


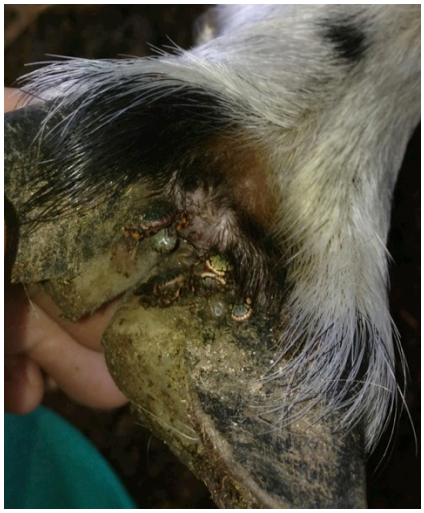

*npact of Amblyomma ticks on the health of goats in the Mnisi area, near Kruger Park, South Africa with the aim to develop a intervention strategy to prevent lameness and exposure to heartwater.*

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**By Tiffany Leenders (3742806), April 2015**

# Contents

**A b s t r a c t**

The seasonal dynamics of ticks on goats in the Mnisi area (Mpumalanga province) was studied using whole body tick collections. The ticks that were collected were later identified using a light microscope. The infection rates of *Amblyomma hebraeum* ticks were determined using polymerase chain reaction (PCR) and reverse line blot hybridization (RLB). The summer collections of this study were compared with four studies in the same area during different periods. (Busser 2014)(Deetman 2014)(Sitter 2015)(Van der Steen 2013).

Seventeen villages from the Mnisi area (Mpumalanga province), South Africa were selected for the collections of ticks. In every village 2 households were selected and at each household 5 goats were selected for the whole body tick collection. The households that were selected were the same households as used during previous collections if possible. In other cases a different household from the same villages with five of goats or more was selected. The goats were randomly selected from the group. A questionnaires survey was performed that gave information about the owner's opinion and knowledge about ticks' and tick-borne diseases. Questions were asked about tick prevention, number of death goats during last year and possible signs of heartwater in these death goats. The ticks that were collected were stored in filled with 70% alcohol and identified the same day. At Utrecht Centre of Tick-borne Diseases (UCTD) *A. hebraeum* ticks were selected for PCR and RLB testing on *Ehrlichia/ Anaplasma, Babesia/ theileria,* and *Borrelia*. From each village 4 adult ticks (2 male and 2 female) ticks and 10 nymphs were randomly selected. One goat with symptoms of heartwater was observed and later euthanized to find heartwater inclusion bodies in brain smears in order to make a definitive diagnosis of heartwater. The dissection of the goat was performed in order to see if there were any signs of heartwater like hydropericardium, hydrothorax and/or pulmonary oedema. The brain was removed from the goat and ten crushed brain smears were made to try and find of *E. ruminantium* inclusion bodies in the endothelial cells.

From the questionnaire survey it became clear that 76% of the goat owners in the Mnisi area do not know about tick-borne diseases. In 23 households (68%) goats died during the last year. The cause of death was often unknown. When the owner had seen the goat when it was dying, very often neurological signs were seen like pedalling movements. This could be a sign of a possible heartwater infected goat. In 62% of the households tick treatment was applied.
The species *Amblyomma hebraeum, Rhipicephalus (Boophilus) microplus, Ixodes rubicundus and Rhipicephalus evertsi* were found during this study. *A. hebraeum* was most commonly found and accounted for 69% of all ticks found. Comparing with the results of studies during different seasons it showed that *A. hebraeum* adult ticks are higher in number during the summer period. This was also found in the study from last year during the same season. The number of immature ticks does not follow the same pattern. The number of larvae seems to be higher during the winter period.
In total 16% of the female and 12% of the male *A. hebraeum* ticks were infected with *E. ruminantium*. From all the nymphs that were tested only the village Thorndale was tested positive for *E. ruminantium*. In a lot of the tick samples there was also an infection seen with a *Babesia* and *Theileria* catch all species of the RLB blot. If this pathogen is already known or a new species needs to be investigated.
During the dissection of the goat no macroscopic signs of heartwater were found. In the ten crushed brain smears that were made no inclusion bodies of *E. ruminantium* were found.

## 1. Introduction

*Ehrlichia ruminantium* (previously known as *Cowdria ruminantium*) is the agent that can cause heartwater disease (also called cowdriosis) in cattle, sheep, goats and some wild ruminants.(Bezuidenhout 2009) *E. ruminantium* is an obligate intracellular gram-negative rickettsia that infects endothelial cells and probably also white blood cells.(Steyn, McCrindle et al. 2010, Allsopp 2010, Jongejan, Wassink et al. 1989) Ticks of the genus *Amblyomma* transmit the disease and it is of serious economic importance in many countries of sub-Saharan Africa and several islands in the Caribbean. Ticks of this species are a really robust reservoir of *E. ruminantium*, and infection can persist in them for at least 15 months. (Fernández, White 2010)

In susceptible animals the disease is very often fatal, with mortalities that are up to 50-90% in sheep and goats.(Ahmadu, Lovelace et al. 2004) However, in most indigenous breeds of cattle and small ruminants the disease is less severe. But animals that have never encountered the disease are highly susceptible, even of these breeds. The disease can present itself in many forms depending on the susceptibility of the animal and the virulence of the *E. ruminantium* isolates. The acute signs are most frequently seen. These signs include a fever reaction, anorexia followed by nervous signs (ataxia, chewing movements, twitching eyelids, circling, different behaviour like aggression and blindness, pedalling movements while recumbent and convulsion which cause death). Heartwater derived the name from a pronounced hydropericardium that is one of the most prominent lesions found in animals with this disease. Other commonly found macroscopic lesions are hydrothorax, pulmonary oedema, oedema of the mediastinal and bronchial lymph nodes, petechiae on the epicardium and endocardium, congestion of the brain and moderate splenomegaly. The egress of fluids that causes hydropericardium, hydrothorax and edema are results from increased vascular permeability because of the disease (Bezuidenhout 2009) (Fernández, White 2010).

Amblyomma ticks are a three-host tick whose life cycles may take from 5 months to 4 year to complete. Ticks can pick up the disease by feeding on acutely ill or subclinical infected animals. Because ticks can acquire the infection as larvae or nymphs and can transmit the disease as nymphs or as adults; the infection can persist in the tick for at least 15 months. The infection is not transmitted from the adult females to her offspring. (Fernández, White 2010).

