

## Wild Cervids Are Host for Tick Vectors of *Babesia* Species with Zoonotic Capability in Belgium

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### Abstract

Babesiosis is a tick-borne disease caused by different species of intraerythrocytic protozoan parasites within the genus *Babesia*. Different species of *Babesia* are described as potentially zoonotic and cause a malaria-like disease mainly in immunocompromised humans. Interest in the zoonotic potential of *Babesia* is growing and babesiosis has been described by some authors as an emergent zoonotic disease. The role of cervids to maintain tick populations and act as a reservoir host for some *Babesia* spp. with zoonotic capability is suspected. To investigate the range and infection rate of *Babesia* species, ticks were collected from wild cervids in southern Belgium during 2008. DNA extraction was performed for individual ticks, and each sample was evaluated for the absence of PCR inhibition using a PCR test. A *Babesia* spp. genus-specific PCR based on the 18S rRNA gene was applied to validated tick DNA extracts. A total of 1044 *Ixodes ricinus* ticks were collected and 1023 validated samples were subsequently screened for the presence of *Babesia* spp. DNA. Twenty-eight tick samples were found to be positive and identified after sequencing as containing DNA representing: *Babesia divergens* (3), *B. divergens*-like (5), *Babesia* sp. EU1 (11), *Babesia* sp. EU1-like (3), *B. capreoli* (2), or unknown *Babesia* sp. (4). This study confirms the presence of potentially zoonotic species and *Babesia capreoli* in Belgium, with a tick infection rate of 2.7% (95% CI 1.8,3.9%). Knowledge of the most common reservoir source for transmission of zoonotic *Babesia* spp. will be useful for models assessing the risk potential of this infection to humans.

**Key Words:** *Babesia* species—Belgium—Cervids—Tick—Zoonotic.

### Introduction

**B**ABESIOSIS IS A TICK-BORNE DISEASE caused by different species of intraerythrocytic protozoa classified within the genus *Babesia*. Different species of *Babesia* are described as potentially zoonotic and cause a malaria-like disease in humans. Interest in *Babesia* species with zoonotic capability has recently increased and babesiosis has been described by some authors as an emergent zoonotic disease (Hunfeld and Brade 2004; Vannier and Krause 2009; Gray et al. 2010). Indeed, since the first case in 1957, the number of identified human babesiosis cases has increased steadily, mostly in the U.S. but also in Europe, and while the majority of cases reported in Europe were in immunocompromised individuals. Cases have also been reported in non-asplenic patients (Martinot et al. 2011).

A new species of *Babesia* has recently been described in Europe: *Babesia* sp. EU1, proposed as *B. venatorum*, is a new candidate species and was identified for the first time in two asplenic patients (Herwaldt et al. 2003). *Babesia* sp. EU1 has also been identified in roe deer (*Capreolus capreolus*), (Duh et al. 2005; Bonnet et al. 2007), and *B. divergens* and *B. capreoli* have been described as occurring in wild European cervids (Malandrin et al. 2010; Zintl et al. 2011). The clinical symptoms rarely caused by these parasites in wild cervids appear to be the same as those described in their domestic counterparts, but have been documented less frequently (Penzhorn 2006). Characterization of these species has essentially been based on a comparison of the complete sequence of the 18S rRNA gene. *Babesia* sp. EU1 seems to be closely related to *Babesia odocoilei* (a parasite of white-tailed deer), with

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divergence of 28 nucleotides (Herwaldt et al., unpublished data), and phylogenetically is placed in a sister group with *B. divergens*, with 31 different nucleotides (Herwaldt et al. 2003). *B. capreoli* has recently been described and seems to only display three different nucleotides in the 18S rRNA gene relative to *B. divergens* (Malandrin et al. 2010). Vectorial competence of *Ixodes ricinus*, the most common tick species in Europe, has been proven for all 3 of these *Babesia* species (Nikol'skii and Pozov 1972; Donnelly et al. 1975; Becker et al. 2009; Bonnet et al. 2009).

Numerous reasons have been suggested for the apparent recent emergence of zoonotic *Babesia* species. These include changes in human behavior, activities that cause closer proximity to tick habitats, and a more systematic approach to diagnosis, with awareness of the possibility of co-morbidity with *Borrelia* spp. or subclinical infection. Expanding populations of wild cervids that act as important hosts maintaining tick populations and acting as reservoir hosts for some zoonotic tick-borne pathogens is also suspected.

