

Foci report on indigenous *Dermacentor reticulatus* populations in Belgium and a preliminary study of associated babesiosis pathogens

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Abstract

The occurrence of indigenous clinical cases of canine (Losson *et al.*, 1999) and equine (Mantran *et al.*, 2004) babesiosis in Belgium during the last two decades suggests that the vector of the pathogens responsible for these diseases, *Dermacentor reticulatus*, could be present in Belgium. Consequently evidence for the presence of this tick species in different locations in Belgium was investigated. Four different locations throughout Belgium were monitored by flagging in 2010 and included the locations where *D. reticulatus* was found on a dog in 2009 and on two red deer in 2007. Two different tick species were identified, *i.e.* *Ixodes ricinus* and *D. reticulatus*. A total of 282 *D. reticulatus* adult ticks (98 males, 184 females) were collected from the 4 sites. Ticks were found mainly from early March until the end of May with a peak of activity in April. A *Babesia* spp. genus-specific PCR test based on the amplification of a 411-452 bp fragment of the 18S rRNA gene was used to investigate the potential presence of *Babesia* spp. All DNA extracts isolated from the total tick samples yielded negative results. Additional studies to accurately determine the distribution and vectorial capacity of this important tick species in Belgium are warranted.

Keywords: Tick-*Dermacentor reticulatus*-*Babesia* spp.-Belgium

Introduction

At least 16 tick species, classified to six genera (*Argas*, *Ixodes*, *Dermacentor*, *Hyalomma*, *Amblyomma*, *Rhipicephalus*) and two families (*Argasidae* and *Ixodidae*) have been recorded in Belgium (Fain, 1990). However, many of these species were recorded on imported animals or on animals with a history of travel and, consequently, cannot be considered to represent permanent indigenous populations. *Ixodes ricinus* and *Ixodes hexagonus* are the two most abundant species in North-Western Europe and of these two species *I. ricinus* is the most important vector of human disease. The distribution of *I. ricinus* in Northern Europe is widespread and determined mainly by the presence of habitats with a high level of humidity.

The distribution of *Dermacentor reticulatus* (marsh tick or ornate cow tick) in Europe is more limited and localised. The geographic range of this species extends –West-East wards- from France and the

southwest of the United Kingdom to central Asia. Its southern limit is the Mediterranean Sea but here it is restricted to humid, medium altitude areas. Until recently the French-Belgian border was considered to be the northern boundary of the distribution of *D. reticulatus* in Western Europe (Heile *et al.*, 2006) and within its entire distribution area, the distribution of this tick species is highly focal (Gray *et al.*, 2009). Recently, several reports indicated an expanding geographical distribution of *D. reticulatus*. Indeed, *D. reticulatus* ticks were collected from the environment during the last decade in The Netherlands (Nijhof *et al.*, 2007) and in different German Länder (Dautel *et al.*, 2006). Evidence for the changing distribution of *D. reticulatus* was also provided by the occurrence of canine babesiosis in new regions in Germany (Heile *et al.*, 2006; Barutzki *et al.*, 2007), Hungary (Sréter *et al.*, 2005), Switzerland (Porchet *et al.*, 2007) and the Netherlands (Nijhof *et al.*, 2007). Several factors could be responsible for the apparent expansion of the distribution area of *D. reticulatus*: increased deer abundance and the availability of more fallow land as a result of EU agricultural policies and a warming climate could, at least partly, be involved (Lindgren & Gustafson, 2001).