The epidemiology of heartwater depends on the interaction between three main factors: the tick vector, causative agent, and vertebrate hosts. Important for the tick vector are the tick infection rates, the seasonal changes influencing abundance and activity, and intensity of tick control. The causative agent (*E.ruminantium*) can have different genotypes that can cause a lot of difference in the virulence or stimulation of cross-protection. When looking at the vertebrate hosts it is important for this disease that a wild animal reservoir is available. Then you also need to know if there is an age or genetic resistance. (Fernández, White 2010)

There is evidence that heartwater is endemically stable in sub-Saharan Africa. (Uilenberg 1997) This is a situation where in the relationship between tick, host and pathogen is stable and low morbidity and mortality is seen.(Uilenberg 1997) It is known that there is age-related resistance to heartwater. This age-related resistance against heartwater is of a short duration (several weeks after birth only). However it is possible that this period is long enough to create endemic stability of heartwater in the Mnisi area.

Another important factor is probably the indigenous breed. It is thought that this breed has developed a high level of resistance to the disease in order to survive in the environment with so many lethal challenges with *E. ruminantium* infected ticks. Other breeds are probably more susceptible. To test this isolates of heartwater strains from the Mnisi area (from goats and /or ticks) need to be tested under laboratory conditions using experimental heartwater/ tick models.

Although the disease is of great importance there is only limited data available about the incidence of the disease. There is need for more information about the monitoring of ticks and their infection rate with *E. ruminantium*. This will also give more information about the seasonal differences in the occurrence of lameness. With the use of brain smears a final diagnosis of heartwater can be made. If rickettsial inclusion bodies can be found in the endothelial cells of the brain it is clear that the animal was infected. However this method can only be used post mortem on clinical cases.(Uilenberg 1997)

Livestock is an important part of agriculture in Mnisi. The sub-tropical climate and high densities of hosts in the Mnisi area provide the right conditions for the survival and maintenance of ticks. Ticks and tick-borne diseases are responsible for a limited development of the livestock industry in this country. Tick feeding can result in a reduction in life weight gain; tick bites can get infected with secondary infections and cause loss of udder quarters in cattle and goats. Goats can develop lameness because of the indirect damage due to ticks. Cattle in the area of Mnisi are already treated against ticks. This is mainly because cattle are more important for the farmers' income in the area. Goats are sometimes treated but usually not.

In previous studies the relative abundance and seasonal dynamics of *A. hebraeum* ticks and the prevalence of *E. ruminantium* has been investigated by four students of the University Utrecht (UU) (Francine van der Steen, Suzanne Busser, Iris Deetman and Barry de Sitter). They were supervised in the area by Dr. H. Stoltsz at the University Of Pretoria (UP) and by Prof. Dr. F. Jongejan at Utrecht Centre for Tick-borne Diseases (UCTD). An overview of the sampling times with the results can be found in table 1.

| Overview of sampling times | | infected adults | Infected nymphs |
|---|---|---|---|
| F. Van der Steen | June and July 2013 | 13% | 11.8% |
| S. Busser | October and November 2013 | 25% | 23.5% |
| I. Deetman | March and April 2013 | 9.8% | 46.7% |
| B. De Sitter | July and Augustus 2014 | 17% | 38% |

## 2.1 Main question

What are the seasonal dynamics with respect to the presence of *A. hebraeum* on goats in the Mnisi area (Mpumalanga Province), South Africa?

Knowledge about the seasonal dynamics is very important in order to determine the right period to start treatment against the ticks. Tick controle the ticks is necessary to prevent tick-borne diseases in animals but also to prevent animals of getting lame from the wounds that are causes by ticks feeding.

## 2.2 Sub-questions

- How many adult *A. hebraeum* ticks are present on the goats during this season and how does this relate to the number of adults found during previous collections from other seasons?
- What is the number of immature (larvae and nymphs) *A. hebraeum* ticks present on the goats and how does this number relate to collections from other seasons?
- What proportion of adult and immature ticks is infected with *E. ruminantium*?
- Is it possible to find heartwater in crushed brain smears of dead goats that have shown signs of heartwater?

# 3. Materials and methods

## 3.1 Study area

This research area is part of the Mnisi Community Programme. The initiative of this study comes from the University of Pretoria and the Mnisi Traditional Authority.[10] Mnisi is situated in the northeast corner of the Bushbuckridge Municipal area, Mpumalanga Province, South Africa. Over 40.000 people live in this area and the community mainly speaks Shangaan. The Mnisi area is located within the savannah ecosystem.

The villages that were studied were the same villages as previous students visited. These are Athol, Eglington, Clare B, Dixie, Gottenburg, Hlalakahle, Hluvukani, Ludlow, Seville B, Share, Shorty, Thlavekisa, Thorndale, Utha B, Welverdiend A and Welverdiend B.[8]

## 3.2 Study animals

Ticks were collected the same way as previous studies. Whenever possible, the same households that were sampled before were used, this way the most correlative results as possible can be obtained. In all seventeen villages 2 households were visited. At each household 5 goats were sampled. In every household that was selected there were more than 4 goats.

## 3.3 Examination of the collected ticks

The ticks were carefully removed from the goats with tweezers in order to keep the ticks mount part attached to the body. When removed the ticks were placed in a cup filed with 70% alcohol to kill it and store it. After the collection the ticks will be identified with a stereoscopic microscope at the Hans Hoheisen Research Station. Only the Amblyomma ticks were used for further examination, ticks other than the Amblyomma genus were counted after identification. Further examination of the Amblyomma ticks and their pathogens happened at the UCTD, Utrecht University. After DNA extraction, the presence of pathogens in the DNA were simultaneously detected and quantified by PCR, while the reverse line blot (RLB) hybridization detected different genera of tick-borne pathogens.[10]The PCR/RLB protocol described by Bekker et al was used for the detection of *E. ruminantium*.(Bekker, de Vos et al. 2002)

## 3.4 Tick identification

All students included in this study were informed about the ticks that can be found in the area. At the University of Utrecht they were able to study the *A. hebraeum and other tick species* and get information about how to identify ticks. Genera, species, sex and stage of the life cycle were determined with use of a stereoscopic microscope. The book: 'Tick of Domestic Animals in Africa: a Guide to Identification of Species' guide by Walker, A. R. & Bouattour was present at the laboratory where the ticks were identified.[13]

## 3.5 Detection of *E. ruminantium* in ticks

At the Utrecht Centre of Tick-borne Diseases (UCTD) at the University Utrecht, DNA was extracted from ticks that were collected in the Mnisi area. PCR and RLB were used to detect whether or not the ticks were infected by *E. ruminantium*. PCR/RLB were used according to the protocol described by Bekker et al.[11] The nymphs were pooled and tested in batches of 10 as described in the study by B. Faburay.(Faburay, Geysen et al. 2007) Ticks that are still partly enlarged with blood were selected and

tested as pools since these contain a great amount of host blood. This is to avoid the collection of a separate blood sample. All the adult ticks will be tested individually. See Appendix C for the protocols that will be used during this research.

