The distribution of *Dermacentor reticulatus* in the Netherlands and Belgium and the prevalence of *Babesia canis* in *Dermacentor reticulatus*

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Abstract

Dermacentor reticulatus plays an important vector role for several pathogens, including for *Babesia canis*. Since 2007 the presence of populations of *D. reticulatus* ticks are confirmed in the Netherlands and since 2010 *D. reticulatus* populations are confirmed in Belgium. Eight locations were surveyed for the presence of *Dermacentor* ticks. In total 612 adult *Dermacentor* ticks were found. 160 adult ticks collected from the vegetation of four locations were selected at random and screened by polymerase chain reaction (PCR) and reverse line blot (RLB) for the presence of *Theileria* and *Babesia*. *Theileria equi-like* (27.5%) and *Babesia caballi* (0.63%) were detected in the *Dermacentor reticulatus* ticks, which appeared to be free from *B. canis* infection. We concluded that *Dermacentor reticulatus* slowly spread within in the Netherlands and Belgium and that a broader spectrum of tick-borne pathogens in *Dermacentor reticulatus* is present in the Netherlands and Belgium.

Introduction

Ticks are important vectors for a large number of pathogens and therefore of great medical and veterinary importance. *Dermacentor reticulatus* (Fabricius, 1794) is the most significant vector of *Babesia canis*, a sporozoite, the causal agent of canine babesiosis (Boozer, Macintire 2003). Besides canine babesiosis *Dermacentor reticulatus* can also be a vector of *Coxiella burnettii* (Q fever) (Estrada-Pena, Jongejan 1999), *Babesia caballi* (equine babesiosis), *Rickettsia sibirica* (Siberian tick typhus) (Jongejan, Uilenberg 2004), *Rickettsia slovaca* and *Rickettsia raoultii* (Tick-borne lymphadenopathy (TIBOLA)) (Parola et al. 2009), and *Theileria equi* (equine piroplasmosis) (Butler et al. 2012). Recently, a study in Poland showed that *Dermacentor reticulatus* also can be a vector of tick-borne encephalitis virus (TBEV) (Wojcik-Fatla et al. 2011). The range of the distribution of the *D. reticulatus* is very broad, from the British Isles in the west to Central Asia in the east. The ticks occur in cold and wet sites, where a high degree of humidity is achieved (Estrada-Pena et al. 2004).

Dermacentor ticks are white with variegated brown splashes. The adult female *Dermacentor* ticks are 3.8-4.2 mm when unfed and approximately 10 mm when engorged. The adult male is about 4.2-4.8 mm. In both sexes festoons are present and the basis capituli is right-angled and the palps short. Also in both sexes the coxa of the first pair of legs has an enlarged spur (Taylor, Coop & Wall 2007). This species is a three-host tick and only the adult ticks feed on dogs, but can also be found on cattle, horses and sheep (Estrada-Pena et al. 2004). Larvae and nymphs feed on smaller mammals and sometimes bird. The life cycle can be completed in 1-2 years, depending on ecological circumstances (Taylor, Coop & Wall 2007).

Dogs can become infected with *B. canis* after a bite of an infective *Dermacentor* tick, which releases sporozoites into the circulation. The tick must feed for 1-3 days to complete the transmission of *B. canis*. The total incubation period for canine babesiosis is 10-21 days (Boozer, Macintire 2003). Canine babesiosis can show a wide range of clinical signs such as lethargy, fever, anorexia, renal failure, vomiting, anaemia, jaundice, and even fatal shock (Boozer, Macintire 2003, Bourdoiseau 2006, Matjila et al. 2005).

In 1985 the first five autochthonous cases of canine babesiosis were reported (Uilenberg et al. 1985). In 2004, 23 cases of autochthonous canine babesiosis were reported. *D. reticulatus* ticks were collected from three different dogs, however at that time no *D. reticulatus* ticks were found in the vegetation in the walking area of these dogs (Matjila et al. 2005). A recent study has confirmed the presence of populations of *D. reticulatus* ticks in the Netherlands (Nijhof et al. 2007). In 1999 three autochthonous cases of canine babesiosis in Belgium were reported (Losson et al. 1999). Recently the presence of *D. reticulatus* tick populations was demonstrated in 4 distinct locations in Belgium (Cochez et al. 2011). The distribution of *D. reticulatus* and thereby the increase in incidence of canine tick-borne diseases has been caused due to the changes in ecosystem management with consequent increased wildlife host abundance. In addition, climatic-related changes may have played a role (Nijhof et al. 2007, Gray et al. 2009). Also it is probable that *Dermacentor* ticks are spread in Europe on dogs travelling to and from endemic areas and on southern European cattle breeds imported directly into the newly formed reserves (Matjila et al. 2005, Nijhof et al. 2007). Besides in Belgium and the Netherlands autochthonous cases of canine babesiosis have been reported in other North-Western European countries; Germany (Beelitz et al. 2012, Dautel et al. 2006), Poland (Welc-Faleciak et al. 2009, Zygner, Gorski & Wedrychowicz 2009) and also in Norway (Oines, Storli & Brun-Hansen 2010).

In this study *D. reticulatus* ticks collected from cattle and from the vegetation in the Netherlands and Belgium have been analyzed. A representative number of ticks are subjected to PCR and RLB for the presence of *Babesia* and *Theileria* species. The purpose of this study is to determine the distribution of *Dermacentor* ticks in the Netherlands and Belgium and to define the prevalence of *Babesia canis* in *Dermacentor reticulatus*.

Materials and methods

Tick collection

Ticks were collected from eight different locations, Egmond aan Zee (A), Kwintelooijen (B), De Maashorst (C), Dintelse Gorzen (D), Slikken van de Heen (E), and St. Philipsland (F) in the Netherlands, and De Panne (G) and Moen (H) in Belgium where *D. reticulatus* were suspected to be present (Figure 1 and Table 2). The ticks were trapped by dragging the vegetation with a flannel cloth.

Also ticks were collected from cattle at a farm, Helenahoeve Charolais, nearby St. Philipsland.

Figure 1. Map of Belgium and the Netherlands showing the locations where *D. reticulatus* was found in the vegetation in 2012. The letters correspond to the locations presented in Table 2.

