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Prevalence of tick-borne encephalitis virus in questing *Ixodes ricinus* nymphs in southern Scandinavia and the possible influence of meteorological factors

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Abstract
Ixodes ricinus ticks are Scandinavia's main vector for tick-borne encephalitis virus (TBEV), which infects many people annually. The aims of the present study were (i) to obtain information on the TBEV prevalence in host-seeking *I. ricinus* collected within the Øresund-Kattegat-Skagerrak (ØKS) region, which lies in southern Norway, southern Sweden and Denmark; (ii) to analyse whether there are potential spatial patterns in the TBEV prevalence; and (iii) to understand the relationship between TBEV

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prevalence and meteorological factors in southern Scandinavia. Tick nymphs were collected in 2016, in southern Scandinavia, and screened for TBEV, using pools of 10 nymphs, with RT real-time PCR, and positive samples were confirmed with pyrosequencing. Spatial autocorrelation and cluster analysis was performed with Global Moran's *I* and SatScan to test for spatial patterns and potential local clusters of the TBEV pool prevalence at each of the 50 sites. A climatic analysis was made to correlate parameters such as minimum, mean and maximum temperature, relative humidity and saturation deficit with TBEV pool prevalence. The climatic data were acquired from the nearest meteorological stations for 2015 and 2016. This study confirms the presence of TBEV in 12 out of 30 locations in Denmark, where six were from Jutland, three from Zealand and two from Bornholm and Falster counties. In total, five out of nine sites were positive from southern Sweden. TBEV prevalence of 0.7%, 0.5% and 0.5%, in nymphs, was found at three sites along the Oslofjord (two sites) and northern Skåne region (one site), indicating a potential concern for public health. We report an overall estimated TBEV prevalence of 0.1% in questing *I. ricinus* nymphs in southern Scandinavia with a region-specific prevalence of 0.1% in Denmark, 0.2% in southern Sweden and 0.1% in southeastern Norway. No evidence of a spatial pattern or local clusters was found in the study region. We found a strong correlation between TBEV prevalence in ticks and relative humidity in Sweden and Norway, which might suggest that humidity has a role in maintaining TBEV prevalence in ticks. TBEV is an emerging tick-borne pathogen in southern Scandinavia, and we recommend further studies to understand the TBEV transmission potential with changing climate in Scandinavia.

KEYWORDS
climate change, flaviviruses, *I. ricinus*, Nordic, tick-borne encephalitis virus

1 | INTRODUCTION

The incidence of tick-borne encephalitis (TBE) has been increasing in Sweden, Norway and Denmark in the last few decades (Jaenson et al., 2018; MSIS, 2022; Public Health Agency of Sweden, 2021; Slunge et al., 2022; Statens Serum Institut, 2020). Sweden had the highest incidence of human cases, with 520 cases in 2021 (Public Health Agency of Sweden, 2021). The number of reported TBE cases in Norway has doubled from 2020 to 2021, with 72 cases reported in 2021 (MSIS, 2022). The incidence of human TBE cases in Denmark has also increased, where 13 TBE cases, the highest number until now, were reported in 2019 (Statens Serum Institut, 2020). TBE is caused by different geographically distributed subtypes of tick-borne encephalitis virus (TBEV). Five subtypes of TBEV have been identified so far: the European (TBEV-Eu), Siberian (TBEV-Sib), Far Eastern (TBEV-Fe), Himalayan (TBEV-Him) and Baikalian (TBEV-Bkl) (Dai et al., 2018; Kovalev & Mukhacheva, 2017). In Scandinavia, *I. ricinus* is the most common tick species and is the primary vector for the European subtype of TBEV (TBEV-Eu). The virus is transmitted to humans by tick bites, mainly by nymphs, and occasionally through the consumption of unpasteurized dairy products (Caini et al., 2012; Hudopisk et al., 2013). TBEV has also

Impacts

- This is one of the most extensive studies aimed to describe the prevalence of TBEV in southern Scandinavia, screening 29,570 questing *Ixodes ricinus* nymphs.
- The virus was detected at 20 out of 50 locations, demonstrating that it is widespread in this region. The study confirms the presence of TBEV in ticks in Denmark, with a higher prevalence in Norway's Oslofjord and Sweden's southern Skåne County.
- This study also highlights the possible influence of relative humidity in sustaining TBEV in the region. TBEV was detected from regions with and without previously reported human TBE cases, which is highly relevant information for public health considerations and risk evaluation.

been detected in raw milk from cows and sheep (Cisak et al., 2010; Paulsen et al., 2019; Wallenhammar et al., 2020). TBEV infection may vary from being asymptomatic to fatal meningitis, encephalitis, meningoencephalitis and meningoencephalomyelitis (Kaiser, 1999); however, infections with TBEV-Eu are often reported to be asymptomatic (Gritsun et al., 2003; Larsen et al., 2014; Marvik et al., 2021; Svensson et al., 2021; Thortveit et al., 2020).TBE has been a notifiable disease in Sweden and Norway since 1969 and 1994, respectively, but is not at present notifiable in Denmark.