### 3.6 Questionnaire

A questionnaire survey was implemented in order to get an overview of the epidemiology of heartwater. The EM's were of great help with translations between the students and the farmers and helped completed the questionnaire. The questionnaire consist several open questions, yes/no questions and the farmers were asked about their opinion and knowledge regarding tick-borne diseases and preventive treatment against ticks.

### 3.7 Statistics

The results of the number of *A. hebraeum* tick were compared with the results of this year and last year. Special interest went out to the differences in numbers during different seasons. The PCR and RLB results were compared and data from the questionnaire. In a final report of all researches a statistical analysis will be performed. The overall tick infestation rate, the tick: goat ratio and the questionnaire results were included in the final report in order to gain a better understanding of the epidemiology of heartwater in the Mnisi area.

### 3.8 Polymerase Chain reaction (PCR)

The protocol that was used for the PCR technique is the same protocol that was used in previous studies. The is the way described by Bekker et al. (2002a), Gubbels et al. (1999) and Schnittger et al. (2004) with some modification made in the UCTD protocols from 2012. The protocol from UCTD can be found in APPENDIX C. From each village a standard number of 2 adult males and 2 adult females were selected for DNA, PCR and then RLB. In the villages Shorty, Eglington and Clare B not enough ticks were found. That is the reason why there are only 3 ticks from Shorty (2 female and 1 male), 4 ticks from Eglington (3 male and 1 female) and 2 ticks from Clare B (2 females) selected for testing. For testing of the nymphs only in Clare B not enough ticks were found. Only 9 ticks were tested from Clare B. A total number of 65 adults and 169 nymphs were tested during this research.
Each sample was tested for *Ehrlichia/Anaplasma, Theileria/Babesia* and *Borrelia* species. A positive and a negative PCR control were added to the test.

Different primers were used for the different pathogens that were research. The forward primer used for Anaplasma/Ehrlichia was Ehr-F (5' – GGA ATT CAG AGT TGG ATC MTG GYT CAG) and the reverse primer Ehr-R (5' – Biotin – CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT)(UCTD, 2012). These primers for Anaplasma/ Ehrlichia are degenerate primers. This means that the primers can attach at serveral parts in de sequence. For Babesia/Theileria, the forward primer, RLB-F2 (5' – GAC ACA GGG AGG TAG TGA CAA G), and the reverse primer, RLB-R2 (5' – Biotin – CTA AGA ATT TCA CTT CTG ACA GT) have been used for amplification (UCTD, 2012). For the Borrelia PCR the forward primer Bor-F (5'-ACC ATA GAC TCT TAT TAC TTT GAC CA -3') and the reverse primer Bor-R (5'- Biotin-GAG AGT AGG TTA TTG GCC AGG G-3')

### 3.9 Reverse line blot hybridization (RLB)

The use of RLB makes it possible to detect and identify several different pathogens at the same times. This is made possible with use of species-specific probes that are attached to a membrane.(Bekker, de Vos et al. 2002, Gubbels, de Vos et al. 1999). Multiple samples can be analysed against multiple probes.

This saves a lot of time. With the use of one blot it is possible to analyse 43 samples. There are 45 slots to fill but the first and the last slot are filled with 2 x SSPE/0.1% SDS, to avoid cross flow. In every blot a positive *Anaplasma/Ehrlichia* (in the second slot) and a positive *Babesia* (in slot number 43) was added. This is done to make it easier to read the results in the end. All the other slots were filled with diluted and denaturized PCR samples. Then the blot was placed in a hybridization oven. After hybridization, all samples were removed from the blot by aspiration. The membrane is than removed from the blot and washed twice with preheated 2x SSPE/0,5% SDS. The membrane is than incubated with preheated 2x SSPE/0,5% SDS + 5 µl streptavidin and washed twice again with the same fluid as before the incubations. After that the membrane is washed twice with 2x SSPE. ECL is than added to the membrane. This substance makes it possible for the probes that are attached to the pathogen to emission light. The membrane is than packed in foil and placed in a film cassette. The membrane is exposed to the film for 10 minutes and after that the film is developed. The results can now be read from the developed film. The membrane is than rinsed and stored in a sealed bag with EDTA. Later the membrane can be stripped so it can be used again.