DNA extraction

DNA was extracted from randomly selected *D. reticulatus* ticks using the NucleoSpin® Tissue Kit (Art. No. 740952.10/.50/.250, Macherey-Nagel, Düren, Germany) with a couple of adjustments. The used ticks were surface sterilised with 70% alcohol and cut into four pieces. After that the material was lysed at 56 °C and further treated following the manufacturer's protocols. The resulting DNA samples were stored at -20 °C.

Polymerase Chain Reaction

On the obtained DNA samples a Polymerase Chain Reaction (PCR) was performed. With PCR pieces of DNA are being amplified using a specific primer set (Table 1). In this study was looked at two types of pathogens, *Theileria* and *Babesia*.

To perform a PCR a master mix was made. A master mix contains buffer, 10 mM dNTPS, forward primer, reverse primer, water, and DNA polymerase. Then the master mix was pipetted in 200 µl Eppendorf tubes. Each tube contained 22.5 µl master mix and 2.5 µl DNA product. Also a positive and negative control was made. A specific PCR program was executed in the thermal cycler.

Pathogen	Primer	Sequence	Orientation	T*m* (°C)
Theileria/ Babesia	RLB-F2	5'-GAC ACA GGG AGG TAG TGA CAA G	+	57.9
	RLB-R2	5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT	-	53.7

Table 1. Sequence primers.

Agarose gel electrophoresis

To validate that the PCR was conducted correctly, an agarose gel electrophoresis with the positive and negative control samples of the PCR was carried out. A 1.125% agarose gel was made following the UCTD protocol. The positive and negative control samples were mixed with a 6x DNA loading dye and loaded into the sample wells. Also as a reference a 100 bp DNA ladder was loaded into the sample wells. The gel was run for 45 minutes. After sufficient migration, the gel was observed by illumination with UV-light. The PCR was performed successfully if the positive sample was visible with a fragment and the negative sample showed no fragment on the gel (Figure 2).

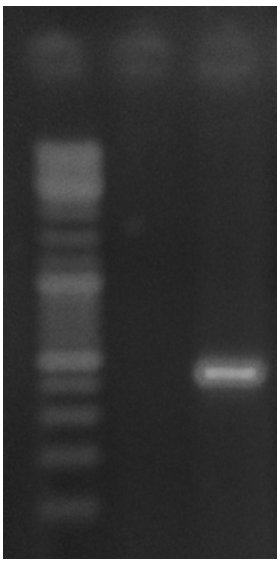

Figure 2. Agarose gel electrophoresis.

Reverse line blot hybridisation

With reverse line blot (RLB) hybridisation multiple samples can be analysed against multiple probes simultaneously. The sensitivity of RLB is up to 1000 fold or higher than PCR only. The membrane used for RLB contains species-specific oligonucleotides, which are applied in lines and covalently linked to the membrane by a 5' terminal aminolinker.

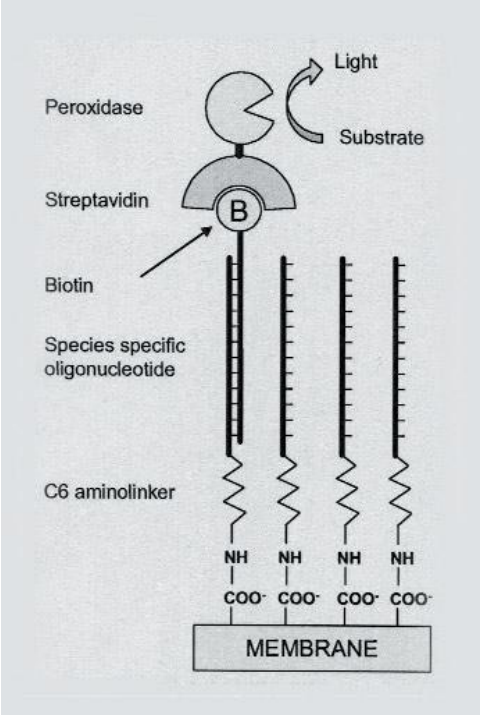

Figure 3. Schematic representation of the hybridisation principle (RLB Hybridisation, 2004).

The PCR products were applied to the membrane, using a miniblotter, so that the direction of the PCR products was perpendicular to the direction of the species-specific oligonucleotides. Also two control oligonucleotides, AE30 and B100, were applied to the membrane. To remove unbound PCR products the membrane was thoroughly washed. The hybridised PCR products were visualized using chemiluminescence. Visualisation makes use of a biotin label attached to the PCR primer. The biotin label was incubated with a streptavidin ligand conjugated to an enzymatic label, HRP. Subsequently the membrane was incubated with the peroxidase substrate, ECL. This results in a reaction producing light which can be detected on a suitable film (Figure 3). After the film had been developed, spots occurred at the sites where species-specific oligonucleotide and PCR-product had hybridised and the identity of the microorganism(s) in the sample was identified (Figure 4) (RLB Hybridisation, 2004).

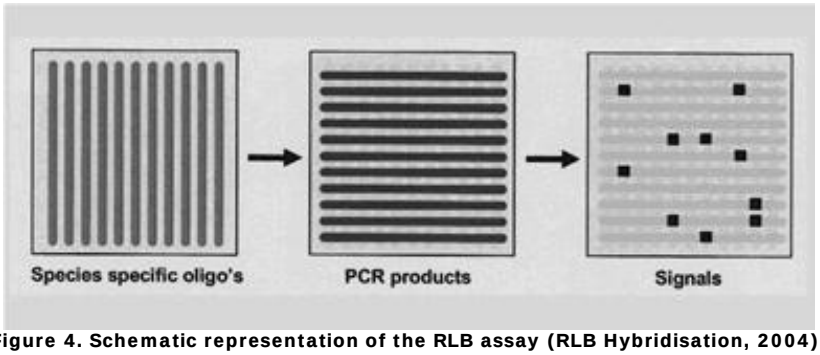

Figure 4. Schematic representation of the RLB assay (RLB Hybridisation, 2004).