In Belgium, adults of *D. reticulatus* were found on a dog in Torgny and from an unspecified animal species in Gérouville in 1945 and 1950 respectively (both localities are located in the south of Luxembourg province very close to the French border) (Fain, 1989). However, these historical observations did not provide any information on the origin of the tick or the travel history of the dog. More recently, low numbers of *D. reticulatus* were reported on dogs in Belgium (Losson *et al.*, 2003). In France *D. reticulatus* is the most important vector of canine babesiosis (*Babesia canis canis*) (Martinod & Gilot, 1991). *D. reticulatus* can also transmit the protozoans *Theileria equi* and *Babesia caballi* of horses (Perez-Eid, 2007) and is also a vector of tick-borne encephalitis virus (TBE), *Rickettsia conori* (boutonneuse fever) (Hillyard, 1996), *Rickettsia slovaca* (Raoult *et al.*, 2002), *Francisella tularensis* (tularemia) and *Coxiella burnetii* (Q-fever) (Estrada-Pena & Jongejan, 1999). *Borrelia* sp. (probably *B. burgdorferi*) was identified in this tick in Saxony in Eastern Germany (Kahl *et al.*, 1992). Presence of *Anaplasma phagocytophilum* DNA was recently detected in *D. reticulatus* ticks isolated from a red deer in Southern Belgium (Wirtgen *et al.*, 2011).

The occurrence of indigenous cases of canine (Losson *et al.*, 1999) and equine (Mantran *et al.*, 2004) babesiosis in Belgium during the last two decades suggests that this tick species could be indigenous

in this country. The present work was carried out to investigate its presence in different locations in Belgium. In addition, to determine potential vectorial capacity, a specific PCR test was applied to check for the presence of *Babesia* spp. in the collected material.

Material and methods

Tick collection sites

Four locations were identified in Belgium where *D. reticulatus* ticks were suspected to be present, namely Beveren (further referred to as location 1) and Moen (location 2) in the northern part of Belgium, Mons (location 3) and Martilly (location 4) in the southern part of Belgium (Figure 1). The Beveren site (location 1, province of East-Flanders) was identified in 2009 when *D. reticulatus* ticks were found repeatedly on a domestic dog that had no history of traveling abroad. The site is suburban, and was established about a decade ago. It is a marshy area with a shallow artificial basin in the middle of the site. It is currently in use as a natural water purification area with an additional function as flood basin in times of heavy rain. It is situated between a railroad track and a provincial road and is often used as recreational area for dogs. The marshy area consists of a mixture of grasses. The dryer patches are occupied mainly by hornbeam and blackberry.

The site in Moen (location 2, province of West Flanders) was selected on the basis of the posting of photographs on an online observations site (www.waarnemingen.be) showing what appeared to be questing *D. reticulatus* ticks that had been misidentified as *Ixodes ricinus* ticks. The location is a natural reserve of about 26 hectares, along a canal that is accessible for recreational purposes.

Flagging on this site was not performed on a regular a basis.

Location 3 is situated in Mons (province of Hainaut) and consists of fallow land flanked by a road. This is close to a leisure area consisting of an artificial lake surrounded by a walking path extensively used for walking and exercising dogs. The vegetation consists of grass, hawthorns, blackthorns, brambles and birch trees. According to local vets this area is a focus of canine babesiosis (Losson *et al.*, 1999).

Location 4 is situated in Martilly (province of Luxembourg) where two areas (referred to as “zone 1” and “zone 2”) at about 600 meters apart from each other were monitored. Both zones are located in a

rural environment consisting of woodland (mainly pine trees) and pastures used for cattle grazing. In zone 1 the trees were cut down a few years ago and the vegetation is now comprised of brambles, ferns, blackthorns and jennets close to a small stream. Zone 2 is a wood comprising mainly birch trees and oak trees, brambles and ferns were also present. In both zones roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*) are present, evident by the presence of numerous faecal deposits. Both zones were selected because *D. reticulatus* were found attached to two red deer killed in these sites by poachers in February 2007.

Tick collection

Ticks were trapped by flagging with a 100 by 100 cm flannel cloth. Flagging was performed in dry or sufficiently dry conditions, wind speed less than 3 Bft and between 10.00 a.m. and 3.00 p.m.

Temperature on the ground (2 cm above surface) and air temperature (1.5 m above surface) were recorded during collection.

Location 1 was monitored on a weekly basis, from the end of February 2010 up to the third week of November. Location 2 was visited only on a few occasions. Locations 3 and 4 were sampled on a weekly basis, from March to August. Ticks were stored in 100% ethanol immediately after trapping and morphologically identified using a standard key (Arthur, 1963). Climatic data were obtained from the Royal Meteorological Institute of Belgium (<http://www.meteo.be>).