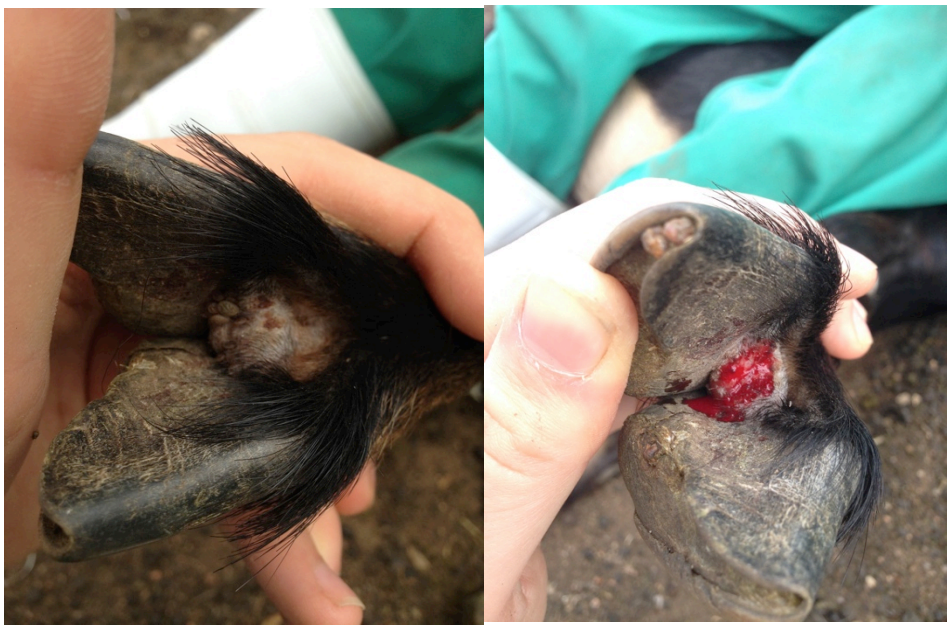
### 3.10 Brain smears and pathology

Animals with clinical signs of heartwater were observed and followed during the progress of the disease. Animals that die or can euthanized were used for pathology research and crushed brain smears were made after permission from the owner.
The crushed brains smears were made with the technique that is described by Jaiswal et al.(Jaiswal, Vij et al. 2012)The alcohol is not added during this research.(Jaiswal, Vij et al. 2012) A small piece of cerebrum or cerebellum is cut off and placed on a slide. Another slide is taken and with this slide the small piece of cerebrum or cerebellum is crushed until there is no structure left and porridge remains. The crush slide is than put a side for the next slides (try to put all the brain mush on the other slide) and a new clean slide is taken. With this slide you pick up the mush to one side of the slide. Now one slide is on the table with cerebrum or cerebellum porridge and the other is in one hand. The porridge is than pushed over the slide that is on the table in the same way as a blood smear. When the porridge is pushed for half a centimetre pick up the slide a little bit so both slides don't touch any more. Then place the slide back on the other slide again and push the porridge the same way as the first time. This is repeated until the end of the slide is reached. On one brain at least 5 brain smears need to be made from different part of the brain. When the slides were finished they were brought to the Hans Hoheisen Research Station where they were fixated and coloured with Giemsa staining.
During the dissection of the goat special interest went out to the signs of heartwater. The abdomen, thorax and pericardium will be opened to see if there is any free fluid in these parts of the body.

## 4. Results

### 4.1 Tick collection

Seventeen different villages of the Mnisi area were included in this research. The village that was named Hluvukani in previous reports is named Eglington in this report because according to the coordinates from these previous reports the villages name is Eglington. The village Utah B no longer has farmers that own goats so this village was excluded from the research.
A total number of 3563 ticks from 170 goats were collected during December 2014 from the area of Mnisi province Mpumalanga, South Africa. On 4 goats from the village Clare B no ticks were found. The species *Amblyomma hebraeum, Rhipicephalus (Boophilus) microplus, Ixodes rubicundus and Rhipicephalus evertsi* were found during this study. *A. hebraeum* was most commonly found and accounted for 69% of all ticks found. Of the *A. hebraeum* ticks 62% was male. Most ticks of the species *A. hebraeum* that were found were nymphs (See table 1). Clare A (323 ticks) and Share (309) were the most infected with ticks. However most of the ticks found here were still nymphs and not *Amblyomma* ticks. The goats in these villages were severely infected with ticks but after removal the damage on the feet of the goats was mild. In Thorndale and Hlalakahle, a lot more adult *Amblyomma* ticks were found, the damage to the feet of the goat was more severe and abscesses were often found on the feet of these goats. (See picture 1 and 2)



Picture 1: Feet of a goat from Clara A before and after removal of *Rhipicephalus microplus* tick.

Picture 2: Feet of a goat from the village Thorndalle before and after the removal of *Amblyomma hebraeum* ticks. Abscess is opened.

An average of 1,7 adult *A. hebraeum* ticks per goat were found during the month December of 2014. Comparing to the previously found numbers from June-July (Winter), March-April (Autumn) and July-august (Winter/Spring) these numbers are at least 30% higher. Comparing to the numbers found a year before (November 2014) almost 40% less adult *A. hebraeum* ticks were found. The number of *A. hebraeum* nymphs found this year was 51% higher than the same season in 2014. (See figure 1).

| Tick species | Adults | | | | Nymphs | | Larvae | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | *Amblyomma hebraeum* | *Rhipicephalus (Boophilus) microplus* | *Ixodes rubicundus* | *Rhipicephalus evertsi evertsi* | *Amblyomma hebraeum* | *Rhipicephalus (Boophilus) microplus* | | | Mean no. of A. hebraeum adult ticks/ host |
| **Ludlow** | 8 | 5 | 1 | 0 | 103 | 3 | 129 | 249 | 0,8 |
| **Share** | 13 | 0 | 4 | 0 | 112 | 9 | 171 | 309 | 1,3 |
| **Athol** | 11 | 0 | 1 | 0 | 124 | 0 | 39 | 175 | 1,1 |
| **Eglington** | 5 | 0 | 0 | 0 | 204 | 8 | 47 | 264 | 0,5 |
| **Shorty** | 3 | 0 | 0 | 0 | 121 | 1 | 4 | 129 | 0,3 |
| **Dixie** | 23 | 0 | 0 | 0 | 136 | 0 | 17 | 176 | 2,3 |
| **Utah A** | 17 | 1 | 0 | 0 | 226 | 1 | 28 | 273 | 1,7 |
| **Serville A** | 11 | 1 | 0 | 0 | 115 | 3 | 100 | 230 | 1,1 |
| **Serville B** | 20 | 0 | 0 | 0 | 159 | 1 | 119 | 299 | 2,0 |
| **Thorndale** | 36 | 14 | 0 | 0 | 119 | 2 | 26 | 197 | 3,6 |
| **Gottenburg** | 28 | 6 | 0 | 0 | 138 | 14 | 64 | 250 | 2,8 |
| **Clare B** | 2 | 2 | 0 | 0 | 9 | 0 | 1 | 14 | 0,2 |
| **Tlhavekisa** | 5 | 0 | 0 | 0 | 151 | 0 | 12 | 168 | 0,5 |
| **Welverdiend A** | 13 | 2 | 1 | 0 | 57 | 0 | 4 | 77 | 1,3 |
| **Welverdiend B** | 24 | 22 | 0 | 1 | 136 | 16 | 47 | 246 | 2,4 |
| **Clare A** | 28 | 112 | 1 | 0 | 131 | 41 | 10 | 323 | 2,8 |
| **Hlalakahle** | 48 | 0 | 0 | 0 | 119 | 3 | 14 | 184 | 4,8 |
| **Totaal** | 295 | 165 | 8 | 1 | 2160 | 102 | 832 | 3563 | 1,7 |