In Southern Scandinavia, the spatial distribution of TBE cases does not fully coincide with the known distribution of TBEV in ticks. TBEV has been detected in ticks from coastal counties in Norway, from Viken in the southeast to Nordland in the north (Andreassen et al., 2012; Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020). However, all human TBE cases from Norway are reported from the southernmost parts of the country, specifically from the counties Agder, Vestfold and Telemark, and Viken (former Buskerud) (MSIS, 2022). The south-western coast of the Oslofjord is a known endemic area with several reported cases, while there are few reported cases on the eastern side, although TBEV has been detected in ticks within the eastern side since 2015 (Larsen et al., 2014; Marvik et al., 2021; MSIS, 2022; Vikse et al., 2020). Studies report TBEV seroprevalence in healthy blood donors from both the eastern and western sides of the Oslofjord with seroprevalence of 0.65% and 0.4%, respectively, suggesting that TBEV infections within the population might have been asymptomatic or undiagnosed (Larsen et al., 2014; Marvik et al., 2021). TBE might not have been diagnosed due to a lack of awareness of tick bites and the distribution range of the virus (Paulsen et al., 2015).

Tick-borne encephalitis virus may be present at very low, potentially undetectable concentrations in questing ticks but might replicate and become detectable under favourable conditions in engorged ticks (Belova et al., 2012; Pettersson et al., 2014). This might explain the lack of correspondence between TBEV sentinel studies and TBEV prevalence estimation from ticks. In Denmark, although TBEV seropositive deer are reported from multiple sites (Andersen, Bestehorn, et al., 2019; Andersen, Larsen, et al., 2019; Skarphédinsson et al., 2005), several tick pools collected in 2010–2011 from northern Zealand, Funen and Jutland were negative for TBEV (Fomsgaard, 2020). Very few human cases have been reported in Denmark, mostly from the Island of Bornholm and sporadic cases from northern Zealand (Agergaard et al., 2019; Fomsgaard et al., 2009). Locally acquired human TBE cases were reported from Jutland and southern Funen in 2018 and one from the island of Falster in 2020.

Sweden has wider known TBEV endemic areas from where human TBE cases, seropositive cervids and TBEV prevalence in ticks have been reported (Jaenson et al., 2012, 2018; Jaenson & Wilhelmsson, 2019; Lundkvist et al., 2011; Pettersson et al., 2014; Wilhelmsson et al., 2020). Human TBE cases are widely reported from the coastal and central areas of Sweden around Stockholm, Örebro and the western Götaland region. The human TBE cases are typically reported from places with high population densities, such as the Swedish capital of Stockholm and lately from around the great Swedish lakes, where TBEV in ticks previously has been reported (Brinkley et al., 2008; Melik et al., 2007).In the later 2010s, studies reported TBEV endemic foci from inland central Sweden to southern and western Sweden (Brinkley et al., 2008; Stjernberg et al., 2008). The virus has been detected in ticks up to Norrbotten in the Gulf of Bothnia in northern Sweden (Jaenson & Wilhelmsson, 2019; Pettersson et al., 2014). However, TBEV prevalence estimation in ticks is still limited in southernmost Sweden (Pettersson et al., 2014).

Tick-borne encephalitis virus primarily circulates among tick populations and rodent hosts that act as reservoirs to persist in the environment (Michelitsch et al., 2019). Climatic factors, such as temperature, humidity, snow cover and rainfall, can influence the distribution of ticks and their hosts, potentially leading to the expansion of TBEV's geographical range as a result of climate change (Jaenson et al., 2012). TBEV transmission in ticks is attributed to tick seasonal activity, which is dependent on the tick life cycle (Randolph et al., 2000). Ticks can acquire infection by feeding on viremic hosts, mostly rodents (Achazi et al., 2011). Larvae can be infected with TBEV from infected adult females through eggs known as transovarial transmission. Mathematical models have shown that when larvae and nymphs feed in close proximity, nonviremic transmission of TBEV can occur from infected to uninfected ticks known as cofeeding transmission (Randolph, 2011; Randolph et al., 1996). This has been identified as a major route of TBEV transmission within a tick population in eastern Europe (Nah & Wu, 2021). Once infected with TBEV, a tick remains infected throughout its life (Jaenson et al., 2012; Kozuch & Nosek, 1980). The prevalence of TBEV in ticks depends on a variety of other factors such as the availability of hosts for feeding opportunities, the type of vegetation for suitable moulting environments and opportunities for transboundary transmission via for example migratory birds. Studies have reported a higher tick abundance in forested areas compared with open meadow areas, due to humid conditions found in forested areas, protecting against desiccation, while open habitats are more exposed to the effects of sun and wind (Jaenson et al., 2018; Lindström & Jaenson, 2003; Medlock et al., 2013). The increase in tick host species, for example, roe deer (*Capreolus capreolus*), has been suggested to cause the increase in human TBE incidence in Sweden (Jaenson et al., 2012). Other studies have found TBEV-infected ticks on migratory birds (Kazarina et al., 2015; Waldenström et al., 2007) thus, migrating birds may play a role in the geographical dispersal of TBEV-infected ticks, and their potential to start a new TBEV foci (Waldenström et al., 2007).

