Ticks and Tick-borne Diseases 10 (2019) 115–123

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Continued expansion of tick-borne pathogens: Tick-borne encephalitis virus complex and *Anaplasma phagocytophilum* in Denmark

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ARTICLE INFO

Keywords:
Tick-borne encephalitis virus
Louping ill virus
Anaplasma phagocytophilum
Ixodes ricinus
Sentinel surveillance
Capreolus capreolus

ABSTRACT

Tick-borne encephalitis virus (TBEV) is a tick-transmitted flavivirus within the tick-borne encephalitis (TBE) complex. The TBE complex is represented by both TBEV and louping ill virus (LIV) in Denmark. *Anaplasma phagocytophilum* is also transmitted by ticks and is believed to play an essential role in facilitating and aggravating LIV infection in sheep.

This study aimed to describe the distribution of TBE complex viruses in Denmark, to establish the possible emergence of new foci and their association with the distribution of *A. phagocytophilum*. We performed a nationwide seroprevalence study of TBE complex viruses using roe deer *(Capreolus capreolus)* as sentinels and determined the prevalence of *A. phagocytophilum* in roe deer. Danish hunters obtained blood samples from roe deer during the hunting season of 2013-14. The samples were examined for TBEV-specific antibodies by virus neutralization tests (NT). *A. phagocytophilum* infection was assessed by specific real-time-PCR.

The overall seroprevalence of the TBE complex viruses in roe deer was 6.9% (51/736). The positive samples were primarily obtained from a known TBE endemic foci and risk areas identified in previous sentinel studies. However, new TBE complex risk areas were also identified. The overall prevalence of *A. phagocytophilum* was 94.0% (173 PCR-positive of 184 roe deer), which is twice the rate observed ten years ago.

These results point to an expansion of these tick-borne diseases geographically and within reservoir populations and, therefore, rationalize the use of sentinel models to monitor changes in transmission of tick-borne diseases and development of new risk areas. We found no association between TBE complex-positive roe deer and the prevalence of *A. phagocytophilum*, as almost all roe deer were infected. Based on our findings we encourage health care providers to be attentive to tick-borne illnesses such as TBE when treating patients with compatible symptoms.

1. Introduction

Tick-borne encephalitis virus (TBEV) is a flavivirus that is currently divided into three genetic subtypes - European (TBE-Eu), Siberian (TBE-S), and Far-Eastern (TBE-Fe) (Ecker et al., 1999). TBEV causes more than 10,000 human cases of tick-borne encephalitis (TBE) annually in Eurasia (European Centre for Disease Prevention and Control, 2018; Gritsun et al., 2003; Haglund and Günther, 2003; Süss, 2011). TBEV is closely related to the other viruses belonging to the TBE complex, e.g., louping ill virus (LIV)which is known to be a common cause of encephalitis in sheep in the British Isles. However, a limited number of humans infected with LIV have been reported (Davidson et al., 1991; Davison and Neubauer, 1948; Reid et al., 1972).

The bacterium *Anaplasma phagocytophilum,* which is also transmitted by *Ixodes* ticks, causes tick-borne fever (TBF) in ruminants and occasionally human granulocytic anaplasmosis (HGA) in humans

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https://doi.org/10.1016/j.ttbdis.2018.09.007
Received 28 April 2018; Received in revised form 30 August 2018; Accepted 13 September 2018
Available online 17 September 2018
1877-959X/

(Bakken et al., 1996; Stuen et al., 2013). *A. phagocytophilum* is known to be immunosuppressive, which permits it to modulate other co-occurring infections. Because ticks can carry numerous microorganisms and often aggregate in large numbers on the blood host, co-infections can easily occur. Animal studies suggest that co-infection with *A. phagocytophilum* and another microorganism tend to run a more severe course than infection with just one of the pathogens (Reid et al., 1986; Thomas et al., 2001). The earliest studies of this phenomenon were performed in the 1930s and were later confirmed by Reid et al. (1986). They showed that sheep dually infected with *A. phagocytophilum,* and LIV almost certainly had a fatal clinical outcome, whereas a single infection with either LIV or *A. phagocytophilum* did not (Gordon et al., 1932a, 1932b; Reid et al., 1986). It can be speculated that similar consideration is relevant in humans, especially in cases with co-occurring *A. phagocytophilum* and TBEV infection, due to the close genetic relationship of TBEV and LIV.