Results

Collected ticks

A total of 612 adult *Dermacentor* ticks, 249 males and 363 females, were collected from the vegetation of eight different locations in the Netherlands and Belgium in March and April 2012 (Figure 1 and Table 2). At Egmond aan Zee one female *Dermacentor reticulatus* was found in dry grass in a deciduous forest. In 2007, a small number of *D. reticulatus* ticks were found at exactly the same location. At Kwintelooijen 204 ticks, 70 males and 134 females, were collected. Kwintelooijen is a recreational area consists of deciduous forest, a small lake, and heathland. Ticks were found in the dry heath land. Sheep were pastured here and this nature reserve is a popular dog walking area. At De Maashorst two female *D. reticulatus* tick were collected. De Maashorst is a typical wetland/woodland with moist deciduous forest where large ruminants are grazing freely. The Maashorst is open to the public. At Dintelse Gorzen 122 ticks, 56 males and 66 females, were collected. The Dintelse Gorzen consists of heathland and is part of a nature reserve. *D. reticulatus* was found to be most abundant in the fresh water tidal marshes and in the field surrounding the water, which consists many of many dry patches. This side is open to the public. At Slikken van de Heen 144 ticks, 65 males and 79 females, were collected. Most ticks were found in the high, dry patches alongside fences separating a walking path from sheep pastures. In St. Philipsland, a suburban area in Zeeland, 55 ticks, 23 males and 32 females, were collected. They were found in high grass alongside a fence separating the provincial road from fields where cattle graze. At De Panne 61 ticks, 25 males and 36 females, were collected. De Panne is a nature area in Belgium close to the sea and the French border. *D. reticulatus* ticks were most found in high dry grass alongside a fence separating the dunes from fields where cattle and horses graze. In Moen 23 ticks, 10 males and 12 females, were found. Moen is a natural reserve of about 26 hectares, along a canal that is accessible for recreational purposes.

Also 308 *Dermacentor* ticks, 297 males and 12 females, were collected from cattle at a farm, Helenahoeve Charolais, nearby St. Philipsland.

Location	Name	Country	Date of inspection	Number of collected ticks
A	Egmond aan Zee	Netherlands	March 2012	1 female
B	Kwintelooijen	Netherlands	March 2012	18 males, 35 females
			April 2012	52 males, 99 females
C	De Maashorst	Netherlands	April 2012	2 females
D	Dintelse Gorzen	Netherlands	March 2012	51 males, 59 females
			April 2012	5 males, 7 females
E	Slikken van de Heen	Netherlands	March 2012	40 males, 49 females
			April 2012	25 males, 30 females
F	St. Philipsland	Netherlands	March 2012	23 males, 32 females
G	De Panne	Belgium	March 2012	16 males, 23 females
			April 2012	9 males, 13 females
H	Moen	Belgium	April 2012	10 males, 13 females

Table 2. Field sites in the Netherlands and Belgium where *D. reticulatus* have been found in 2012.

Pathogen detection

Ticks from the vegetation

A total of 160, 80 (50%) males and 80 (50%) females, adult ticks collected from the vegetation of four locations were screened by RLB for the presence of *Theileria* and *Babesia* (Figure 5 and Table 3).

An amount of 44 (27.5%) ticks were positive for *Theileria equi-like*. One (0.63%) tick, collected from De Panne, was positive for *Babesia caballi*. 115 (71.88%) ticks were negative for any *Theileria* or *Babesia* species.

	Amount	Proportion
Total number of *D. reticulatus* ticks	160	100%
Males	80	50%
Females	80	50%
Theileria equi-like positive	44	27.5%
Babesia caballi positive	1	0.63%
Negative	115	71.88%

Table 3. Pathogens detected in *D. reticulatus* collected from the vegetation of the Netherlands and Belgium.

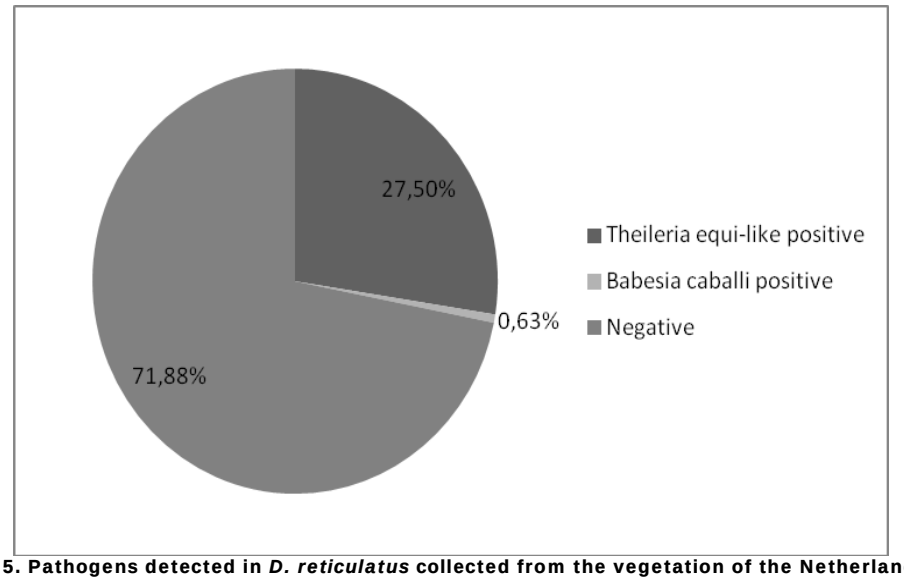

Figure 5. Pathogens detected in *D. reticulatus* collected from the vegetation of the Netherlands and Belgium.

Ticks from Slikken van de Heen

From Slikken van de Heen a total of 40 randomly selected adult ticks were screened by RLB for the presence of *Theileria* and *Babesia* (Figure 6, Table 4 and Appendix A). 20 (50%) ticks were male and 20 (50%) ticks were female. Ten (25%) ticks were positive for *Theileria equi-like*. 30 (75%) ticks were negative for any *Theileria* or *Babesia* species.

	Amount	Proportion
Total number of *D. reticulatus* ticks	40	100%
Males	20	50%
Females	20	50%
Theileria equi-like positive	10	25%
Negative	30	75%

Table 4. Pathogens detected in *D. reticulatus* collected from the vegetation of Slikken van de Heen.

