The Microbe 8 (2025) 100467

Contents lists available at ScienceDirect

The Microbe

journal homepage: www.sciencedirect.com/journal/the-microbe

Can *Ixodes trianguliceps* sustain a transmission cycle of tick-borne encephalitis virus?

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ARTICLE INFO

Keywords:
Ixodes ricinus
Ixodes persulcatus
Ixodes trianguliceps
Tick-borne encephalitis virus
Clethrionomys glareolus
Transmission dynamics

ABSTRACT

The transmission dynamics of the tick-borne encephalitis virus (TBEV) remain enigmatic. New lineages of the European TBEV-variant have been emerging in areas where it was not expected to circulate, questioning their origins. Although *Ixodes ricinus* and *I. persulcatus* are primary vectors for TBEV, other tick species have also been proposed to transmit the virus. Under natural conditions, *I. trianguliceps* can maintain *Anaplasma phagocytophilum* and *Babesia microti* circulation, but not *Borrelia burgdorferi* sensu lato. As *I. trianguliceps* is reported to be a vector for TBEV, we investigated whether it can sustain a cryptic TBEV transmission in areas without *I. ricinus* and/or *I. persulcatus*. Sera from 951 bank voles from six sites in Finland, where *I. trianguliceps,* but neither *I. ricinus* nor *I. persulcatus*, have been observed, were negative for TBEV antibodies, and *B. burgdorferi* s.l., but positive for *A. phagocytophilum* and *B. microti*. In contrast, 32 bank voles from two endemic TBEV-foci, where *I. ricinus/ I. persulcatus* are abundant, were positive for TBEV-antibodies as well as *B. burgdorferi* s.l., *A. phagocytophilum* and *B. microti*. Our results provide no evidence that TBEV would circulate alone by *I. trianguliceps* under natural conditions. Our results corroborate previous findings that *A. phagocytophilum* and *B. microti*, but neither TBEV nor *B. burgdorferi* s.l., can be sustained in the absence of *I. ricinus/I. persulcatus*. Whether TBEV can circulate in cryptic cycles or whether *I. trianguliceps* contributes to the maintenance of TBEV in the rodent - tick cycle, remains unresolved, but warrants further investigations.

Introduction

The geographical range of tick-borne encephalitis virus (TBEV) has been expanding in Europe (The TBE Book, 2024). This partially coincides with the latitudinal expansion in Fennoscandia and the altitudinal spread in central Europe of its most prominent vectors: *Ixodes ricinus* and *I. persulcatus* (Medlock et al., 2013; Tokarevich et al., 2011) that have a higher propensity to bite humans than other European Ixodes species (Hofhuis et al., 2017). New TBEV foci have been reported in locations in Norway and Finland up to more than 65°N latitude (Soleng et al., 2018) and from altitudes up to 2100 m above sea level (Holzmann et al., 2009; Briggs et al., 2011; Lukan et al., 2010). Also, in areas where *I. ricinus* populations have long been established, but where the virus was not expected to circulate (Randolph et al., 2000), new TBEV foci emerged, such as in the Netherlands, United Kingdom and Northern Germany (Holding et al., 2020; Topp et al., 2022; Esser et al.,

Abbreviations: IP, *Ixodes persulcatus*; IR, *Ixodes ricinus*; IT, *Ixodes trianguliceps*; TBEV, tick-borne encephalitis virus.

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https://doi.org/10.1016/j.microb.2025.100467
Received 7 February 2025; Received in revised form 10 June 2025; Accepted 16 July 2025
Available online 18 July 2025

2022; Holding et al., 2019). New foci have also emerged recently in Finland (Smura et al., 2019; Jääskeläinen et al., 2006, 2011). Due to the low prevalence and highly focal presence of TBEV in ticks, new TBEV-foci are typically identified when human infections arise. More recently, TBEV-foci are also found by serological screening of (wild) ungulate populations (Holding et al., 2019; Jahfari et al., 2017; Garcia-Vozmediano et al., 2022).