The vector, *I. ricinus* ticks are greatly influenced by temperature and show analogous seasonal variation; however, very less is known about the variation of TBEV and its relationship with the prevailing microclimate (Daniel et al., 2018). The vegetation period has been prolonged in Scandinavia, which has been identified as a key factor contributing to the increased abundance and activity of ticks in Sweden and Norway (Hvidsten et al., 2020; Jaenson et al., 2012). The impacts of climate change in Nordic countries are estimated by wetter and warmer climate with an increase in the length of growing season in the future (Hanssen-Bauer et al., 2017; Randolph, 2001). Although *I. ricinus* may expand its northern range in Scandinavia with climatic changes, it was also found that a potential range expansion in Scandinavia would only affect a small additional fraction of the human population as most of the population already live close to established tick areas (Kjær et al., 2019). TBEV poses a health risk to people living close to areas with TBEV presence in ticks (Vikse et al., 2020). TBE is a climate-sensitive disease, and the risk of virus transmission can be influenced by environmental factors such as temperature, humidity and saturation deficit (Jaenson et al., 2012; Medlock et al., 2013; Randolph et al., 2000). It is important to note that the direct effect of temperature and humidity conditions on TBEV is practically unknown (Korenberg, 2009). It is usually agreed that microorganisms in ticks are generally well adapted to the variability of temperature and humidity as well as other environmental conditions that are important for reproduction, vertical and horizontal transmission (Sirotkin & Korenberg, 2019). Although the TBEV infection in ticks is affected by both temperature and relative humidity (RH), RH has been suggested to be the major determinant for infection rate rather than temperature (Danielová et al., 1983). This highlights the importance of identifying regions with high pathogen prevalence. This study aims to provide updated knowledge on TBEV distribution and prevalence in ticks in relation to climatic parameters in southern Norway, southern Sweden and Denmark.

2 | MATERIALS AND METHODS

2.1 | Tick collection and stratification of study sites

We collected ticks at each site between 15 August and 30 September 2016, from 11 sites along the Oslofjord and southern Norway, 30 sites in Denmark and nine sites in southern Sweden as part of the ScandTick Innovation project. Ticks were collected from areas below 450 m above sea level (masl). A total of 29,570 tick nymphs were collected according to the tick identification key by Hillyard (1996) while larvae and adult ticks were excluded from the analysis. The collected nymphs were pooled in groups of 10, totaling 2957 tick pools. The procedures for vegetation stratification, site selection, tick collection and storage have been described in previous studies (Jung Kjær et al., 2019; Kjær et al., 2019, 2020). In short, each country within the study region was divided into a northern and southern part (of equal sizes) and furthermore divided into high and low values of the maximum normalized difference vegetation index (NDVI, from Fourier processed satellite imagery 40). Lastly, these stratified regions were further divided into forest and meadow, using Corine land cover (all one × 1 km resolution 40), based on the Corine definitions; forest: broad-leaved forest, coniferous forest, mixed forest and meadow: land principally occupied by agriculture with significant areas of natural vegetation, natural grasslands, moors and heathland, transitional woodland-shrub (Jung Kjær et al., 2019; Kjær et al., 2019, 2020). Tick collection sites were then selected at random within the stratified regions (80% forest and 20% meadow).

2.2 | Laboratory methods

2.2.1 | TBEV detection

Tick-borne encephalitis virus was detected as previously described by Andreassen et al. (2012). Briefly, ticks were homogenized as described previously by Klitgaard et al. (2019) and sent to the Norwegian Institute of Public Health on dry ice. The samples were stored at −80°C until further analysis. Total RNA was extracted from homogenized nymphs using the RNeasy mini kit (QIAGEN Inc.) with an automated QIAcube instrument (Qiagen). Immediately after the extraction process, the RNA was reversely transcribed to cDNA with random primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems). An in-house real-time reverse transcriptase RT-PCR was performed targeting a 54-base pair (bp) fragment on the envelope gene of TBEV. As positive controls, RNA from the TBEV strains 'Soukup' or 'Hochosterwitz' (kindly provided by Christian Beuret, Spiez lab, Switzerland and Franz-Xaver Heinz, University of Vienna, Austria, respectively) were used, and nuclease-free water was used as a negative control. All the RT-PCR-positive TBEV pools were pyro-sequenced and compared with a positive control for confirmation as described earlier (Andreassen et al., 2012). A pyro run was deemed valid when all TBEV controls were positive, all water controls were negative, and PCR-positive pools show pyrogram plots, which followed the positive control pattern. PCR-positive pools that could not be confirmed by pyrosequencing were excluded from the prevalence calculation. A few PCR-positive samples could not be confirmed by pyrosequencing due to technical errors. These comprised 8 tick pools from Denmark. The total number of pools included in the study was 2957 (1790 from Denmark, 660 from Norway and 507 from Sweden).