TBEV circulation is dependent on a complicated relationship between virus, hosts, and environmental factors. Small mammals can function both as reservoirs for microorganisms and amplifier hosts for ticks. Ticks serves as vectors but may also transmit the virus directly to uninfected ticks by co-feeding on nonviremic small mammals (Labuda et al., 1997). It is believed, that the dependency on co-feeding transmission may account for the patchy distribution of TBEV risk areas, that can be restricted to foci as small as 0.5 km^2 (Dobler et al., 2011; Kupca et al., 2010).

In the past decade, the incidence of TBE has increased rapidly in nearly all European countries where TBE is endemic, and new foci have emerged (Jaenson et al., 2012; Süss, 2011). The changes in TBEV occurrence are believed to be driven primarily by changes in the ticks' host population and climate. The distribution of TBEV has consistently shifted further north and to higher altitudes. Changes in human behavior favoring outdoor recreational activities are also thought to play a significant role (Jaenson et al., 2012; Medlock et al., 2013).

Surveillance of human TBE cases is, on its own, inadequate when trying to identify and monitor TBEV foci accurately. Instead, detection of TBEV in ticks or small rodents is crucial for confirmation of focal TBEV circulation (Süss, 2011). The prevalence of TBEV in ticks collected from endemic areas are typically less than 1%, even in areas with a TBE prevalence of ≥ 10/100,000 in humans. Hence, TBEV detection in ticks may produce false-negative results (Pettersson et al., 2014; Stefanoff et al., 2013). The highest risks of false-negative results arise from an inability to locate smaller TBEV foci, therefore, it has been suggested to use sentinel animals to monitor the distribution of TBEV (Imhoff et al., 2015). The prerequisite for being a good sentinel animal for TBEV surveillance is to have a long-lasting immunity after natural infection, to be frequently exposed to ticks, to have a well-defined home range, to be available in large numbers and to be dispersed within the surveillance area (Gerth et al., 1995; Imhoff et al., 2015). Small mammals are, as mentioned earlier, reservoirs of TBEV, and therefore serve as good sentinels (Achazi et al., 2011; Tonteri et al., 2011). Due to their relatively long-lasting viremia, they also have the potential advantage of having detectable TBEV RNA in tissue which enables molecular characterization of the virus (Achazi et al., 2011; Pintér et al., 2014). However, ethical concerns, limited home range, and difficulty of trapping and sampling small mammals make them less attractive in large-scale screening (Imhoff et al., 2015). Ungulates such as deer and sheep feed large numbers of ticks but do not support the transmission of TBEV to ticks (Randolph et al., 1999). Sentinel surveys using roe deer to estimate TBEV risk areas have been successfully performed in Austria, Belgium, Denmark, Croatia, Czech Republic and Germany. These studies show a good correlation between TBE incidence in humans and seroprevalence in ungulates (Balling et al., 2014; Borcic et al., 1990; Duscher et al., 2015; Hubalek et al., 1993; Kiffner et al., 2012; Linden et al., 2012; Skarphédinsson et al., 2005). Domestic animals such as dogs, horses, and livestock as well as wild birds have also been used as sentinels, each having particular advantages and shortcomings (Klaus et al., 2013, 2012; Lindhe et al., 2009; Mikryukova et al., 2014).

In Denmark, TBE was first reported in the late 1950s on the island of Bornholm in the Baltic Sea (Freundt, 1963). For the next five decades, Bornholm was thought to represent an isolated focus with a TBE incidence of 4/100,000 inhabitants per year (Laursen and Knudsen, 2003; Ocias et al., 2017). TBE risk areas had, however, been predicted in other parts of Denmark, e.g., north Zealand, from where human cases had not previously been reported (Randolph, 2001; Skarphédinsson et al., 2005). From 2008 to 2009 two human cases of TBE from north Zealand (Tokkekøb Hegn) were identified, and a new microfocus of TBEV was confirmed by two identical TBEV E gene sequences obtained from ticks collected in the Tokkekøb Hegn area (Fomsgaard et al., 2013; Fomsgaard et al., 2009). However, no TBE cases have been reported from this area since 2009 (Andersen et al., 2013; Ocias et al., 2017).

