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The relative abundance of *Amblyomma* tick infestations in relation to the prevalence of heartwater (*Ehrlichia ruminantium* infection) in goats in the area of Mnisi (Mpumalanga), South Africa

Amblyomma hebraeum (male)

Amblyomma hebraeum (female) (Stoltsz)

Research Internship Veterinary Medicine

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Abstract

The main goal of this study was to determine the relative abundance of *Amblyomma* tick infestations in relation to the prevalence of heartwater (*Ehrlichia ruminantium* infection) in goats in the area of Mnisi (Mpumalanga), South Africa. Indigenous goats belonging to small-scale farmers in Mnisi (Mpumalanga), South Africa, were sampled for ticks during the period June to July 2013. Five Ixodid tick species, of which the majority were immature ticks, were collected from the goats (n = 117) in Mnisi (Mpumalanga). *Amblyomma hebraeum* was the most numerous of these, followed by *Rhipicephalus (Boophilus) microplus*. Forty samples with *Amblyomma hebraeum* ticks were assessed for *Ehrlichia ruminantium* infection with Polymerase Chain Reaction (PCR) and Reverse Line Blot hybridization (RLB). The prevalence of infection detected by the PCR-RLB in adult ticks was 13% (3/23) and for the pooled nymphs 11,8% (2/17). The substantial numbers of *Amblyomma hebraeum* and the relative high prevalence of *Ehrlichia ruminantium* in this area will make the import of high-producing animals difficult.

1 Introduction

Ticks play an important role as vectors in the transmission of diseases. An important example of such disease is heartwater also known as cowdriosis (Allsopp, Bezuidenhout & Prozesky 2004). Heartwater is a common disease of domestic ruminants and causes great economic losses (Mukhebi et al. 1999). Ticks in the genus of *Amblyomma* transmit the disease, which is caused by the rickettsia *Ehrlichia ruminantium* (Allsopp, Bezuidenhout & Prozesky 2004). In Southern Africa *Amblyomma hebraeum* is the main vector (Walker et al. 2003). Heartwater affects cattle, sheep and goats and also susceptible wild antelope species (Peter et al. 1999a). Disease symptoms caused by heartwater include high fever, nervous signs and accumulation of fluid in the lungs, brain, thoracic cavity and pericardial sac (Bezuidenhout 2009). Transmission of the disease to a susceptible goat often results in acute fatal disease (Peter et al. 1999a).

In endemic areas the prevalence of heartwater is often under-reported, because definitive diagnosis is often not performed (Allsopp, Bezuidenhout & Prozesky 2004). The heartwater endemic areas include most of sub-Saharan Africa and the Caribbean (Allsopp 2010, Peter et al. 1999a). Especially goats are affected, and in some part of the rural farming sector the number of infected goats with heartwater may reach 30 per cent of the population (Allsopp, Bezuidenhout & Prozesky 2004). It is unknown how many animals die, but this percentage is considered to be high. Certain breeds are more resistant than others, making the introduction of high-producing animals more difficult (Allsopp, Bezuidenhout & Prozesky 2004).

Besides ticks of the genus of *Amblyomma,* it is important to gain insight in the overall species composition and distribution of ticks infesting goats in the area of Mnisi (Mpumalanga), South Africa. Ticks that are prevalent on goats may play an important role in the transmission of diseases, not necessarily only for goats, but also for other livestock (Nyangiwe, Horak 2007). Horak et al. (2009) found that the tick species most common infesting goats and cattle in Eastern Cape Province and Maputo Province were *Amblyomma hebraeum, Rhipicephalus (Boophilus) microplus, Rhipicephalus appendiculatus* and *Rhipicephalis evertsi evertsi.* Goats are good (alternative) hosts of most of the economically important ticks that infest cattle (Nyangiwe, Horak 2007). Nyangiwe and Horak (2007) concluded that goats play an important role in maintaining tick infestations and should be included in tick control programmes applied to cattle (Nyangiwe, Horak 2007).

One example of such economic important cattle tick is *Rhipicephalus (Boophilus) microplus. R. (B.) microplus* uses cattle almost exclusively as hosts and is usually only found on other animals provided infested cattle are present at the same location (Nyangiwe, Horak 2007). Nyangiwe and Horak (2007) concluded that *R. (B.) microplus*, which was considered to be a cattle tick, might be in process of adapting to goats. They found that a large proportion of female *R. (B.) microplus* ticks that they collected from goats were 5 mm or more in length. Meaning that these ticks successfully completed their life cycle on the goats and that they perhaps even do this in the absence of infested

cattle (Nyangiwe, Horak 2007). Furthermore, Horak et al. (2009) found that in Eastern Cape Province and Maputo Province there was a partial or complete displacement of the indigenous tick *Rhipicephalus (Boophilus) decoloratus* by the introduced species *R. (B.) microplus*.