DNA extraction and PCR

Tick DNA extraction was performed according to the proteinase K protocol (20mg/ml) of Boom *et al.*, (1990). To discount potential false-negative results due to polymerase chain reaction (PCR) inhibition and to validate the efficiency of the DNA extraction, an initial PCR test targeting a 325-bp DNA fragment corresponding to the tick 16S rRNA gene was performed. This PCR was set up using 16S+1 and 16S-2 primers (Baumgarten *et al.*, 1999). Only tick-DNA positive samples were further analyzed for the presence of *Babesia* spp.

The *Babesia* spp. genus-specific PCR was developed according to Casati *et al.* (2006) using BJ1 and BN2 primers and generated amplification of a 411–452 bp fragment of the 18S rRNA gene (Lempereur *et al.*, 2011).

Statistical analysis

Possible correlations between environmental factors (temperature and humidity) and numbers of collected ticks were investigated using the Pearson correlation test. $P < 0.05$ was considered to indicate a significant correlation.

Results

Two different tick species -*Ixodes ricinus* and *D. reticulatus*- were identified on all sites except the Beveren site where only *D. reticulatus* was trapped. A total of 282 *D. reticulatus* adult ticks were collected from the 4 sites (98 males, 184 females). The majority of the *D. reticulatus* ticks were found from early March until June with a peak in March. A few specimens were also found in July, August and October. Although *Ixodes ricinus* is the most prevalent tick species in Belgium, only *D. reticulatus* was obtained in location 1 (Beveren) with a total of 139 adult ticks collected (47 males and 92 females). The weekly number of trapped ticks indicated that questing activity was highest in March-April (Figure 2). A period of heavy rain with flooding in the last weeks of November and subsequently freezing temperatures and snow in December obliged us to halt our survey. The most relevant climatic parameters are summarized in Table 1.

No correlation was found between the air and ground temperature at the time of sampling for male, female or total number of ticks flagged, but the majority of the ticks were collected at an air temperature of approx 16°C and a ground temperature of 6°C, from the second week of March up to the third week of April.

Four out of 234 available tick DNA extracts remained negative for the tick 16S rRNA gene PCR test even after diluting the samples 10 and 100 X and were discarded. The remaining 230 DNA extracts were all scored negative for *Babesia* spp using the *Babesia* specific PCR test.

Discussion

D. reticulatus is known to occur in Europe but in the North of Western Europe, its distribution is considered as scarce and has not been accurately recorded. Recently, questing populations of this tick were discovered by flagging in the Netherlands (Nijhof *et al.*, 2007). Its presence has also been documented in Germany (Dautel *et al.*, 2006), in Poland (Zygner *et al.*, 2009) in Austria (Sixl *et al.*, 2003) and in the western part of Switzerland (Porchet *et al.*, 2007). In central France it is the commonest tick present on dogs but on this host is replaced in the south of France by *Rhipicephalus sanguineus* and in the north by *Ixodes canisuga* (Hillyard, 1996). In this survey indigenous questing populations of *D. reticulatus* were found in Belgium. These results confirm the results of a previous survey which indicated the presence of low numbers of feeding adults of *D. reticulatus* on dogs in the country (Losson *et al.*, 1999). As Belgium is situated between France and the Netherlands, it seems likely from this study and others that the species has enlarged its distribution area in recent years from Northern France to the Netherlands, invading Belgium in the process. Different factors such as landscape use and climate changes, human activities, increased host population densities (such as cervids) could explain this spreading as summarized by Dautel *et al.* (2006).