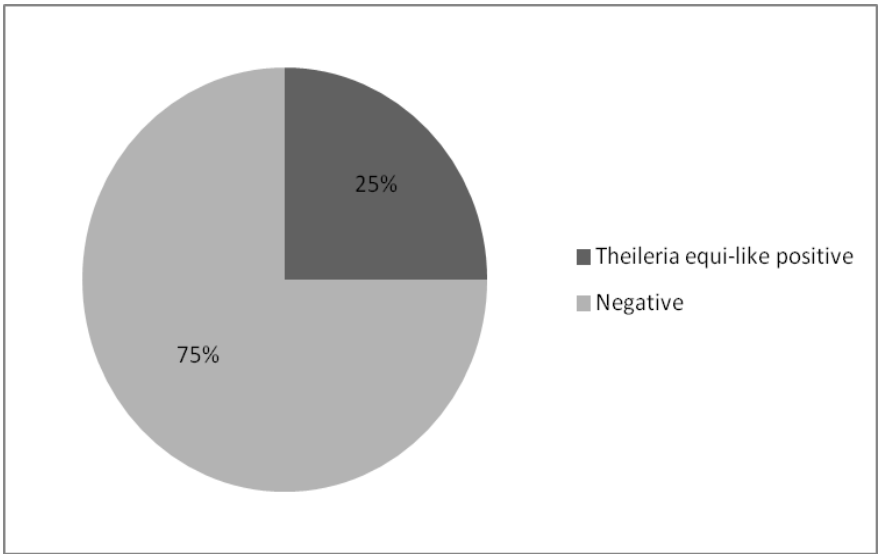

Figure 6. Pathogens detected in *D. reticulatus* collected from the vegetation of Slikken van de Heen.

Ticks from St. Philipsland

From St. Philipsland a total of 40 randomly selected adult ticks were screened by RLB for the presence of *Theileria* and *Babesia* (Figure 7, Table 5 and Appendix B). Half of the ticks were male and half of the ticks were female. Three (7.5%) ticks were positive for *Theileria equi-like*. 37 (92.5%) ticks were negative for any *Theileria* or *Babesia* species.

	Amount	Proportion
Total number of *D. reticulatus* ticks	40	100%
Males	20	50%
Females	20	50%
Theileria equi-like positive	3	7.5%
Negative	37	92.5%

Table 5. Pathogens detected in *D. reticulatus* collected from the vegetation of St. Philipsland.

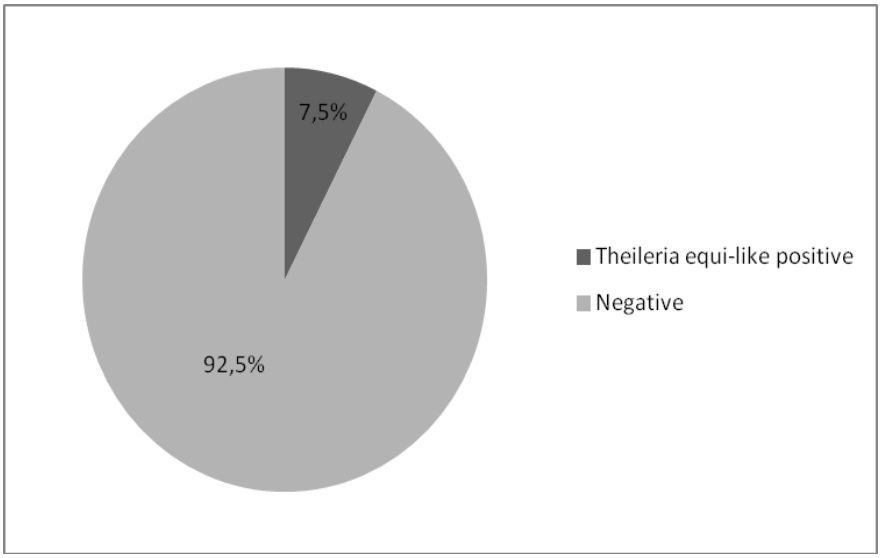

Figure 7. Pathogens detected in *D. reticulatus* collected from the vegetation of St. Philipsland.

Ticks from Kwintelooijen

From Kwintelooijen a total of 40 randomly selected adult ticks were screened by RLB for the presence of *Theileria* and *Babesia* (Figure 8, Table 6 and Appendix C). 20 (50%) ticks were male and 20 (50%) ticks were female. 25 (62.5%) ticks were positive for *Theileria equi-like*. 15 (37.5%) ticks were negative for any *Theileria* or *Babesia* species.

	Amount	Proportion
Total number of *D. reticulatus* ticks	40	100%
Males	20	50%
Females	20	50%
Theileria equi-like positive	25	62.5%
Negative	15	37.5%

Table 6. Pathogens detected in *D. reticulatus* collected from the vegetation of Kwintelooijen.

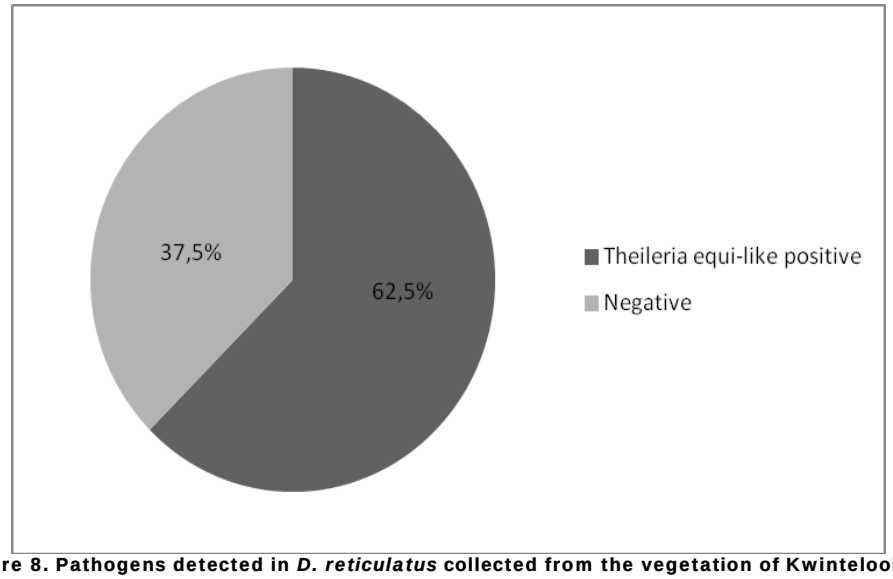

Figure 8. Pathogens detected in *D. reticulatus* collected from the vegetation of Kwintelooijen.