The Mnisi area is suitable for both species (Walker et al. 2003). Unpublished data of ticks collected from cattle in this area showed that *R. (B.) decoloratus* has been almost completely displaced by *R. (B.) microplus*. If *R. (B.) decoloratus* and *R. (B.) microplus* appear in the collection of ticks obtained from goats, it might shed some light on the prevalence of these tick species in the area of Mnisi (Mpumalanga), South Africa. As the study area borders on wildlife areas, and *R. (B.) microplus* is not known to feed on other hosts, the presence or absence of this species on goats may also be of significance.

The main goal of this study was to determine the relative abundance of tick infestations on goats in the area of Mnisi (Mpumalanga), South Africa with emphasis on the vectors of heartwater (*Amblyomma* ticks). Information on the tick species composition and distribution of ticks infesting goats may provide a better understanding of the prevalence of heartwater in this area and their role in transmission of disease to the local livestock population.

2 Research questions

2.1 Main research question

What is the relative abundance of *Amblyomma* tick infestations in relation to the prevalence of heartwater (*Ehrlichia ruminantium* infection) in goats in the area of Mnisi (Mpumalanga), South Africa?

2.2 Sub-questions

In an attempt to answer the main question, the following sub-questions were examined:

- What is the tick composition on goats in this area?
- What is the prevalence of heartwater in this area?
- Is there also a displacement of *Rhipicephalus (Boophilus) decoloratus* by *Rhipicephalus (Boophilus) microplus* seen on goats, in this area?

3 Materials and Methods

3.1 Study area

Collection of ticks took place in Mnisi, Mpumalanga Province. The Mnisi area is situated in the north-eastern corner of the Bushbuckridge Municipal Area (University of Pretoria 2011). More than 40.000 people divided over 8.555 households are included in the study area (Kriek, Burroughs 2009). In this area approximately 6.000 goats are kept by 917 owners (Kriek, Burroughs 2009). The communal farming area borders on the adjacent Andover and Manyeleti provincial game reserves (University of Pretoria 2011). A fence is the only barrier between livestock and abundant wildlife populations (University of Pretoria 2011). Many tick species, including known vectors of important diseases such as Corridor disease, redwater, heartwater and anaplasmosis, flourish in the Mnisi area (University of Pretoria 2011).

In the area described above the Mnisi Community Programme (MCP) is established in 2008 (University of Pretoria 2011). This program is an initiative by the University of Pretoria, and the Mnisi Traditional Authority (University of Pretoria 2011). The aim of MCP is improving the health and welfare of the community, within the 'One Health' philosophy (Kriek, Burroughs 2009). Research in the MCP is aimed at being beneficial to the major stakeholders, which include the community, conservation areas, various authorities and the ecosystem (University of Pretoria 2011).

3.2 Tick collection

From June 17th 2013 till July 12th 2013 ticks were collected from goats at seventeen different villages in the Mnisi area. In every village 1 to 5 households were visited. A total of 39 households were used for sampling. From every household 1 to 8 goats (depending on the group size) were searched for ticks. In total 117 goats were inspected for ticks. Ticks specimens were obtained by examining the whole body of the goats. Ticks obtained from each village were placed in a labelled bottle containing 70% alcohol. Bottles were labelled with date, host species and collection site.

The ticks were identified to species level with a stereoscopic microscope. Identification took place by identification of genera, geographical area and species identification (Walker et al. 2003). Since collection took place during the cold season a lot of immature ticks were collected. A differentiation has been made between adults, nymphs and larvae. Adults and nymphs were identified to species level and the larvae were only counted. The tick identification was randomly re-identified/reconfirmed by Dr. Stoltsz and Prof. Horak of the Onderstepoort Veterinary Institute, South Africa.

3.3 Questionnaire survey

A questionnaire was submitted to every household that participated in this study. The aim of the questionnaire was to get an insight in the disease symptoms, mortality, use of medication and tick prevention and their familiarity with tick-borne diseases. The questionnaire involved open and closed questions for the farmers. In total 38 questionnaires were completed by the livestock owners. The questionnaire used for this study is included in appendix 1.

3.4 DNA extraction

For DNA extraction twenty-three adult *A. hebraeum* ticks and hundred and sixty-six *A. hebraeum* nymphs were used. All the adults were tested separately and the immature ticks were pooled with a maximum of 10 immature ticks per pool. From every village 10 nymphs were pooled for DNA extraction, with the exception of the village Gottenberg were only six nymphs were used because of their size. A total of forty samples where examined by PCR and RLB after DNA extraction.

The ticks were washed with distilled water in a sonification bath, put in an eppendorf tube with 70% ethanol for several seconds (including vortexing) and dried on a clean tissue, according to UCTD protocols (2012). After this the dried ticks were placed in a sterile tube with 180µl T1 lysis buffer. The samples were frozen for 15 minutes at -80°C. Metal beats of 7 mm were added to the frozen samples. For disruption of the ticks the samples were put into the TissueLyser LT. DNA was extracted from ticks by using the protocol of NucleoSpin® Tissue kits (Machery-Nagel 2012).