New lineages of the European TBEV have been emerging in the last few years. For example, the TBEV-Salland in the Netherlands (Jahfari et al., 2017) and United Kingdom (Holding et al., 2020), the DEN09_-Tokkekoeb in Denmark (Agergaard et al., 2019) and TBEV-Ain in France (Gonzalez et al., 2022). In one study from Finland (Smura et al., 2019), sequence analyses indicated that at least four distinct virus lineages independently emerged, but were probably introduced to these foci several decades ago. These findings raise the question of why these newly emerging lineages haven not been detected before, and from where they originate. The TBEV genome is a positive single-stranded RNA molecule, approximately 11,000 bases in length, which has a single reading frame encoding a polyprotein. The only demonstrated mechanism of genetic variation in TBEV is a mutation process via single nucleotide substitutions, and new subtypes/lineages can only occur through (gradual) evolution, rather than recombination (Kovalev and Mukhacheva, 2014). The rate of mutations in RNA viruses is high and estimated to be about 10^{-2}–10^{-5} nucleotide substitutions per site per year (Kovalev and Mukhacheva, 2014). The observed genetic divergence together with the estimated mutation rate suggests that the newly emerging lineages of TBEV have been circulating for a very long time without being detected.

One possible explanation is that TBEV can be sustained in a cryptic cycle with nidicolous tick species, which generally do not bite humans. Cryptic cycles of vector-borne microorganisms with (potential) spill-over to *I. ricinus,* where *I. ricinus* can act as a vector, have been demonstrated for *Babesia microti* (Randolph, 1995), *Borrelia turdi* (Heylen et al., 2017) and more recently with Usutu virus (Bakker et al.,

