression (Stuen & Longbottom 2011). Other clinical signs are mild thrombocytopenia or other cytopenias (such as neutropenia, lymphopenia and mild anemia) (Carrade et al.), reduced weight gain (Stuen et al. 2002), rigors, headache, myalgia and malaise (Goldman & Green 2009).

A clinical diagnosis can be made based on the clinical signs such as a sudden onset of high fever. But most importantly are the typical cytoplasmatic inclusion bodies in especially neutrophilic granulocytes which are known as morulae. Light microscopy of blood smears taken in the initial fever period are usually sufficient to confirm the diagnosis by demonstrating the morulae, which present as blue inclusions (Stuen & Longbottom 2011). This microscopic detection of the inclusions may be difficult and prolonged examination is often required to accurately detect *A. phagocytophilum*, as less than 0,1% of the neutrophils may show these morulae (Thomas et al. 2009).
For definitive diagnosis, laboratory confirmation is required (Woldehiwet 2010). Laboratory methods include immuno-histochemistry of tissue samples and PCR. Serology can also be used to support the diagnosis, for example with indirect immunofluorescent antibody (IFA) test. However, it may not be straightforward to use IFA to diagnose acute infection in sheep, as IFA titers remain persistent for months after the primary *A. phagocytophilum* infection. There is a commercially available SNAP 4Dx test for diagnostics in dogs. Pathology can also be useful, as an enlarged spleen (up to 4-5 times the

normal size) can be regarded as indicative of TBF in sheep (Stuen & Longbottom 2011). Of these diagnostic tests, PCR and IFA provide the highest sensitivity for the diagnosis of *A. phagocytophilum* (Stuen et al. 2013).

Therapy consists of antibiotics, of which tetracycline or doxycycline are preferred. Prevention of disease in domestic animals is done by reduction of tick infestation by use of chemical acaricides (such as pyrethroids) (Stuen & Longbottom 2011)

Ixodes ricinus

Of the Ixodidae family, the genus *Ixodes* has the most species. The most important species is *ricinus*, which is widespread in Europe: part of the British isles and from Norway southward to Iran and Turkey, Italy, Bulgaria and the Pyrenes. *I. ricinus* is also known as ''sheep tick'' and is considered the ''most important multi-potent vector in Europe'' (Randolph 2009; Capinera 2008). This makes *I. ricinus* an important hematophagous vector of zoonotic disease of both veterinary and public health importance (Collini et al. 2015).

Males are 2,5-3 mm and females (Fig. 1) are with their size of 3-4 mm a little bit larger, especially when engorged (10 mm). When engorged, the color of females is light grey, but male and unfed females will appear red-brown (Wall & Shearer 2001).

Figure 1: *I. ricinus,* female
(Hubálek & Rudolf 2010)

During spring/early summer and late summer/autumn, *I. ricinus* is most active (Jongejan & Kaufman 2003). *I. ricinus* is known as the primary vector of *A. phagocytophilum* on a wide range of hosts, including small rodents, hedgehogs, birds, cats, dogs, deer, horses, cattle, sheep and humans (Reppert et al. 2014). The immature stages feed on birds, but sheep can be host of all three stages (Capinera 2008)

Dermacentor reticulatus

This ornate dog tick belonging to the family of Ixodidae, is the second most important hard tick species in central Europe, after the *Ixodes ricinus*, in terms of their number and impact on the economy (Karbowiak 2014). *D. reticulatus* ticks are ornate, white with variegated brown spots (Fig. 2) (Wall & Shearer 1997).

Figure 2: *D. reticulatus*. Left: male, right: female (UCTD 2014)

D. reticulatus is distributed in many parts of Europe (from Atlantic ocean to Kazakhstan), particularly in wooded areas (Wall & Shearer 1997; Taylor et al. 2007).

The most important hosts for the adult ticks are dogs, horses and wild and domestic ruminants,. Humans are also a possible host, but are very seldom affected. Rodents and insectivores are infected by larvae and nymphs. Mating of adult ticks takes place on the host (Wall & Shearer 1997; Karbowiak

2014). Feeding of the ticks results in damage to skin and stress, but most importantly, *D. reticulatus* is the main vector of *Babesia canis* and other piroplasm species. They are also able to transmit tularemia, rickettsioses and other pathogens. Based on these major infection risks, *D. reticulatus* has great epidemiological importance in Europe (Karbowiak 2014).

Amblyomma hebraeum

Amblyomma hebraeum is family of Ixodidae and are relatively large ticks which are ornate, variegated and have long robust mouthparts (Fig. 3 and 4). Due to this, they are difficult to remove (Spickler et al. 2010).

Figure 3: *A. hebraeum*; female

Figure 4: *A. hebraeum*; male (Kaufmann 1996)

Amblyomma hebraeum (the South African bont tick) is a common endemic tick in the rural areas of southern Africa (Fig. 5) (including South-Africa, Zimbabwe and Mozambique). Almost 100% of the *A. hebraeum* population may be infected with a major important disease caused by *Rickettsia africae* which is known as ''African tick bite fever''. *Rickettsia africae* is transmitted transstadially and transovarially and happens in all feeding stages of the tick. *A. hebraeum* might also be a possible vector for tick-borne encephalitis which affects humans, but this is not completely sure yet. This all makes *A. hebraeum* a notorious tick species in southern Africa (Mabey et al. 2013).

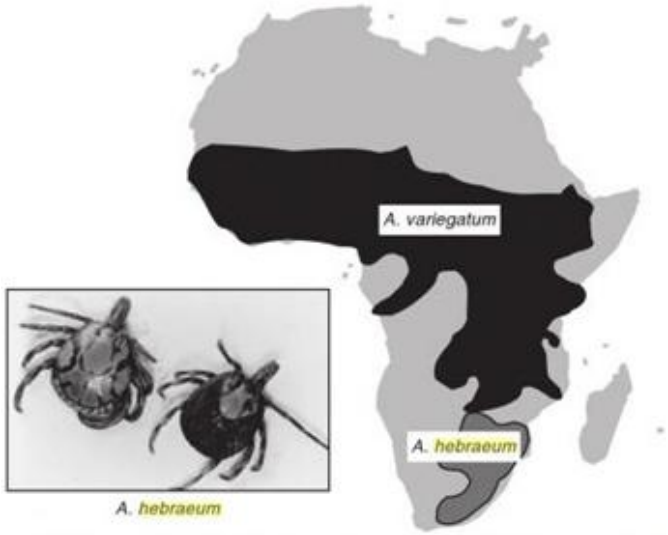


Figure 5: Geographical distribution of *A. hebraeum* and *A. variegatum*, both vectors of *Rickettsia africae* (Mabey et al. 2013)

Most Ixodid ticks (e.g. *Amblyomma, Dermacentor, Ixodes*) have a three-host cycle in which each active stage (larva, nymph, adult) parasitizes a different animal of the same or different species (Cook & Zumla 2009). Immature *A. hebraeum* stages usually feed on smaller mammals such as birds and reptiles, while the adult stages usually tend to be found on large animals, including livestock and wildlife (Spickler et al. 2010).

Main goal of this study

In this study, there are two main goals to achieve. At first, an experimental design will be followed which will test if *A. hebraeum* and *D. reticulatus* are capable of transmitting *A. phagocytophilum* in sheep, since there have been positive results of transmitting *A. phagocytophilum* in *I. ricinus*.
In order to study *A. phagocytophilum* more closely, it is essential to do this in an experimental model where sheep are only infected with *A. phagocytophilum* and studied under standardized laboratory conditions. To this end, DEC permission has been granted in order to use sheep for experimentally infection with *A. phagocytophilum* and to conduct tick transmission studies.
If there is proof of a transmission of *A. phagocytophilum* by *A. hebraeum* and *D. reticulatus*, there is a significant possibility for the occurrence of *A. phagocytophilum* in *A. hebraeum* and *D. reticulatus* in areas of the world where these ticks are common (southern Africa and Western-Europe, as mentioned before in the introduction).

The second goal of this study is to investigate if blood samples from different animals coming from different areas in the Netherlands or faeces from infected ticks will cause the same symptoms in each individual sheep and contain the same strain of *Anaplasma phagocytophilum*. Through the years of research at UCTD, many blood samples from animals infected with *A.phagocytophilum* were collected and stored in the freezer (first they were frozen at -80 ^{o}C after which the samples were placed in nitrogen). Thereby, infected tick faeces was collected from ticks which were infected with *A. phagocytophilum*. This faeces product and the blood samples from the collection at UCTD (blood samples from the freezer and new/fresh blood samples), will be taken from different animals and will each be injected in a sheep from the experimental model.

Main research question

Researching the relative importance of transstadial transmission (vertical) of *A. phagocytophilum* in *A. hebraeum* and *D. reticulatus* and the effect of different blood strains and infected tick faeces with *A. phagocytophilum* in sheep.

Hypothesis

Experiment 1: *A. hebraeum* and *D. reticulatus* used as vectors for the transmission of *A. phagocytophilum*
H_0 = *A. hebraeum* and *D. reticulatus* are not capable of transmitting *A. phagocytophilum* in sheep
H_1 = *A. hebraeum* and *D. reticulatus* are capable of transmitting *A. phagocytophilum* in sheep

Experiment 2: Testing of different blood samples and *Anaplasma phagocytophilum* contaminated products in sheep
H_0 = Blood from different animals coming from different areas in the Netherlands or faeces from infected ticks will cause the same symptoms in each individual and contain the same strain of *Anaplasma phagocytophilum*.
H1 = Blood from different animals coming from different areas in the Netherlands or faeces from infected ticks will cause different symptoms in each individual and contain not the same strains of *Anaplasma phagocytophilum*.

For the design of each experimental model, see Appendix I, II and III.

Materials and Methods

Materials

Sheep

For this research, sheep of the Department of Farm Animal Health from the Faculty of Veterinary Medicine in Utrecht were used. Permission of the DEC is required to use these sheep.
The sheep were tested in an experimental model and under laboratory conditions, which was called Mx (x stands which number of model it is). UCTD already performed two experimental rounds (M1-M2) before, so in this research, experimental rounds M3-M5 were executed.
At the beginning of each experimental model, five sheep from the herd of the Faculty of Veterinary Health were selected and blood samples were collected at day -7 of the experiment. These blood samples were tested for various diseases to make sure the sheep that would be used in the experimental model were free from any diseases or pathogens. Eventually, the four sheep that were tested negative for diseases or pathogens were selected for the experiment. In case all sheep were Specific Pathogen Free (SPF), the sheep that were the easiest to handle were selected.
During the experiment (from day 0, the inoculation, until day 14 or until the fever was over), animal caretakers from the Department of Farm Animal Health checked twice a day each day with the sheep if they were in good health and to take care of them (feeding, cleaning the stable). Every morning at 09.00 o' clock, the rectal temperature of the sheep was measured and noted. When the temperature was > 40 °C, fever was diagnosed and special measurements would be executed. Blood was collected in order to further investigate the presence of *A. phagocytophilum.* The blood was collected by jugular vein punction (after disinfection of the skin overlaying the vein) in tubes containing EDTA anticoagulant. These blood samples were transported in a transport safe Tupperware box to UCTD laboratory for research in the lab. The febrile period was most of the times self-limiting, but in case of severe illness or when odd symptoms would occur, the sheep would be treated with doxycycline, since there had already been proven by temperature measurement that the infection was present in the sheep.
The sheep were fed twice a day 0,25 kg KV/animal and 0,75 kg artificial dried grass/animal. Hay or silage was not preferred by the animal caretakers.

Stable

In the stable, a list was present to write down the temperature of the sheep. There were protocols which were signed by the animal caretakers after each time a step in the protocol was finished (for example, see Appendix IX). Also, there was an observation list to write down odd symptoms or deviations in behaviour.
The sheep were kept in individual compartments (separated by fences). There was a gutter around the stable in which Delladet was poured to create a barrier and avoid the ticks from escaping the stable. There was a protocol which described that 3x 1L Delladet should be poured in the gutter at the beginning of the experiment and this should be repeated after 3-4 days (see Appendix VIII).

Ticks and tick faeces

Ticks were provided from UCTD. All materials used to collect the ticks were provided by UCTD and collection was done by UCTD's protocols for collecting ticks.
Tick stages used in the experiment were nymphs and adults. Nymphs of different species (depending of the experimental round) were used to introduce to an infected host (which was infected by a injection of a blood sample with *A. phagocytophilum*) in order to infect the nymphs with *A. phagocytophilum*. When they fed on the infected sheep, assumed was that the nymphs would get infected with *A. phagocytophilum* and therefore were able to transmit the infection to a naive host

when adult. Eventually, adults, that were infected as nymphs on an infected host, were placed on a naive host to investigate if transmission by this specific tick species was possible.

The tick faeces used in experimental model M5 was coming from the *I. ricinus* nymphs used in experimental model M3. This tick faeces consisted of different components (Fig. 6). There were white, crystal-like fractions (which consists of guanine, coming from the Malpighian tubules), red particles (undigested blood) and coil-like black material (known as the digested fraction, which includes hematin from the digestion of the blood) (Sonenshine 1991).
All described particles of the faeces were used and were dissolved in serum buffer. After a briefly spin in the centrifuge, supernatant was transferred to clean tubes. Hereafter, this fluid (± 1ml) was injected subcutaneous between the shoulders of one of the sheep.

Figure 6: The infected tick faeces from *I.ricinus.* The different colored particles are distinguishable.

Blood samples

In order to infect the sheep with *A. phagocytophilum,* different blood samples were used in each experimental round.
In M3, Frozen blood from UCTD collection was used to infect the sheep with *A. phagocytophilum*: stabilate number CR 346, *A. phagocytophilum* infected sheep from Ameland (a Dutch island), collected on 7-3-1999.
In M4, a sample from the same blood as in M3 was used to infect he sheep with *A. phagocytophilum*: stabilate number CR 346*, A. phagocytophilum* infected sheep from Ameland (a Dutch island), collected on 7-3-1999.
In M5, three different blood samples were used to see if they had the same outcome in symptoms:
- Sample 1: Fresh blood with *A. phagocytophilum* collected at 29-6-2015, Bargerveen (Drenthe, The Netherlands)
- Sample 2: Frozen blood with *A. phagocytophilum* collected at 11-6-2015, Oosterbos (Drenthe, the Netherlands)
- Sample 3: Frozen blood with *A. phagocytophilum* collected at 24-6-2015 from dog Tiba in Den Haag (Zuid-Holland, the Netherlands).