3.5 Polymerase Chain Reaction (PCR)/Reverse line blot hybridization (RLB)

The Reverse Line Blot (RLB) hybridization assay makes it possible to analyse multiple samples against multiple probes at the same time (Bekker et al. 2002). This advantage has been used not only to test the samples for *E. ruminantium* but also for other *Anaplasma, Ehrlichia, Babesia* and *Theileria* species. The results can give an insight in the diseases in this area and the possible role of *A. hebraeum* in the transmission of these diseases. The prevalence of *E. ruminantium* in the ticks was determined by the analysis of each sample with a PCR/RLB protocol as described by Bekkers et al. 2002. The probes

used to test for *Anaplasma, Ehrlichia, Babesia* and *Theileria* species are the same as described by Bekkers et al (2002), Gubbers et al (1999) and Schnittger et al. (2004).

The primers used for PCR were RLB-F2 (5'- GAC ACA GGG AGG TAG TGA CAA G) and RLB-R2 (5'- Biotin – CTA AGA ATT TCA CTT CTG ACA GT) for *Babesia/Theileria* (Utrecht Centre for Tick-borne Diseases (UTCD) 2012). For *Anaplasma/Ehrlichia* the primers Ehr-F (5'- GGA ATT CAG AGT TGG ATC MTG GYT CAG) and Ehr-R (5'- Biotin – CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT) have been used (Utrecht Centre for Tick-borne Diseases (UTCD) 2012). The following symbols indicate degenerate positions: M = A+C and Y = C+T (Utrecht Centre for Tick-borne Diseases (UTCD) 2012). After processing the samples with PCR the negative and positive control samples have been analysed with Agarose gel electrophoresis. The exact protocols used for PCR, Agarose gel electrophoresis and RLB were conform the protocols of Utrecht Centre for Tick-borne Diseases (UCTD) and are included in appendix 2.

4 Results

4.1 Tick collection

A total of 188 adult ticks, 1.267 nymphs and 1.932 larvae were collected over the study period from 117 goats. Five Ixodid tick species were recovered from the area of Mnisi (Mpumalanga), South Africa. *Amblyomma hebraeum* and *Rhipicephalus (Boophilus) microplus* were the most prevalent on goats. All nymphs were *Amblyomma hebraeum*. The larvae were not differentiated to species level but it seemed that different species were present. The tick species composition and number of ticks collected from goats are summarized in Table 1.

Collection site (n= number of goats)	Tick species: Adults: Amblyomma hebraeum	Adults: Boophilus microplus	Adults: Rhipicephalus appendiculatis	Adults: Rhipecephalus simus	Adults: Rhipicephalus zambeziensis	Nymphen: Amblyomma hebraeum	Larvae	**Total**
Ludlow (n=8)	0	11	0	0	0	38	55	104
Utha B (n=5)	0	2	0	0	0	46	31	79
Shorty (n=14)	5	1	0	0	1	72	13	92
Clare B (n=3)	0	1	0	0	0	32	77	110
Athol (n=11)	0	3	0	0	0	23	236	262
Share (n=11)	0	2	0	0	0	35	77	114
Gottenburg (n=2)	0	0	0	0	0	14	2	16
Hlalakahle (n=14)	0	0	0	0	0	115	85	200
Clare A (n=3)	1	16	1	0	0	21	60	99
Hluvukani (n=7)	2	4	0	0	0	70	92	168
Welverdiend A (n=14)	1	71	0	1	1	113	108	295
Thorndale (n=4)	1	1	0	0	0	36	38	76
Thlavakisa (n=8)	9	0	0	0	0	135	62	206
Seville (n=3)	0	1	0	0	0	59	0	60
Welverdiend B (n=6)	0	6	0	0	0	37	106	149
Utha A (n=4)	3	30	0	0	0	327	650	1010
Dixie (n=1)	1	12	0	0	0	94	240	347
Total (n=117)	23	161	1	1	2	1267	1932	3387

Table 1: Species and total number of ticks collected from goats in seventeen different villages in the area of Mnisi (Mpumalanga), South Africa.

4.2 Questionnaire survey

Fifty-five per cent of the interviewed livestock owners (n = 38) indicated that they experienced problems with ticks. About 37% indicated that the tick problems were seasonal and most severe in the summer. Only 37% treat against ticks with pour-on. The frequency in use of pour-on varies tremendously between livestock owners, some owners use it every week others use it only twice a year. Sixty per cent experienced disease problems with the goats and 26% treated their animals when they were sick. Treatment consisted of alternative methods or antibiotics (Tetramycine). Disease symptoms mentioned by the owners are summarized in figure 1. Disease symptoms most mentioned were death (45%), malaise (29%) and lameness (16%). Sixteen per cent of the livestock owners think that ticks can cause these symptoms.