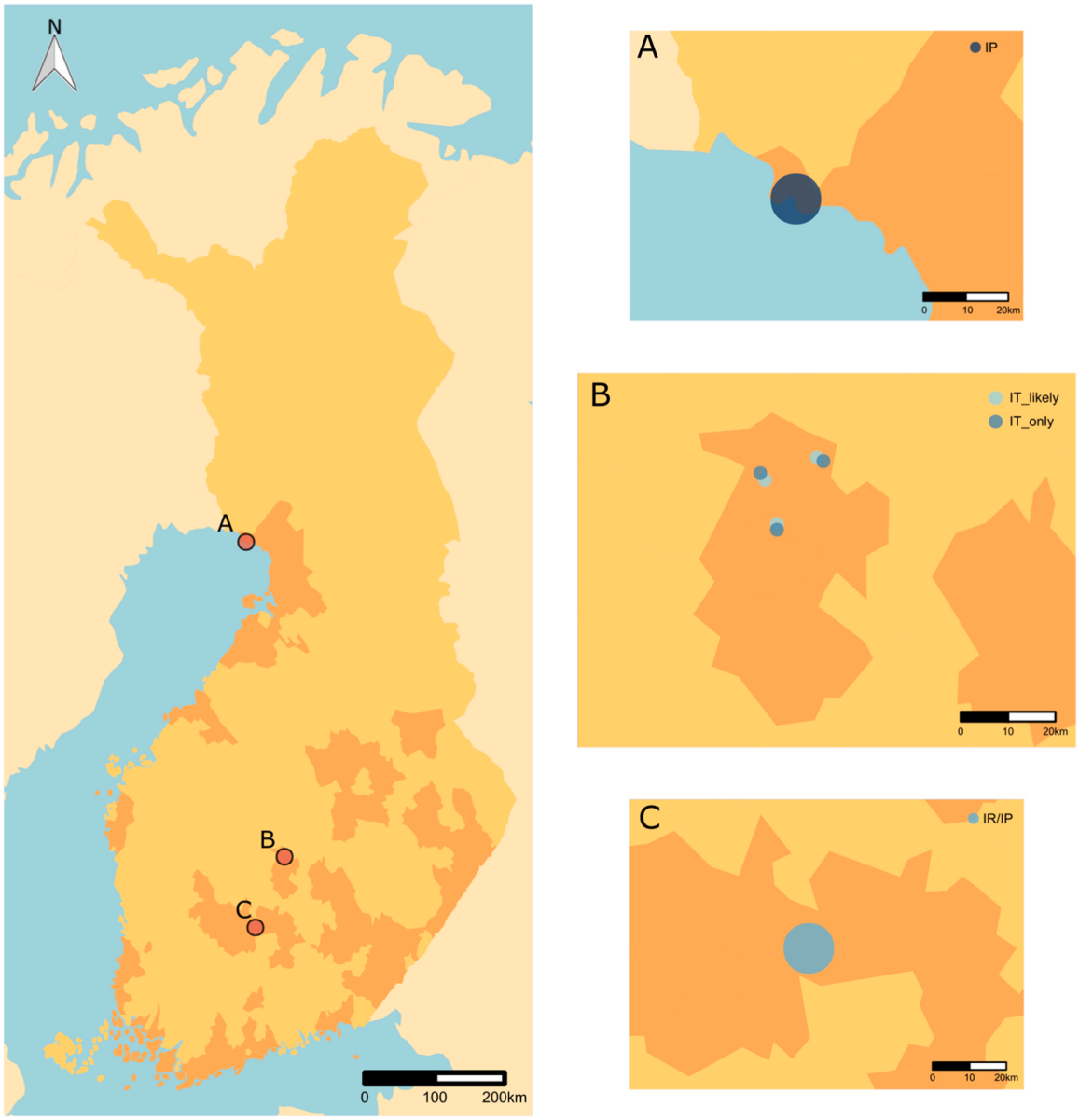

Fig. 1. Map of Finland indicating the sampling areas of the bank voles: A – Simo, B - Jyväskylä and C - Kuhmoinen. Finnish municipalities where human cases of tick-borne encephalitis have been diagnosed between 2019 and 2023 are indicated in dark orange. Dots show the sampling sites of bank voles, with the colour denoting the tick species (as in Table 1). TBE prevalence based on the data from the Finnish Institute for Health and Welfare, THL (https://www.thl.fi/ttr/gen/atlas/html/atlas.html?show=tbe_riskienarviointi.

2024). About 18 tick species have been implicated as (potential) vectors for TBEV, including several nidicolous tick species, such as *I. trianguliceps (*Korenberg and Kovalevskii, 1994; Alekseev and Chunikhin, 1990). Unfortunately, these findings are difficult to assess due to the limited availability of the original publications (The TBE Book, 2024). Also, whether a competent vector significantly contributes to the transmission of the virus under natural conditions needs to be established as well.

Ixodes trianguliceps is a specialised nest-dwelling tick that predominantly infests small mammals and, hence, does not pose a direct risk to humans (Cotton and Watts, 1967). Interestingly, ecological studies indicate that *I. trianguliceps* is capable of transmitting and sustaining *Anaplasma phagocytophilum* ecotype III and *B. microti,* but probably not *B. burgdorferi* sensu lato, in enzootic cycles, in the absence of the generalists *I. ricinus* or *I. persulcatus* (Bown et al., 2008a; Cayol et al., 2018; Jaarsma et al., 2019). Neither *A. phagocytophilum* ecotype III nor the European variant of *B. microti* are considered to cause disease in humans or domesticated animals (Jaarsma et al., 2019; Azagi et al., 2020), but their detection in small mammals can serve as an indication that *I. trianguliceps* is present there.

To test whether *I. trianguliceps* is sustaining TBEV in a cryptic cycle in nature in areas without *I. ricinus* and/or *I. persulcatus*, we examined bank voles (*Clethrionomys glareolus*) from six plots from the Jyväskylä region, Central Finland, for the presence of TBEV-antibodies. Bank voles are the most common rodent TBEV reservoir in the northern Fennoscandia, as well as well-known hosts for both *I. trianguliceps* and *I. ricinus/I. persulcatus*. Those plots are considered positive for *I. trianguliceps* and negative for *I. ricinus/I. persulcatus* (Cayol et al., 2018). Furthermore, these are located adjacent to TBEV-risk areas, based on reported TBE cases (Fig. 1 (dark orange colour in the Maps). Bank voles were also captured from two known TBEV foci as positive controls and to estimate the minimal number of rodent reservoirs to be tested to affirm the presence of TBEV circulation in a TBEV-endemic area. Moreover, we assessed the presence of *A. phagocytophilum* ecotype III and *B. microti*, which are known to transmitted by *I. trianguliceps*, and *B. burgdorferi* s.l., which is not transmitted by *I. trianguliceps*, in the study region (Cayol et al., 2018).