Each blood sample which is stored at UCTD, undergoes the same procedure for freezing:
Before the blood samples were frozen, 10% DMSO was added to the blood to cryoprotect them. So if there was 15 ml blood, 1,5 ml DMSO was added to preserve the blood in the freezer. By adding the DMSO, the blood was kept in an Erlenmeyer on ice because adding DMSO results in an exothermic reaction. After the blood with the added DMSO was distributed in 1ml tubes, they were kept in the freezer at -80^{o}C. To let the temperature from the blood decrease gradually, the tubes were placed in a small box felt with isopropanol (isopropyl alcohol). When completely frozen, they were eventually transferred and stored in liquid nitrogen.

Methods

Design of the experimental models

Experimental models M3, M4 and M5 each consisted of four sheep. Each sheep was introduced with *A. phagocytophilum* differently:

Experimental model M3 (see Appendix I):
- Sheep 1 (57179): *A. hebraeum* nymphs were released on day 1 (males) and day 3 (females) after injection with *A. phagocytophilum* strain. Male ticks were released first. This was necessary because otherwise, the female ticks would not attach (*A. hebraeum* female ticks only attach when there are already male ticks present and attached).
- Sheep 2 (72273): *D. reticulatus* nymphs were released after injection with *A. phagocytophilum* strain.
- Sheep 3 (72382): *I. ricinus* adult ticks infected with *A. phagocytophilum* as a nymph, were placed in small bags on the sheep's back.
- Sheep 4 (57337): *I. ricinus* adult ticks infected with *A. phagocytophilum* as a nymph, were placed in small bags on the sheep's back.

Experimental model M4 (see Appendix II):
- Sheep 1 (72086): *A. hebraeum* adult ticks infected with *A. phagocytophilum* as a nymph, were placed in the small bags. At day 0, 25 male ticks were set free on the sheep's back. In that case, the female ticks would hatch at day 2.
- Sheep 2 (56970): *D. reticulatus* adult ticks infected with *A. phagocytophilum* as a nymph, were placed in the small bags on the sheep's back.
- Sheep 3 (72253): An *A. phagocytophilum* strain (CR 346*, A. phagocytophilum* infected sheep from Ameland (a Dutch island), collected on 7-3-1999) was injected in the vena jugularis. Short after the injection, nymphs of *Ixodes ricinus* were set free in the small bags.
- Sheep 4 (72261) did undergo the exact same procedure as sheep 3.

Experimental model M5 (see Appendix III):
Four sheep were selected, but no ticks were used during this experiment. Just before the commencement of the experimental model 5, a dog was diagnosed with Anaplasmosis. To further investigate the specific strain of *A. phagocytophilum*, the composition of the upcoming study was altered: instead of testing *A. phagocytophilum* transmission by ticks (like in M3 and M4), the intention became to inject other blood samples from the collection at UCTD with *A. phagocytophilum* to monitor if all *A. phagocytophilum* was the same and if each strain would cause the same symptoms. Therefore, the composition of experimental model 5 was as follows:
- Sheep 1 (57782): Fresh blood that was collected at 29-6-2015 from a lamb (earnumber: 85928570) which was part of the sentinel study from UCTD in Bargerveen (Drenthe, The Netherlands) was injected into the vena jugularis.
- Sheep 2 (52317): Frozen blood (-80^{o}C) that was collected at 11-6-2015 from a lamb (earnumber: 85828542, which had a temperature of 41,4^{o}C that day) which was part of the sentinel study from UCTD in Oosterbos (Drenthe, the Netherlands), was injected intraveneus in the vena jugularis.
- Sheep 3 (57783): A blood sample was taken at 24-6-2015 from dog Tiba in Den Haag (Zuid-Holland, the Netherlands) after Anaplasmosis was diagnosed. The tick on this dog probably infested the dog at Terschelling (a Dutch island). This blood sample was frozen (-80^{o}C) and injected in the vena jugularis of this sheep.
- Sheep 4 (52310): Faeces from *I. ricinus* nymphs that were fed on a sheep with *A. phagocytophilum* (sheep 57337 from M3), was dissolved in buffer, centrifuged and eventually injected subcutaneous in this sheep to see if the *A. phagocytophilum* detected in these faeces can still cause infection and symptoms.

Blood smear

After blood was collected from the sheep with fever, the blood samples were taken to the laboratorium at UCTD where a blood smear was made instantly. This happened in a laminar flow cabinet. After the blood on the object-glass was dried, the blood smear was stained with the Kwik™diff stain kit (which consists of three coloring steps). The smear was dipped for five seconds in the first two colors and four seconds in the last one. After staining, the blood smear was washed in distilled water and dried before it could be examined. Determination of the blood smear occurred with light microscopy with the 100x oil immersion objective. If there were morulae (micro-colonies of *Anaplasma phagocytophilum*) present in the neutrophil granulocytes (Fig. 7), the blood from which the blood smear was made, was noted as ''positive'' until the RLB results would prove otherwise.

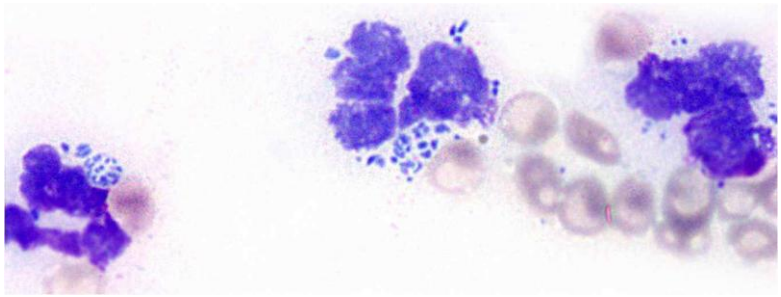


Figure 7: *A. phagocytophilum* present as morulae in the neutrophil granulocytes

Infection rate in ticks

To investigate to what extent transmission of *A. phagocytophilum* is effective by ticks, the infection rate was determined. After tick collection in each experimental round, the ticks were stored in the freezer at UCTD until they were tested.

Blood DNA extraction

Before performing a PCR amplification and RLB to examine if the blood really contains *A. phagocytophilum*, DNA extraction from the blood is necessary. From each blood sample, DNA was extracted and after the extraction, the product (with DNA) was frozen at -20°C at UCTD. The DNA extraction was done according to a protocol, which is included in the Appendix (IV). During the DNA extraction process, the blood cells are lysed so the DNA is set free. Then, the DNA is separated from the other particles by using different buffers and the Nucleospin Tissue Kit (Art. No. 740952.10/.50/.250, Macherey-Nagel).

Tick DNA extraction

The process of DNA extraction from ticks or sheep tissue samples is practically the same as for blood. However, some additional steps are necessary in order to lyse all tissue cells (for instance, adding 5mm stainless steel beads to the ticks to crush them and set all the DNA free). A protocol of DNA extraction of ticks is added in the Appendix (IV).

PCR

PCR was performed after tick or DNA extraction. PCR is a process whereby specific selected strands of DNA are amplified (Fig. 8). By using a specific set of primers complementary to a certain part of the pathogen's DNA (target sequence), only the pathogen's DNA will be amplified. In addition, by increasing the amount of a specific part of the pathogen's DNA (target sequence), it will be more likely to detect the DNA target sequence and thus the sensitivity and the specificity of the subsequent RLB outcome will increase.

In this study, primers complementary to *Anaplasma* spp. and *Ehrlichia* spp. were used, since only the presence of *Anaplasma phagocytophilum* in the ticks and blood was investigated (Table 1). Because of their relative similarity to one another concerning the PCR-protocol, primers of *Anaplasma* and *Ehrlichia* were put together (a forward and a reverse primer).

Pathogen	Primer	Sequence
Ehrlichia/Anaplasma	Ehr-F	5' - GGA ATT CAG AGT TGG ATC MTG GYT CAG - 3'
	Ehr-R	5' - Biotin - CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT - 3'

Table 1: PCR primers and sequences

For the primer set, a mastermix was made, in order to be able to execute the PCR. Besides the primer set, this mastermix contained PCR grade water, 5x Phire buffer, 10 mM dNTPs and 2U/µl Phire hot start II DNA polymerase. The mastermix was then pipetted into eppendorf tubes and shortly after, the extracted DNA samples were added. A positive and negative control were made for every pathogen that was tested. *Ehrlichia chaffeensis* counted as the positive control DNA sample in the RLB, so this sample also went through the PCR process. The mastermix alone counted as the negative control. Eventually, the eppendorf tubes were placed in the PCR machine and the settings were adjusted in order to ensure maximum DNA replication (Table 2). After PCR amplification, the PCR products were stored at 4°C until used for RLB hybridization. The protocol used to perform PCR is included in the Appendix (V).

Number of cycles	Time	Temperature
1 cycle	30 sec.	98 °C
10 cycles	5 sec. 5 sec. 7 sec.	98 °C 67 – 57 °C 72 °C
40 cycles	5 sec. 5 sec. 7 sec.	98 °C 57 °C 72 °C
1 cycle	1 min.	72 °C

Table 2: The PCR computer program for *Anaplasma/Ehrlichia* in the PCR machine (temperature cycle).

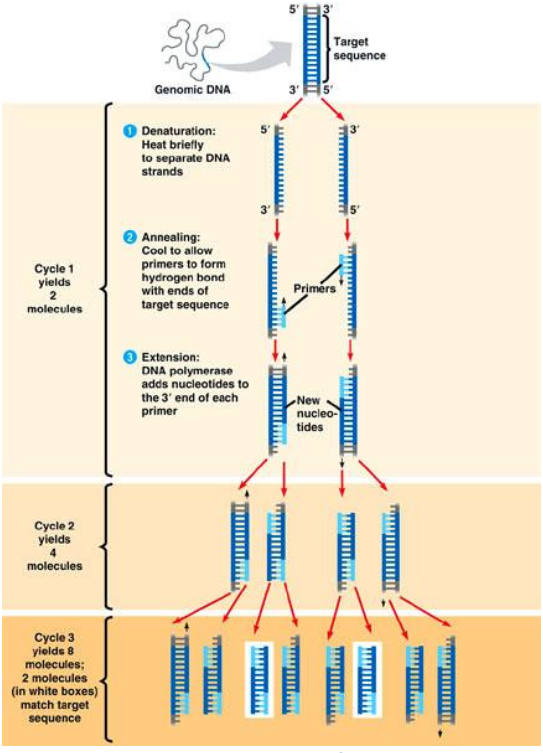


Figure 8: Schematic representation of consecutive PCR steps in order to amplify a target DNA sequence (Ablett et al. 2015)

RLB

After PCR was performed, the PCR products were used in the RLB. Reverse Line Blotting (RLB) hybridization is a process in which target DNA strands amplified by PCR are introduced onto a membrane containing covalently-bound probes (oligonucleotides) complementary to the amplified DNA sequences of the specific pathogens (Fig. 9). So if the amplified DNA in the PCR product is complementary to one of the probes on the membrane, it will match with the probe and attach. They are applied to the membrane using a miniblotter in such a way that the direction of the PCR products were perpendicular to the direction of the species-specific oligonucleotides (Fig. 10). Two control oligonucleotides were also applied to the membrane: *Ehrlichia* and *Babesia*.

The amplified DNA strands in the PCR product are labeled with biotin. After binding of the PCR product to the membrane and thoroughly washing to remove unattached PCR products, Streptavidin labeled with peroxidase will be added and will bind to the biotin label. When additionally ECL reagents are added, the peroxidase catalysis a reaction with the ECL reagents. This chemical reaction results in luminal (a substance in ECL reagent 2) becoming oxidized and producing light. A light-sensitive film is subsequently placed on top of the membrane in a dark room in order to visualize the binding of the PCR products, after developing the film. A protocol of the RLB procedure is included in the Appendix (VI). The membrane will be washed for reuse.

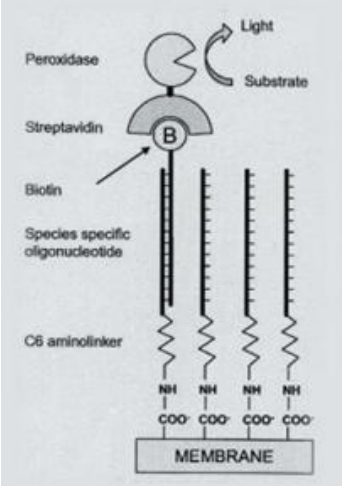


Figure 9: Schematic representation of the hybridization principle (Isogen Life Science 2004)

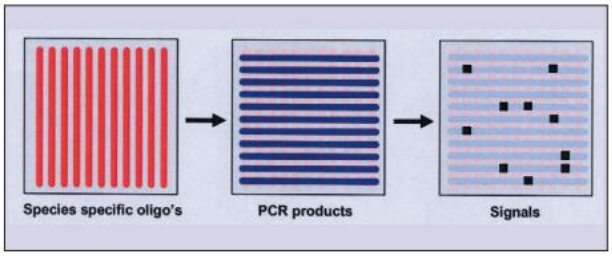


Figure 10: Schematic representation of the RLB assay (Isogen Life Science 2004)

The RLB used in this study contains probes for detection of the following tick-borne pathogens: *Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Theileria* spp. and *Borrelia* spp., the most common tick-borne diseases. Probes used for detection of *A. phagocytophilum* are shown in Table 3. These different primers used in the RLB are now able to detect different *A. phagocytophilum* strains in the blood samples (hypothesis 2).

Besides the species-specific probes, catch-all probes are included on the membrane in order to screen more general for the presence of pathogens. These catch-all probes consist of highly conserved parts of the DNA. The RLB membrane used in this study contained catch-all probes for *Ehrlichia/Anaplasma*, *Theileria/Babesia, Babesia, Theileria* and *Rickettsia*.