2.2.2 | TBEV prevalence in ticks

We calculated Estimated Pooled Prevalence (EPP) using the online Epitools epidemiological calculator with fixed pooled size and perfect test (https://epitools.ausvet.com.au/ppfreqone). We used EPP in all further analysis except for the spatial analyses. To test for differences in TBEV pool prevalence between sites, we used Pearson's chi-squared test statistics (test of equal or given proportions). This test is nonspatial and only tests whether there is a statistically significant difference between the site-specific pool prevalences.

2.3 | Spatial analyses

We tested Global clustering, in the context of a regional study 'global clustering refers to the identification of larger-scale patterns or trends within the study area', by Global Moran's *I* to test spatial patterns between the TBEV pool prevalence at each of the

TABLE 1 Tick-borne encephalitis virus (TBEV) in *Ixodes ricinus* tick nymphs in southern Scandinavia.

Country	Number of PCR-positive pools/ total tick pools analysed	Positive sites/ total sites tested	Number of confirmed TBEV-positive pools by pyrosequencing	Estimated pooled prevalence (%) by country
Southern Norway	10/660	3/11	8	0.1 (0.1–0.2)
Southern Sweden	11/507	5/9	8	0.2 (0.1–0.3)
Denmark	21/1790	12/30	13	0.1 (0.1–1.5)
Total	42/2957	20/50	29	0.1 (0.1–0.2)

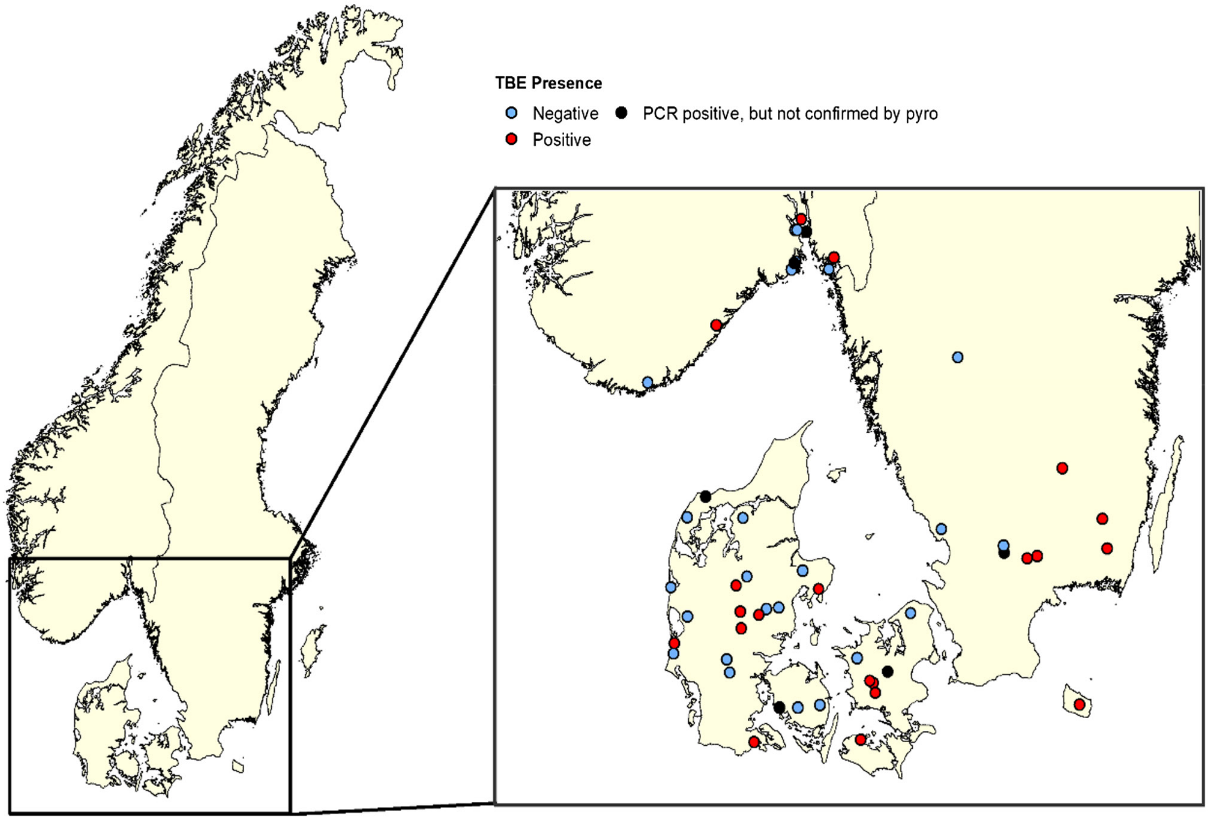

FIGURE 1 Visualization of TBEV-positive areas in Denmark, southern Norway and southern Sweden. Areas with unconfirmed TBEV-positive sample are marked in black, blue dots are negative sites and red dots are positive sites.

50 sites. Global Moran's *I* measure spatial autocorrelation based on site location and the TBEV prevalence at the sites and evaluates whether the observed prevalence patterns are clustered, dispersed or random.