While the establishment of *I. ricinus* might take 2 or more years, due to a life cycle that can take 3-4 years to complete (Gray, 1991), the life cycle of *D. reticulatus* is probably shorter. The ovipositioning activity of the species in spring seems to be a determining factor for flagging success (Zahler & Gothe, 1995). Although the year 2010 was globally the warmest year ever (0.62 °C above the 1901-2000 mean) it was for Belgium a colder-than-normal year (yearly mean 9.5°C, compared to 11.0°C in 2009 and 12.3 °C in 2008). Climatic and geographical differences between N-Belgium (atlantic climate, altitude on average below 50 meters) and S-Belgium (a more continental climate, altitude on average above 200 meters) are most probably responsible for the earlier questing activity in Northern Belgium. Despite the evidence for possible expansion, the study results suggest that *D. reticulatus* has a rather focal occurrence in Belgium and is localized in different fairly specific environments. The best example is, in this study, the difference of the habitat between areas. Thus, *D. reticulatus* was collected from rural areas with presence of deer and cattle (location 4) or cattle and horses (location 2)

but was also located in sub-urban environments used for leisure activities and accessible to walkers and dogs (locations 1 and 3). It may be noteworthy that all the positive sites are fairly humid with the presence of water (marshes, river, canal, lake) nearby.

Given its vectorial capacity, spread of *D. reticulatus* can affect the occurrence of certain diseases.

Indeed, the prevalence of *B. canis canis* in *D. reticulatus* was estimated to be 1% and 4% in Slovenia and Russia respectively (Duh *et al.*, 2004) (Rar *et al.*, 2005). A similar observation was made in the Netherlands where autochthonous canine babesiosis foci are known to exist (Matjila *et al.*, 2005). No evidence for presence of *Babesia* spp. specific DNA was found in any of the tick samples analyzed.

However, the possibility that *D. reticulatus* can act as a vector for *B. canis canis* cannot be ruled out completely given the low prevalence of tick infection and the relatively low number of ticks analyzed.

In conclusion, the presence of indigenous populations of *D. reticulatus* was demonstrated for the first time in several distinct areas of Belgium. A more detailed monitoring of *D. reticulatus* populations and screening for *Babesia canis canis* and other viral, rickettsial and bacterial pathogens is warranted in future studies.

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Fig 1. Map showing the four investigated locations in Belgium. Beveren = location 1, Moen = location 2, Mons = location 3, Martilly = location 4.

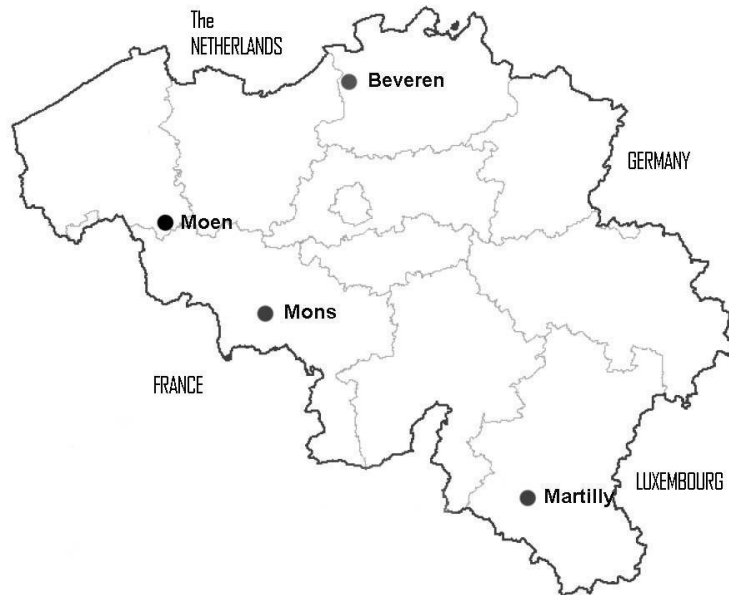


Fig 2. Number (N) of *D. reticulatus* (male and female) flagged at location 1 (N total = 139)