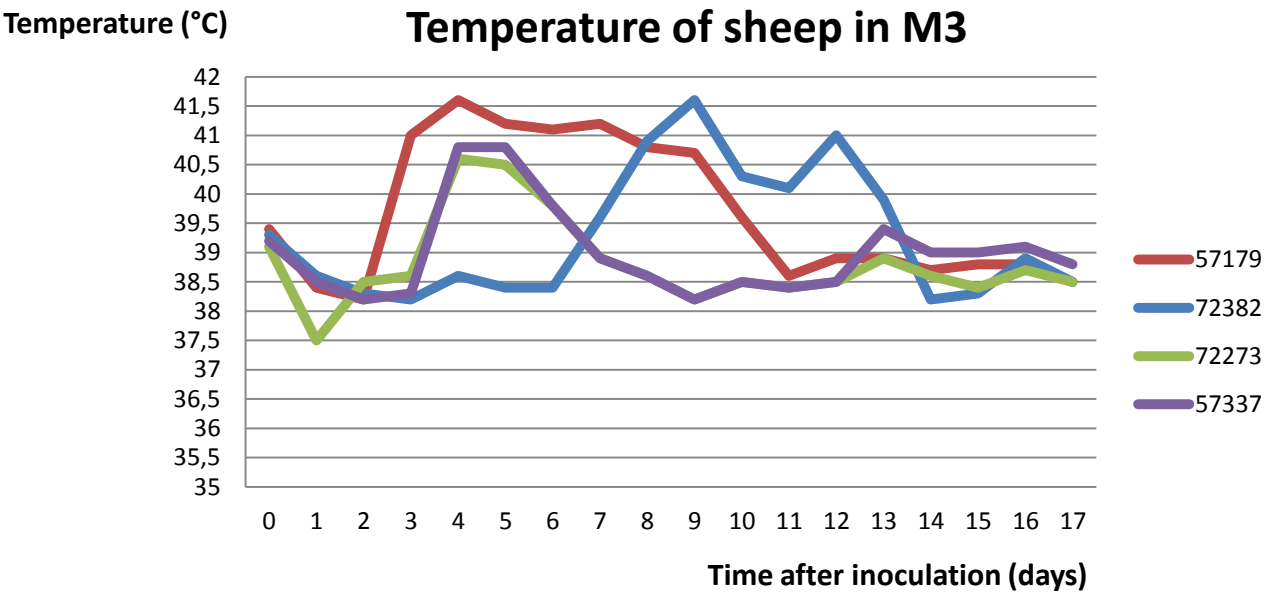
Pathogen	Primer DNA-sequence
***Anaplasma phagocytophilum* 1**	TTG CTA TAA AGA ATA ATT AGT GG
***Anaplasma phagocytophilum* 3**	TTG CTA TGA AGA ATA ATT AGT GG
***Anaplasma phagocytophilum* 5**	TTG CTA TAA AGA ATA GTT AGT GG
***Anaplasma phagocytophilum* 7**	TTG CTA TAG AGA ATA GTT AGT GG

Table 3: Primers used for the detection of *Anaplasma phagocytophilum*. Four different primers were used on the RLB. Nucleotides that deviate from the other DNA sequences are marked in red.

Results

Effect of *Anaplasma phagocytophilum* on sheep

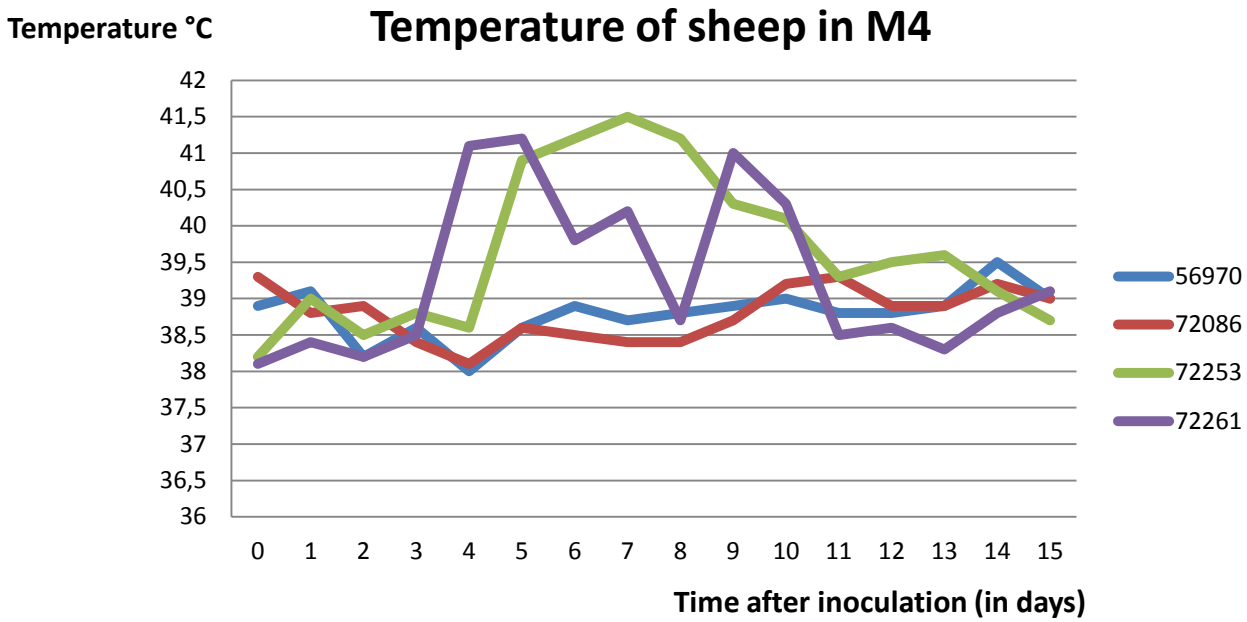
In the materials and methods and the Appendix I, II and III already was explained how each sheep was exposed to *A. phagocytophilum*. To make clear how each sheep reacted on *A. phagocytophilum* by means of temperature, it will again be summarized shortly before interpreting the graphics.



M3 consisted of (see Appendix I):
Sheep 57179: *A. hebraeum* nymphs + injection with *A. phagocytophilum* strain.
Sheep 72273: *D. reticulatus* nymphs + injection with *A. phagocytophilum* strain.
Sheep 72382: *I. ricinus* adult ticks infected with *A. phagocytophilum* as a nymph.
Sheep 57337: *I. ricinus* adult ticks infected with *A. phagocytophilum* as a nymph.

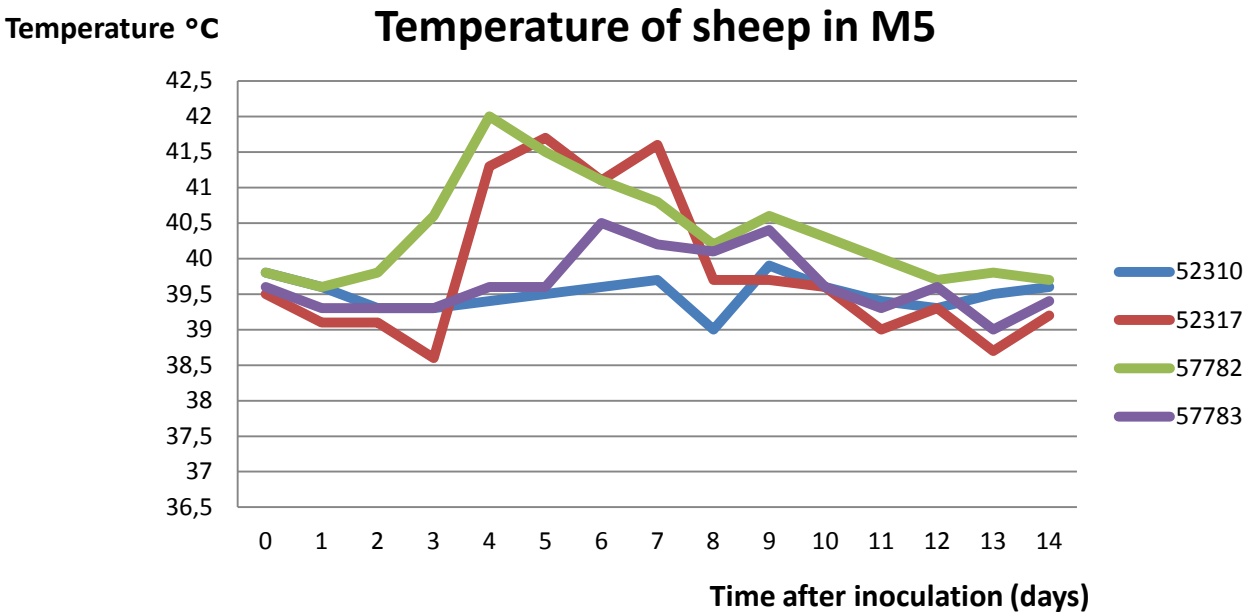
Results of the course of temperature in M3, show a febrile period for sheep 57179, 72273 and 57337 which started at approximately day 3 and peaked at day 4. For sheep 72273 and 57337 the peak lasted for approximately 2 days while sheep 57179 had a much longer febrile period: for approximately 7 days. The fever for sheep 72382 started much later. After 8 days, fever was measured for the first time. This febrile period lasted for 5 days.

Proven is that the injection with *A. phagocytophilum* caused fever in both sheep. Thereby, *I.ricinus* adult ticks infected with *A. phagocytophilum* as a nymph were able to transmit the infection, since sheep 72382 and 57337 both had a febrile period. RLB results should confirm if the infection truly transmitted correctly, but by means of the febrile period, transmission of *A. phagocytophilum* is likely.



M4 consisted of (see Appendix II):
Sheep 56970: *D. reticulatus* adult ticks infected with *A. phagocytophilum* as a nymph.
Sheep 72086: *A. hebraeum* adult ticks infected with *A. phagocytophilum* as a nymph.
Sheep 72253: *I. ricinus* nymphs + injection with *A. phagocytophilum* strain.
Sheep 72261: *I. ricinus* nymphs + injection with *A. phagocytophilum* strain.

This graphics show that both sheep 72253 and 72261 had a febrile period due to the injection with *A. phagocytophilum*. In sheep 72253, fever was first detected at day 5 and this peak lasted for 6 days. During her febrile period, the sheep did show some signs of a nosebleed for one morning. It is not known if *A. phagocytophilum* could result in such kind of symptoms, but maybe it was due to a secondary infection or to the fact that *A. phagocytophilum* results in thrombocytopenia (Carrade et al.). Sheep 72261 was subject to fever for three short periods: the first peak took place at day 4 and 5, the second peak was at day 7 and lasted for only 1 day. The third and last temperature rise began at day 9 and lasted for 2 days. Despite of the fluctuation in the febrile period, this sheep is noted as ''infected''.
In sheep 56970 and 72086 was no fever measured. The adult ticks which were infected with *A. phagocytophilum* as a nymph did not transmit the infection. This means that transmission of *A. phagocytophilum* did not occur.



M5 consisted of (see Appendix III):
Sheep 52310: Faeces dissolved in buffer from *I. ricinus* nymphs that were fed on a sheep infected with *A. phagocytophilum* (sheep 57337 from M3).
Sheep 52317: Injection with frozen blood with *A. phagocytophilum* from a lamb from the sentinel study from UCTD in Oosterbos (Drenthe, the Netherlands).
Sheep 57782: Injection with fresh blood with *A. phagocytophilum* from a lamb from the sentinel study from UCTD in Bargerveen (Drenthe, The Netherlands).
Sheep 57783: Injection with frozen blood with *A. phagocytophilum* from UCTD collection from a dog (Tiba) in Den Haag (Zuid-Holland, the Netherlands).

During M5, three sheep showed a febrile period. In sheep 57782, fever was measured first at day 3 and lasted for 9 days, which is a long period. Probably this could be due to the fact that the sheep was infected with fresh blood, which still contains many intact pathogen parts. One day later (at day 4), sheep 52317 showed a fever peak which lasted for 4 days. Sheep 57783 became febrile at day 6 which was gone after 4 days. So the different blood samples did all cause a notable infection with *A. phagocytophilum*. Sheep 52310 was the only sheep that did not show a febrile period. Tick faeces from an infected tick did not cause a notable infection in the sheep, which assumes that tick faeces tested positive for *A. phagocytophilum* does not contain living *A. phagocytophilum*, but only DNA.

Blood smears

During each experimental round, except M3, immediately after blood collection a blood smear was made to search for *A. phagocytophilum* inclusion body's. In the following tables, the result of each blood smear is shown.

Blood smears of blood samples during M4

Sheep nr.	Sample nr.	Study	Collection date	Day of study	Examination date	Blood smear: *A. phagocytophilum* + or -	Temp. of the sheep
72253	M15048	M4	25-5-2015	6	26-5-2015	Not examined	41,2 °C
72253	M15049	M4	26-5-2015	7	26-5-2015	Positive	41,5 °C
72261	M15050	M4	26-5-2015	7	26-5-2015	Positive	40,2 °C
72253	M15051	M4	27-5-2015	8	27-5-2015	Positive	41,2 °C
72253	M15052	M4	28-5-2015	9	28-5-2015	Positive	40,3 °C
72261	M15053	M4	28-5-2015	9	28-5-2015	Positive	41,0 °C
72253	M15054	M4	29-5-2015	10	29-5-2015	Positive	40,1 °C
72261	M15055	M4	29-5-2015	10	29-5-2015	Positive	40,3 °C

Table 4: Results blood smears from the blood samples collected during experimental round M4. One blood sample was not examined because the blood was not in good condition anymore.

Blood smears of blood samples during M5

Sheep nr.	Sample nr.	Study	Collection date	Day of study	Examination date	Blood smear: *A. phagocytophilum* + or -	Temp. of the sheep
57782	M15109	M5	2-7-2015	3	2-7-2015	Positive	40,6 °C
57782	M15110	M5	3-7-2015	4	3-7-2015	Positive	42,0 °C
52317	M15111	M5	3-7-2015	4	3-7-2015	Negative	41,3 °C
52317	M15112	M5	4-7-2015	5	6-7-2015	Positive	41,7 °C
57782	M15113	M5	4-7-2015	5	6-7-2015	Positive	41,5 °C
52317	M15114	M5	5-7-2015	6	6-7-2015	Positive	41,1 °C
57782	M15115	M5	5-7-2015	6	6-7-2015	Positive	41,1 °C
57783	M15116	M5	5-7-2015	6	6-7-2015	Positive	40,5 °C
52317	M15117	M5	6-7-2015	7	6-7-2015	Positive	41,6 °C
57782	M15118	M5	6-7-2015	7	6-7-2015	Positive	40,8 °C
57783	M15119	M5	6-7-2015	7	6-7-2015	Positive	40,2 °C
57782	M15120	M5	7-7-2015	8	7-7-2015	Positive	40,2 °C
57783	M15121	M5	7-7-2015	8	7-7-2015	Positive	40,1 °C
57782	M15122	M5	8-7-2015	9	8-7-2015	Positive	40,6 °C
57783	M15123	M5	8-7-2015	9	8-7-2015	Positive	40,4 °C
57782	M15124	M5	9-7-2015	10	9-7-2015	Positive	40,3 °C
57782	M15125	M5	10-7-2015	11	10-7-2015	Positive	40,0 °C

Table 5: Results blood smears from the blood samples collected during experimental round M5.