To identify potential local clusters of TBEV within the study region, we used the program SatScan (Kulldorff, 2018) and the package *rsatscan* (Kleinman, 2015) in R 3.5.2 (R Development Core Team, 2018). We selected an elliptical scanning window and the Bernoulli probability model along with a maximum spatial window size of less than or equal to 50% of the total population at risk. The analysis looks for significant geographical clusters within circular or ellipsoid areas and tests whether EPP at sites included in the cluster on average has higher (hotspots) or lower (cold spots) prevalence compared with sites outside the clusters. The relative risk was used as a measure of clustering, which is the estimated risk within the cluster divided by the estimated risk outside the cluster. We used the Gini coefficient (Han et al., 2016) to evaluate the clusters, which measures the heterogeneity of the clusters, and thus determines whether to report multiple smaller clusters or a large joint cluster. In this analysis, we used the observed proportion of TBEV (RT real-time-PCR) positive pools rather than the estimated pooled prevalence (EPP). Before running the analysis, we transformed site coordinates into a flat UTM projection (UTM zone 32 N).

2.4 | Climatic analysis

We obtained climatic data from the Norwegian Meteorological Institute (www.met.no/), the Swedish Meteorological and Hydrological Institute (www.smhi.se) and the Danish Meteorological Institute (www.dmi.dk/) and compiled the data over the period 2015–2016. We chose data from weather stations, based on the closest distance to the sampling site. From these weather stations, we acquired air temperatures (°C) and RH (%) along with their monthly extremes (mean, maximum and minimum) and we calculated saturation deficit (SD), which is the measurement of drying power of the air according to Randolph and Storey (1999) (Andreassen et al., 2012; Estrada-Peña et al., 2004; Perret et al., 2004; Randolph & Storey, 1999). Months were grouped into the following seasons: March, April and May were considered Spring; June and July were considered Summer; August, September and October were considered Autumn and November, December, January and February were considered Winter. A Pearson's Correlation analysis was conducted to examine the correlation between the EPP and several meteorological factors: temperature, RH and SD, as well as the extremes of these factors. This analysis included only TBEV-positive sites and classified the variables according to country, season and year. The analysis was conducted using SPSS version 22, and statistical significance was assessed at either $p<0.05$ or $p<0.01$.

3 | RESULTS

3.1 | TBEV prevalence in southern Scandinavia

Of the 50 sites tested, 20 (40% of sites) were found to be positive for TBEV (Table 1). In total, we tested 2957 tick pools of which 29 pools were positive resulting in an overall TBEV prevalence of 0.1% in questing *I. ricinus* nymphs in southern Scandinavia (Figure 1, Table 2). The nonspatial Pearson's chi-squared test statistics testing for differences in EPP between sites was statistically significant ($\chi^2_{49}=70.9$, $p=0.02$) and thus not randomly distributed. All the TBEV-positive pools came from forested habitats.

3.2 | TBEV prevalence in tick nymphs from southern Sweden

In southern Sweden, five of nine sites were positive for TBEV. A large proportion of the positive pools (five out of eight) came from two sites in northern Skåne County located only 11 km apart. Blekinge, Kalmar and Jönköping Counties all had one positive site. Among the two real-time RT-PCR-positive pools from Blekinge, one was lost due to technical errors during pyrosequencing. The overall TBEV prevalence in ticks in southern Sweden was 0.2%.

3.3 | TBEV prevalence in tick nymphs from southern Norway

Tick-borne encephalitis virus was detected in eight pools out of 660 pools tested giving an EPP of 0.1% in southern Norway. A total of three of 11 sites were positive for TBEV and two of those sites from Oslofjord showed a relatively high EPP of 0.7% and 0.5%, respectively (Table 2). One positive tick pool came from the coastal area of Agder County in the southern part of Norway.

3.4 | TBEV prevalence in tick nymphs from Denmark

In Denmark, a total of 12 of 30 sites were positive and 13 tick pools out of 1790 were positive, resulting in an overall TBEV prevalence of 0.1% (Table 1). Seven of the positive sites were from Jutland, three from Zealand, one from Falster and one from Bornholm. Among the 12 positive sites, 11 sites had one positive pool, while the last location from southern Denmark, close to the North Sea, had two positive pools (Figure 1, Table 2). We lost five positive pools from three additional sites in Denmark during pyrosequencing (shown by black-filled dots, Figure 1). These contained two pools from one site on Funen, two pools from one site from western Zealand and one pool from one site from northern Jutland (Table 2). We also lost three pools out of four from one site on Bornholm, which site is included in the 12 positive sites above.

3.5 | Spatial patterns in TBEV prevalence

We found no evidence of global clustering (Moran's $I=0.017$, $z=0.38$, $p=0.71$) for site-specific pool prevalence, and none of the local clusters found within the study region using SatScan were statistically significant ($p>0.1$). The results show that the amount of prevalence varied between the sites, but it did not seem to be grouped in any specific area within the region.