Because of the recent first report of potentially zoonotic *Babesia* species in the tick *Ixodes ricinus* in Belgium (Lempereur et al. 2011), combined with sparse information regarding the prevalence of the different *Babesia* species and their potential zoonotic impact, this study was performed to investigate the range of *Babesia* species found in ticks collected from wild cervids in Belgium.

## Materials and Methods

In 2008, visible ticks were systematically collected from wild cervids (*Cervus elaphus* and *Capreolus capreolus*) found dead, hunted, or killed for sanitary reasons in the context of the disease monitoring activities of the WildScreen Network in southern Belgium (Linden et al. 2011). Ticks were preserved in ethanol 70% and morphologically identified to stage and species level using a standard key for morphological identification (Arthur 1963). Sex and repletion level were also recorded. Ticks were frozen in liquid nitrogen and immediately homogenized in a Tissue Lyser® (Qiagen, Hilden, Germany). DNA extraction was performed for individual ticks using a NucleoSpin tissue kit (Macherey-Nagel GmbH, Dueren, Germany). DNA of males was extracted with the XS version of the kit.

DNA quantity and quality was evaluated for each extracted sample using a spectrophotometer. To remove 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 the tick 16S rRNA gene was performed, using primers TQ16S+1F (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and TQ16S-2R (5'-ACG CTG TTA TCC CTA GAG-3') (Black et al. 1994; Halos et al. 2004). The *Babesia* spp. genus-specific PCR based on the amplification of a 411- to 452-bp fragment located at position 488–912 of the 18S rRNA gene, and using BJ1 (5'-GTC TTG TAA TTG GAA TGA TGG-3') and BN2 (5'-TAG TTT ATG GTT AGG ACT ACG-3') primers, was then applied to validated DNA extracts (Casati et al. 2006; Lempereur et al. 2011).

*Babesia* spp.-positive amplicons generated from the tick DNA samples were sequenced following purification with the Qiaquick PCR purification kit (Qiagen). Cycle sequencing reactions were performed in both directions with BigDye terminator v3.1 (3730 DNA analyzer; Applied Biosystems,

Carlsbad, CA) by the Giga Genomics Facility (Liège University, Liège, Belgium), and by DBS Genomics (Durham University, Durham, U.K.). A consensus sequence representing each PCR amplicon was generated and sequence comparison was performed using BLASTn searches in GenBank ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) and alignment with ClustalW software (Thompson et al. 1994).