The results from the blood samples from both M4 and M5 show that in almost every blood sample collected during the febrile period, *A. phagocytophilum* was detected in the blood smear (Table 4 and 5). To further define the significance of this result and the reliability of the blood smears, a comparison between the outcome of the blood smears and the RLB hybridization was made. From the blood smears of the blood samples of sheep in M4, all blood samples contained *A. phagocytophilum* after RLB hybridization. Table 6 shows that none of the blood smear that was made, was considered negative. So the blood smear results were in all blood samples 100% reliable. In M5, in one blood smear no inclusion bodies were found (morulae within neutrophil granulocytes) after investigating the blood smear. But all samples were tested positive for *A. phagocytophilum* after RLB hybridization.

		Condition (as determined by 'Golden Standard' = RLB outcome)			
	Total population	*Condition positive*	*Condition negative*		
Test outcome (blood smear)	*Test outcome positive*	23 (true positive)	0 (False positive)	23	positive predictive value = …
	Test outcome negative	1 (False negative)	0 (true negative)	1	Negative predictive value = …
		24	0	24	
		Sensitivity 95,8% (23/24)	Specificity 0% * (0/0)		

Table 6: Comparing the outcome of blood smears (M4 + M5) with RLB hybridization as Golden Standard.

* The specificity is 0%, which is as expected. The specificity is a value which indicates the proportion of negatives that are correctly identified as such (the percentage of healthy sheep who are correctly identified as not being infected by *A. phagocytophilum*). Since in this study, only blood samples were collected from sheep with fever (most likely positive for *A. phagocytophilum*), there were (almost) no negative blood samples collected. This explains a specificity of 0%.

Infection rate ticks

Tested ticks M3

Since *I. ricinus* adults were the only ticks that were responsible for the tick transmission in this experimental round, their infection rate was determined (Table 7). After tick DNA extraction, PCR and RLB hybrydization (all testing for *A. phagocytophilum*), only 1 tick was found positive for *Ehrlichia/Anaplasma* (E/A Catch-all). After calculation, there was found that approximately 6,25% of the *I. ricinus* adults was infected during M3.
The nymphs of *D. reticulatus* and *A. hebraeum* could have been tested too, but chosen was to not do so because M4 showed that there was no transmission possible by these ticks as an adult. So infection rate determination in these nymphs would not be informative.

Sheepnr. in M4	Tick species	Date ticks collected from sheep	Total ticks	Total ticks tested	Ticks positive for *A. phagocytophilum*	% of infected ticks	Total % infected
72273	*D. reticulatus* (nymphs)	23-3-2015 + 25-3-2015 + 26-3-2015	female: 703 male: 519	0	x	x	x
72086	*A. hebraeum* (nymphs)	23-3-2015 + 25-3-2015 + 26-3-2015	female: 89 male: 46	0	x	x	x
72382	*I. ricinus* (adults)	23-3-2015	female: 63 male: 23	4 male, 4 female	0	0	
		25-5-2015	female: 15 male: 10				
57337	*I. ricinus* (adults)	23-3-2015	female: 262 male: 166	4 male, 4 female	1 (E/A catch-all)	12.5%	**6.25%**
		25-3-2015	female: 24 male: 13				

Table 7: Tested and infected ticks M3

Tested ticks M4

In this experimental round, the infection rate in the *I. ricinus* nymphs was determined since for *I. ricinus* already has been proven in several studies that these ticks are able to transmit *A. phagocytophilum*. An infection rate of 53,1% was found (Table 8). Interesting fact is that in sheep 72253, 5/8 of the dead nymphs were tested positive for *A. phagocytophilum* (the other three nymphs were tested positive for the *Ehrlichia/Anaplasma* catch-all, but this was due to the presence of *Rickettsia helvetica*) and in the nymphs that were still alive, this range was 3/8 (and 3 were tested positive for *Ehrlichia/Anaplasma)*. In sheep 72261, there were also more nymphs tested positive in the nymphs who did already die: 6/8 of the dead nymphs were infected with *A. phagocytophilum* and 0/8 of the alive nymphs were tested positive for *Ehrlichia/Anaplasma*.

Sheepnr. In M4	Tick species	Date ticks collected from sheep	Total ticks	Total ticks tested	Ticks positive for *A. phagocytophilum*	% of infected ticks	Total % infected
56970	*D. reticulatus* (adults)	29-5-2015	female: 109	0	x	x	x
72086	*A. hebraeum* (adults)	29-5-2015	0 (no ticks engorged during M4)	0	x	x	x
72253	*I. ricinus* (nymphs)	24-5-2015	± 777	8 living and 8 dead nymphs	11 (originally 14, but three *E/A* catch-all's were due to *Rickettsia*)	68,8%	
		25-5-2015	± 961				
72261	*I. ricinus* (nymphs)	24-5-2015	± 443	8 living and 8 dead nymphs	6 (originally 7, but 1 *E/A* catch-all was due to a *Rickettsia*)	37,5%	**53,1%**
		25-5-2015	± 456				

Table 8: Tested and infected ticks M4

RLB results

This is a summary of the RLB results. For the original RLB results (the sheets), see Appendix VII. Before presenting and discussing the results, one thing should be made clear: a signal for the catch-all's on the sheet are interpreted as follows: there is a DNA sequence match for a part of the total DNA sequence of *Ehrlichia/Anaplasma*, but when it is the only positive result, it is not completely similar to *A. phagocytophilum* or another pathogen on the membrane.

RLB results ticks M3 and M4

After the RLB hybridization performed for the ticks collected during M3 and M4, pathogens showed in table 9 and in figure 11 were found. In M3, only one tick was tested positive for the *Ehrlichia/Anplasma* catch-all (which represents 6,25% of the population).
Tested ticks from M4 were tested positive for a lot more pathogens. 21 nymphs (65,6%) were tested positive for *Ehrlichia/Anaplasma*, 14 (43,8%) tested positive for *A. phagocytophilum* 1, 13 (40,6%) tested positive for *A. phagocytophilum* 3, 12 (37,5%) tested positive for *A. phagocytophilum* 5, 3 (9,4%) tested positive for *Rickettsia* catch-all and also 3 (9,4%) tested positive for *Rickettsia helvetica* and 11 (34,4%) were tested negative.

RLB ticks M3 and M4	Number of ticks M3	Proportion M3	Number of ticks M4	Proportion M4
Total number of ticks	16	100%	32	100%
Males	8	50%		
Females	8	50%		
Nymphs			32 (16 †, 16 alive)	100%
Ehrlichia/Anaplasma catch-all	1	6,25%	21	65,6%
A. phagocytophilum 1			14	43,8%
A. phagocytophilum 3			13	40,6%
A. phagocytophilum 5			12	37,5%
Rickettsia catch-all			3	9,4%
Rickettsia helvetica			3	9,4%
Negative	15	93,75%	11	34,4%

Table 9: Pathogens detected in the *I. ricinus* ticks collected during M3 and M4. Note: several ticks tested positive for more than one pathogen, which has consequences for the proportions.

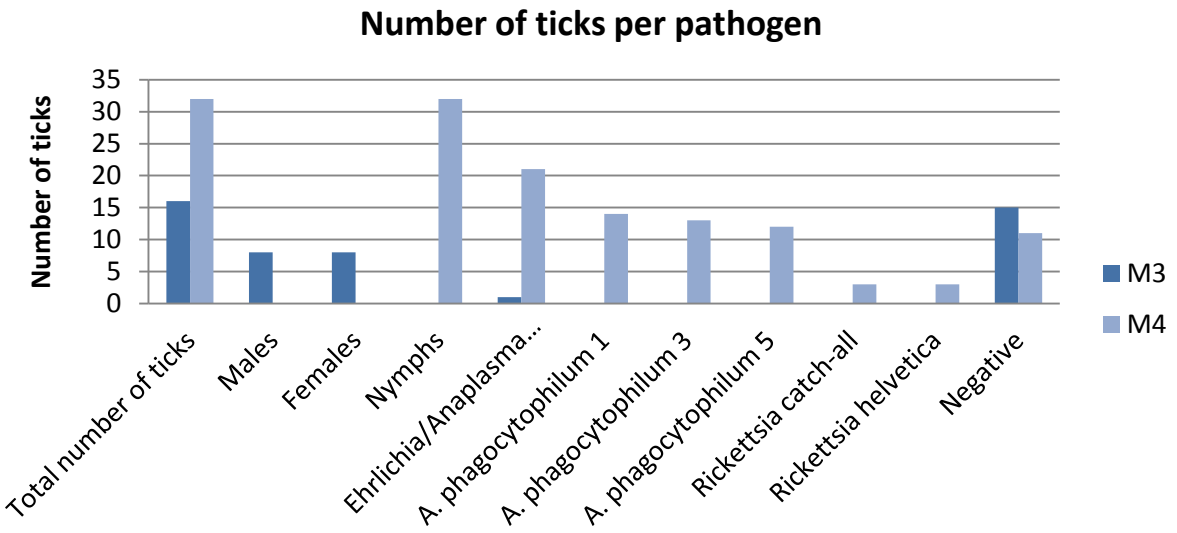


Figure 11: Pathogens detected in the *I. ricinus* ticks, collected during M3 and M4.

RLB results blood samples

Results from blood samples M3

After RLB hybridization of the blood samples collected during M3, the following pathogens were detected: 12 (100%) samples were tested positive for *Ehrlichia/Anaplasma* catch-all and also 12 (100%) were tested positive for *A. phagocytophilum* 1. 8 blood samples (66,7%) were tested positive for *A. phagocytophilum* 3, 2 (16,7%) were tested positive for *A. phagocytophilum* 5 and 0 blood samples were tested negative (see Table 10 and Fig. 12).

RLB Blood samples M3	Number of blood samples	Proportion
Total number of blood samples	12	100%
Ehrlichia/Anaplasma catch-all	12	100%
A. phagocytophilum 1	12	100%
A. phagocytophilum 3	8	66,7%
A. phagocytophilum 5	2	16,7%
Negative	0	0%

Table 10: Pathogens detected in the blood samples collected from the sheep during their febrile period in M3.*

Results from blood samples M4

In table 11 and figure 12, the pathogens found in the blood samples from M4 are shown. 8 (100%) samples were tested positive for *Ehrlichia/Anaplasma* catch-all and also 8 (100%) were tested positive for *A. phagocytophilum* 1. 6 blood samples (75%) were tested positive for *A. phagocytophilum* 3, 6 (75%) were tested positive for *A. phagocytophilum* 5 and 0 blood samples were tested negative.

RLB Blood samples M4	Number of blood samples	Proportion
Total number of blood samples	8	100%
Ehrlichia/Anaplasma catch-all	8	100%
A. phagocytophilum 1	8	100%
A. phagocytophilum 3	6	75%
A. phagocytophilum 5	6	75%
Negative	0	0%

Table 11: Pathogens detected in the blood samples collected from the sheep during their febrile period in M4.*

Results from blood samples M5

The blood samples collected from the sheep during M5 contained the pathogens (Table 12):
17 (100%) blood samples were tested positive for *Ehrlichia/Anaplasma*, 16 (94,1%) tested positive for *A. phagocytophilum* 1, 12 (70,6%) tested positive for *A. phagocytophilum* 5, 10 (58,8%) tested positive for *A. phagocytophilum* 7 and 0 tested negative.

RLB Blood samples M5	Number of blood samples	Proportion
Total number of blood samples	17	100%
Ehrlichia/Anaplasma catch-all	17	100%
A. phagocytophilum 1	16	94,1%
A. phagocytophilum 5	12	70,6%
A. phagocytophilum 7	10	58,8%
Negative	0	0%

Table 12: Pathogens detected in the blood samples collected from the sheep during their febrile period in M5.*

* Note: several blood samples tested positive for more than one pathogen, which has consequences for the proportions.

The following table (Table 13) shows which strain of *A. phagocytophilum* was found in the blood samples collected from the sheep during M5, since one of the goals of this research was to look

more closely what strains are present in each blood sample from the different animals (which was tested during M5).

	Sheep nr. M5	*A. phagocytophilum* 1	*A. phagocytophilum* 3	*A. phagocytophilum* 5	*A. phagocytophilum* 7
DNA sequence from the primer for detecting the strain	-	TTG CTA TAA AGA ATA ATT AGT GG	TTG CTA TGA AGA ATA ATT AGT GG	TTG CTA TAA AGA ATA GTT AGT GG	TTG CTA TAG AGA ATA GTT AGT GG
Fresh blood sheep, Bargerveen	57782	times detected: 9	times detected: 0	times detected: 5	times detected: 7
Frozen blood sheep, Oosterbos	52317	times detected: 4	times detected: 0	times detected: 3	times detected: 4
Frozen blood dog ''Tiba'', Den Haag	57783	times detected: 4	times detected: 0	times detected: 4	times detected: 0

Table 13: Overview of the different *A. phagocytophilum* strains detected by RLB hybridization in the different blood samples collected during M5.