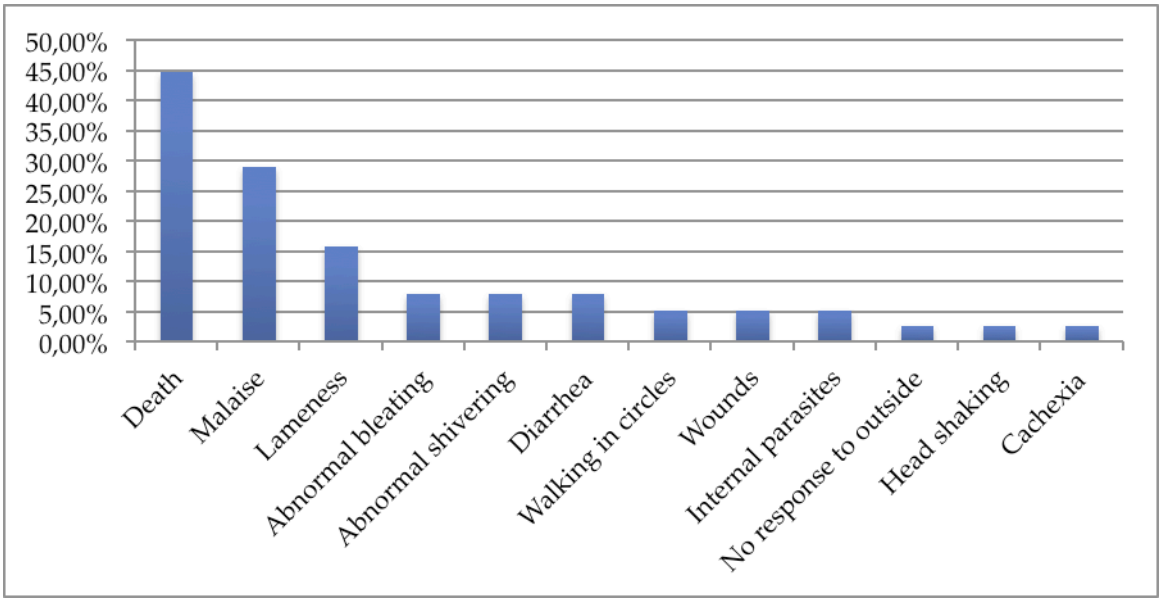

Figure 1: Overview of the disease symptoms that were mentioned by the livestock owners.

4.3 Polymerase Chain Reaction (PCR)/Reverse line blot hybridization (RLB)

After DNA extraction the forty samples were tested with RLB-PCR for several *Anaplasma, Ehrlichia, Babesia* and *Theileria* species. The RLB-PCR was performed on the species shown in figure 2. *E. ruminantium* was detected in 5 samples of which there were 3 samples from adult ticks and 2 samples from pooled nymphs. In addition, four samples were positive for *Rickettsia* catch-all and *Rickettsia massilliae.* Five samples were positive for *Babesia* catch-all 1. Negative control samples were negative in the RLB assay. The RLB assay detected 13% (3/23) of the adult ticks and 11,8% (2/17) of the samples with pooled nymphs *E. ruminantium* positive. The exact results of the RLB-PCR are displayed in figure 2.

