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Detection of four species of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected from roe deer (*Capreolus capreolus*) in the Netherlands

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SUMMARY

Roe deer (*Capreolus capreolus*) were investigated for their value as sentinel animals for Lyme borreliosis in the Netherlands. Serum was obtained from 114 roe deer, and 513 *Ixodes ricinus*, predominantly females (72%), were obtained from 47 animals (41%). The polymerase chain reaction was used to detect DNA of *Borrelia burgdorferi* sensu lato in a total of 190 ticks, comprising 106 engorged ticks and 84 non-engorged ticks. Borrelia DNA was detected in 24 engorged ticks (23%) and 26 non-engorged ticks (31%). This difference was not significant ($P = 0{\cdot}25$). Four species of *B. burgdorferi* sensu lato were identified in the ticks: *B. burgdorferi* sensu stricto, *Borrelia garinii*, *Borrelia afzelii* and group VS116. *B. afzelii* was most commonly found and present in 13 mixed infections, and in 28 single infections. Fifteen sera (13%) contained antibodies to *Borrelia* spp. Ticks are more appropriate sentinel animals for Lyme borreliosis than roe deer, an important host for *I. ricinus*. Although the viability of borrelia spirochaetes in engorged ticks collected from roe deer was not assessed, a bloodmeal taken from roe deer did not eliminate borrelia spirochaetes from the tick. The relevance of this finding for transovarial transmission of borrelia spirochaetes in ticks is discussed.

Lyme borreliosis (LB) is a tick-borne infection caused by *Borrelia burgdorferi* sensu lato, a group of genetically diverse spirochaetes. The principal vectors of these spirochaetes are ticks belonging to the *Ixodes ricinus* complex [1]. Since 1992, several species have been recognized within *B. burgdorferi* sensu lato on the basis of DNA hybridization, rRNA gene restriction fragment length patterns, protein electrophoresis patterns and monoclonal antibody reactivity [2–4]. In Europe, clinical LB has been associated with three species: *B. burgdorferi* sensu stricto has been related to arthritis while *Borrelia garinii* has been isolated from cerebrospinal fluid of neuroborreliosis patients and *Borrelia afzelii* from skin lesions of patients with acrodermatitis chronica atrophicans [5]. These species have been isolated from Dutch LB patients and *I. ricinus* ticks [5, 6].

I. ricinus ticks infest various mammals including roe deer (*Capreolus capreolus*) and small rodents, such as *Apodemus sylvaticus* (woodmouse) and *Clethrionomys glareolus* (bank vole), which are competent reservoirs of *B. burgdorferi* sensu lato [7, 8] while deer are regarded as important hosts for *Ixodes* ticks [9]. In Europe, larvae infected transovarially [10, 11] contribute substantially to the basic reproduction rate (R_0) of *B. burgdorferi* sensu lato in nature [8, 12, 13]. Transmission of *B. burgdorferi* sensu lato from deer to ticks remains a matter of debate. In Sweden, engorged Ixodes larvae collected from deer were free of borrelia [14], while borrelia DNA was detected in Japanese

Table 1. *Presence of* Borrelia burgdorferi *sensu lato DNA in engorged and non-engorged* Ixodes ricinus *collected from roe deer* (Capreolus capreolus)

	Number of ticks positive in PCR/number tested (%)		
	Engorged	Non-engorged	Engorged and non-engorged
Female ticks	24/105 (23)	8/40 (20)*	32/145 (22)
Male ticks	0/1 (0)	18/44 (41)	18/45 (40)†
Total	24/106 (23)	26/84 (31)‡	50/190 (26)

* $P = 0{\cdot}82$.
† $P = 0{\cdot}02$.
‡ $P = 0{\cdot}25$.

deer [15]. The prevalence of infection in ticks fed on deer was lower than in ticks collected in the field [16], which suggests that deer blood may contain spirochaetecidal factors which eliminate *B. burgdorferi* sensu lato from feeding ticks [17]. The antibody response of deer may reflect the presence of *B. burgdorferi* sensu lato in the local tick population, and deer have been proposed as sentinel animals for LB [18]. In Denmark, a large proportion of roe deer contained antibodies to *B. burgdorferi* sensu lato [19] while a study performed in Connecticut (USA) showed that the prevalence of seroconversion among deer correlated well within the proportion of borrelia-infected ticks collected from these animals [20].