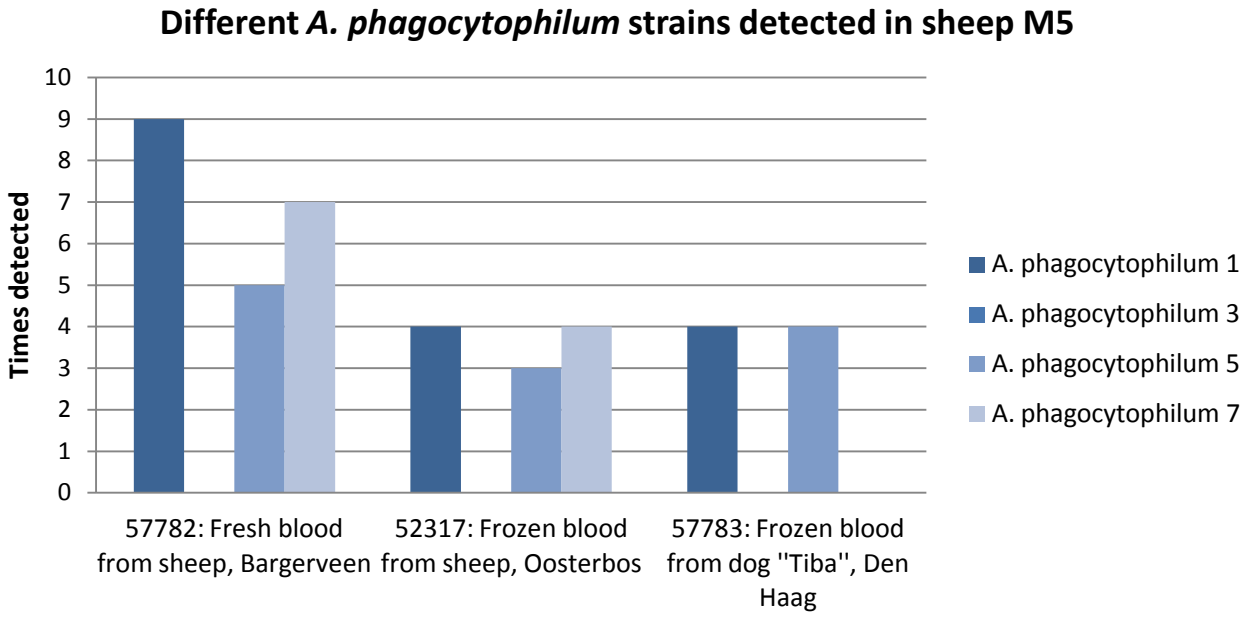


Figure 12: The different *A.* phagocytophilum strains detected in the blood samples collected during M5. Which blood was injected, is showed in the figure as well. There was no *A. phagocytophilum* 3 found.

This figure (Fig. 12) and table 13 show that in each blood sample, taken from the different animals in the Netherlands, different strains of *A. phagocytophilum* were found after RLB hybridization. The blood sample from the sheep (57782) which was injected with the fresh blood from a sheep in Bargerveen (Drenthe, collected at 29-6-2015) contained three different strains: *A. phagocytophilum* 1, 5 and 7. Sheep 52317 was injected with the frozen blood collected from a sheep in Oosterbos (Drenthe) at 11-6-2015 and contained also three different strains: *A. phagocytophilum* 1, 5 and 7. Frozen blood collected at 24-6-2015 from dog ''Tiba'' living in Den Haag, was injected in sheep 57783 and after blood collection in the sheep's febrile period, the RLB hybridization tested the blood sample positive for two different strains: *A. phagocytophilum* 1 and 5.

A complete overview of which pathogens were found in M3, M4 and M5 (already shown in table 10, 11 and 12) is illustrated in the following figure (Fig. 13).

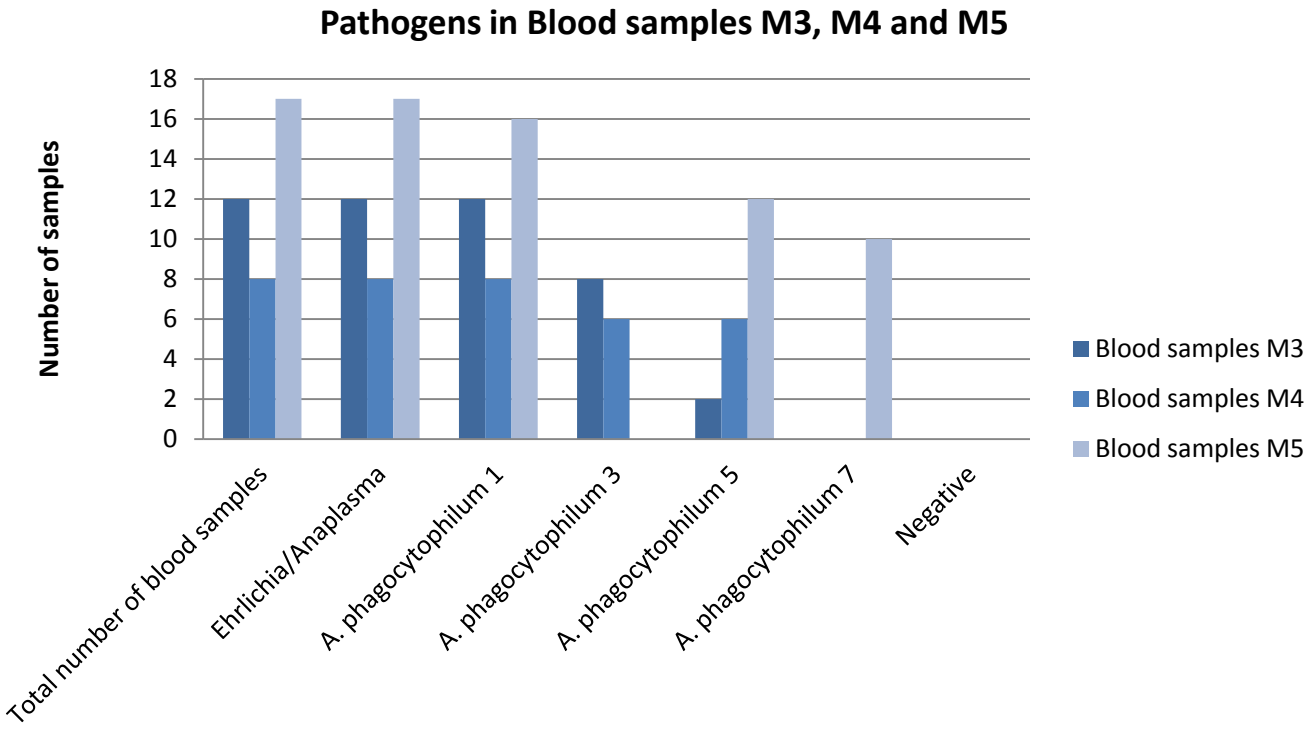


Figure 13: Pathogens found in the blood samples during M3, M4 and M5

Discussion

This study was designed for two reasons. At first, this study was done to further investigate *A. phagocytophilum* transmission by *I. ricinus* in sheep and furthermore if *D. reticulatus* and *A. hebraeum* were also possible vectors for a successful transmission of *A. phagocytophilum.* This transmission took place by means of a tick transmission model, which was designed especially for this research. This model contained four sheep, which were all exposed to the infection and ticks differently (see Appendix I, II and III). Three experimental model designs were followed during this research.
As expected, *I. ricinus* did transmit the infection successfully. This already has been reputed elsewhere (Stuen et al. 2013; Goldman & Green 2009), so the results were not really new. The sheep got fever, did lose some of their appetite and their blood contained *A. phagocytophilum* (which was proved by blood smears and RLB Hybridization). However, when *D. reticulatus* and *A. hebreaum* were fed on the sheep as adults to transmit the disease (these ticks were brought in contact with the infection as a nymph who fed on an infected sheep in the experimental model before), the sheep did not get any symptoms. There was no febrile period, so no blood was collected from these sheep. The transmission did, unfortunately, not succeed.

Ticks from the models in which the ticks did transmit the disease (*I. ricinus* from M3 and M4), were taken to determine whether they were infected with *A. phagocytophilum* or not. This happened by RLB hybridization. Thereby, the infection rate of *A. phagocytophilum* in these ticks could be calculated. The RLB results show that not all ticks were tested positive for *A. phagocytophilum.* Tick M15091 (a living *I. ricinus* nymph from M4) shows a positive signal for the *Rickettsia* catch-all and M15081, M15083 and M15086 (dead *I.ricinus* nymphs from M4) all show a positive signal for the *Rickettsia* catch-all and *Rickettsia helvetica*. When there is no *Anaplasma* or *Ehrlichia* species present in the sample, but there is a *Rickettsia* species (in this case a *Rickettsia helvetica*), the *Ehrlichia/Anaplasma* catch-all could have a positive signal because the *Ehrlichia/Anaplasma* primer used during the PCR could also react with *Rickettsia* species. So these four nymphs had a positive signal for *Ehrlichia/Anaplasma* because there was a positive signal for a *Rickettsia* species. Tick M15099 (a dead *I. ricinus* nymph from M4) shows a smeared positive signal for *Babesia motasi* or *Babesia ovis* (see Appendix VII).
It is very unlikely that these pathogens (*Rickettsia helvetica* and *Babesia motasi* or *Babesia ovis*) would be present in the ticks, since the ticks and sheep on which they were placed were SPF and the stable was a closed area with a hygiene protocol. The presence of a smeared positive signal for *Babesia motasi* or *Babesia ovis* could be due to the positive RLB control (for *Babesia*), which probably leaked over the membrane during the transportation into the hybridization oven (see RLB protocol, Appendix IV). However, if that is the case, it is weird that the *Babesia* catch-all did not show a positive signal. Another reason might be that the membrane, used during this RLB hybridization test, was not washed properly before use. But in both cases, it is more likely that contamination has occurred since the lab conditions were just restored (this will be explained later in the discussion).

From the *I. ricinus* nymphs in M4, there were more dead nymphs tested positive for *A. phagocytophilum* than nymphs that were still alive before testing. A reason for this interesting fact could be that, somehow, *A. phagocytophilum* also has some influence on the tick's health. But further research is necessary.

By means of the results of the ticks used in M3 and M4, a infection rate for *A. phagocytophilum* was calculated. In M3, the infection rate was 6,25%. Despite this low rate, the transmission did succeed: both sheep from M3 who were infested with these adult *I. ricinus* ticks did show symptoms similar to

an infection with *A. phagocytophilum*. This low infection rate could be due to the number of ticks which were tested (16 in total, 8 from each sheep), then the survey was to unpredictable. But at least, the experimental model showed that an infection took place, as do the blood samples tested by RLB hybridization. The infection rate of the *I. ricinus* nymphs in M4 was 53,1%, which represents the presence of the infection better. Both sheep were injected with *A. phagocytophilum* in order to infect the nymphs. So the high infection rate in the nymphs is not surprising, since the blood of the sheep was infected. This shows that this model is highly suitable in order to infect *I. ricinus* ticks with *A. phagocytophilum*.

That was originally the reason that, to further investigate if *D. reticulatus* and *A. hebraeum* are able to transmit *A. phagocytophilum*, this model was used to infect the ticks as nymphs and set them free as adults in the upcoming model. Nevertheless, these tick species were not able to transmit the disease. Why this transmission did not take place, is not clear yet. Maybe, there is something in the tick species that blocks the transmission of *A. phagocytophilum* (for instance, something in the anatomy of the tick). But further research is necessary to investigate the possible reasons of the impossible transmission of *A. phagocytophilum* by *D. reticulatus* and *A. hebraeum*.

Only during febrile periods, blood samples were collected from the sheep, since this is described in the protocol. This means there was no blood collected from the sheep with *D. reticulatus* and *A. phagocytophilum* in M4. The blood samples from the sheep were tested with RLB Hybridization to determine if *A. phagocytophilum* was present during the febrile period. The RLB results (see Appendix VII), show that all blood samples from M3 and M4 contained *A. phagocytophilum*, which was in some blood samples even present with different strains.

There was a second goal to achieve by this study. In this research several blood samples and tick faeces were tested, which were collected by UCTD and were kept in collection. These products (the blood samples as well as the tick faeces, which was dissolved and centrifuged) were injected in sheep to determine which *A. phagocytophilum* strains were present in the different blood samples from animals from different areas in the Netherlands. Thereby, there was interest in whether these different samples (including the tick faeces sample) did cause the same symptoms as expected in an infection with *A. phagocytophilum*.

In M5, the sheep that was injected subcutaneously with the tick faeces (tested positive for *A. phagocytophilum*) did not develop symptoms for an *A. phagocytophilum* infection. The faeces did not contain living *A. phagocytophilum* anymore. But it is interesting to do further research to this phenomenon, because it is theoretically possible that *A. phagocytophilum* transmission could also occur by intruding the skin as a ''porte d'entrée'' (for instance after falling which caused skin-damage). When there is infected tick faeces in the environment, the same mechanisms could occur as in ''cat-scratch disease'' (which is caused by *Bartonella henselae,* and could even be transmitted by *I. ricinus*!) (Mazur-Melewska et al. 2015). To repeat this test with some alterations to investigate this hypothesis could be worthwhile.

So no positive results were found in the sheep that was injected subcutaneously with the dissolved tick faeces. There were only blood samples collected from the three other sheep during their febrile periods. Every blood sample tested positive for at least one of the different *A. phagocytophilum* strains. And each blood sample did contain different *A. phagocytophilum* strains, which was one of the sub-questions in this study. Interesting is the fact that the blood samples taken from the sheep which were injected with the fresh blood sample from Bargerveen (sheep 57782) and from the sheep injected with the frozen blood sample from Oosterbos (57783) did contain the same strains of *A. phagocytophilum* (1, 5 and 7). These areas are very close to each other, so the presence of the

same strains in both blood samples is maybe due to that fact (the areas are approximately 15 km apart from each other).

The value of this single result is not very high. But if all different *A. phagocytophilum* strains are known, there can be a detailed survey of the different isolated/samples made. This could help improving prevention and control of *A. phagocytophilum* in the Netherlands and elsewhere.

The positive and negative PCR control was not only made for *Ehrlichia/Anaplasma*, since this study was performed to investigate which strains of *A. phagocytophilum* were present in each blood sample. But, as shown in the RLB results of M5, there also was a positive and negative PCR control made for *Borrelia and Babesia/Theileria*. This is due to the fact that there were also some ticks tested for the ''Tickbusters'' (UCTD) in this RLB too.

The RLB results of M5 show that 9 (52,9%) blood samples tested positive for *A. marginale*. This bacterium is known in cattle and survives and replicates in the erythrocytes (Hunter 2012; Nene & Kole 2008). *A. marginale* causes bovine anaplasmosis (Zivkovic et al. 2009). It occasionally occurs in sheep and goat (Brenner & Krieg 2006). But *A. marginale* is not a common pathogen in the Netherlands. It is most seen in tropical and subtropical regions in America, Europe, Asia, Africa and Australia (Kocan et al. 2004).
So the reason why this pathogen was detected in the RLB results is probably due to contamination during the procedure of the RLB or a test fault. However, the RLB controls are working properly, therefore the results for the blood samples of M5 are still valid.