On the island of Bornholm TBEV and LIV are thought to co-exist. LIV was described by a 250-nucleotide long LIV-specific sequence found in ticks collected on Bornholm in 1999 (Jensen et al., 2004). This finding has so far not been confirmed in other studies. Human infection with LIV has never been reported in Denmark. However, on the island of Bornholm sheep are known to fall ill, with symptoms of encephalitis and antibodies against the TBE complex in serum. The causative agent has, however, not been further characterized (personal communication: Veterinarian Inga Stamphøj).

A. phagocytophilum has been found in multiple Danish animals, i.e., ticks and roe deer (*Capreolus capreolus*) (Michelet et al., 2014; Skarphédinsson et al., 2005). In contrast to TBEV, roe deer are considered to be the primary reservoir of certain strains of *A. phagocytophilum* (Alberdi et al., 2000). These strains, however, do not seem to be pathogenic to humans (Al-Khedery et al., 2012; Barbet et al., 2013; Jahfari et al., 2014; Stuen et al., 2013), and thus roe deer do not serve as an appropriate sentinel for clinically relevant HGA infection. Seroconversion in humans is thought to be relatively common in Denmark with a seroprevalence of about 20% in tick-exposed adults (Skarphédinsson et al., 2001). Unfortunately, *A. phagocytophilum* strain characterization and detailed clinical studies are not yet available, and the role of *A. phagocytophilum* as a human pathogen in Denmark has therefore yet to be elucidated.

In Denmark, *I. ricinus* is by far the most abundant tick species, and it is thought to represent more than 90% of all Danish ticks feeding on vertebrate hosts (Jensen et al., 2000). The tick host interaction has likely been changing in Denmark due to changes in the abundance of wildlife populations. Roe deer are the most abundant cervid, and within recent decades there has been a sizeable increase in the roe deer population to an estimated 300,000–400,000 individuals (Asferg et al., 2016; Jensen and Jespersen, 2005; Olesen et al., 2002). In Denmark roe deer serve as the primary host of adult *I. ricinus* but also feed both larva and nymphs, and therefore, an increase in roe deer abundance can boost the tick population (Andersen et al., 2018b; Jensen et al., 2000; Kiffner et al., 2011; Medlock et al., 2013; Vor et al., 2010). At the same time, people are more frequently engaging in outdoor recreational activities, which increase their tick exposure. These factors all contribute to an increased risk of human tick-borne disease (Jensen, 2012; Jensen et al., 2000). We, therefore, have an interest in continuing and intensifying the surveillance of known tick-borne pathogens in Denmark and the possible emergence of new TBE complex foci, as Denmark is surrounded by known TBE endemic countries such as Norway, Sweden, and Germany (European Centre for Disease Prevention and Control, 2018).

The aim of this study was to describe the distribution of TBE complex viruses in Denmark, to establish the possible emergence of new foci and their possible association with the distribution of *A. phagocytophilum*. To accomplish this, we performed a large-scale seroprevalence study of the TBE complex viruses TBEV and LIV using roe deer (*Capreolus capreolus*) as sentinels and determined the prevalence of *A. phagocytophilum* in Danish roe deer.

2. Materials and methods

2.1. Sample collection

Danish hunters were invited by e-mail to participate in our study during the regular hunting seasons (October-January, May–July) of 2013 and 2014. They were asked to obtain blood samples from the thoracic cavity of roe deer when field dressing the freshly bagged animals. Additionally, some roe deer were killed as part of a tracking hound operation for wounded or traffic injured game. Therefore, a small portion of the roe deer was sampled between the two hunting seasons.

Hunters were supplied with information on how to collect the blood, pre-numbered EDTA tubes (BD-vacutainer, K2E (EDTA) 7,2 mg, BD Plymouth, UK), and a questionnaire. Prepaid, addressed, and insulated return envelopes were provided.

The questionnaire required information on the location of the kill, the age of the roe deer (Fawns ≤ 11 months, Yearlings 12–23 months, Young adults 24–35 months and Adults ≥ 36 months), sex, and tick infestation levels (< 10 ticks, 10–50 ticks, > 50 ticks). No information on tick development stage or species was obtained.