Although precise numbers are lacking, the Dutch roe deer population has increased considerably during the last decade due to changes in land use and this study sought to establish whether roe deer can be used as sentinel animals for LB, and if these animals spread borrelia-infected ticks, thereby increasing the risk for humans contracting LB infection in areas where roe deer are present.

During the summer hunting season of 1995, 113 male and 1 female roe deer were shot in the provinces of Friesland ($n = 38$), Flevoland ($n = 57$), and Limburg ($n = 19$). The majority of the animals (77%) were between 1 and 2 years old. The physical condition of roe deer was assessed according to a questionnaire. Both engorged ticks and non-engorged ticks were collected from 47 animals (41%) stored in 70% ethanol at 4 °C and prepared for the polymerase chain reaction (PCR) as described previously [21]. Borrelia DNA was detected by PCR, and hybridization of the PCR product to species-specific probes identified *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii* and group VS116 [21, 22]. A PCR on *Ixodes* DNA was performed to determine whether components of tick lysates inhibited the PCR [21, 23]. Sera from the 114 roe deer were analysed for borrelia genus-specific flagellar antibodies by an inhibition ELISA [24, 25]. The statistical significance of data was determined by the Fisher exact test (two-tailed, version 1.14. GraphPad Software, San Diego CA).

A total of 513 *I. ricinus* ticks were collected, and this group consisted of 331 female ticks, 88 male ticks, 18 nymphs and 38 pairs *in copulo*. A total of 190 ticks, comprising 106 engorged ticks and 84 non-engorged ticks were investigated by PCR (Table 1). The PCR on Ixodes DNA amplified a fragment of the expected size in all lysates (results not shown) indicating that all the ticks belonged to the genus *Ixodes* [23]. The PCR was not inhibited either by tick components or components from deer blood. Borrelia DNA was detected in 50 ticks (26%, see Table 1). The prevalence of *B. burgdorferi* sensu lato infection among male ticks was significantly higher compared to female ticks ($P = 0{\cdot}02$). Engorged and non-engorged ticks had different prevalences of borrelia infection, but these differences were not significant (Table 1). The prevalence of borrelia infection of ticks collected in three provinces varied (Table 2), but this difference was not statistically significant.

Analysis of the PCR products revealed that *B. afzelii* was the dominant species among ticks with single infections (64%). This observation was supported by the presence of *B. afzelii* in 13 ticks which carried double infections (Table 2). Two ticks carried borrelia spirochaetes which could not be categorized as a species.

Borrelia antibodies were detected in 15 of 114 roe deer (13%). The prevalence of seropositivity was significantly lower (1·7%) in Flevoland province ($P < 0{\cdot}05$) than in Friesland (21%) or Limburg (32%). Ticks were collected from seropositive roe deer ($n = 9$) and seronegative roe deer ($n = 38$). Ticks which were positive for *B. burgdorferi* sensu lato DNA by PCR were found on four seropositive animals (44%) and 21 seronegative animals (55%). This difference was not significant ($P = 0{\cdot}71$).

The physical condition of roe deer could not be related to seropositivity, the number of ticks per deer or the presence of borrelia spirochaetes in ticks (results not shown).