Table 1. Species composition and total number of ticks collected from goats in the area of Mnisi (Mpumalanga), South Africa during November and December (collected by Tiffany Leenders)

Combining the results from different seasons you can see that during the summer and the beginning of summer (November till February) more adult *A. hebraeum* ticks are found (table 2). During autumn (March till May) less adult *A. hebraeum* are found and this decreases even more during the winter (June till August). The most *A. hebraeum* nymphs were found at the end of the winter and at the beginning of summer. The most larvae were found during the winter.

| Tick species | adults | | Nymphs | | Larvae | Total | |
|---|---|---|---|---|---|---|---|
| | *Amblyomma hebraeum* | *Rhipicephalus (B.) microplus* | *Amblyomma hebraeum* | *Rhipicephalus (B.) microplus* | Larvae | | Mean no. of *A. hebraeum* adult ticks/ host |
| June/July 2013 (n=117) | 23 | 161 | 1267 | 0 | 1932 | 3384 | 0,2 |
| November 2013 (n=184) | 522 | 35 | 1430 | 19 | 726 | 2732 | 2,8 |
| March/April 2014 (n=169) | 219 | 202 | 1272 | 292 | 878 | 2884 | 1,3 |
| July/Augustus 2014 (n=160) | 114 | 25 | 3147 | 30 | 732 | 4129 | 0,7 |
| December 2014 (n=170) | 295 | 165 | 2160 | 102 | 832 | 3563 | 1,7 |
| **total (n=800)** | 1173 | 588 | 9276 | 443 | 5100 | 16692 | |

Table 2: Species and number of ticks found on goats from the Mnisi area during different periods of the year.

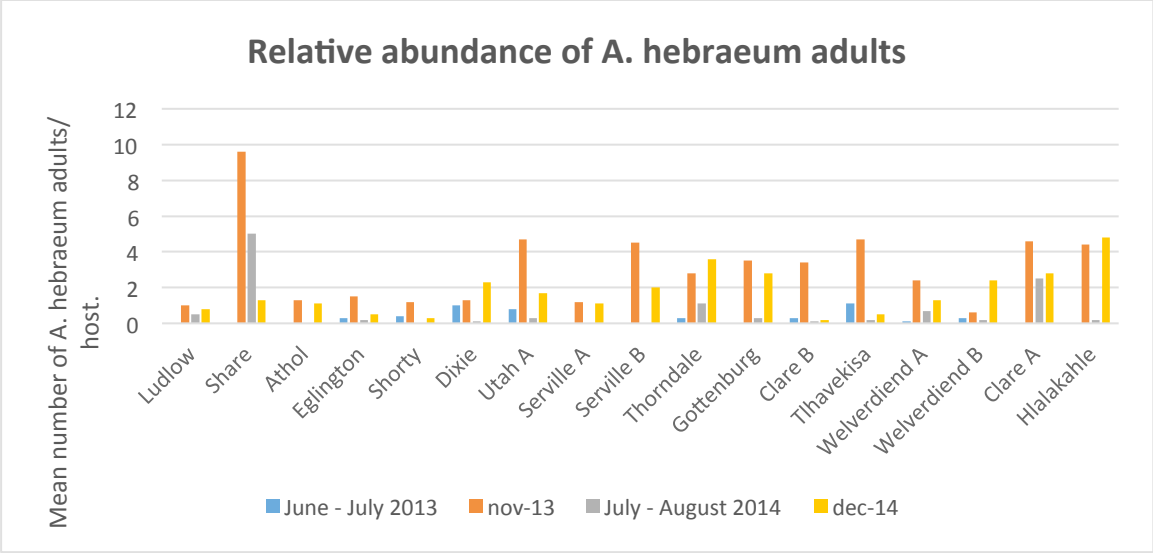



Figure 1. Results of a comparison of the mean number of A. hebraeum adults/number of sampled hosts per village collected in June-July 2013, November 2013 and December 2014.

## 4.2 Questionnaire survey

A total number of 34 households from the region of Mnisi were selected. Together these owners owned 395 goats and of each household 5 goats where selected for a full body tick inspection and removal of al ticks that are found. In 23 of these households (68%) goats died during the last year. The reason why the goats died was often unknown because the owner did not go to the veterinarian for diagnosis. (See figure 2) The owners often said that the goats died of dog bites because they found the goat the next morning dead with wounds that look like dog bites or the dog was still eating the goat. There was one case where the owner said that the goat died of heartwater disease. The veterinarian told him about heartwater and the symptoms that the goat showed just before it died looked like heartwater. The goat was treated against heartwater but died the next day. The owner did not know what kind of medication was used. In six of the households the owner had seen the goat just before it died and saw some neurological signs (like quick movements of the legs, tight muscles and chewing), which can be an indicator of heartwater. The owners often slaughtered the goat after it dies and eat the meat. Water around the heart or inside of the thorax or abdomen was not noticed.

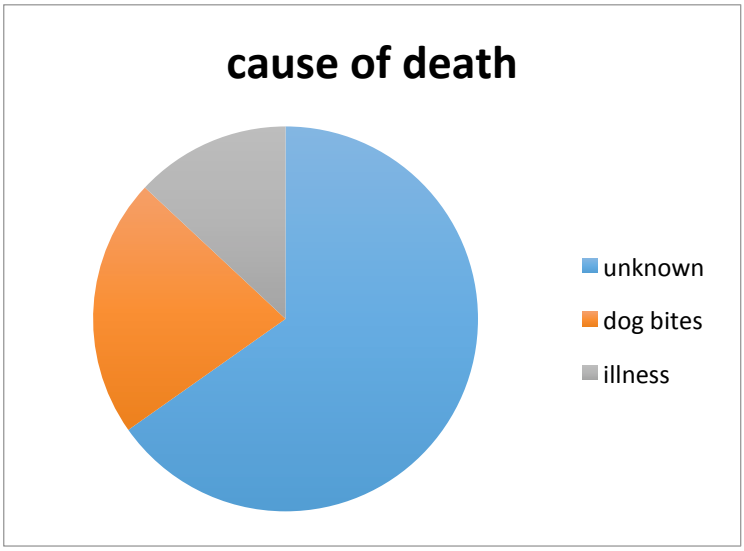



Figure 2: The possible causes of the death of the goats, according to the owners.