2.2. Serological test

Upon the arrival of the blood samples at the laboratory plasma was retrieved and frozen at – 80 °C. The samples were examined for TBEV-specific antibodies by TBEV neutralization tests (NT) at the Center for Virology, Medical University of Vienna, Austria. NT was carried out in microtiter plates using baby hamster kidney cells (ATCC BHK-21). Twofold serial dilutions of polyclonal sera were mixed with 25 plaque-forming units (PFU) of TBEV strain Neudoerfl (starting dilution of the serum in the mixture, 1:10) and incubated for 1 h at 37 °C. BHK cells were added, and incubation was continued for three days. Four-layer enzyme-linked immunosorbent assay (ELISA) determined the presence of virus in the supernatant. The virus neutralization titer was defined as the reciprocal of the serum dilution that gave a 90% reduction in the absorbance readout in the assay compared to the control without antibody. NT titers ≥10 were considered positive (Stiasny et al., 2009).

Due to cross-neutralization within the TBE complex, it is not possible to separate TBEV from LIV in the NT, despite this being the gold standard of TBE analysis (Calisher et al., 1989). For this reason, the term TBE complex viruses are used hereafter for NT antibodies against TBEV and/or LIV in this study.

2.3. Nucleic acid extraction

Nucleic acids from a selected group of roe deer plasma were purified using “DNA and Viral NA Large Volume” kit (#06374891001; Roche) (Input:500 µl, Output:100 µl) and protocol name “Pathogen Universal 500″ on the MagNA Pure 96 Instrument (#06541089901; Roche).

2.4. A. phagocytophilum polymerase chain reaction (PCR)

The presence of *A. phagocytophilum* DNA was determined by real-time PCR using the primers AnaF (5′-ATGGAAGGTAGTGTTGGTTATG GTATT-3′), AnaR (5′-TTGGTCTTGAAGCGCTCGTA-3′), and the probe AnaP (5′-FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-BHQ1-3′) sequences generating a 77-bp fragment of the msp2 gene specific for *A. phagocytophilum* as described by Courtney et al. (2004) PCR was performed in a 384-well PCR plate format with a reaction volume of 15 µl using LightCycler® 480 Probes Master (#04887301001; Roche) and a primer and probe concentration of 800 nM and 2400 nM, respectively, and 5 µl template. The PCR was performed in a LightCycler® 480 Instrument System II (Roche) with one cycle of pre-incubation at 95 °C for 10 min followed by 45 cycles of DNA denaturation at 95 °C for 10 s, primer annealing and DNA elongation at 60 °C for 30 s and 72 °C for 1 s, and one cycle of cooling at 40 °C for 30 s.

2.5. Data management

The geographical location of bagged roe deer was reported using GPS-coordinates. Original data on bagged roe deer from the study by Skarphédinsson et al. (2005) were retrieved, and the GPS-coordinates were registered. Results were plotted using the geographical information system software ArcGIS version 10.3 by Esri (Environmental Systems Research Institute, California, USA).

2.6. Statistical analysis

Data were analyzed using STATA 14.2 (StataCorp LP, College Station, Texas, USA). Pearson’s chi-square was used to establish if there was an association between TBE complex virus NT results or *A. phagocytophilum* infection and potential risk factors such as gender, age, hunting season or tick infestation, but also between TBE complex virus NT results and *A. phagocytophilum* prevalence. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Roe deer study population

In total 840 blood samples from roe deer were collected. 60 samples were immediately discarded, either due to the poor quality of the blood or missing data. Of the remaining 780 samples, 360 were from bucks (46.2%), and 420 from does (53.8%). In the autumn/winter hunting season, from October 2013 to January 2014, 578 samples (74.1%) were collected, while 188 samples (24.1%) were collected in the spring/summer hunting season from May to July 2014. The remaining 14 samples (2%) were collected between the two seasons. Tracking hound operation for wounded or traffic injured game supplied 53 (7%) samples.

Roe deer heavily infested with ticks (more than 50 feeding ticks per deer) was found in 18 samples (2%). Of these, 14 roe deer (74%) were bagged during the summer hunt. Detailed information on tick infestation is stated in Table 1. The original observations can be obtained from Andersen et al., 2018a.