Ticks from De Panne

From De Panne a total of 40 randomly selected adult ticks were screened by RLB for the presence of *Theileria* and *Babesia* (Figure 9, Table 7 and Appendix D). Half of the ticks were male and half of the ticks were female. Six (15%) ticks were positive for *Theileria equi-like*. One (2.5%) tick was positive for *Babesia caballi*. 33 (82.5%) ticks were negative for any *Theileria* or *Babesia* species.

	Amount	Proportion
Total number of *D. reticulatus* ticks	40	100%
Males	20	50%
Females	20	50%
Theileria equi-like positive	6	15%
Babesia caballi positive	1	2.5%
Negative	33	82.5%

Table 7. Pathogens detected in *D. reticulatus* collected from the vegetation of De Panne.

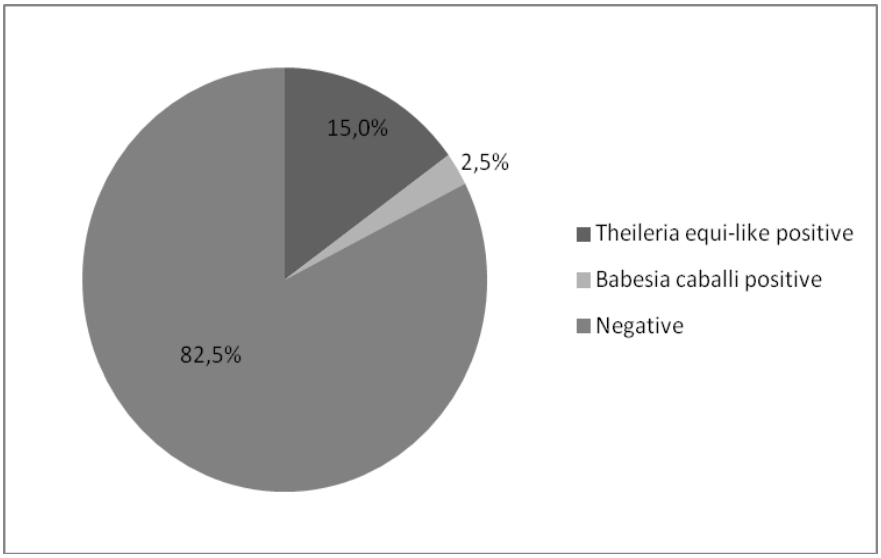

Figure 9. Pathogens detected in *D. reticulatus* collected from the vegetation of De Panne.

Ticks from cattle at Helenahoeve Charolais

From cattle at Helenahoeve Charolais a total of 40 randomly selected adult ticks were screened by RLB for the presence of *Theileria* and *Babesia* (Figure 10, Table 8 and Appendix E). Six (15%) ticks were female and 34 (85%) ticks were male. 23 (57.5%) ticks were positive for *Theileria equi-like*. 17 (42.5%) ticks were negative for any *Theileria* or *Babesia* species.

	Amount	Proportion
Total number of *D. reticulatus* ticks	40	100%
Males	34	85%
Females	6	15%
Theileria equi-like positive	23	57.5%
Negative	17	42.5%

Table 8. Pathogens detected in *D. reticulatus* collected from cattle at Helenahoeve Charolais.

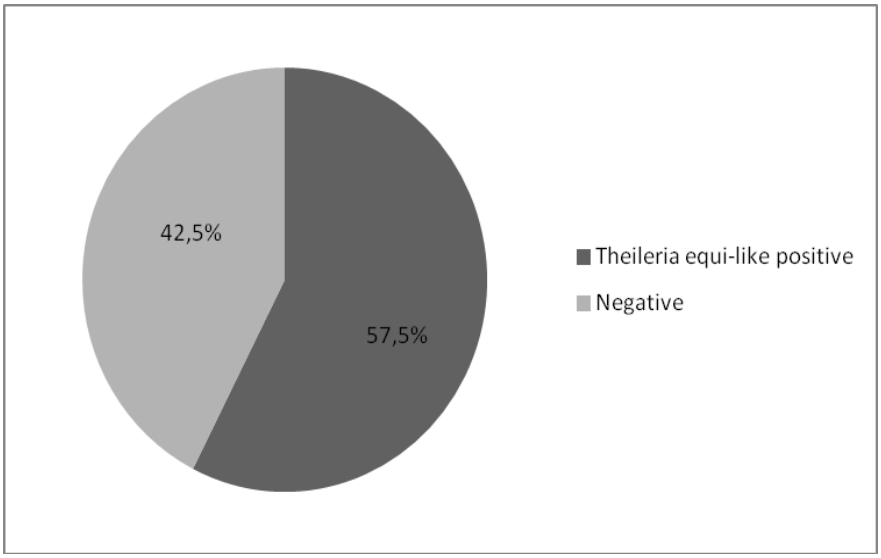

Figure 10. Pathogens detected in *D. reticulatus* collected from cattle at Helenahoeve Charolais.

Discussion

When the first five autochthonous cases of canine babesiosis were reported in 1985 no *Dermacentor reticulatus* was found in the Netherlands (Uilenberg et al. 1985). In 2004, when 23 cases of autochthonous canine babesiosis were reported *Dermacentor reticulatus* was only found on three different dogs (Matjila et al. 2005). In 2007 *D. reticulatus* was collected from the vegetation in six locations in the Netherlands (Nijhof et al. 2007). In 2010 *D. reticulatus* was collected from the vegetation in four locations in Belgium (Cochez et al. 2011). In 2012 *D. reticulatus* was found in eight locations. All of the localities where *Dermacentor* ticks were found from November 2005 to October 2006 were visited again in 2012. In four of the six locations *Dermacentor* ticks were found again. Also two localities in Belgium where *Dermacentor* ticks were found in 2010 were visited again. Only in Moen *D. reticulatus* was found. The area in Beveren where *Dermacentor* ticks were found in 2010 is rather small, therefore it is probable that all the *D. reticulatus* were scavenged in 2010. Another explanation could be that the area is too small to maintain a population of *Dermacentor* ticks. De Panne, Kwintelooijen and Egmond aan Zee were selected on basis of posts on an internet site that collects flora and fauna observations (www.waarneming.nl and www.waarnemingen.be). In particular Kwintelooijen is very interesting, because at this location the most ticks were found in 2012. Furthermore it is interesting that this location is much farther north than other locations where such quantities of *Dermacentor* ticks were found.