## Results

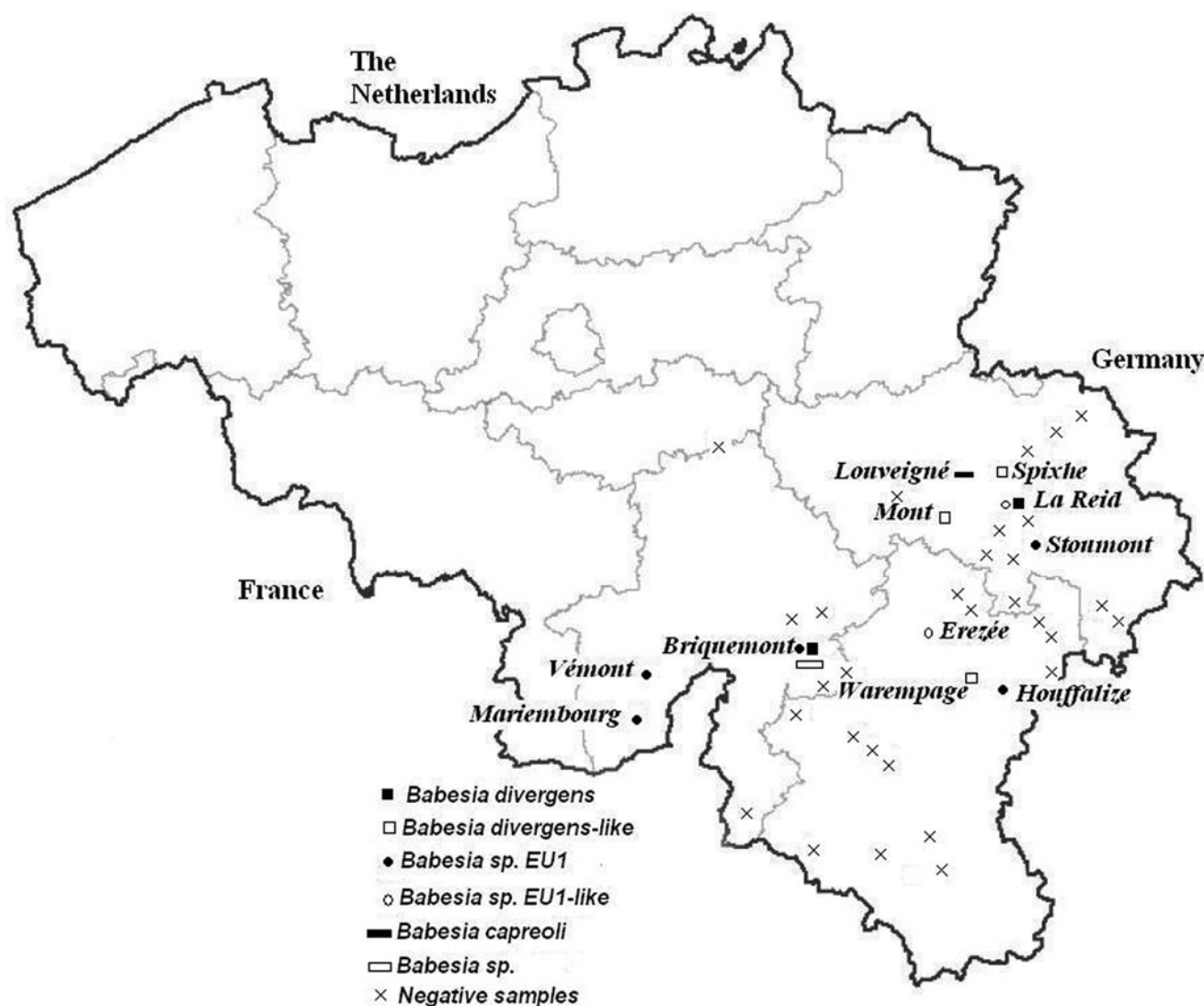
A total of 1044 ticks were collected from 47 cervids (34 *Cervus elaphus* and 13 *Capreolus capreolus*) found dead, hunted, or killed for sanitary reasons in 41 locations situated in 4 different provinces (Liège, Luxembourg, Namur, and Brabant Wallon) (Fig. 1). The number of ticks per animal ranged between 1 and 89. These ticks were all identified as *Ixodes ricinus* (23 nymphs, 678 females, and 343 males). From these ticks, 1023 DNA extracts were validated and subsequently tested by PCR for the presence of *Babesia* spp. DNA.

From these extracts 28 generated *Babesia* PCR amplicons and were designated as positive, giving a tick infection rate of 2.7% (95% CI 1.8,3.9%). The positive results were found in a nymph (1) and adult ticks (15 in females and 12 in males), collected from 12 different cervid hosts (7 roe deer and 5 red deer). From the analysis of the sequences derived from the *Babesia*-positive amplicons, three samples (GenBank: JF776397–JF776398, and JF776408) were identified to contain *B. divergens*, with 100% similarity to the GenBank sequence (AY046576), five samples (GenBank: JF776393, JF776399, and JF776400–JF776402) showed 98% and 99% homology, and were designed as *B. divergens*-like (Table 1). Eleven samples (GenBank: JF776394, JF776406, JF776407, and JF776410–JF776417) were identified as *Babesia* sp. EU1 after demonstrating 100% similarity with the reference sequence (GenBank: AY046575). Three samples (GenBank: JF776395, JF776396, and JF776405) were identified as *Babesia* sp. EU1-like. *Babesia divergens* and *Babesia* sp. EU1 DNA was identified in extracts of ticks collected from both roe and red deer. Two *Babesia* amplicons generated from independent tick extracts (GenBank: JF776403 and JF776404) showed 100% sequence identity with the *B. capreoli* reference sequence (GenBank: FJ944828). Both ticks were collected from the same roe deer in the province of Liège. *Babesia* sequences derived from four tick extracts could not be identified at the species level and were identified as *Babesia* sp. (GenBank: JF776409) (Table 1). The geographical distribution of the validated and the positive samples for *Babesia* spp. DNA found in the provinces of Liège, Luxembourg, and Namur are shown in Figure 1.

## Discussion

This study confirms the presence of *Babesia capreoli* together with two potentially zoonotic *Babesia* species in Belgium, most notably *Babesia* sp. EU1, in ticks collected from wild ruminants. The estimated *Babesia* spp. infection rate (2.7%) is in agreement with other previous publications (Iori et al. 2010; Lempereur et al. 2011).