At the beginning of this study, there were some problems in the laboratory. The results from several RLB blots (when the ticks from the 'Tickbusters' were tested) were not reliable because they tested positive for many catch-all's and pathogens. These were most likely false results due to contamination or defective equipment. There could also have been a problem with the equipment used in the PCR procedure. Decided was to first clean all labs and equipment thoroughly with sodium hypo chloride. The lab coats were washed in the washing machine and two weeks later again with sodium hypo chloride (by hand) and in the washing machine at 90°C. New supplies, such as buffers, were made and supplies (PCR primers and polymerase) were also replaced. Certain steps in the protocols were being rewritten to ensure a better clean-up. For instance, from now on, the RLB lab will be cleaned with sodium hypo chloride instead of ethanol (which must be adjusted in the protocol in Appendix VI). New PCR control samples were made by performing a DNA extraction, PCR and RLB on several blood samples that were known for being infected with *E. chaffeensis, E. canis, E. ruminantium, A. centrale, A. marginale, B. burgdorferi, B. bigemina, B. canis, B. canis canis, B. major, B. vogeli, T. annulata and T. parva*. Unfortunately, after all this was altered the blots still continued to test positive for many catch-all's and pathogens. That was when the help of a external professional specialist was requested. She said that our polymerase for the PCR was probably too nonspecific. Again some alterations in the lab were executed: the lab coats were again washed (first with sodium hypo chloride and then in the washing machine at 90°C). After cleaning them, the coats had to stay in their own specific place (each in a different lab), so there would be a clear differentiation between the different labs and contamination would be reduced as much as possible. Thereby, in some labs disposable coats were introduced (in the RLB lab and DNA extraction lab) which would be replaced every two weeks. Visitors would from now on be forced to wear visitor-lab coats which are situated in the corridor. Door closers were installed, in order to keep the labs clean. Also, walking routes were designed (clean room (PCR lab) → dirty room (DNA extraction lab) → PCR computer room). If all these alterations still did have no effect, advice was to replace the membranes from the RLB because they could be damaged. A lot of tests were executed in which was worked with different concentrations of polymerase. Fortunately, after all this measurements everything worked as it should.

On the RLB blot, spots appear were species specific oligonucleotides and PCR products were hybridized. The interpretation of these spots is subjective, since some spots appear lighter than others. Therefore, the prevalences of the pathogens mentioned in this survey may be different than reported.

The fact that there are more *Ehrlichia/Anaplasma* catch-all signals in the RLB than there are specific pathogen signals, means that either the pathogen is yet unknown and not present on the membrane, or that the pathogen is just not present on the membrane.

For every experimental round which has been performed in this study counts: in order to exclude coincidences in the results, each sample should be tested twice at least. Thereby, the experimental group could be made bigger in order to make the sample as accurate as possible.

In this study, *D. reticulatus* and *A. hebraeum* were not capable of transmitting *A. phagocytophilum.* It is not very attractive to repeat the experimental model which has been used in M4, because it demonstrated that the transmission did not occur, despite the fact that the model has been used successfully before in studies with *I.ricinus*.

Not many results have yet been found in order to prove that *D. reticulatus* is a possible vector for *A. phagocytophilum*. Tijsse-Klasen et al. (2013) tested 61 *D. reticulatus* ticks which were collected from the vegetation of Wales and England, but none of them tested positive for *A. phagocytophilum* (Tijsse-Klasen et al. 2013). Also Bonnet et al. (2013) tested whether *Dermacentor* ticks are a possible vector for *A. phagocytophilum*. But this pathogen was not found in the ticks collected in that study either (Bonnet et al. 2013). However, there are some recent studies which actually did find *A. phagocytophilum* in *D. reticulatus* (Szekeres et al. 2015; Karbowiak et al. 2014). But these studies did not prove the vector competence of *D. reticulatus* for *A. phagocytophilum*

It is possible to perform a study regarding this research to investigate the possibility of carriers for *A. phagocytophilum* after the infection is eliminated. The question that arose during this study was: are the sheep that were infected and treated completely sterile from *A. phagocytophilum*? There might be a possibility that ticks living in the same area as the sheep could feed on these sheep and then infect other uninfected sheep in the herd with *A. phagocytophilum*. Carriers of *A. phagocytophilum* have been described before. In the study by Hornok et al., blood samples from non-pet dogs in southern Hungary were collected. In 11% of the blood samples from the dogs, *A. phagocytophilum* was discovered (Hornok et al. 2013). The carriers of *A. phagocytophilum* do not show any clinical signs, but maybe they are a threat for the other animals in their direct environment.

Conclusion

This study was set up to investigate two hypotheses, so there were two main goals to achieve. The first goal of this study, was to investigate if *A. hebraeum* and *D. reticulatus* were capable of transmitting *A. phagocytophilum* in sheep. Experimental model M3 and M4 were performed in order to research these hypotheses. After analyzing the results, it is clear that *A. hebraeum* and *D. reticulatus* are in fact not capable to do so. The sheep did not get symptoms which are expected when a sheep is infected with *A. phagocytophilum,* so the ticks did not transmit the disease. Further research is necessary in order to determine why the transmission by these ticks species is not possible.
The second goal of this study was to see if different blood samples collected from different animals from the Netherlands and infected tick faeces did contain the same strain of *A. phagocytophilum* and if the sheep would develop the same symptoms after injection.
After the infection was revived in the sheep in experimental model M5 and blood was collected during their febrile period, the blood was tested by RLB hybridization. Results show that the blood samples did indeed contain different strains of *A. phagocytophilum* (which differed by their nucleotides-code at a specific part) and did all cause similar symptoms which are expected in an infection with *A. phagocytophilum*. Tick faeces collected during M3 (which tested positive by RLB hybridization for *Anaplasma phagocytophilum*) seems to cause no harm when the faeces particles will enter the body through the skin. No symptoms were found, but further research could be interesting.

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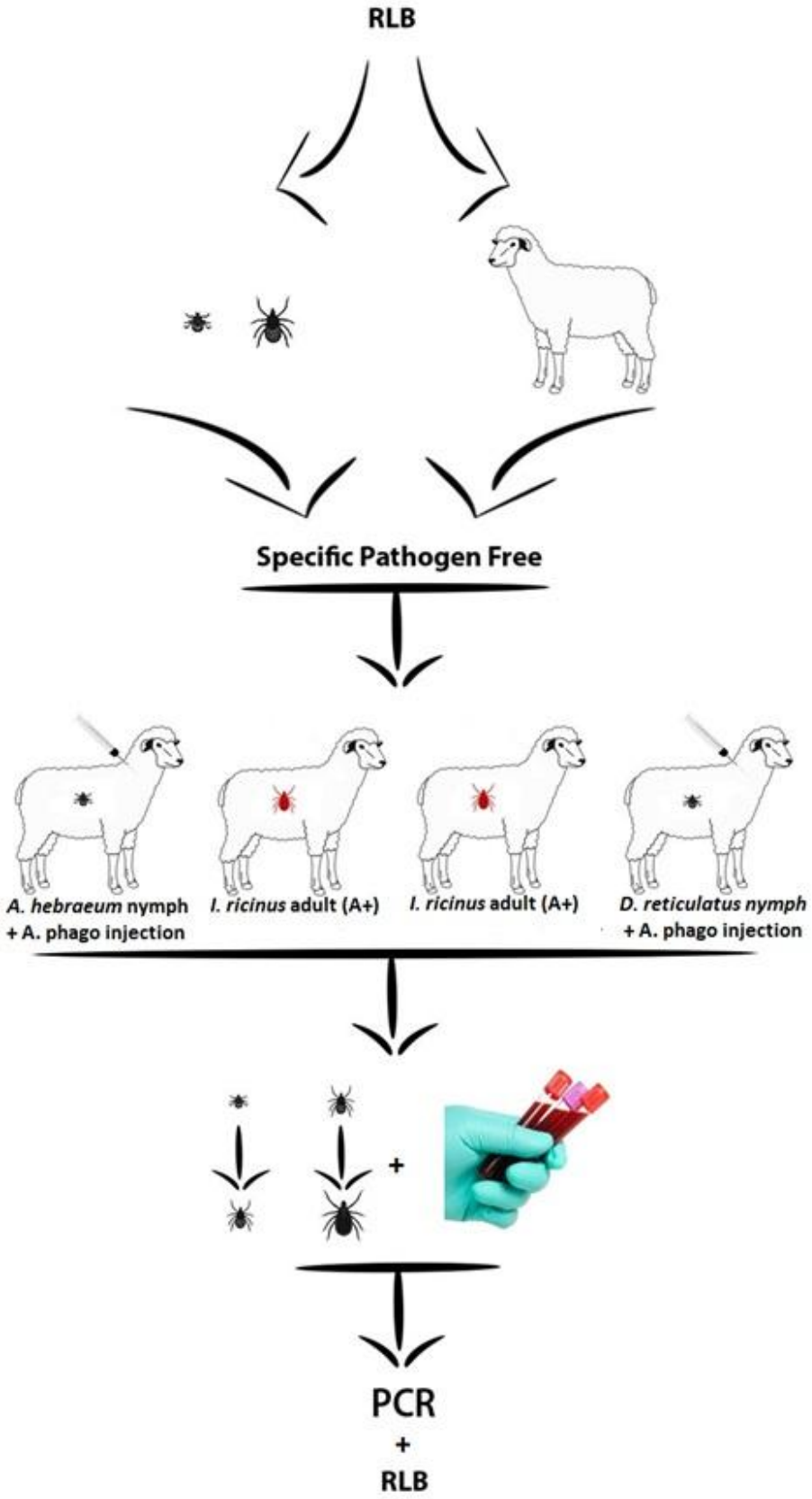
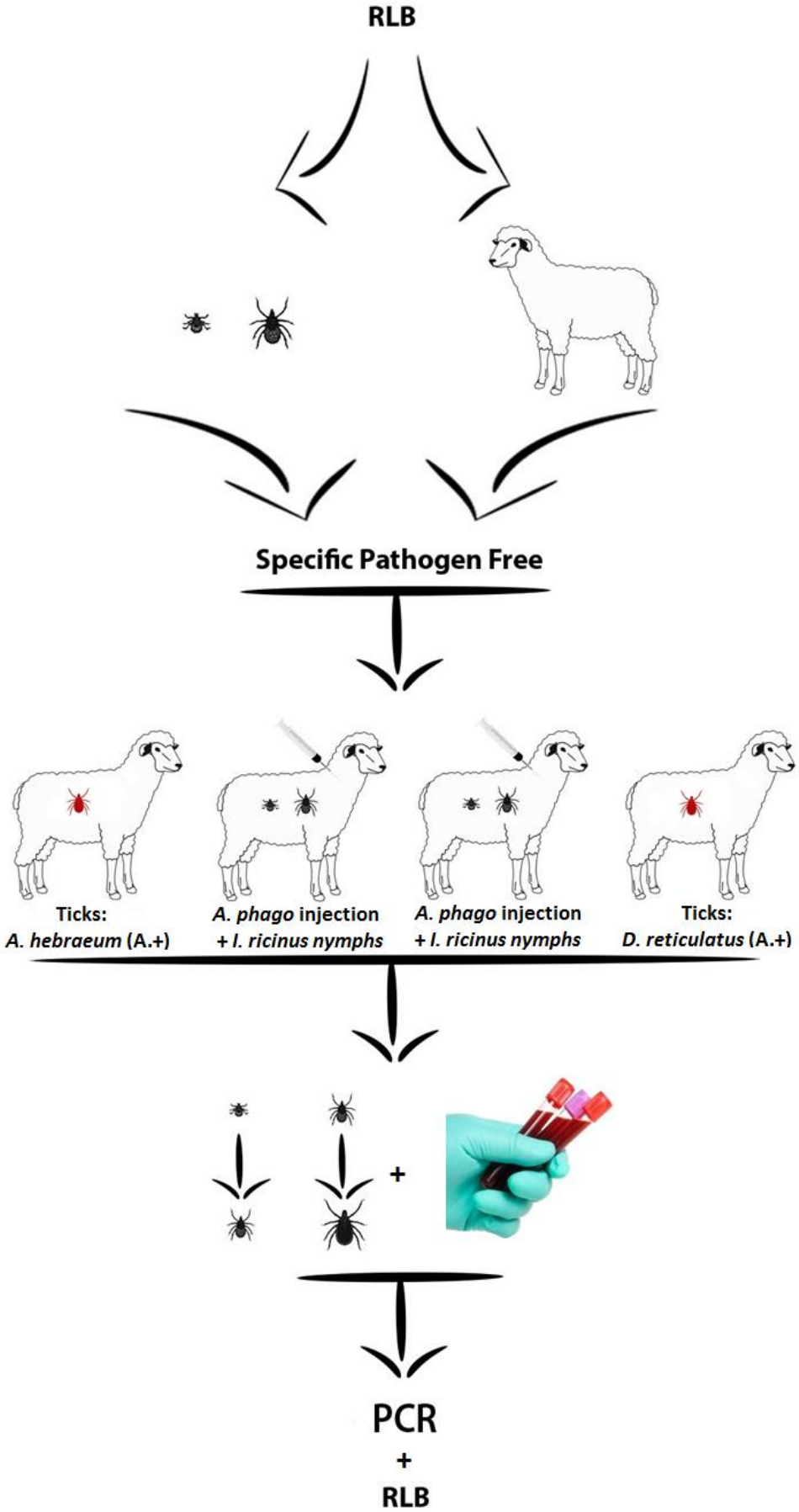
Appendices