Babesia canis, the causal agent of canine babesiosis, was not found in any of the 160 analyzed *Dermacentor* ticks in this study. This is not surprising considering the low number of ticks that were examined. Also the natural infection rates of *B. canis* in *D. reticulatus* ticks range between 1% to 3.6% (Duh et al. 2006, Rar et al. 2005). Ergo the possibility that *D. reticulatus* acts as a vector for *B. canis* cannot be ruled out. Further survey is necessary to determine the vector role of *Dermacentor reticulatus*.

In this study *T. equi-like* was detected in 27.5% of *D. reticulatus* ticks. Also 23 ticks collected from cattle were positive for *T. equi-like*. At this moment little is known about what *T. equi-like* is. Maybe it can cause equine piroplasmosis just as *T. equi*. Further research to what *T. equi-like* is should be done to increase our knowledge about *T. equi-like* and the possible risks of *T. equi-like* for dogs, cattle, and other animals.

Furthermore *B. caballi* was detected in one tick collected from the vegetation of De Panne. Wild horses graze next to the collection site, so those horses and the positive result for *B. caballi* might be connected. *B. caballi* can cause equine babesiosis.

With RLB spots occur at the sites where species-specific oligonucleotide and PCR-product have hybridised. Because you have to interpret the spots, the results are a little bit subjective. In the results of this study both weak and strong signals are interpreted positive. Therefore in reality the positive percentage could be lower.

Conclusion

This study showed that *Dermacentor reticulatus* slowly spread within in the Netherlands and Belgium. New locations where *D. reticulatus* ticks live have been found and at most known locations the population of *Dermacentor* ticks is maintained.

This research did not confirm that *Dermacentor reticulatus* is the vector of *Babesia canis* in the Netherlands and Belgium. However this study did confirm the vector role of *D. reticulatus* for *Theileria equi-like* and *B. caballi*. The prevalence of *Theileria equi-like* (27.5%) and *B. caballi* (0.63%) has been determined.

In conclusion, further survey is necessary to monitor the distribution of *Dermacentor reticulatus* in the Netherlands and Belgium and to determine the vector role of *Dermacentor reticulatus* in the Netherlands and Belgium.

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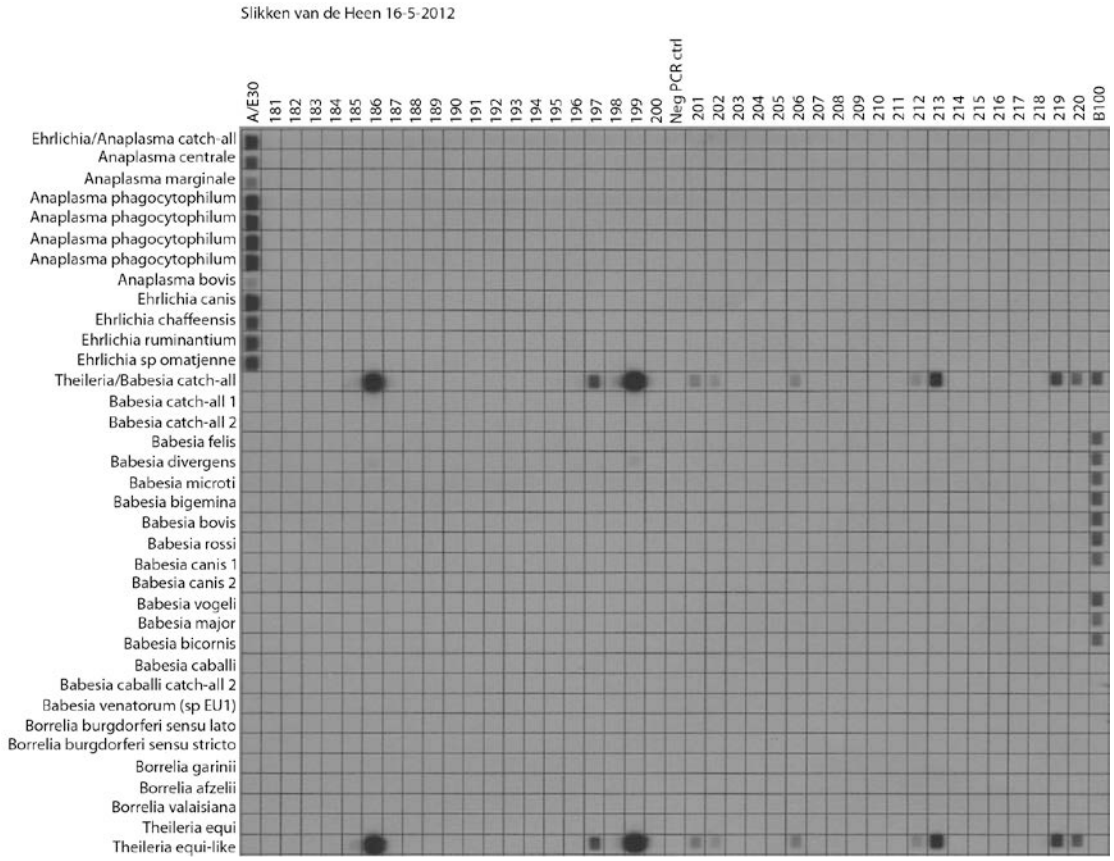
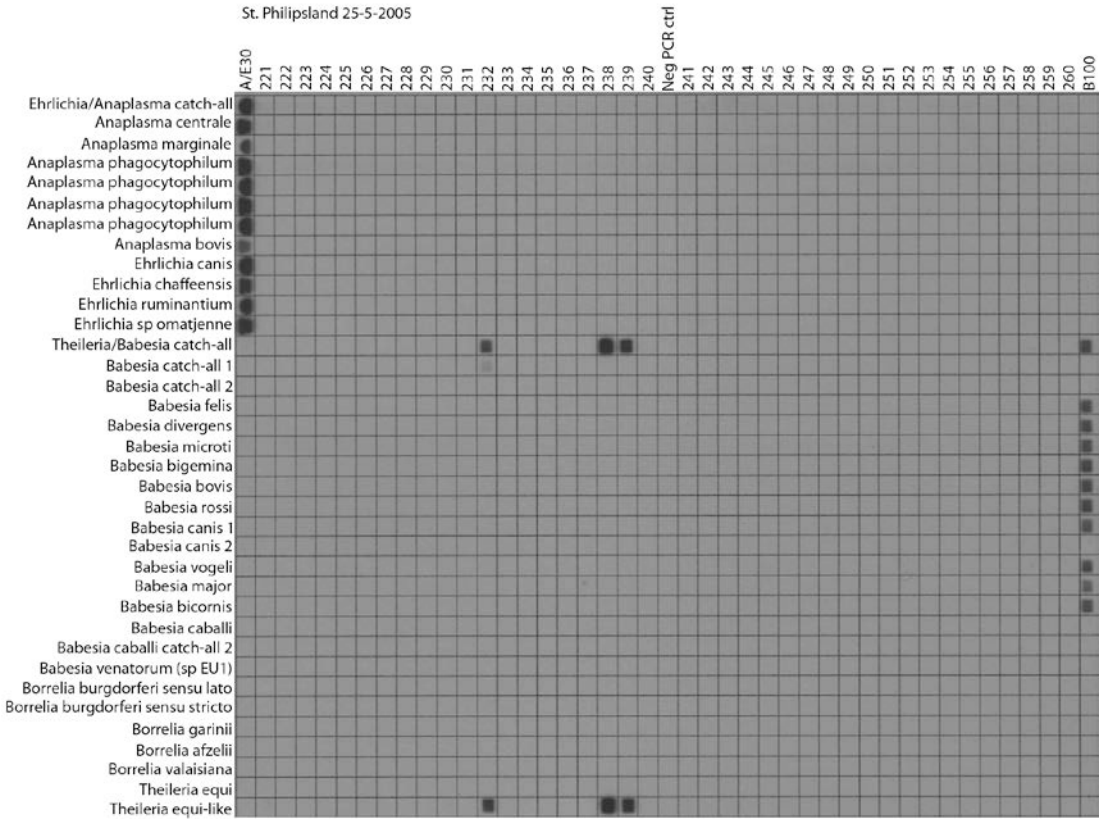
Appendix A