The abundance of engorged female ticks, and the presence of tick-couples, on roe deer support the assumption that these animals are important re-

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Table 2. *Identification of* Borrelia burgdorferi *sensu lato species in* Ixodes ricinus *ticks collected from roe deer* (Capreolus capreolus) *in three Dutch provinces*

Province	Number of ticks positive in PCR/number tested (%)	Species of *B. burgdorferi* sensu lato identified*							
		AF	GA	SS	VS	NT	AF+VS	AF+GA	AF+SS
Friesland	16/72 (22)	11	0	1	1	1	1	0	1
Flevoland	24/75 (32)	13	0	1	2	0	6	1	1
Limburg	10/43 (26)	4	1	0	1	1	0	0	3
Total	50/190 (26)	28	1	2	4	2	7	1	5

* Abbreviations of species: AF, *B. afzelii*; GA, *B. garinii*; SS, *B. burgdorferi* sensu stricto; VS, Group VS116; NT, not typeable.

productive hosts for *I. ricinus* [14]. A considerable proportion (26%) of ticks collected from roe deer were infected with borrelia spirochaetes. However, not all roe deer which carried infected ticks showed evidence of previous exposure to borrelia spirochaetes, and a relation between the immune status of roe deer and the presence of *B. burgdorferi* sensu lato in ticks was not observed. The seroprevalence of animals varied in the three provinces, whereas the prevalence of borrelia infection in ticks was similar in all regions. This may be explained, in part, by the age of the deer or by the lack of an antibody response against the borrelia genus-specific flagellar epitope. Therefore, serologic findings in deer should be interpreted with care when they are used to verify the presence of borrelia spirochaetes in a designated area. An earlier study in northern Croatia also revealed that ticks were more appropriate sentinel animals for LB than roe deer [21].

The highest prevalence of *B. burgdorferi* sensu lato infection was found among male ticks, which were nearly all non-engorged. However, among female ticks, borrelia DNA was commonly present in both engorged and non-engorged ticks. These findings suggest that deer may not be the source of the borrelia spirochaete and demonstrate that spirochaetal infection is not eliminated from ticks feeding on ungulates. In contrast, Lacombe and colleagues [16], and Matuschka and colleagues [17], who used indirect immunofluorescence rather than PCR to detect borrelia spirochaetes in ticks, reported a substantial reduction in the proportion of infected ticks after engorgement. The use of different techniques may explain the discrepancy with our results. Since the sensitivity of an immuneserum used for indirect immunofluorescence may be affected by a change in antigen expression in borrelia spirochaetes as a result of the bloodmeal taken by the tick [26], whereas PCR may still detect borrelia DNA in these samples. Although, the viability of borrelia spirochaetes in engorged ticks collected from deer remains to be proven, the results of this study indicate that ticks remain infected after consuming deer blood.

Ticks collected from deer carried four species of *B. burgdorferi* sensu lato, and double infections occurred frequently. *B. afzelii* was commonly present among ticks, supporting findings in questing ticks [5]. Representatives of *B. garinii*, *B. burgdorferi* sensu stricto and group VS116 were isolated regularly from Dutch ticks [6, 22], and double infections occurred with regularity [22]. Borrelia DNA fragments which could not be typed, were obtained by PCR from two tick lysates. These PCR products will be sequenced to determine the relationship of these with known species of *B. burgdorferi* sensu lato. In conclusion, ticks rather than roe deer should be used as sentinel animals to study the presence of *B. burgdorferi* sensu lato in a designated area and PCR may be a more suitable method for identifying of borrelia spirochaetes in an engorged tick than indirect immunofluorescence.

The presence of naturally infected *I. ricinus* larvae infers that transovarial transmission may occur in ticks which have fed on ungulates. Indeed, borrelia spirochaetes have been detected in unfed larval ticks derived from engorged ticks collected from deer [13]. Subsequently, infected larvae may establish new foci of borrelia infection in small rodents or maintain its presence in a region. More investigations are, therefore, needed to define the role of roe deer in the spread of borrelia-infected ticks. The viability of *B. burgdorferi* sensu lato in engorged female ticks will be determined by culture and the efficiency of transovarial transmission of *B. burgdorferi* sensu lato will be assessed.

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