3.6 | Correlation analysis of TBEV prevalence in ticks with meteorological factors

We used Pearson's correlation analysis to investigate the relationship between EPP with meteorological factors on data from the autumn and winter of 2015 and spring, summer and autumn of 2016. The meteorological factors included mean, minimum and maximum of temperature; mean, minimum and maximum of RH; and mean, minimum and maximum of SD during the autumn and winter of 2015 and spring, summer and autumn of 2016.

We found a statistically significant correlation between the EPP and RH in Sweden and Norway. There was a positive relationship between EPP and monthly minimum RH in all seasons in Sweden: autumn of 2016 (df = 13, $r=0.7$, $p=0.01$), winter (df = 18, $r=0.6$, $p=0.00$), spring (df = 13, $r=0.6$, $p=0.02$), summer (df = 8, $r=0.63$, $p=0.05$) and autumn (df = 13, $r=0.58$, $p=0.02$). This relationship was also present for mean RH in winter 2015 (df = 18, $r=0.7$, $p=0.01$) and autumn 2016 (df = 13, $r=0.6$, $p=0.02$) (Table S1). In Norway, the EPP showed statistically significant negative correlation with maximum RH and statistically significant positive correlation with minimum SD in the spring, summer and autumn of 2016. Specifically, in spring (df = 7, $r=-0.9$, $p=0.00$; df = 7, $r=0.8$, $p=0.00$), summer (df = 4, $r=-0.9$, $p=0.00$; df = 4, $r=0.7$, $p=0.01$) and autumn (df = 7, $r=-0.9$, $p=0.00$; df = 7, $r=0.9$, $p=0.00$), there was a strong negative relationship with monthly maximum RH and a strong positive

TABLE 2 Detailed description of the sites where TBEV was detected in host-seeking *Ixodes ricinus* nymphs in southern Scandinavia.

Country	County	SiteID	Longitude	Latitude	Habitat type	No. of PCR-positive pools/ pools tested	No. of confirmed PCR pools[a]	EPP% (range of EPP)
Norway	Vestfold and Telemark	NO-124	8.92	58.61	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Norway	Viken	O-111	11.1	59.19	Forest, high NDVI	4/60	3	0.5 (0.10–1.5)
Norway	Viken	O-238	10.51	59.53	Forest, low NDVI	4/60	4	0.7 (0.20–1.8)
Denmark	Jutland	DK-002	8.22	55.79	Forest, low NDVI	2/60	2	0.3 (0.0–1.2)
Denmark	Zealand	DK-025	11.55	55.42	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Falster	DK-120	9.56	54.92	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Zealand	DK-121	11.59	55.33	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Bornholm	DK-123	14.96	55.11	Forest, high NDVI	4/60	1, 3 lost[b]	0.2 (0.0–0.9)
Denmark	Zealand	DK-124	11.5	55.44	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Falster	DK-201	11.32	54.92	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-485	9.36	55.93	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-521	10.68	56.26	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-554	9.65	56.04	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-604	9.27	56.3	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Denmark	Jutland	DK-616	9.33	56.08	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Sweden	Skåne	SE-005	14.44	56.45	Forest, low NDVI	3/60	3	0.5 (0.10–1.5)
Sweden	Skåne	SE-011	14.26	56.43	Forest, low NDVI	2/60	2	0.3 (0.04–1.2)
Sweden	Jonkoping	SE-064	14.99	57.2	Forest, low NDVI	1/60	1	0.2 (0.0–0.9)
Sweden	Kalmar	SE-122	15.61	56.72	Forest, high NDVI	1/60	1	0.2 (0.0–0.9)
Sweden	Karlskrona	SE-221	15.64	56.45	Forest, low NDVI	2/44	1, 1 lost[b]	0.2 (0.0–0.9)
Denmark	Central Zealand	DK-237	11.81	55.51	Forest, high NDVI	2/60	2 lost[b]	-
Denmark	Western Funen	DK-122	9.98	55.22	Forest, high NDVI	2/60	2 lost[b]	-
Denmark	North Jutland	DK-721	8.74	57.09	Meadow, low NDVI	1/60	1 lost[b]	-

[a]Pyrosequencing.

[b]TBEV RT-PCR-positive pools that were lost due to failure in pyrosequencing and could not be confirmed.

relationship with minimum SD. There was also a statistically significant positive correlation between EPP and mean temperature (df = 4, $r = 0.89$ and $p = 0.02$) and minimum SD (df = 4, $r = 0.98$ and $p = 0.00$) in summer (Table S2). In Denmark, we did not see any clear pattern. However, during the winter of 2015/2016, we observed a weak negative correlation between EPP and mean RH (df = 46, $r = -0.33$, $p = 0.02$) and a weak positive correlation between EPP and mean SD (df = 46, $r = 0.41$, $p = 0.00$). There was also a weak positive correlation between EPP and mean SD (df = 34, $r = 0.366$, $p = 0.03$) in the spring of 2016 (Table S3).