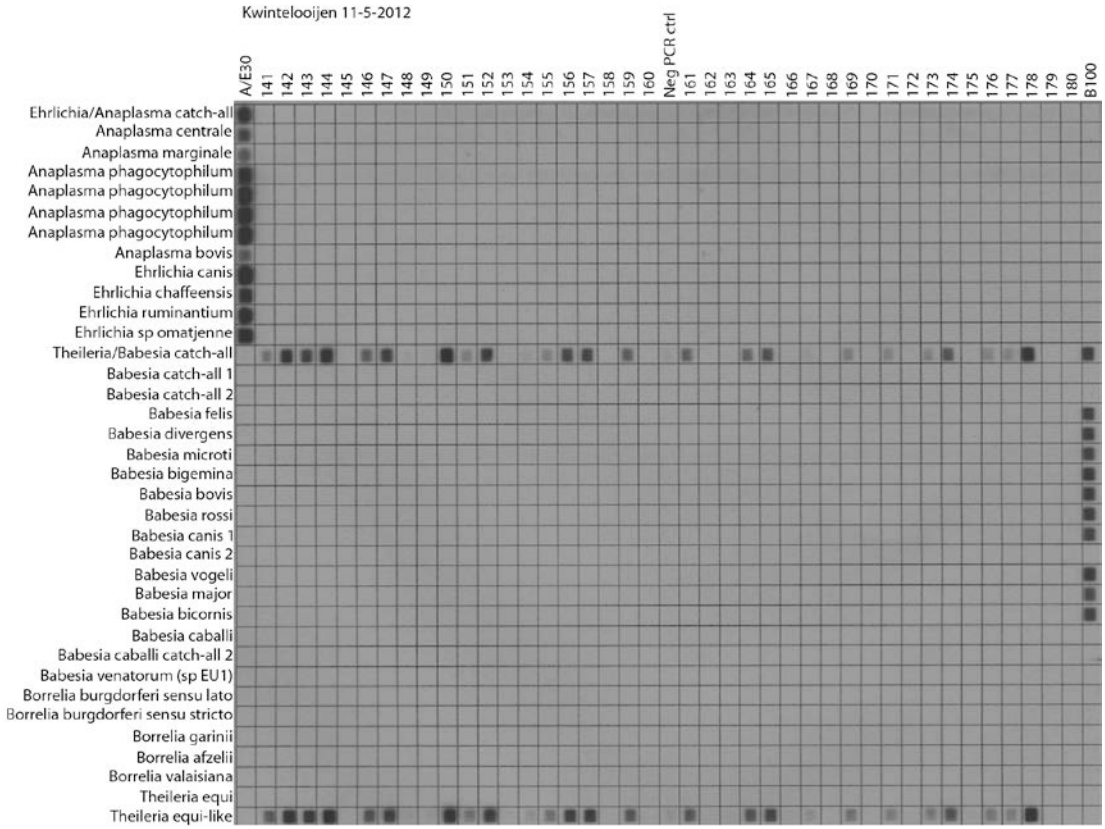
RLB results of ticks collected from Slikken van de Heen. Numbers 181 to 200 are female ticks and numbers 201 to 220 are male ticks.