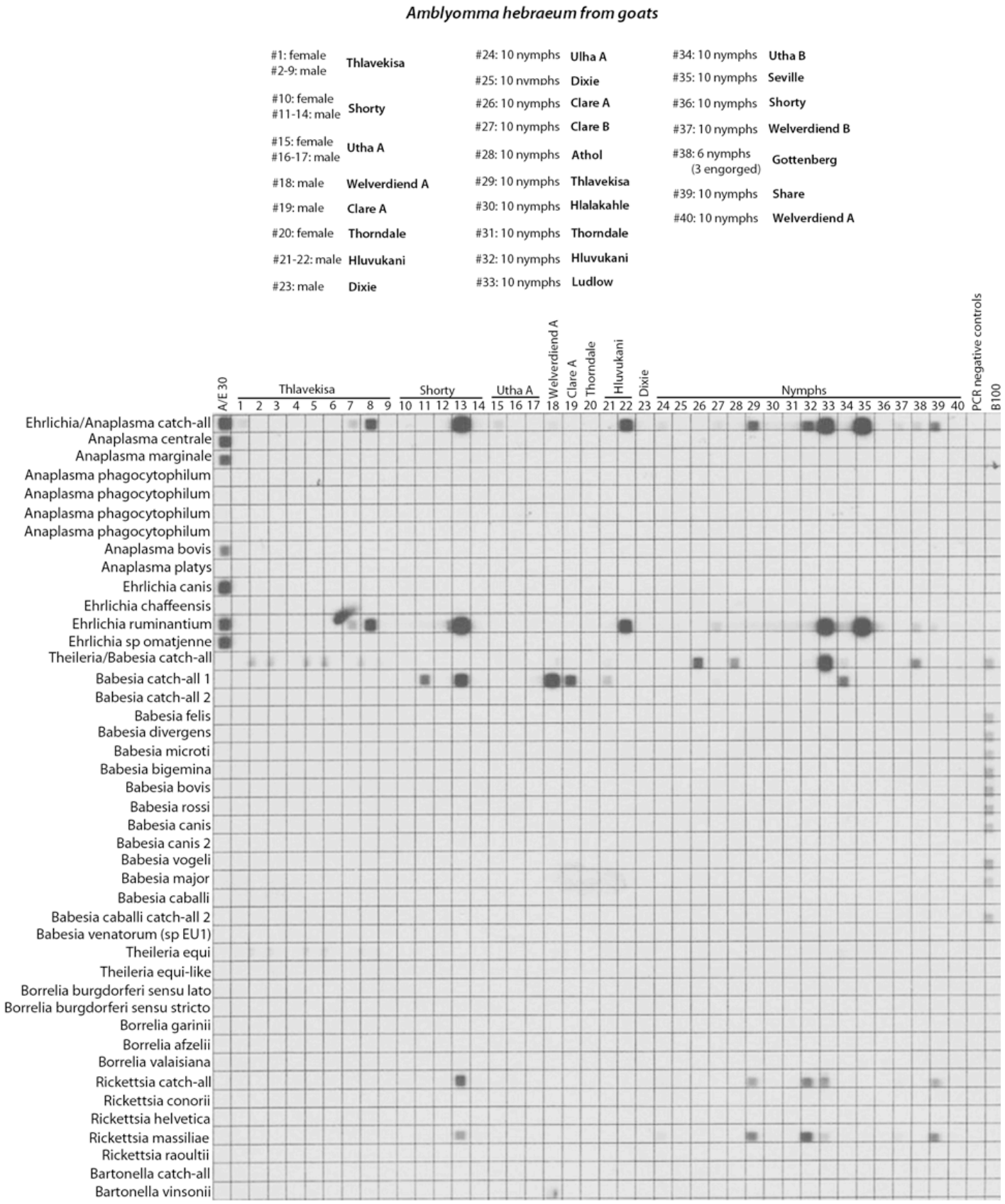

Figure 2: RLB with PCR products derived from DNA extracted from ticks collected from goats in Mnisi (Mpumalanga), South Africa.

5 Discussion

A total of 5 Ixodid tick species were recovered from goats in the area of Mnisi (Mpumalanga), South Africa. Namely, *Amblyomma hebraeum, Rhipicephalus (Boophilus) microplus, Rhipicephalus appendiculatus, Rhipicephalis simus* and *Rhipicephalus zambeziensis*. *A. hebraeum* was the most abundant species. *A. hebraeum* is most known as the vector of *E. ruminantium* and was the main focus of this study. *R. (B.) microplus* was also frequently present and can act as vector for *Anaplasma marginale*, *Babesia bigemina* and *Babesia bovis* (Estrada-Peña et al. 2006). The difference with *R. (B.) decoloratus* in vector-borne diseases is that *Babesia bovis*, the cause of Asiatic redwater in cattle, is not transmitted by *R. (B.) decoloratus* (Nyangiwe, Horak 2007). The high presence of *A. hebraeum* and *R. (B.) microplus* on goats is in agreement with findings from other studies (Nyangiwe, Horak 2007, Horak et al. 2009).

As mentioned earlier Nyangiwe and Horak (2007) concluded that *R. (B.) microplus*, which was considered to be a cattle tick, might be in process of adapting to goats. They came to this conclusion because they found a large proportion of female *R. (B.) microplus* ticks of 5 mm or more in length on goats (Nyangiwe, Horak 2007). This means that these ticks successfully completed their life cycle on the goats and that they perhaps even do this in the absence of infested cattle (Nyangiwe, Horak 2007). In this study the length of the *R. (B.) microplus* ticks were not measured. Nevertheless it seemed that some of the ticks collected were more than 5 mm. This implies that, although domestic cattle are the most efficient hosts of *R. (B.) microplus,* goats also play a significant role as host (De Matos et al. 2009, Nyangiwe, Horak 2007, Nyangiwe, Harrison & Horak 2013).

Only one adult *R. appendiculatis* and none *R. evertsi evertsi* were collected in this study. Most likely, the reason for this is that sampling took place during the cold season. In the summer the abundance of ticks and activity is much greater than in winter (Mbati et al. 2002). It might be possible that these two species were present among the larvae, since these were not identified to species level.