In eleven households (32%) the owner has never seen any ticks on the goats and often said that their goats do not have ticks. Of the owners that did see ticks on their goats 90% (20/22) said that there are more ticks during the summer. Twenty-six owners (76%) did not know that it is possible for ticks to transmit diseases to the goat. Twenty-one (62%) of the households used prevention tick control. Most goats from the area were treated with dip from the dip tank or with Tictac (Amitraz). (See figure 3) Often no standard method of prevention was used. Most often the treatment was only applied during summer or if they noticed any ticks on the goats. Many owners where interested in a preventive method for the ticks. Only one owner does not want treatment because he already treats the goats himself and almost all these goats were not infected with ticks.

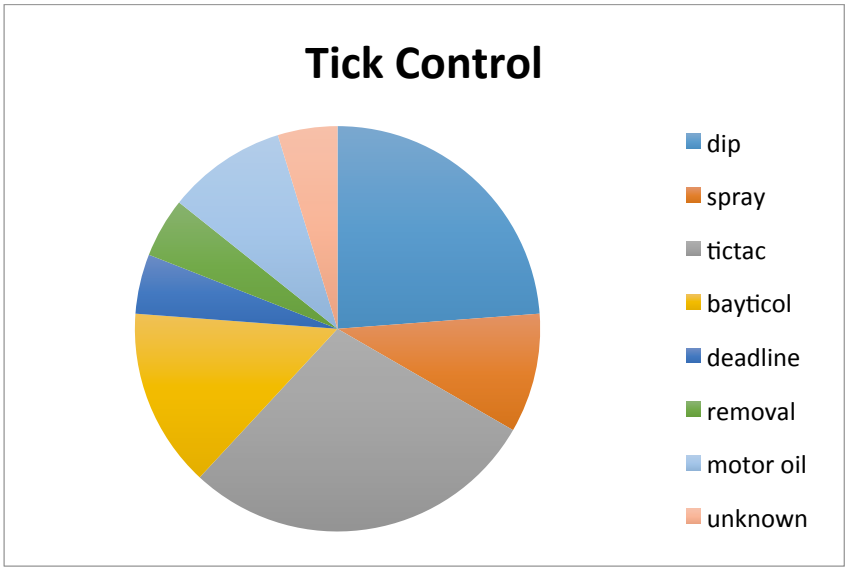


Figure 3: Prevention methods used in the selected households from the area of Mnisi.

### 4.3 Polymerase chain reaction (PCR) and Reverse line blot hybridization (RLB)

The PCR products that were obtained were used in a RLB to find out what kind of DNA these ticks were carrying. The main purpose was to find out how many of these ticks were infected with *E. ruminantium*. Of the sixty-five adult *A. hebraeum* ticks that were tested nine ticks were detected as positive for *E. ruminantium*. These ticks came from the villages Eglington, Welverdiend A, Athol, Share, Hlalakahle, Gottenburg, Serville B and Welverdiend B. Four of these ticks were males and five were females. In total 16% of the females and 12% of the males was infected with *E. ruminantium*. From all the nymphs that were tested only the village Thorndalle was tested positive for *E. ruminantium* (See table 2). In many of the tick samples there was also an infection seen with a *Babesia* and *Theileria* species of the RLB blot. It is still investigated if this pathogen is new or already known. See Appendix D for the results of the RLB.

| Villages | Nymphs infected | Infected/ no. analysed | | Overall |
|---|---|---|---|---|
| | | Males | Females | |
| Shorty | No | 0/1 | 0/2 | 0/3 |
| Eglington | No | 1/3 | 0/1 | 1/4 |
| Clare B | No | 0/2 | 0/0 | 0/2 |
| Welverdiend A | No | 0/2 | 1/2 | 1/4 |
| Athol | No | 1/2 | 0/2 | 1/4 |
| Serville | No | 0/2 | 0/2 | 0/4 |
| Share | No | 1/2 | 0/2 | 1/4 |
| Tlhavekisa | No | 0/2 | 0/2 | 0/4 |
| Hlalakahle | No | 0/2 | 1/2 | 1/4 |
| Gottenburg | No | 0/2 | 1/2 | 1/4 |
| Utah A | No | 0/2 | 0/2 | 0/4 |
| Serville B | No | 0/2 | 2/2 | 2/4 |
| Welverdiend B | No | 1/2 | 0/2 | 1/4 |
| Dixie | No | 0/2 | 0/2 | 0/4 |
| Clare A | No | 0/2 | 0/2 | 0/4 |
| Thorndale | Yes | 0/2 | 0/2 | 0/4 |
| Ludlow | No | 0/2 | 0/2 | 0/4 |
| Total | 1/17 | 4/34 | 5/31 | 9/65 |

Table 2: Result of the infection rate with *E.ruminantium* in ticks collected from goats from the Mnisi area (Mpumalanga), South Africa, during December 2015.

Comparing the infection rates from December 2015 with the rates found in previous studies the nymphs were a lot less infected. Fewer adults were infected when comparing with the findings of last year November.

| Infection rates *A. hebraeum* | Nymps | Adults |
|---|---|---|
| June/ July 2013 v/d Steen | 2/17 12% | 3/23 13% |
| November 2013 Busser | 4/17 24% | 17/68 25% |
| March/April 2014 Deetman | 7/15 47% | 5/51 10% |
| July/Augustus 2014 De Sitter | 6/16 38% | 6/36 17% |
| December 2014 Leenders | 1/17 6% | 9/65 14% |

Table 3: comparison of the infection rate of *A. hebraeum* ticks from the area of Mnisi during different seasons.

### 4.4 Brain smear and pathology

A total of ten brain smears were made from one goat that had shown signs of heartwater and was suspected of heartwater by the local veterinarian. This goat showed signs of heartwater like tetanus, nystagmus, pedalling movements and chewing. The goat did not have a fever. Blood was taken from the goat but no signs of heartwater inclusion bodies were found. The goat was then bought from the owner and euthanized. After the euthanasia the goat was brought to the clinic at Hluvukani for pathologic research. There was a little bit more fluid in the pericardium than normal (4 ml). There was no fluid in the thorax or the abdomen of the goat. No macroscopic signs of heartwater were found during this dissection.

In the brain smears that were made from this goat there were some suspicions looking figure. Prof. Dr. Jongejan looked at these slides and *E.ruminantium* inclusions bodies were not found.