Appendix B



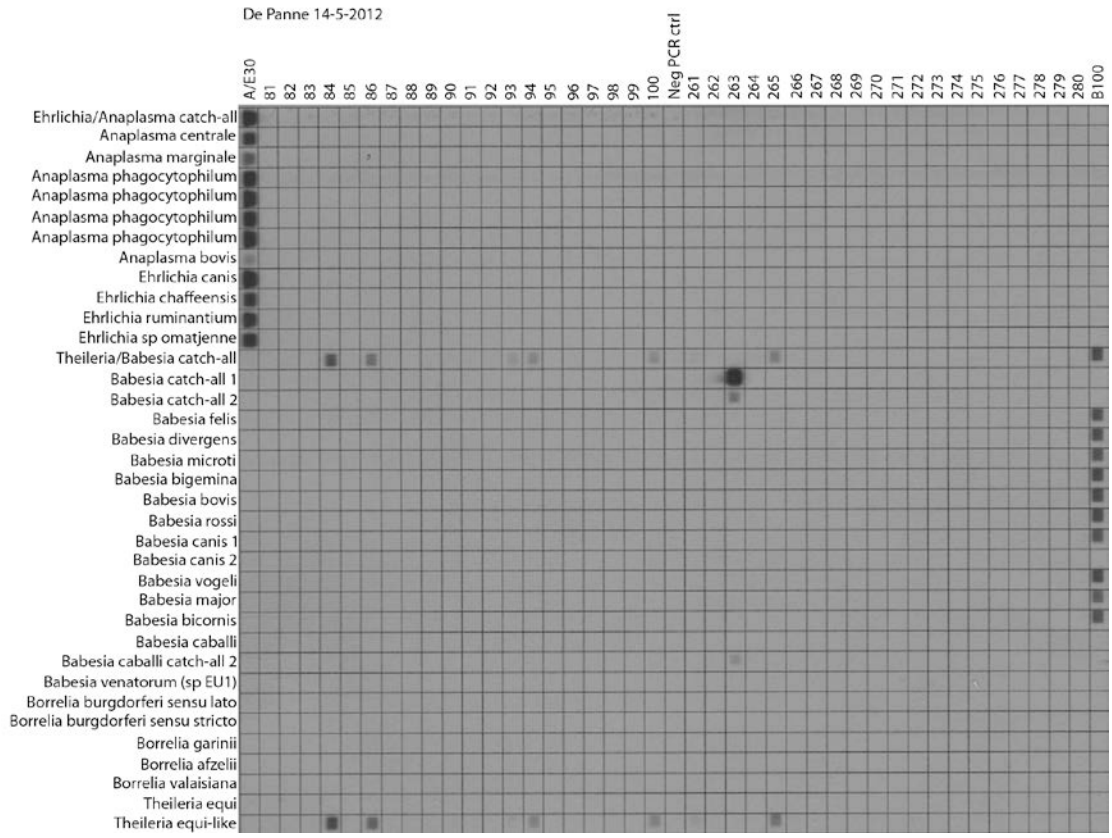
RLB results of ticks collected from St. Philipsland. Numbers 221 to 240 are female ticks and numbers 241 to 260 are male ticks.

Appendix C



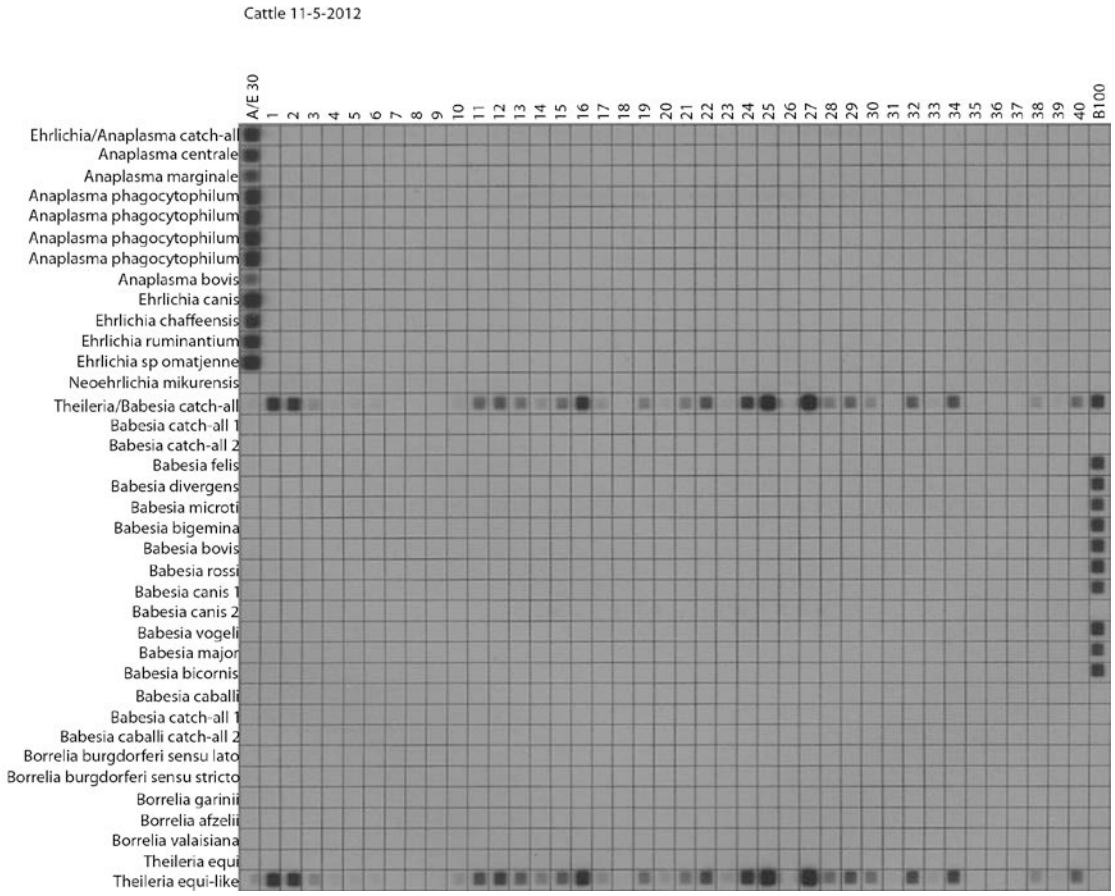
RLB results of ticks collected from Kwintelooijen. Numbers 141 to 160 are female ticks and numbers 161 to 180 are male ticks.

Appendix D



RLB results of ticks collected from De Panne. Numbers 81 to 100 are female ticks and numbers 261 to 280 are male ticks.

Appendix E



RLB results of ticks collected from cattle at Helenahoeve Charolais. Numbers 1 to 6 are female ticks and numbers 7 to 40 are male ticks.