3.2. Prevalence of TBE complex virus neutralizing antibodies

Of the 780 analyzed samples, 51 were positive, and 685 were negative for antibodies against the TBE complex, giving a national seroprevalence of 6.9%. Due to the toxic effect of the serum on the cells in the NT, 44 samples could not be analyzed. The highest seroprevalence of 8.8% (30/341) - was found in the young adults (age: 24–35 months), and the lowest 4% (3/72) was found in adults (age ≥ 36 months). However, there was no association between TBE complex seroprevalence and the age of the roe deer, χ^2 (3, n = 736) = 4.29, p = 0.23. Similarly, no association was found for the sex of the roe deer χ^2 (1, n = 736) = 0.16, p = 0.69, the hunting season in which the roe deer was bagged χ^2 (2, n = 736) = 3.85, p = 0.15, or the observed tick infestation level, χ^2 (2, n = 736) = 1.84, p = 0.4. Since the fawns had less than one year to develop antibodies, they could skew the analysis, but we found the same lack of association when the fawns (≤ 11 months) were excluded from the calculation (data not shown). The positive NT titer values differed between location and roe deer. Four roe deer samples scored the cut-off titer 10, while 45 samples scored a titer between 15 and 160 (titer 15: n = 7; titer 20: n = 17; titer 30: n = 3; titer 40: n = 11; titer 60: n = 2; titer 120: n = 4; titer 160: n = 1). Two of the roe deer samples had extremely high NT titers of 640 and 940 respectively (Fig. 1).

Table 1
Tick infestation, TBE complex virus antibody prevalence and *Anaplasma phagocytophilum* prevalence in Danish roe deer, grouped by age, 2013–2014.

		Tick Infestation[b]			TBEV NT	*Anaplasma* PCR
Age group	n[a] (%)	< 10 (%)	10–50 (%)	> 50 (%)	Positive (%)	Positive (%) (n = 184)
Fawns (≤ 11 mo)	177 (24.0)	153 (86.4)	23 (13)	1 (0.6)	8 (4.5)	37/39 (95)
Yearlings (12–23 mo)	146 (19.8)	106 (72.6)	38 (26.0)	2 (1.4)	10 (6.8)	33/35 (94)
Young adults (24–35 mo)	341 (46.3)	227 (66.6)	100 (29.3)	14 (4.1)	30 (8.8)	96/99 (97)
Adults (≥ 36 mo)	72 (9.8)	48 (67)	23 (32)	1 (1)	3 (4)	7/11 (64)
All age groups	736	534 (72.6)	184 (25.0)	18 (2.4)	51 (6.9)	173/184 (94.0)

PCR = Polymerase chain reaction.
[a] Of the 780 eligible samples, 44 samples giving a toxic reaction in the TBE neutralization test are excluded from this table.
[b] < 10, 10–50 and > 50 equals the number of feeding ticks observed on the roe deer. There are no available data on tick development stage or species.

3.3. The geographical distribution of roe deer with neutralizing antibodies against TBEV

The NT-seropositive roe deer were primarily distributed along the coast of all major islands and mainland Denmark (Fig. 1). On the TBEV-endemic island of Bornholm (area 1, Fig. 1) 39% (7/18) of the roe deer were NT-seropositive with titers ranging from 10 to 160.

The highest local seroprevalence of TBE complex viruses and the highest NT-titers originated from an area on the island of Falster known as Bøtø plantation (area 2, Fig. 1), where 89% (8/9) of roe deer were NT-seropositive, with titers ranging from 20 to 960.

At other locations, the NT-seropositive roe deer were geographically further apart, but some locations had more than one NT-seropositive roe deer within a distinct smaller area. On the north-eastern coast of Jutland 2/11 roe deer were NT-seropositive (Mariagerfjord area 3, Fig. 1). West of this location inland, but still with direct contact with the sea via Limfjorden, 2/5 roe deer were NT-seropositive (Højslev area 4, Fig. 1). On the east coast of Jutland, alongside Vejlefjord, 2/4 roe deer were NT-seropositive (Daugård, area 5, Fig. 1). In all these distinct smaller areas, the highest NT-titer was 40.