In this study, many ticks that have been collected from goats were female. Two studies of Horak et al. (1992, 2003) revealed that for every *R. (B.) decoloratus* female there were at least 2 males. There is no reason to believe that this pattern would be different for *R. (B.) microplus* (Horak et al. 2009, Nyangiwe, Harrison & Horak 2013). Which means that the number of adult male *R. (B.) microplus* should have been at least twice as high than the number of adult females *R. (B.) microplus* (Horak et al. 2009, Nyangiwe, Harrison & Horak 2013). This suggests that the collections were by no mean exhaustive (Nyangiwe, Harrison & Horak 2013). It is virtually almost impossible to collect all ticks from living animals because of the numerous sites they attach (Nyangiwe, Harrison & Horak 2013). In addition, the number of larvae and nymphs collected would probably have been higher if the goat hair had been combed with a coarse metal comb (Bryson et al. 2002b). Consequently, the numbers of the species collected from the goats do not represent the true burdens of these ticks (Nyangiwe, Harrison & Horak 2013).

Moreover, the ticks collected from each goat were not kept separately for the identification of tick species and counting. Therefore the number of ticks recovered from each animal cannot be compared.

Many adult *R. (B.) microplus* were recovered from the goats and no *R. (B.) decoloratus*. It seems that *R. (B.) microplus* displaced *R. (B.) decoloratus* completely, which is in accordance with previous studies (Horak et al. 2009, Nyangiwe, Harrison & Horak 2013, Tønnesen et al. 2004). Tønnesen et al. (2004) and Horak et al. (2009) already discussed several reasons for the displacement of *R. (B.) decoloratus* by *R. (B.) microplus*. One of these reasons was that excess *R. (B.) microplus* males would mate with female *R. (B.) decoloratus* (Horak et al. 2009, Tønnesen et al. 2004). Seventeen of such couplings were observed in a study of Nyangiwe et al. (2013). As *Boophilus* females mate only once (Horak et al. 2009, Tønnesen et al. 2004) and interspecific mating between *R. (B.) microplus* and *R. (B.) decoloratus* leads to the production of sterile eggs (Spickett, Malan 1978), the numbers of *R. (B.) microplus* will only increase. The shorter life cycle and higher reproductive potential of *R. (B.) microplus* enables it to compete successfully against *R. (B.) decoloratus* where these ticks occur together (Walker et al. 2003). This collection was restricted to ticks of indigenous goats in cattle rearing areas and did not extend into the surrounding National Parks and wildlife reserves, which might have led to some underrepresentation of *R. (B.) decoloratus* in the present collection (Lynen et al. 2008). Since *R. (B.) decoloratus* is less host-specific than *R. (B.) microplus* and often infest wildlife wherever available.

The prevalence of *E. ruminantium* in adult *A. hebraeum* collected from goats in the Mnisi area was 13% (3/23). The prevalence of *E. ruminantium* in the tested *A. hebraeum* nymphs was 11,8% (2/17). These percentages fall within the wide prevalence range observed in *A. hebraeum* in other studies (Bryson et al. 2002a, Norval, Andrew & Yunker 1990, Peter et al. 1999a, Peter et al. 1999b). The prevalence for *E. ruminantium* in the nymphs is high in relation to the prevalence of the adult ticks. Normally, the prevalence is expected to be higher in adult ticks as transstadial infection can occur (Andrew, Norval 1989b). In addition, intrastadial infection can occur when a male *A. hebraeum* moves from an infected animal to a susceptible animal (Andrew, Norval 1989b, Camus, Barre 1992).

The relative high prevalence in the nymphs was probably due to the pooling of the nymphs in groups of ten, which makes the chance of detecting *E. ruminantium* much higher. Besides, the overall sample size was very small, which means that the estimated prevalence might greatly differ when more *A. hebraeum* ticks are tested for *E. ruminantium*.

These ticks were collected from goats and most likely became infected during the preceding life cycle stage(s). Free-living nymphs are likely to have lower infection rates because larvae feed on a wide range of hosts, including those not susceptible to *E. ruminantium* (Norval, Andrew & Yunker 1990, Peter et al. 1999b).

Multiple hosts for *A. hebraeum* (including domestic and wildlife) are present in and around the Mnisi area and may have been a source of *E. ruminantium* infection. Cattle and goats were probably a dominant source, due to their greater abundance in the direct area. Infected animals remain carriers of

heartwater for a long period after recovery (Andrew, Norval 1989a) and are therefore symptomless reservoirs of heartwater. In endemically stable areas many carriers of *E. ruminantium* are present (O'Callaghan et al. 1998).