Material and methods

Study sites, rodent trapping and sampling

The samples were collected from bank voles captured in three regions in Finland (Fig. 1). In two of the study regions, Simo (Fig. 1, A) and Kuhmoinen (Fig. 1, C) the trappings were carried out in known TBEV foci in August 2021. The predominant generalist tick species in Simo region is *I. persulcatus* (Jääskeläinen et al., 2011), whereas in Kuhmoinen both *I. ricinus* and *I. persulcatus* are commonly found (Uusitalo et al., 2022). In Kuhmoinen, adult *I. trianguliceps* have been found feeding on bank voles (not shown). In rural Jyväskylä region (Fig. 1, B), the trappings were carried out for the purposes of a longitudinal study conducted in three pairs of sites (six sites in total) with a one-month interval between May-December in 2013 and 2014. In half of the sites, called hereafter as IT_only sites, all ticks were removed from the captured bank voles and identified under a microscope using identification keys (Hillyard, 1996). In addition, all captured bank voles were treated with antiparasitic treatment (Fipronil) in these sites (IT_only sites). In the other half of the sites, called hereafter as IT_likely sites, the ticks attached to bank voles were counted but not removed. Neither *I. ricinus* nor *I. persulcatus* was found with tick collections carried out in the rural Jyväskylä sites (Cayol et al., 2018).

In Jyväskylä sites, all voles were marked with microchip after the handling (tick counts or removal, treatment) and sampling (ear biopsy and blood), which after they were released back to the field (first capture). In Simo and Kuhmoinen, the captured bank voles were euthanised. Blood and ear tissue samples were collected. Blood samples were centrifuged to separate plasma/serum. All samples were stored at minus 20 until further analyses.

TBEV antibody detection

Sera from *C. glareolus* were incubated at 56°C for 30 min in a heating block for virus inactivation prior to serological analysis. The enzyme immunoassay for TBE Virus IgG (Testline, Brno, Czech Republic) was used according to manufacturer's instruction with the following modifications: bank vole sera were diluted 1:50, and Protein G IgG HRP-conjugate (1:200000) was used (Thermo scientific, Waltham, MA). As a positive control, a serum from a previously found positive animal was used (Esser et al., 2022). The cut-off value was calculated by adding 3 standard deviations (SD) to the mean optical density (OD) of the negative sera (Saraswati et al., 2019).

For confirmation of the ELISA results, the bank voles captured from the TBEV-positive sites detected by the TBE ELISA, were also examined for the presence of TBEV-specific antibodies using an immunofluorescent assay (Kallio-Kokko et al., 2006). PBS-diluted (1:20) serum samples were placed on the wells of 10-well slides with TBEV-infected cells. Antibodies that bound on the infected cells were visualized using conjugated using FITC-conjugated mouse IgG secondary antibodies (Agilent, Santa Clara, CA) and detected under a microscope.

Detection of Borrelia burgdorferi s. l., Anaplasma phagocytophilum and Babesia microti

DNA from blood were used to detect *A. phagocytophilum* and *B. microti* in Jyväskylä sites. DNA from ear biopsies (2 mm diameter ear sample) were used to detect *B. burgdorferi* s.l. in all sites, and *A. phagocytophilum* and *B. microti* in Simo and Kuhmoinen sites. DNA was extracted from using the method described (Laird et al., 1991) with modifications (see (Cayol et al., 2018)) or using DNEasy blood and tissue kit Qiagen (Hilden, Germany). Real-time PCR assays were used to detect *A. phagocytophilum, B. microti* and *B. burgdorferi* s.l. infections in the bank vole samples (Cayol et al., 2018).

Statistical analyses

Seroprevalence (+/- 95 % confidence intervals) for each of the sites (IT_Only, IT_Likely, IR/IP sites) were calculated based on OD values from TBEV-specific ELISA. No overlap with confidence intervals can be considered as statistically significant difference. Moreover, we examined OD values in association with the sites. While OD results from all observations are included in the visualisation of the data (Fig. 2), in the statistical analyses only the first capture observations were used to