4 | DISCUSSION

This is the first study of TBEV in Scandinavia covering the whole Øresund-Kattegat-Skagerrak (ØKS) region. TBE is an emerging zoonosis in Scandinavia, which constitute the northernmost part of the distribution range of *I. ricinus* (Riccardi et al., 2019; Süss, 2011). Colder and dry winters and limited vegetation periods are a limiting factor for sustainable tick populations (van Oort et al., 2020) and thus the presence of TBEV in most of this region. The possible expansion of ticks into new regions in Scandinavia has been documented in earlier studies (Hvidsten et al., 2020; Lindquist & Vapalahti, 2008; Randolph, 2001). This study reports the presence of TBEV in tick populations in most parts of Denmark, which to our knowledge is reported for the first time. According to our analyses, TBEV prevalence in ticks in Denmark is widespread as we found 12 confirmed TBEV-positive sites, seven sites were in Jutland, three on Zealand and one each on Lolland and Bornholm (Figure 1, Table 2). Our PCR results suggest TBEV presence at several other sites, but these could not be confirmed due to technical issues (Table 2).

It appears that TBEV circulation in Denmark is not a recent development. Roe deer (*C. capreolus*) serum from 2003 to 2005 was analysed for the prevalence of tick-borne encephalitis complex virus and positives were found from southern Jutland, Lolland, Falster, northern Zealand and Bornholm (Skarphédinsson et al., 2005). The researchers indicated that although the TBE complex virus had emerged in new areas, the infection was still rare and focal in its distribution (Skarphédinsson et al., 2005). A follow-up study on the same deer species, roe deer sampled in 2013–2014 found that TBEV seropositive deers were found all over Denmark with a national seropositivity of 6.9%. Compared with the results from the study conducted in 2005, the seropositive roe deers had expanded to most areas of northern and central Jutland and Funen (Andersen, Larsen, et al., 2019; Skarphédinsson et al., 2005). The sentinel study also reported the presence of roe deer sera with high antibody titers from northern and central Jutland, which shows that the infection response in the deer could be a recent infection or it can be triggered by bites from multiple positive ticks. Our results confirm the findings that TBEV is more widespread in Denmark than previously anticipated. This study found seven positive sites from Jutland and supports TBEV foci being prevalent in Jutland. A seropositive roe deer was found on Funen (Andersen, Larsen, et al., 2019). In the present study, one PCR-positive pool was detected from Funen. Although the PCR-positive pool could not be confirmed positive due to technical difficulties during sequencing, this indicates that further assessment is needed to identify TBEV foci. The island of Bornholm in the Baltic Sea is a well-known TBEV endemic area since the 1950s (Kristiansen, 2002). The phylogenetic analysis of TBEV strains isolated from TBEV-infected patients from Bornholm showed relatedness to the eastern and central European strains indicating the expansion of TBEV infection from around the Baltics (Andersen, Bestehorn, et al., 2019). Besides Bornholm, human TBE cases have been reported from Tokkekøb Hegn and Tisvilde Hegn in northern Zealand, where the infecting TBEV strain clustered with the Norwegian strain, Mandal 2009 indicating possible expansion of TBEV from southern Norway or vice versa (Agergaard et al., 2019; Fomsgaard et al., 2013).

The previous roe deer and tick studies combined with the present study show that TBEV is circulating in Denmark and is more widespread than previously anticipated, although human TBE cases are rare. The low amount of human TBE cases could potentially be explained by TBEV not being a notifiable disease in Denmark. However, in Norway, TBEV is also prevalent in ticks and cervids (*C. capreolus*, *Cervus elaphus*, *Alces alces*) along the coast until 65°N (Paulsen et al., 2020; Soleng et al., 2018; Vikse et al., 2020), but human cases occur only in restricted foci along the southern coast (Andreassen et al., 2012; MSIS, 2022). Jutland and the western coast of Norway are both influenced by the North Sea, and it might be important to understand climatic influences on ticks and TBEV that can have special relevance to climate-sensitive zoonoses in the future. It is possible that a virus strain causing less severe disease circulates in these areas (Paulsen et al., 2015; Soleng et al., 2018; Vikse et al., 2020). The awareness of the general practitioners on the presence of TBEV might also play a role when diagnosing milder cases. TBEV detection methods also play a role in the detection of TBEV from questing ticks as the virus concentration is low (Schwaiger & Cassinotti, 2003). The length of the target sequence in the PCR might also be important to consider when the virus concentration is low in samples (Andreassen et al., 2012; Schwaiger & Cassinotti, 2003). False negatives due to limitations in the detection methods might be a reason for the lack of coherence in TBEV prevalence studies in ticks and sentinel studies in Denmark and Norway.

The present study found several new sites with relatively high TBEV prevalence along the Oslofjord in Norway. The Oslo and Viken counties in Norway are heavily populated areas where people drive to the city for work and live away from city areas. One location 45 km south of Oslo centrum showed EPP of 0.7% and another location near Sarpsborg, showed EPP of 0.5%. Both these locations are new locations where ticks were not screened for TBEV before. In the last few years, the incidence of TBE has been reported from new areas in Viken County (MSIS, 2022). As mentioned earlier, TBEV is endemic on the southern coast of Norway and there is a concern about the virus establishing in ticks in the more northeastern parts where Oslo is located. TBEV EPP of 0.2%–0.4% has been reported in Viken County from the former counties of Østfold, Akershus and Vestfold (Vikse et al., 2020) and the present study reports higher TBEV EPP along the Oslofjord in Norway than previously detected.