From the questionnaire survey farmers indicated that they experienced disease problems and losses in their goats. The reason for this was often unknown. Some of the symptoms like the neurological symptoms and (sudden) deaths would fit well with the disease caused by *E. ruminantium*. Sometimes there was a suspicion that heartwater was the cause of the symptoms but definitive diagnostics were not performed. Thirty-seven per cent of the households indicated that most problems occur during the summer months, which is when most of the ticks are active (Mbati et al. 2002). Resulting in an increased incidence of heartwater because the peak numbers of ticks that are present (Allsopp 2010). Only a small percentage of the farmers use pour-on and if they use the pour-on the frequency is often so low that it can be questioned if it is effective. The absence of many clinical cases would indicate that this area is clinical stable for heartwater (Mbati et al. 2002). In areas were heartwater is endemic, the local livestock has been exposed to a long process of natural selection, and has developed a certain resistance to the disease (Bryson et al. 2002b, Jongejan, Uilenberg 2004). Since the Mnisi area seems to be a heartwater-endemic region import from susceptible animals will be difficult. For the control of heartwater vaccination and strategic tick control measures are essential (Deem 2008).

Besides the five samples that were positive for *E. ruminantium* the PCR-RLB detected some additional pathogens. Four samples were positive for *Rickettsia* catch-all and *Rickettsia massiliae* and five samples were positive for *Babesia* catch-all 1. *R. massiliae* was recently recognized as a human tick-borne spotted fever rickettsiosis (Cazorla et al. 2008, Parola et al. 2013). In Sub-Saharan Africa this *Rickettsia* has been found in *Haemaphysalis paraleachi* and several *Rhipicephalis spp.* (Parola et al. 2013). *A. hebraeum* has not yet been mentioned as a recognized or potential vector of *R. massiliae* (Parola et al. 2013). The finding of *R. massiliae* in this area may be of interest from a zoonotic perspective. The *Babesia* catch-all 1 positives suggest the presence of an uncharacterized *Babesia* species. The positive signals were very strong, so it was expected that if one of the tested *Babesia* species was present, it would give a signal on the RLB.

6 Conclusion

This study provides insight in the tick species composition on goats and the prevalence of heartwater in the area of Mnisi (Mpumalanga), South Africa during the winter season.

A high number of *A. hebraeum* species that can serve as a vector were observed and quantified during the tick collection. The substantial numbers of *A. hebraeum* and the relative high prevalence of *E. ruminantium* in this area make the import of high-producing animals difficult. Research during different seasons is necessary because the species composition and prevalence of *E. ruminantium* in *Amblyomma* ticks is likely to differ between seasons. Furthermore it seems that *R. (B.) microplus* is adapting to goats and has displaced *R. (B.) decoloratus*. Further research is necessary to support this conclusion. It is interesting to see if *R. (B.) decoloratus* is present on goats in other seasons and if *R. (B.) microplus* can complete his life cycle on goats in absence of cattle.

7 Acknowledgements

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9 Appendix 1

9.1 Question survey - Goats in the Mnisi area

House number/name owner:

1. How many goats do you have?

2. Do you experience tick problems with the goats?

    2.1. What time of the year (seasonal)?

3. Do you treat the goats for ticks?

    3.1. With what?

    3.2. How often?

4. Do you experience disease problems with the goats?

4.1 Could you describe the symptoms?

4.2 What time of the year (seasonal)?

5. Do you treat for any diseases?

    5.1. With what?

    5.2. How often?

6. Do you associate any of the diseases described above with ticks?

10 Appendix 2

10.1 RLB-PCR

- Put frozen DNA samples at 4 °C the day before use (including positive PCR controls).
- Thaw the PCR reagents.
- Turn on the UV in the DNA workstation and flow cabinet for 30 minutes prior to working in them.
- Strictly follow the one way route: clean room ➔ dirty room ➔ PCR room.
- PCR reagents for 1 sample are as follows:

- 5.0 µl 5x Phire reaction buffer
- 0.5 µl 10mM dNTPs
- 0.5 µl F primer (20 pmol/µl)
- 0.5 µl R primer (20 pmol/µl)
- 0.125 µl 2U/µl Phire Hot Start II DNA polymerase
- 15.875 µl PCR grade H_2O

y µl (usually 2,5 µl) cDNA or DNA

End volume of every PCR individual sample is: 25 µl

Leave the polymerase in the freezer until the very last moment.

1. Prepare the PCR mix in an eppendorf tube, multiply the individual reagents by the number of samples. To compensate for pipet accuracy, advisable is that you add 10% additional samples to the total number of PCR samples:
   40 DNA samples + 1 positive PCR control = 41
   41 + 10% = 45 samples.
2. Pipet 22,5 µl of the master mix in every PCR tube, add the leftover mix to an additional tube, which will become the negative PCR control.
3. Close the PCR tubes and clean up everything in the clean room, you are not allowed to come back to the clean room after setting up the PCR.
4. Take the closed PCR tubes to the dirty room and get the DNA samples from the fridge.
5. Pipette 2,5 µl of the DNA sample to the corresponding PCR tube.
6. Pipette 2,5 µl of the positive PCR control, corresponding to the PCR to be performed, to the positive control PCR tube.
7. Vortex and spin down the samples briefly before placing them into the PCR machine.