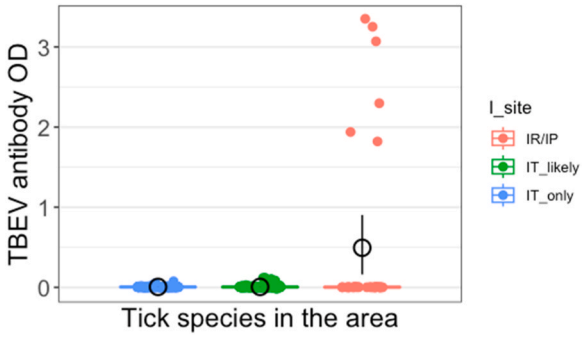

Fig. 2. Optical density (OD) values in TBEV antibody ELISA values in bank vole sera samples (dots) with mean (circles +/- 95 % confidence intervals) over the tick species in the study areas (I_site: IR/IP = *I. persulcatus* and/or *I. ricinus* known to be present, IT_only =only *I. trianguliceps* detected on rodents and no *I. ricinus/I. persulcatus* detected in drag flagging, IT_likely =sites close to IT_only sites and no *I. ricinus/I. persulcatus* detected by flagging).

ensure independence of observations. We used Kruskal-Wallis Test to examine whether the OD values differed depending on the trapping sites (IT_Only, IT_Likely, IR/IP sites) and posthoc Dunn's pairwise comparison with Bonderfoni correction to test the site-by-site differences in the OD values.

Results

A total 983 plasma samples (Table 1), collected from 526 bank voles, were included in this study. From location A (Simo) where *I. persulcatus* is present, 16 bank voles were tested and 3 of which were positive for TBEV antibodies. From location C (Kuhmoinen) where *I. ricinus/ I. persulcatus* are present, three out of 16 tested bank voles were TBEV antibodies. Thus, of the 32 bank voles captured from the known TBEV foci (Simo and Kuhmoinen) with *I. ricinus* and//or *I. persulcatus* (IR/IP_sites), six bank voles (three in both regions) had anti-TBEV antibodies. From location B (Jyväskylä) 494 bank voles (951 samples) were tested, of these 231 (432 samples) were from "IT_only" and 263 (519 samples) were from "IT_likely" sites. None of the bank voles captured from the IT_only or IT_likely sites were found to be seropositive against TBEV. Thus, individuals that were captured several times, always tested negative for TBEV antibodies.

In both TBEV foci (location A and C), TBEV seroprevalence among the captured bank voles was 18.8 % (95 % confidence interval: 4–45 %), whereas no TBEV seropositives were identified in IT_only or IT_likely sites (TBEV seroprevalence 0 %; 97.5 % one sided confidence interval: 0–1.6 %). The OD values differed between the sites (Kruskal-Wallis chi-squared = 23.704, df = 2, p-value < 0.001), with individuals captured from IR/IP sites having significantly higher OD values (mean= 0.493, s.d. = 1.07) than individuals captured from IT sites (IT_only: mean = 0.005, s.d.=0.005; IT_likely: mean=0.006, s.d. = 0.010) (Fig. 2). Dunn's posthoc pairwise test showed significant differences in the mean OD values between IT_only and IR/IP sites (Z-value= 4.664, adj. p < 0.001) and IT_likely and IR/IP sites (Z-value=3.477, adj. p = 0.002).

Similarly, none of the bank voles captured from the IT_only or IT_likely sites was found to be positive for *B. burgdorferi* s.l. DNA, whereas 9 of the 32 bank voles captured from the two *I. ricinus/ I. persulcatus*-positive sites (TBEV foci) were positive for *B. burgdorferi* s.l. DNA (Table 1). Regardless of the presence/absence of *I. ricinus/ I. persulcatus,* bank voles from all sites were positive for both *A. phagocytophilum* and *B. microti* (Table 1).

Discussion

One possible explanation for the discovery of multiple, phylogenetically distinct TBEV-Eu strains is the existence of long-standing, cryptic TBEV cycles within tick species that rarely interact with humans. Given that the primary hosts of *I. trianguliceps* are also known TBEV reservoirs, and that *I. trianguliceps* rarely bites humans, investigating the potential for TBEV circulation between rodents and *I. trianguliceps* is warranted. This is especially important considering the historical assertions of *I. trianguliceps* as a possible TBEV vector.