Although the presence of TBEV endemic foci along the western and southeastern coast was reported long ago, TBE foci in the inland part of southern Sweden is still a new emergence area (Brinkley et al., 2008; Fält et al., 2006; Lundkvist et al., 2011; Melik et al., 2007; Pettersson et al., 2014; Stjernberg et al., 2008; Waldeck et al., 2022). The nine sites in this study are from the seven neighbouring counties; Skåne, Kronoberg, Halland, Jönköping, Blekinge, Kalmar and Västragötaland (Figure 1). We found two positive sites from the northern part of Skåne County with five of eight positive pools resulting in TBEV EPP of 0.5% (Table 1). Several human TBE cases have also been reported in this area (Fält et al., 2006). A TBEV prevalence above 0.5% in questing ticks for more than 1 or 2 years has been used to define an endemic focus (Andreassen et al., 2012; Gäumann et al., 2010; Pettersson et al., 2014). Endemic foci refer to a geographical location in space and time where TBEV circulation is persistent in nature (Dobler et al., 2011). In other words, this area may be a risk area for possible TBEV infections in the future.

The positive sites in Blekinge and Kalmar counties in Sweden are close to the southeastern coast, where migrating birds may have introduced TBEV to the areas. Öland Island along the coast of Kalmar County, is a well-known bird migration stop (Waldenström et al., 2007) and it could potentially be a hotspot for the dispersal of TBEV-infected ticks by birds. TBEV isolated from a patient in Kalmar in 1993 has been whole genome sequenced and showed the closest resemblance to the NL/UH strain, isolated from *I. ricinus* ticks in the Netherlands and the Ljubljana strain, isolated from a patient in Slovenia (Paulsen et al., 2021). This suggests that there is a relationship between the long transport of ticks with TBEV infection and tick/human infection.

A potential study limitation is the single sampling between August 15th and September 30th, possibly missing peak infection rates in ticks due to differences in feeding cycles and how it aligns with seasonal spikes in reported cases. We might not capture peak infection rates within the ticks since these differences in detection rates may be based on feeding cycles in April to July versus August to November. However, according to the Norwegian Surveillance

System for Communicable Diseases (MSIS), TBE cases in Norway starts to appear in May, but the peak season is typically in August and September. This makes the timing of our sampling particularly fitting, as it aligns with the peak of TBE incidence.

We did not find any geographical clustering of TBEV in southern Scandinavia, unlike other tick-borne pathogens (Kjær et al., 2020). This could be attributed to a unique transmission potential of TBEV during cofeeding. Cofeeding transmission is identified as one of the major routes of TBEV transmission and maintenance in foci despite the low prevalence in tick population (Nah & Wu, 2021; Randolph et al., 2000). The formation of TBEV foci is a result of the complex interaction between ticks, their host species and the environment and hence might not exhibit a specific pattern.

Relative humidity is a crucial factor in deciding tick survival as ticks are very sensitive to desiccation, which might also affect the virus in the tick; however, very little is known about the effect of RH in TBEV prevalence in ticks (Danielová, 1990; Danielová et al., 1983; Korenberg, 2009; Sirotkin & Korenberg, 2019). This study suggests that humidity may play a major role in influencing TBEV prevalence in ticks in southern Scandinavia. In the northern part of Skåne in Sweden, TBEV prevalence in ticks was positively correlated with minimum humidity levels in all seasons. This suggests that higher air humidity may support more TBEV-positive ticks in areas with a continental climate like Sweden. TBEV virus was negatively correlated with maximum humidity and positively correlated with minimum dryness in coastal regions of southeastern Norway. This suggests that in regions where humidity levels are already high, lower humidity levels may be favourable. Overall, the study suggests that humidity might play an important role in TBEV virus prevalence; however, the effect may vary according to local microclimatic conditions. Further studies on understanding the influence of climatic parameters particularly the RH might be of special relevance under changing climatic conditions in Scandinavia. It is important to note that the obtained climatic parameters were from the nearest meteorological stations and provide a general relationship. The impact of the difference in scales in climate and microclimate has been discussed as ticks mostly live close to the ground where microclimatic conditions are modified by vegetation (Estrada-Peña et al., 2004; Randolph & Storey, 1999). It is important to consider that the relationship observed is based on few positive sites from Norway and Sweden and a larger number of study locations might give more information. It may be necessary for future studies to consider environmental data from local microclimate measurements rather than an aggregation of data collected from the national meteorological stations covering larger areas. This study confirms that TBEV is circulating in many locations throughout southern Scandinavia, pointing out that people acquiring tick bites in these areas are at risk of developing TBE and as such implying a public health concern. Future studies should aim to assess the impacts of climate change and monitor TBE foci in the region.

AUTHOR CONTRIBUTIONS

All authors revised and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical approval is not required for collecting and analysing tick samples in Norway.

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