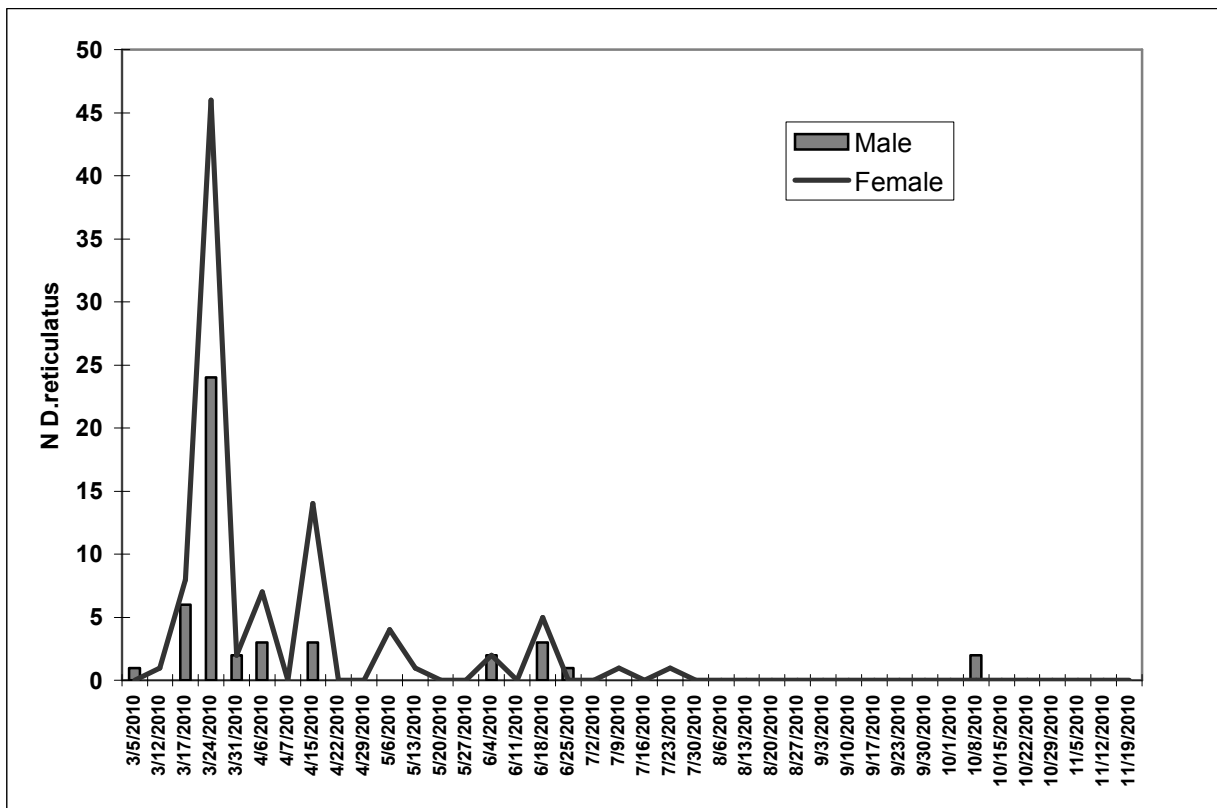


Table 1. Climatic conditions for 2010 as recorded by the Royal Meteorological Institute

(<http://www.meteo.be>) and number of *D. reticulatus* flagged at location 1 (Beveren, East-Flanders).

2010	hrs sunshine	Rain days	mm rain	mean t°	min t°	max t°	<i>D. reticulatus</i>
Jan	48,7	18,0	43,9	0,1	-2,0	2,2	-
Feb	28,9	24,0	76,1	2,5	0,0	5,0	-
Mar	118,0	13,0	50,2	6,7	3,1	10,4	90
Apr	223,1	8,0	15,7	10,3	4,7	15,7	27
May	171,8	14,0	66,6	11,2	7,0	15,4	5
Jun	258,6	9,0	30,0	17,4	12,3	22,4	13
Jul	252,0	12,0	62,8	20,5	15,6	25,8	2
Aug	136,5	23,0	187,4	17,0	13,1	21,5	0
Sep	142,6	18,0	109,8	14,2	10,2	18,6	0
Oct	118,6	16,0	70,8	10,6	6,5	14,7	2
Nov	23,9	21,0	124,7	6,1	4,0	8,2	0
Dec	33,1	26,0	76,2	-0,7	-3,0	1,2	-