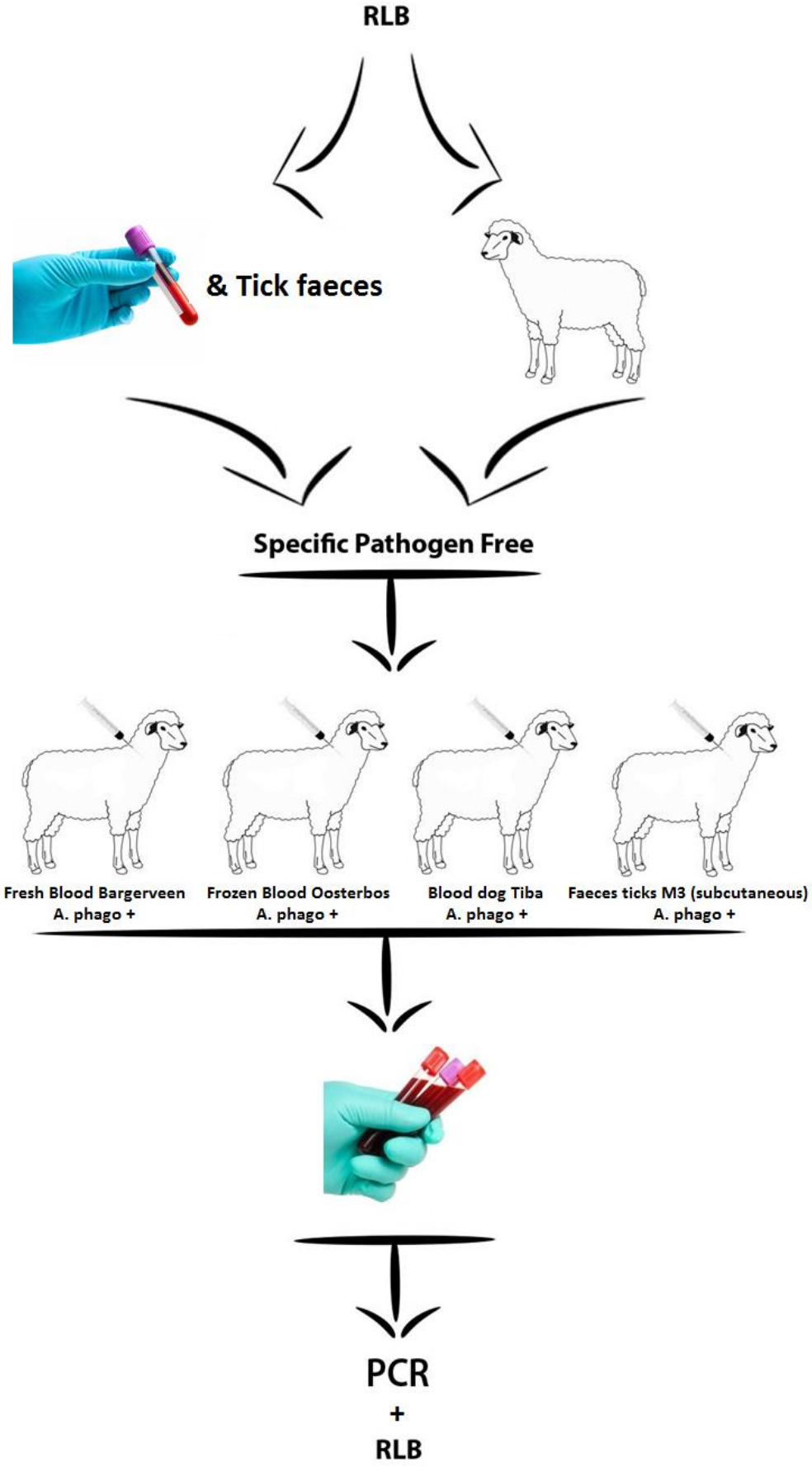
I: The experimental model M3



II: The experimental model M4



III: The experimental model M5



IV Protocol for DNA extraction Blood and Ticks

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)
FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

DNA EXTRACTION FROM BLOOD PROCEDURE

Sample description	
Number of samples	

Wear gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with sodium hypochlorite.	
2	Take the proteinase K solution from the freezer and store at 4°C.	
3	Turn on the heating block at 70°C and preheat the BE buffer.	
4	Vortex the blood samples and add 200µl of each to sterile 1.5ml tubes.	
5	Add 25µl proteinase K.	
6	Add 200µl B3 buffer and vortex vigorously (10-20 seconds).	
7	Incubate the tubes at room temperature for 5 minutes.	
8	Incubate the tubes at 70°C for 15 minutes. (The samples should turn brown/black. If this does not happen, extend incubation time up to 30 minutes and vortex vigorously several times.)	
9	Briefly spin down the tubes.	
10	Add 210µl 96% ethanol, vortex and briefly spin down the tubes.	
11	Transfer the supernatant to spin columns.	
12	Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
13	Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
14	Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
15	Centrifuge the columns at 11,000x g for 1 minute.	
16	Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly**.	
17	Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.	

18	Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.	
19	Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.	
20	Turn off all equipment and clean working space with sodium hypochlorite.	

DNA extraction done:

by _____ on _____ _____

Signature

Comments:

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

DNA EXTRACTION FROM TICKS

Sample description	
Number of samples	

Wear gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with sodium hypochlorite.	
2	Turn on a water bath at 56°C.	
3	Take the proteinase K solution from the freezer and store at 4°C.	
4	Wash the ticks in a sonofication bath with demineralized water for up to 30 seconds.	
5	Put the ticks, with cleaned forceps, in 1.5ml tubes with 70% ethanol and vortex for several seconds.	
6	Wash the forceps in 70% ethanol followed by washing in demineralized water after each tick.	
7	Take the ticks from the tubes and let it dry on a clean tissue paper and place the dried ticks in a sterile 2ml tube with 180µl T1 lysis buffer.	
8	Freeze the samples at -80°C for 15 minutes.	
9	Add a 5 or 7mm (depending on tick size) metal bead to the frozen samples.	
10	Disrupt the ticks in the TissueLyser LT at 50 oscillations per second for 3 minutes.	
11	Briefly spin down the tubes. **1000x g maximum!**	
12	Add 25µl proteinase K and vortex.	
13	Prelyse the samples at 56°C in a water bath for 3 hours and vortex every hour.	
14	**During the incubation**; empty and clean the sonification bath.	
15	**During the last incubation hour** ; turn on the heating block at 70°C and preheat the BE buffer.	
16	Briefly spin down the tubes. **1000x g maximum!**	
17	Add 200µl B3 buffer and vortex.	
18	Incubate the tubes at 70°C for 15 minutes.	

19	Briefly spin down the tubes. **1000x g maximum!**	
20	Add 210µl 96% ethanol, vortex and briefly spin down the tubes. **1000x g maximum!**	
21	Transfer the supernatant to new sterile 1.5ml tubes. (Tick parts are allowed to be transferred.)	
22	Centrifuge the tubes at 11,000x g for 2 minutes.	
23	Transfer the supernatant to spin columns. Avoid pipetting tick parts, as it can block the spin column.	
24	Centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
25	Add 500µl BW buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
26	Add 600µl B5 buffer and centrifuge the columns at 11,000x g for 1 minute. Discard the flow through.	
27	Centrifuge the columns at 11,000x g for 1 minute.	
28	Place the spin columns in sterile 1.5ml tubes. **Label the tubes accordingly.**	
29	Add 100µl preheated BE buffer directly on the membrane of the spin columns and incubate at room temperature for 1 minute.	
30	Centrifuge the columns at 11,000x g for 1 minute. Discard the spin columns.	
31	Store the DNA samples at 4°C for use within the next few days or store at -20°C for long term preservation.	
32	Turn off all equipment and clean working space with sodium hypochlorite.	

DNA extraction done:

by _____ on _____ _____

Signature

Comments:

V Protocol for PCR RLB procedure

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

PCR RLB PROCEDURE

Sample description	
Number of samples	

Wear (green) gloves and use filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

Primers:	***Anaplasma Ehrlichia***	***Babesia Theileria***	***Borrelia***	***Rickettsia***	**Other:**

Reagent	**1x**	**Number of samples + 10%**	
PCR grade H_2O	15.875µl		
5x Phire reaction buffer	5.0µl		
10mM dNTPs	0.5µl		
Forward primer (20pmol/µl)	0.5µl		
Reverse primer (20pmol/µl)	0.5µl		
2U/µl Phire Hot Start II DNA polymerase	0.125µl		

		Done	
1	Put DNA samples a (few) day(s) before the PCR at 4°C.		
2	Turn on the DNA workstations in the clean room and the dirty room.		
3	Clean workspace in both DNA workstations with sodium hypochloride.		
4	Label the PCR and Eppendorf tubes and put them in the DNA workstation in the clean room		
5	Turn on the UV-light in both DNA workstations for 20 minutes.		
6	**During the UV-light**; thaw the PCR reagents at room temperature, except the polymerase.		
7	Prepare the PCR mix in the Eppendorf tube(s). Multiply the reagent volumes by the number of samples plus 10% of the number of samples: 40 DNA samples + 1 PCR control = 41 + 10% = 45 samples.		

8	Pipet the master mix gently up and down to mix well.		
9	Pipet 22,5µl master mix to each PCR tube and add the leftover mix to an additional tube which will be the negative PCR control.		
10	Close the PCR tubes and remove them from the workstation, clean the workspace with sodium hypochloride and turn on the UV-light for 20 minutes.		
11	Take the closed PCR tubes to the dirty room and place them in the workstation.		
12	Vortex the DNA samples, spin them down briefly at 11,000x g and place them in the workstation.		
13	Add 2.5µl DNA sample to the corresponding PCR tube.		
14	Add 2.5µl of the positive control (, corresponding to the PCR to be performed,) to the positive PCR control tube.		
15	Vortex and spin down briefly.		
16	Clean the workstation with sodium hypochloide and turn on the UV-light for 20 minutes.		
17	Run the corresponding PCR program.		
18	Store the PCR products at 4°C for use within the next few days or store at -20°C for long term preservation.		
19	Turn off both DNA workstations after the UV-light is switched off.		

PCR done:

by _____ on _____ _____

Signature

Comments:

VI Protocol for RLB Hybridization procedure

UTRECHT CENTRE FOR TICK-BORNE DISEASES (UCTD)

FAO REFERENCE CENTRE FOR TICKS AND TICK-BORNE DISEASES

REVERSE LINE BLOT HYBRIDIZATION PROCEDURE

Sample description	
Number of samples	
Membrane ID	

Wear gloves and use non-filter pipet tips

Strictly follow the one-way route: Clean room → Dirty room → PCR room

		Done
1	Clean workspace with 70% ethanol.	
2	Turn on a heating block at 100°C.	
3	Turn on the hybridization oven at 42°C en preheat 50ml 2x SSPE/0.5% SDS solution.	
4	Turn on the water bath at 50°C en preheat the bottle with 2x SSPE/0.5% SDS solution.	
5	Combine and dilute the PCR products per DNA sample in a 1.5ml tube. Take 10µl of every PCR product and add 2x SSPE/0.1% SDS to a final volume of 160µl. (10µl *Anaplasma/Ehrlichia* PCR + 10µl *Babesia/Theileria* PCR + 140µl 2x SSPE/0.1% SDS.)	
6	Take 10µl of the RLB positive controls and add 150µl 2x SSPE/0.1% SDS to a 1.5ml tube,	
7	Denature the diluted PCR samples and controls at 100°C for 10 minutes.	
8	**During the denaturation step**; wash the membrane at room temperature with 2X 2SSPE/0.1% SDS for 5 minutes under gentle shaking and fill a bucket with ice.	
9	Immediately transfer the samples in order on ice after the denaturation.	
10	Prepare the miniblotter by placing the membrane on the lanes, with the line pattern of the membrane perpendicular to the lanes of the blotter. Place de support cushion on the membrane followed by the other half of the blotter. Turn the blotter right-side up without moving the membrane and turn the screws hand-tight,	
11	Remove residual fluid in the slots by aspiration.	
12	Briefly spin down the tubes at 4°C and place them back on ice in order.	
13	Fill the slots with the samples (150µl) and fill the first, last and other empty slots with 2x SSPE/0.1% SDS. **Avoid air bubbles.**	
14	Hybridize the blotter at 42°C for 60 minutes in the hybridization oven without shaking.	
15	Remove the samples by aspiration.	
16	Dissemble the blotter and remove the membrane from the blotter.	

17	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS at 50°C for 10 minutes under gentle shaking.		
18	**During the washing step**; clean the blotter and the support cushion.		
19	Incubate the membrane with 50ml 2x SSPE/0.5% SDS + 5µl streptavidin at 42°C for 30 minutes in the hybridization oven under gentle shaking. **Discard the streptavidin solution in a tube and into the bio-waste bin. Do not pour it in the sink.**		
20	**During the streptavidin hybridization**; change the water bath temperature to 42°C and preheat the bottle with 2x SSPE/0.5% SDS solution. Keep the lid open.		
21	Wash the membrane **twice** with preheated 2x SSPE/0.5% SDS solution at 42°C for 10 minutes under gentle shaking.		
22	Change the water bath temperature to 80°C and preheat the bottle with 1% SDS solution.		
23	Wash the membrane **twice** with 2x SSPE at room temperature for 5 minutes, under gentle shaking.		
24	**During the washing step**; prepare the foil and film cassette and check if the developing machine is on (5th floor).		
25	Add 10ml ECL (5ml ECL1 + 5ml ECL2) to the membrane and gently shake by hand until the whole membrane is covered. **Discard the ECL in a tube and into the bio-waste bin. Do not pour it in the sink.**		
26	Cover the membrane in foil and place it in the film cassette. **Avoid air bubbles.**		
27	Go to the dark room and expose a film to the membrane for 10 minutes.		
28	Develop the film with the developing machine.		
29	Remove the foil and wash the membrane **twice** with preheated 1% SDS at 80°C for 30 minutes under gentle shaking.		
30	Wash the membrane with 20mM EDTA at room temperature for 15 minutes under gentle shaking.		
31	Store the membrane in a seal bag with 20mM EDTA at 4°C.		
32	Turn off all equipment and clean workspace.		