## 5. Discussion

In this research *A. hebraeum* and *R. (B.) microplus* were the tick species that were found the most in the area. This was also found during the previous studies in the area (Busser 2014)(Deetman 2014)(Sitter 2015)(Van der Steen 2013). Earlier studies also found *Rhipicephalus appendiculatus* but not during this study. During the collection in November the year before *R. appendiculatus* was also not found. A possible explanation for this is that this species prefers cooler and wetter places. In a study in Zimbabwe they found that the species *R. appendiculatus* is more dominant in regions with a cool and wet climate.(Sungirai, Madder et al. 2015) *R. appendiculatus* was not found during collections that took place in the summer period in South Africa. This means that the temperatures were high and lots of rain. During the other seasons the temperature is lower and less rain. It is possible that *R. appendiculatus* moves to cooler places during the summer and comes back in the other seasons. This can be a reason why this species is not found during summer in the area but is found during the other seasons.
The species *Ixodes rubicundus* and *Rhipicephalus evertsi* were found during this study but not in any of the previous studies. Only a couple of ticks were found. It is possible that these species also have a preference for climate and that these species like the summer climate. It is also possible that these species do occur in the area, but only in small numbers.
A larger number of male ticks were found on the goats than females. The reason for this is that male ticks often stay longer on the host. (Bryson, Tice et al. 2002)The male ticks also look for a partner to mate with. When collecting the ticks it was often found that multiple males surrounded a feeding female.

The collections of the villages Welverdiend A and Welverdiend B took place during a very wet day. The goats that were research were very muddy which made the collection of ticks really hard. It is very likely that many sampled nymphs and larvae were missed that day. A relatively small amount of nymphs and larvae were found to last year.

A difference between the seasonal abundance of ticks in the Mnisi area was found during the first four researches. When comparing the results of the summer this year and last year less adult ticks were found during this year. Because not a standardized number of goats were used during the research of November 2013 the absolute number cannot be compared with the absolute numbers of this year's research. This is the reason why the results were compared using the mean number of ticks per host. The mean number per host was also lower than found the year before. It is possible that this was just a coincidence and that the same total number of ticks was present in the area during both season but

because of the selection of household less ticks were found. Another option the goats in the area are now more often treated. During this research a couple of households just treated the goat a couple of days or a week before the collection. On these goats often very small number of ticks were found. The ticks that were found on these goats were often dead or immature stage. These dead ticks were still collected identified and included in the collection. The villages were the owners just treated the goats were Clare A and B, Shorty and Athol.

The number of immature ticks that were collected during the different studies does not follow the same pattern as that of the adults. Also comparing the results of this year with that of the year before a lot of differences were found. The reason for this different finding can be that the immature ticks are many harder to find on the goat and that some immature ticks were missed. Also in the research of November 2013 it was mentioned that some goats were so heavily infected that not all the ticks were removed. The large ticks are often the first ticks to be removed. When the large ticks are removed the immature ticks are easier to find and remove. Some small ticks are attached to larger ticks so when the larger tick is collected you automatically collect smaller ticks. If some large nymphs were left on the goat it is possible that also al lot of larvae were not collected. Also it was not mentioned when a goat was called heavily infected and not possible to collect al ticks. During this study only in the village Clare A was there was one goat were not all the immature ticks were fully removed because there were to many ticks. When comparing to different studies it is possible that one researcher will stop collecting sooner than the other in this situation. This can be a reason why different numbers of immature ticks were found in the same season.

During this research only one goat was presented for further research of Heartwater. This goat did show signs of heartwater and was treated for heartwater by the veterinarian of the Hluvukani clinic. This goat was later euthanized and further research by dissection but no definitive diagnosis of heartwater could be made. It is possible that this goat did not have heartwater but a different disease. Possible other diseases that look like heartwater are rabies, tetanus, bacterial meningitis or encephalitis, babesiosis, anaplasmosis or cerebral trypanosomiasis. The ticks that were collected and used for PCR and RLB did show infections with *Babesia* and *Theileria* and rabies is common in the area of the goats. It is also possible that the heartwater inclusion bodies were not found but that they were inclusion bodies present in a different slide or different part of the brain. During pathology only a small amount of extra fluid was found around the heart. It is possible that this was normal for this goat. The goat did not reacted to the heartwater treatment so there is a change that this goat did not have heartwater but another disease.

For further research a different method could be used to diagnose the goats for heartwater when they are still alive. In a report from F. Jongejan et al. (Jongejan, Wassink et al. 1989)it is described that it is possible to cultivate *Cowdria ruminantium* in caprine or ovine neutrophil granulocytes. If during the next studies students can be thought how to perform this technique it will be easier to diagnose heartwater. For this test blood needs to be collected from the goat when it is showing signs of heartwater and preferably before it has had the fever peak. For the cultivation more preparation is needed. It is also not easy to find the inclusion bodies even if the goat is showing lots of signs. Maybe this technique can be used together with the pathology investigation.

## 6. Conclusion

It became clear from the results of these studies that there is a seasonal dynamic in the presence of *A. hebraeum* ticks on goats of the Mnisi area (Mpumalanga Province), South Africa. With the mean number of adult ticks per host it is seen that more ticks are found during the beginning of summer and during summer. During the winter period the number of adult ticks reduced.

If these results are compared with the results from last year (the same season) both found higher numbers of adults ticks than during the other seasons.

The number of immature ticks did not follow this pattern. Most nymphs and larvae where found during the winter periods. But during the whole year high numbers of larvae and nymphs were found.

During the whole year ticks were infected with *E. ruminantium*. The percentage of infected ticks did not show a pattern. It was found that goats in one village of the Mnisi area had nymphs that were infected and goats in eight villages had adults that were infected with *E. ruminantium*.

One goat with signs of heartwater was necropsied. No hydrothorax was found. There was little fluid in the pericardium but not an extensive amount. Ten brain smears where made but no inclusions bodies were found.