Assuming *I. trianguliceps* can acquire TBEV from and transmit to bank voles, we sought to investigate whether *I. trianguliceps* is maintaining the virus without *I. ricinus* and/or *I. persulcatus* present. Study sites in TBEV endemic regions of Finland were selected. Serological screening maximised the opportunity to detect any (endured) infection with TBEV, in addition to repeated sampling across a range of sites in the *I. trianguliceps* area. Despite this, we found no evidence that *I. trianguliceps* is circulating TBEV under natural conditions in the absence of *I. ricinus/I. persulcatus*. Since absence of evidence is not the same as evidence of absence, we could neither confirm nor disprove the role of *I. trianguliceps* as competent vector for TBEV. Meanwhile, our study corroborates previous findings that the circulation of *A. phagocytophilum* ecotype III and *B. microti* can rely on *I. trianguliceps* only (Cayol et al., 2018), rather than the coexisting *I. ricinus* (Bown et al., 2008b), but that the presence of *I. trianguliceps* is not sufficient, at least alone, in maintaining the circulation of *B. burgdorferi* s.l. in wild hosts (Cayol et al., 2018). The latter further indicates that the conditions for enzootic cycles of (some) tick-borne pathogens between *I. trianguliceps* and bank voles were favourable in the selected study areas. We are aware that *I. ricinus/I. persulcatus* are primary vectors for TBEV (see introduction), and that *I. trianguliceps* might only play a secondary/supportive role in TBEV transmission, that is only in the presence of *I. ricinus/I. persulcatus*, but that is beyond the scope of this study.

Despite its wide distribution, TBEV has a highly focal nature, which is not fully understood (Martello et al., 2022; Pustijanac et al., 2023). To address this, multiple sites within Jyväskylä area were selected. On a wider scale, areas surrounding Jyväskylä are suitable for TBEV circulation (Fig. 1). It is unlikely that our sample size would have been insufficient to detect TBEV, as 30-fold more samples were screened from IT sites than from the two known TBEV foci sites. Based on 19 % seropositivity in bank voles in both 'control' IR/IP sites, the sample in IT sites of 30 times more will have been sufficient to detect TBEV exposure should the virus have been circulating within the sites. To further

Table 1
Bank voles captured in Simo (Site A on map), Jyväskylä (Site B) and Kuhmoinen (C) with the information of tick species present in the site (IP= *Ixodes persulcatus*, IR= *I. ricinus*, IT= *I. trianguliceps*) were tested on the presence of tick-borne pathogens. Total number of tested samples per study area and site (Tested) together with samples positive for TBEV antibodies (TBEV), and presence of DNA from *A. phagocytophilum* (Ap), *B. microti* (Bm) and *B. burgdorferi* s/l/ (Bb) in blood or ear tissue samples.

Study area	Plot*	Tick species**	Year	Tested (n)	TBEV (n)	Ap (n)	Bm (n)	Bb*** (n)
Simo (A)		IP	2021	16	3	5	4	10 (16)
Jyväskylä (B)	1 A	IT_likely	2013	131	0	23	25	0 (3)
Jyväskylä (B)	1 A	IT_likely	2014	96	0	25	37	0 (10)
Jyväskylä (B)	1B	IT_only	2013	79	0	11	41	0 (5)
Jyväskylä (B)	1B	IT_only	2014	33	0	9	23	0 (9)
Jyväskylä (B)	2 A	IT_likely	2013	91	0	22	36	0 (9)
Jyväskylä (B)	2 A	IT_likely	2014	49	0	19	27	0 (6)
Jyväskylä (B)	2B	IT_only	2013	110	0	23	34	0 (6)
Jyväskylä (B)	2B	IT_only	2014	103	0	22	50	0 (6)
Jyväskylä (B)	3 A	IT_likely	2013	132	0	23	56	0 (9)
Jyväskylä (B)	3 A	IT_likely	2014	20	0	7	15	0 (7)
Jyväskylä (B)	3B	IT_only	2013	37	0	10	18	0 (6)
Jyväskylä (B)	3B	IT_only	2014	70	0	17	34	0 (6)
Kuhmoinen (C)		IR/IP	2021	16	3	12	5	9 (16)

**Tick species detected/previously identified in the site (Cayol et al., 2018).
*** *Borrelia burgdorferi* s.l. was tested from a subset (34) of samples collected from study area B and additional 48 bank voles that were captured from the same sites. The total number of samples tested are provided in parenthesis.
* Site: In Jyväskylä six permanent trapping plots established for the purposes of another study.

investigate whether *I. trianguliceps* is able to sustain TBEV transmission, evidence should be sought in more and different locations across Europe.