10.2 Agarose gel electrophoresis

Be careful! Ethidiumbromide is carcinogenic! Wear gloves during all procedures involving the electrophoresis

Preparation of the gel:

Prepare 1x TAE solution by diluting the 10x stock. (100ml stock solution 900ml demineralized water):

1. Add 2,25 grams of agarose to an erlenmeyer and add 150ml 1x TAE buffer for a large gel. Add 0.563 grams of agarose to an erlenmeyer and add 37.5ml 1x TAE buffer for a small gel.
2. Heat the solution in a microwave until the agarose is fully dissolved.
3. Let the agarose solution cool down until about 60 °C and add 2,5µl ethidiumbromide solution (10mg/ml).
4. Prepare the gel tray by putting the rubber sides on the edges of the tray and place the comb(s).
5. Pour the gel onto the tray (air bubbles can be removed using a pipet tip).
6. When the gel has solidified, the comb(s) can be gently removed and the gel can be placed in the electrophoresis unit.
7. If needed, fill up the 1x TAE level in the electrophoresis unit until it fully covers the gel.

Preparation of the PCR samples

1. Pipette 1µl of the 6x loading buffer a 0,2ml PCR tube or in one of the wells of a 96 well plate.
2. Add 5µl PCR sample to the loading buffer, mix by pipetting and load onto the gel.
3. Pipette 5µl of the DNA marker and load onto the gel.
4. Run the gel for 30-45 minutes and check the gel using the gel-dock system (LabWorks program)

10.3 Reverse Line Blot (RLB) hybridization

Wear gloves and use non-filter pipet tips.

1. Turn on the heating block at 100 °C.
2. Turn on the hybridization oven at 42°C. Preheat 50ml 2x SSPE/0.5% SDS solution in the hybridization oven at the same time.
3. Turn on the water bath at 50°C. Preheat in the water bath the 2x SSPE/0.5% SDS solution at the same time.
4. Clean working space with 70% ethanol.
5. Combine and dilute the PCR products, per DNA sample, in a 1,5 ml eppendorf tube. Use of every PCR sample 10 µl and add 2x SSPE/0,1% SDS until 160 µl. (For example: 10 µl *Anaplasma/Ehrlichia* PCR + 10 µl *Babesia/Theileria* PCR + 140 µl 2x SSPE/0,1% SDS.)
6. Add 10 µl of the RLB positive controls to 150 µl 2x SSPE/0,1% SDS to give a final amount of 160 µl.
7. Denature the diluted PCR samples at 100 °C during 10 minutes using the heating block and cool down the samples rapidly on ice after. After the samples have cooled down, briefly spin down the samples before opening. Keep the samples on ice.
8. Wash the membrane, during the denaturation step, at room temperature with 2x SSPE/0,1% SDS for 5 minutes under gentle shaking.
9. Place membrane on a support cushion in miniblotter, with slots perpendicular to line pattern of applied probes.
10. Remove residual fluid by aspiration.
11. Fill the slots with the diluted and denaturized PCR samples (150 µl), avoid air bubbles. Fill empty slots with 2 x SSPE/0.1% SDS, to avoid cross flow.
12. Hybridize the blotter at 42°C for 60 minutes in the hybridization oven, without shaking.
13. Turn screws hand tight and remove the samples by aspiration.
14. Remove the membrane from the blotter.
15. Wash the membrane twice with preheated 2x SSPE/0,5% SDS during 10 minutes at 50°C under gentle shaking.

16. Clean the blotter and the support cushion during the washing step. Use the appropriate cleaning product.
17. Incubate the membrane with 50 ml preheated 2x SSPE/0,5% SDS + 5 µl streptavidin during 30 minutes at 42°C in the hybridization oven under gentle shaking. Discard the streptavidin solution in a tube and into the yellow bin. Do not pour it in the sink.
18. Turn down the water bath to 42 °C during the streptavidin hybridization with the 2x SSPE/0,5% SDS solution inside. Keep the lid of the water bath open.
19. Wash the membrane twice with preheated 2x SSPE/0,5% SDS during 10 minutes at 42 °C under gentle shaking.
20. Change the water bath temperature to 80 °C and place the 1% SDS solution inside the water bath.
21. Wash the membrane twice with 2x SSPE at room temperature for 5 minutes under gentle shaking.
22. Prepare the film cassette and check if the developing machine is on.
23. Dispose the 2x SSPE solution.
24. Add 10 ml ECL (5 ml ECL1 + 5 ml ECL2) to the membrane and gently shake the membrane by hand until the whole membrane is covered with ECL. Collect the ECL in a tube and dispose in the yellow bin, do not pour it in the sink.
25. Place the membrane in foil and place it in the film cassette.
26. Go to the dark room and expose the membrane to the film for 10 minutes.
27. Develop the film using the developing machine.
28. Strip membrane or store membrane in a seal bag wit 20 mM EDTA at 4 °C until stripping.
29. Turn off all equipment and clean working space.