## 7. Acknowledgement

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## 9. APPENDIX A

Overview the villages

The villages that will be visited will be the same as in the previous studies conducted by UU students:

Ludlow (24°40'54.5"S 31°16'37.7"E), Utha A (24°41'36.0"S 31°26'49.5"E), Utha B (24°42'33.9"S 31°24'13.1"E), Shorty (24°40'22.2"S 31°22'28.2"E), Clare A (24°37'59.0"S 31°18'05.4"E), Clare B (24°38'43.5"S 31°20'11.7"E), Athol (24°42'34.2"S 31°20'45.8"E), Share (24°40'36.0"S 31°18'46.5"E), Gottenburg (24°38'21.6"S 31°24'38.8"E), Hlalakahle (24°37'13.1"S 31°25'50.9"E), Hluvukani (24°38'42.9"S 31°20'47.5"E), Welverdiend A (24°34'46.3"S 31°19'26.7"E), Welverdiend B (24°35'18.4"S 31°20'22.9"E), Thorndale (24°39'01.1"S 31°26'58.4"E), Dixie (24°41'40.3"S 31°28'29.6"E), Tlhavekisa (24°37'30.9"S 31°22'31.6"E) and Seville (24°40'10.1"S 31°25'01.9"E).

## 10. APPENDIX B

Questionnaires

These surveys will be conducted with the owner of the goats:

| | | |
|---|---|---|
| How many goats does the farmer own? | | |
| How many goats died during last year? | | |
| *When did they die last year? | | |
| | | |
| **Signs before death** | **YES** | **NO** |
| Ticks seen on goats? | | |
| Falling on the ground? Quick movements of legs? Tight muscles? Chewing just before goats died? | | |
| Has owner slaughtered any goats that suddenly died? | | |
| Noticed water around hart/in thorax? | | |
| Lameness on goats? Infected feet on goats? | | |
| | | |
| **General questions about ticks** | **YES** (score 1,2,3,4,5) | **NO** |
| Does owner see ticks on goats? | | |
| *Does owner see difference in amount of ticks during the different seasons? | | |
| Does owner know the role of ticks in diseases? | | |
| Can owner treat goat when it's ill? | | |
| | | |
| **Questions about practical preventive methods** | **YES** | **NO** |
| Does owner use tick prevention? And what? | | |
| Is owner interested in implementing tick preventive methods? | | |
| *When does owner use preventive methods? (which time of year) | | |

This survey is a continuation of the survey conducted by Suzanne Busser and Iris Deetman. The questions with an asterisk are new and try to gain more insight in the general knowledge of the epidemiology of ticks, tick-borne diseases and possibilities of treatment of goats by the owner.

## 11. APPENDIX C

**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

**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

**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 ($5^{th}$ 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. | | |

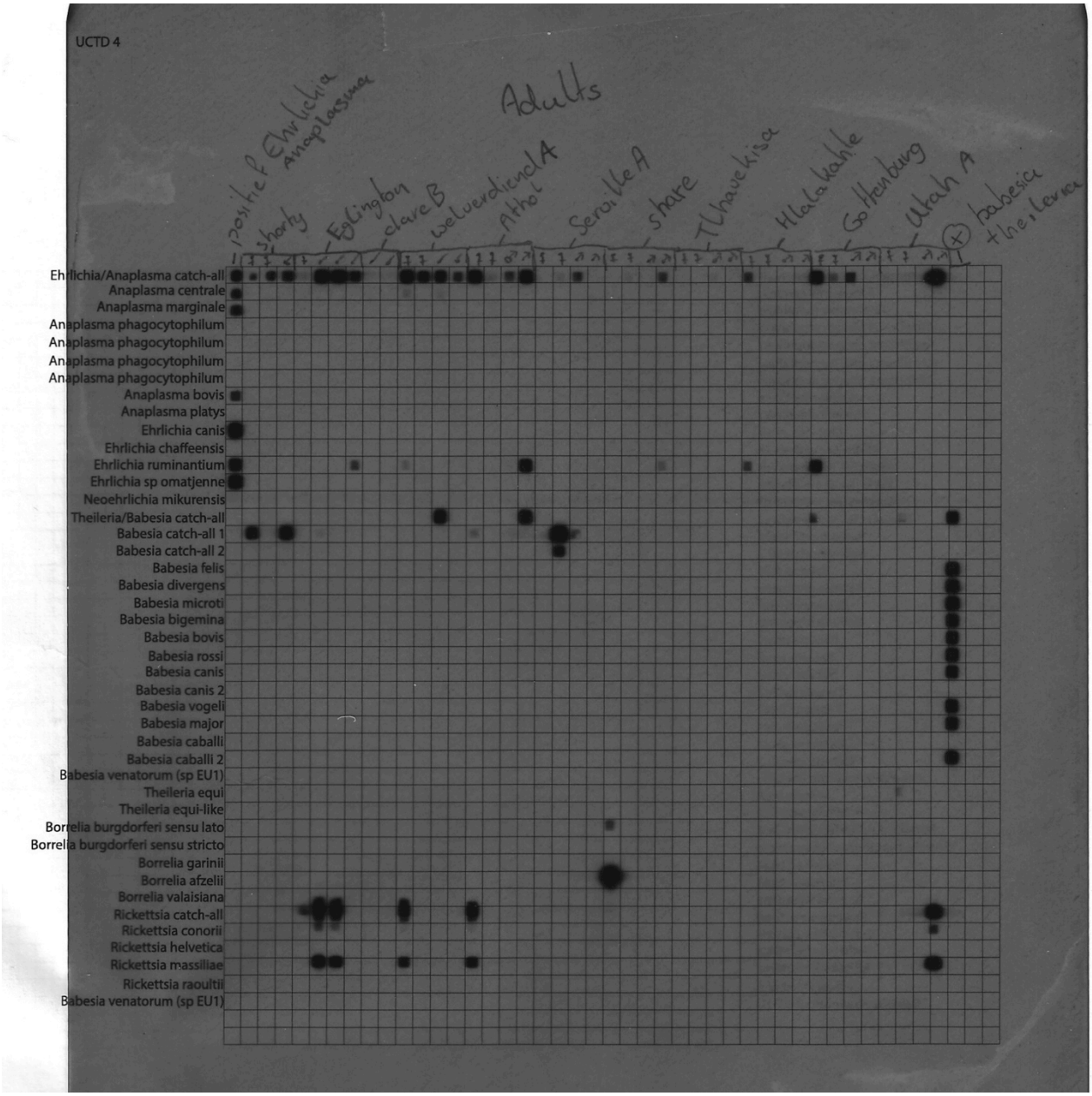
RLB hybridization done:

by _____ on _____ _____

Signature

## 12. APPENDIX D

Results RLB blot number 1



Results RLB blot number 2