RLB hybridization done:

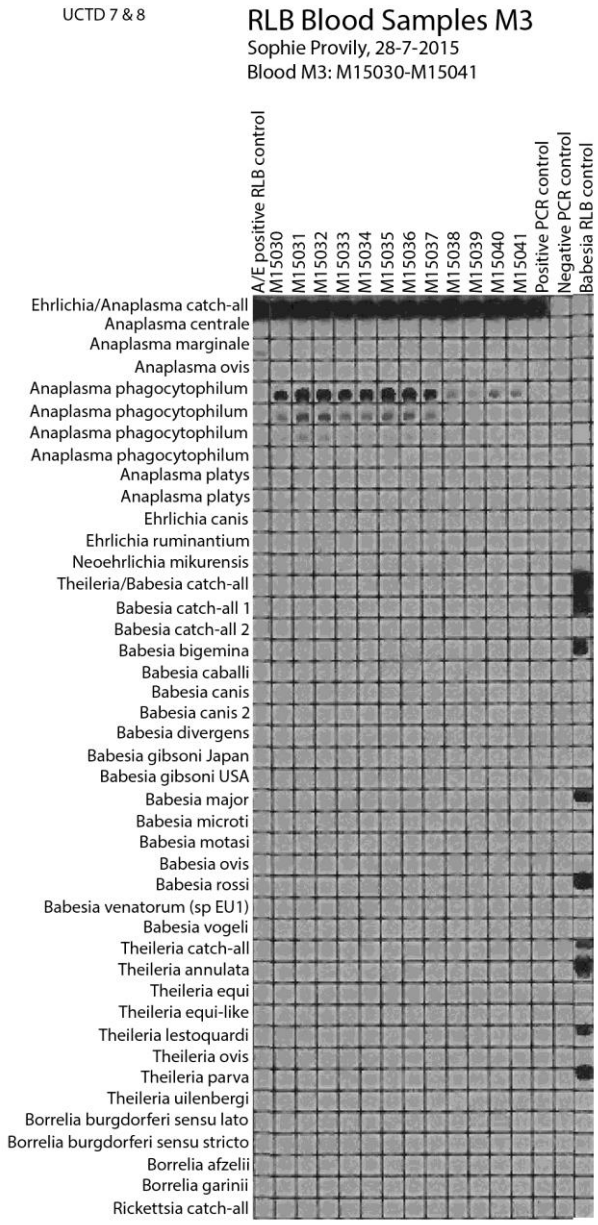
by _____ on _____ _____

Signature

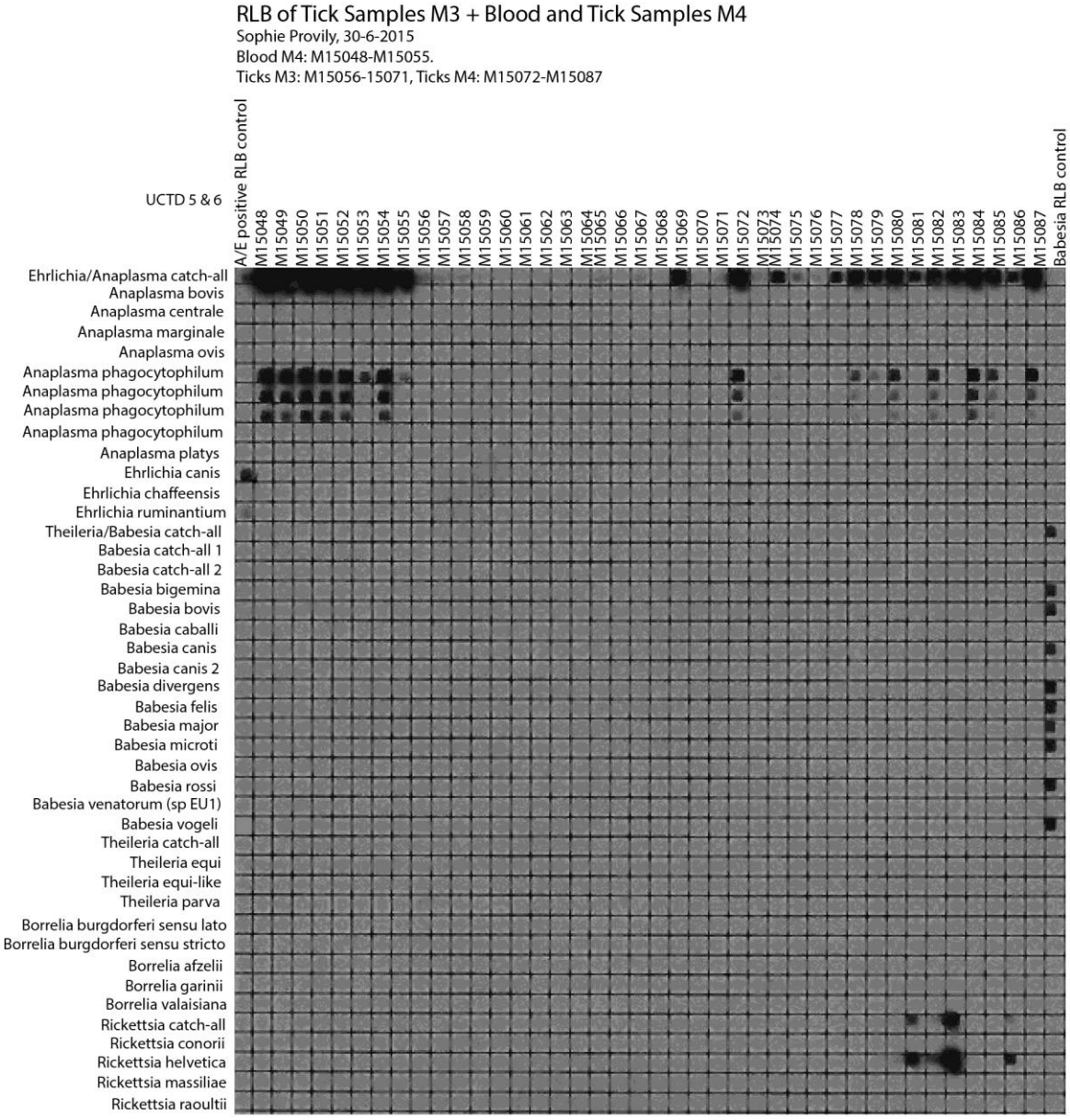
Comments:

VII: RLB results

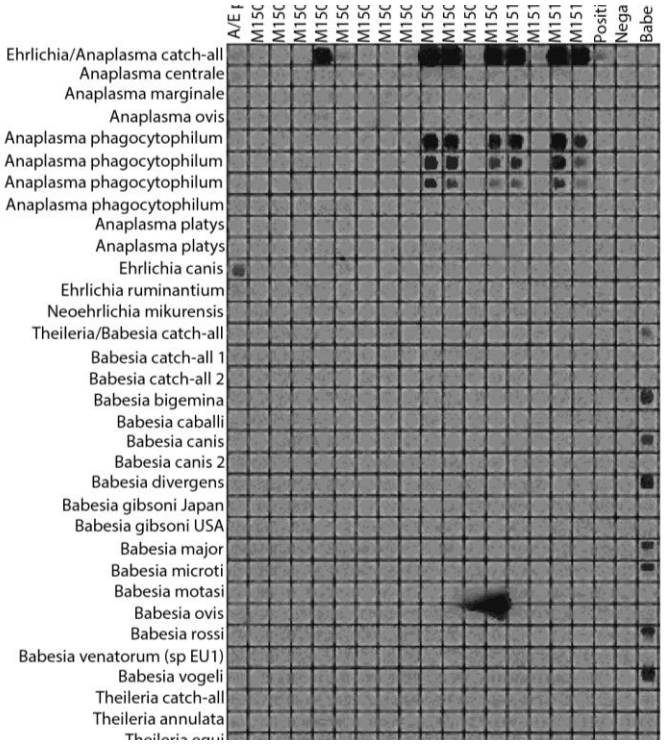
RLB results of blood samples M3



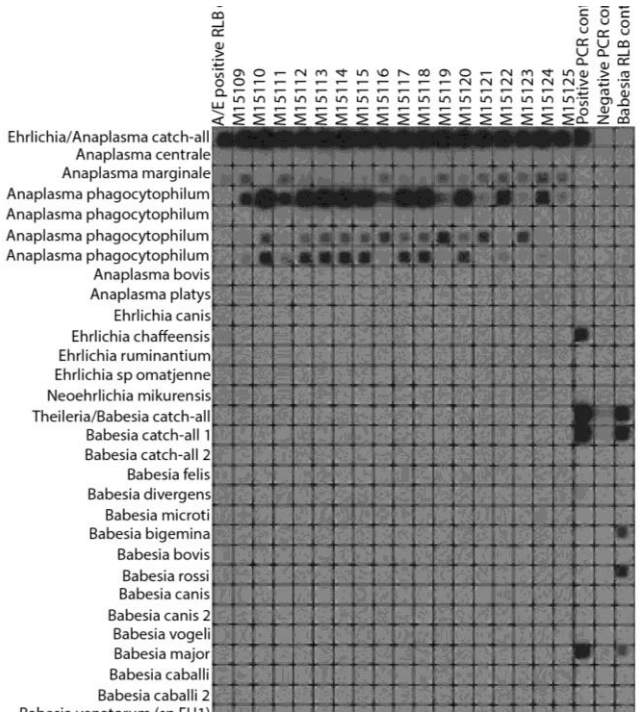
RLB results of tick samples M3 + M4 and blood samples M4



RLB results of tick samples M4



RLB results of blood samples M5



VIII Delladet protocol

DEC: 2013.II.08.087

Protocol: Goot schapenverblijf vullen met Delladet

Utrecht Centrum for Tick-borne diseases (UCTD), Faculty of Veterinary Science, Utrecht University, The Netherlands.

Om te voorkomen dat eventueel ontsnapte teken uit de proefopstelling kunnen ontsnappen en elders in het gebouw terecht komen, maken we gebruik van een goot gevuld met Delladet.

Stappenplan:

1. Trek handschoenen aan.
2. Vul de goot volledig met water.
3. Voeg **3 flessen** Delladet van 1 Liter (met een blauwe dop) toe in de geul. Verdeel de inhoud van de 3 flessen over de volledige lengte van de goot. De Delladet mengt vervolgens vanzelf.

Tijdschema:

Gedurende het experiment moet *iedere donderdag en maandag* de volledige inhoud van de goot verwijderd worden, deze schoongespoeld worden en vervolgens opnieuw gevuld worden met Delladet volgens bovenstaand stappenplan. In de tabel hieronder kan worden afgetekend door wie dit is gedaan.

Datum	Verwijder inhoud uit de goot	Vul de goot opnieuw volgens bovenstaand stappenplan
18-5-2015 (donderdag)		
21-5-2015 (maandag)		
25-5-2015 (donderdag)		
28-5-2015 (maandag)		
1-6-2015 (donderdag)		

IX Example : Protocol for experimental model (ticks and *Anaplasma phagocytophilum*)

Realisatielijst Departement Gezondheidszorg Landbouwhuisdieren Page 2 of 3
Schaap: 72261

Anaplasma

Plannings/realisatielijst (alle bevindingen bij handelingen/controle dienen te worden afgetekend in het welzijnslogboek)

NR	Week	Datum	Dag/tijd	Handeling: mbt de teken op schapen	Verantwoordelijke	Realisatiedatum	Paraaf
1	21	18-5-2015	-1	Scheren van de rug	Dierenverzorger	18 mei 2015	FJ
2	21	18-5-2015	-1	Aanbrengen lijm en zakjes	F.Jongejan	18 mei 2015	FJ
3	21	19-5-2015	0	Loslaten van de teken	F.Jongejan	20 mei 2015	FJ
3		21/5/15	2	Loslaten van de teken	F.Jongejan	21 mei 2015	FJ
4		24/5/15	5	Verzamelen van de volgezogen teken	F.Jongejan	24 mei 2015	FJ
4		25/5/15	6	Verzamelen van de volgezogen teken	F.Jongejan	25 mei 2015	FJ
4		29/5/15	10	Verzamelen van de volgezogen teken	F.Jongejan	29 mei 2015	FJ
4				Verzamelen van de volgezogen teken	F.Jongejan		
4				Verzamelen van de volgezogen teken	F.Jongejan		
4				Verzamelen van de volgezogen teken	F.Jongejan		
5	23	~~2-6-2015~~ 29/5/15	~~24~~ 10	Verwijderen van de laatste teken, verwijderen zakjes (natmaken) plus acederm	F.Jongejan	29 mei 2015	FJ
5	23	~~3-6-2015~~ 30/5/15	~~25~~ 11	Acederm behandeling	Dierenverzorger		
6	23	~~4-6-2015~~ 31/5/15	~~26~~ 12	Acederm behandeling plus anti-teken middel	Dierenverzorger		

Realisatielijst Departement Gezondheidszorg Landbouwhuisdieren Page 3 of 3
Schaap: 72261

Anaplasma

NR	Week	Datum	Dag	Handeling mbt Anaplasma in schapen	Verantwoordelijk	Realisatiedatum	Paraaf
1	20	12-5-2015	-7	Bloedafname dag -7 voor PCR/RLB (schoon)	Dierenverzorger	12/5/2015	YZ
2	21	19-5-2015	0	Infectie met Anaplasma	Dierenverzorger/FJ.	19 mei 2015	FJ
3	21	19-5-2015	0	Rectaal temperaturen	Dierenverzorger	19/5/2015	YZ
4	21	20-5-2015	1	Rectaal temperaturen	Dierenverzorger	20/5/2015	YZ
5	21	21-5-2015	2	Rectaal temperaturen	Dierenverzorger	21/5/2015	YZ
6	21	22-5-2015	3	Rectaal temperaturen	Dierenverzorger	22/5/2015	YZ
7	21	23-5-2015	4	Rectaal temperaturen	Dierenverzorger	23/5/2015	YZ
8	21	24-5-2015	5	Rectaal temperaturen	Dierenverzorger	24/5/2015	YZ
9	22	25-5-2015	6	Rectaal temperaturen	Dierenverzorger	25/5/2015	YZ
10	22	26-5-2015	7	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	26/5/2015	YZ
11	22	27-5-2015	8	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	27/5/2015	YZ
12	22	28-5-2015	9	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	28-5-15	MAW
12	22	29-5-2015	10	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	29-5-15	MAW
14	22	30-5-2015	11	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	30-5-15	MAW
15	22	31-5-2015	12	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	31-5-15	MAW
16	23	1-6-2015	13	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	1-6-15	SQ
17	23	2-6-2015	14	Rectaal temperaturen/bloedafname bij koorts	Dierenverzorger	2-6-'15	RLH
18	23	2-6-2015	14	Behandeling van Anaplasma met doxycycline	Dierenverzorger	2-6-'15	BH