Given that *I. ricinus/I. persulcatus* is absent in the rural forests in Jyväskylä region (Cayol et al., 2018) and that *I. ricinus/I. persulcatus* are present in localities north, south, east and west (Uusitalo et al., 2022), it appears that macroclimatic conditions are present, however specific ecological or microclimatic conditions may be absent to support *I. ricinus* populations. This in itself may present a bias in exhibiting conditions that are not suitable for TBEV circulation. This is particularly difficult to establish given the uncertainty in environmental and microclimatic conditions required for TBEV microfoci establishment. The presence and/or abundance of *I. ricinus/I. persulcatus* propagation hosts, mainly deer, will heavily affect the presence and/or abundance of *I. ricinus/I. persulcatus* (Dickinson et al., 2020; Hofmeester et al., 2017). Studies investigating the contributors important for *I. ricinus/I. persulcatus* presence in Fennoscandia have produced mixed results: one study found open water coverage important, while another indicated temperature during tick activity period having the greatest contribution followed by precipitation, fox, and deer densities (Cayol et al., 2018; Uusitalo et al., 2022). In Fennoscandia, TBEV foci are typically close to bodies of water. Whether this is due to areas also being suitable for *I. ricinus* or whether this is due to specific microclimatic conditions required for TBEV circulation is unclear (Zeimes et al., 2014; Tonteri et al., 2016, 2015). Therefore, despite this study not detecting TBEV circulating in sites where only *I. trianguliceps* are present, this does not mean that *I. trianguliceps* cannot maintain TBEV elsewhere in Europe and is worthy of investigation.

Many different tick species have been proposed as TBEV vector with little follow up research (The TBE Book, 2024). A tick can be a vector and become infected with a virus and transmit it transstadially and even transmit it to animal hosts, however this may not be sufficient for virus maintenance with solely by that tick species. Thus, this study highlights the need to reassess whether they are firstly truly a vector, and secondly investigate whether circulation can be maintained by these vectors under natural conditions, either in the presence of *I. ricinus/I. persulcatus* or in their absence. To date, empirical evidence supporting TBEV infection in *I. trianguliceps* or its primary role in the circulation as a competent vector remains insufficient. Further research in the vectoral capacity of *I. trianguliceps* and other nidicolous vectors is highly necessary. Ideally, the complete the transmission cycle of TBEV in *Ixodes trianguliceps* with a natural host (*Clethrionomys glareolus*) is investigated under laboratory (and natural) conditions. Furthermore, it would be advantageous if more genome sequences of TBEV isolates from more (new/old) locations will become available, in order to explain the emergence of previously undetected lineages of TBEV in new areas.

CRediT authorship contribution statement

Holding Maya: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Conceptualization. **Hein Sprong:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Kallio Eva:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Saana Sipari:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Tapio Mappes:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Esa Koskela:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis, Data curation. **Heikki Henttonen:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Ankje de Vries:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Claire Cayol:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis. **Ilze Brila:** Writing – review & editing, Methodology, Formal analysis, Data curation.

Ethics approval and consent to participate

The trapping and handling of wild bank voles was carried out in accordance with the Finnish Act on the Use of Animals for Experimental Purposes (62/2006). The methods applied on wild bank voles were approved by the Finnish Animal Experiment Board and the Finnish Ministry of the Environment, under the authorisation ESAVI/3834/04.10.03/2011, ESAVI/3981/2018 and ESAVI/3981/20218 Muutos 1.

Consent for publication

Not applicable.

Funding

HS and AdV are financially supported by the Dutch Ministry of Health, Welfare, and Sports, The Netherlands (VWS). The research was financially supported by the Research Council of Finland (grant numbers 250524, 335651, 354988 to ERK). The funders had no role in the study design and interpretation, or the decision to submit the work for publication. Views expressed in this publication are those of the authors and do not necessarily reflect those of the employing institutes.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are very grateful to Saija Vuorenmaa and Anja Siukkola for their help in the field.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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