Two samples were identified as *B. capreoli* in the province of Liège. The differentiation between *B. capreoli* and *B. divergens* is difficult due to their morphological similarities and strong serological cross-reactivity, and it is also complicated due to the high percentage of identity between the sequences of their respective 18S rRNA genes. In this survey, species



**FIG. 1.** Map of Belgium showing 41 localities situated in four different provinces (Liège, Luxembourg, Namur, and Brabant Wallon), where the 47 cervids originated. *Babesia*-positive samples collected from 12 different deer are localized on the map. One *Babesia*-positive tick sample was collected from a deer of unknown origin. In two localities (La Reid and Briquemont), more than one *Babesia* species was found in different ticks collected from the same deer. Negative samples are designated by X.

identification was based on the difference of 2 nucleotide bases on the amplified fragment of the 18S rRNA gene at positions 631 and 663, following the recent description of *B. capreoli* by Malandrin and associates (2010) (Table 1). Although *B. capreoli* is not considered to be a zoonotic species of *Babesia*, this parasite can infect various members of the cervidae family (Adam et al. 1976; Gray et al. 1990), and also alpine chamois (Hoby et al. 2009), but the roe deer seems to be most susceptible to infection (Malandrin et al. 2010). Based on the results of our sequence analysis we conclude that *B. capreoli* is present in Belgium, as suggested by the results of a previous serological study (Lonueux et al. 1991).

Identification of the potentially zoonotic *Babesia* sp. EU1 in ticks collected in Belgium in this study validated previous findings of Lempereur and colleagues (2011). The finding that one adult male tick collected from red deer was positive for *Babesia* sp. EU1 might be considered surprising, as *Babesia* sp.

EU1 has been identified in humans and in roe deer, but never in red deer (Duh et al. 2005; Tampieri et al. 2008; Zintl et al. 2011). While it is possible that this result does reflect a genuine infection of red deer with this parasite, it is also possible, due to the propensity of adult male *I. ricinus* to feed often and intermittently and to transovarial transmission, that the positive identification represents a previous acquisition from a different host species. Further work analyzing blood samples from the deer population would be required to support the conclusion that the red deer is a reservoir host for *Babesia* sp. EU1.

*Babesia* sp. EU1 and *B. capreoli* are known to have cervids as hosts (Duh et al. 2005; Zintl et al. 2011), but the role of deer as a reservoir for *B. divergens* is still controversial. In the genus *Babesia*, *B. divergens* has one of the largest ranges of host species, and is considered to be the main causal agent of human babesiosis in Europe. *Babesia divergens* has been found in

[illegible]

Additions of nucleotides are shown by an additional column following the nucleotide position. Slashes indicate deletions and asterisks indicate samples with 100% similarity to the reference species.



roe and red deer (Duh et al. 2005; Garcia-Sanmartin et al. 2007; Zintl et al. 2011); however, since it is difficult to distinguish this species from *B. capreoli*, misidentification is possible, and further molecular-based studies with a high level of species-specific fidelity are required. In cattle, this species is endemic in some areas of southern Belgium, and its distribution seems to be spreading (Losson 1989; Losson and Lefevre 1989; Everaert et al. 2007). *Ixodes ricinus* is also the vector of other human and animal pathogens, such as *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum*. While small rodents act as competent reservoirs for *B. burgdorferi*, wild cervids may play a similar role for *Anaplasma phagocytophilum*. Co-infections with *Babesia* spp. are probably fairly common and could enhance the severity of these protozoal infections in humans and animals (Welc-Faleciak et al. 2010; Hildebrandt et al. 2011). Within Europe it is generally accepted that the risk of tick-borne diseases has increased, and has the potential to become of greater significance due to climate change and additional factors, such as altered human recreational activity and an increasing number of immunocompromised patients. An important factor that could increase the risk of infection with zoonotic *Babesia* species is the expansion of reservoir host populations, as these promote maintenance of the parasite and allow transmission to the next generation of feeding ticks. Since 1975, wild ruminant populations have doubled in southern Belgium, despite an increase in hunting activity (SPW-DGO3 Agriculture-Ressources naturelles et Environnement-Département de la Nature et des Forêts 2011). Such a situation has the potential to alter the balance between the parasite and its additional, potentially human, hosts. Therefore, knowledge of the most common reservoir source for transmission of zoonotic *Babesia* spp. will be useful for modeling the risk potential of this infection to humans.

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### Author Disclosure Statement

No competing financial interests exist.

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