The identified TBE complex risk areas appear to be overlapping with previous assessments performed in 2002/2003 (Fig. 2). New risk areas were also identified, as illustrated from the comparative map of the geographical distribution of NT-seropositive roe deer bagged during the hunting seasons (May-July, October-January) of 2002/03 (Skarphédinsson et al., 2005) (Fig. 2).

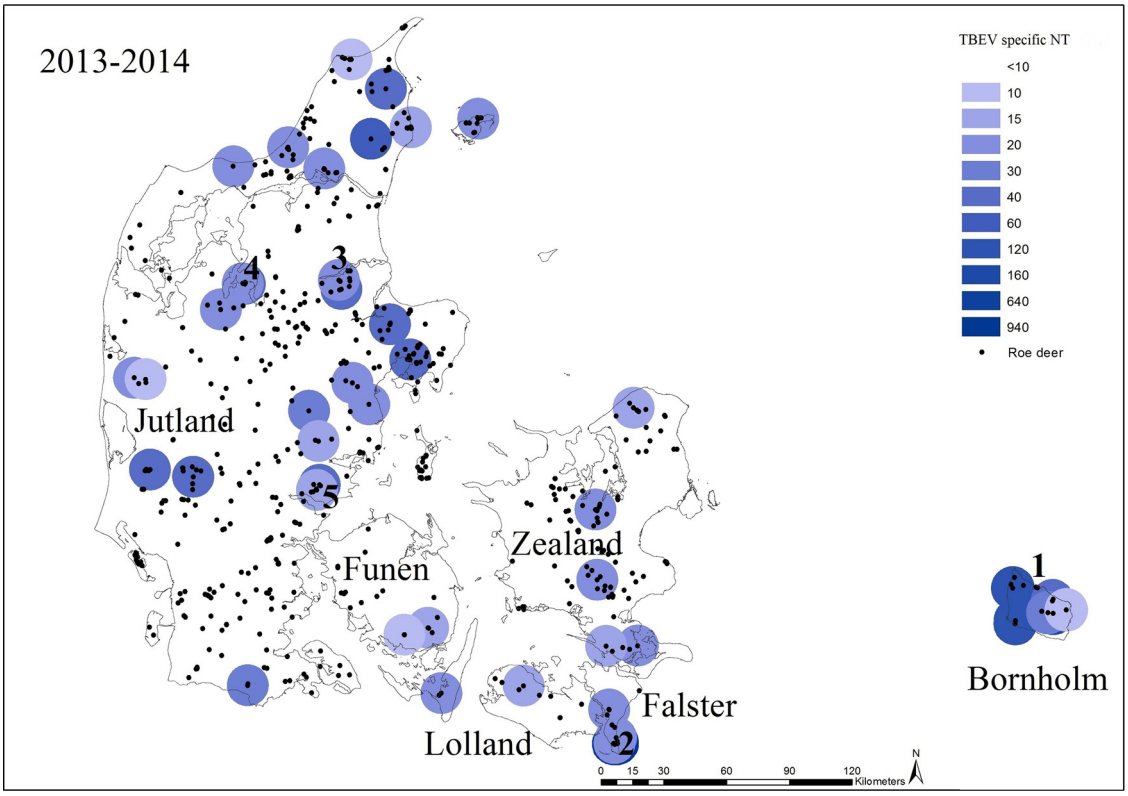

Fig. 1. The geographical distribution of Danish roe deer (*C. capreolus*) with neutralizing antibodies against tick-borne encephalitis virus.2013–2014.
Geographical distribution by GPS-coordinates of Tick-borne encephalitis virus (TBEV/LIV) antibody positive and negative roe deer, in Denmark, based on virus neutralization test (NT) (Stiasny et al., 2009). The black dots represent the location of the bagged roe deer. The blue shaded areas represent NT-seropositive roe deer and its corresponding titer with a 10 km buffer radius, reflecting the possible maximum migrating range of the roe deer (Strandgaard, 1972; Wahlström and Liberg, 1995). Area: 1) Bornholm; 2) Bøtø Plantation, Falster; 3) Mariagerfjord, Jutland; 4) Højslev, Jutland and 5) Daugård, Jutland. The test was carried out at the Department of Virology, Medical University Vienna, Austria.

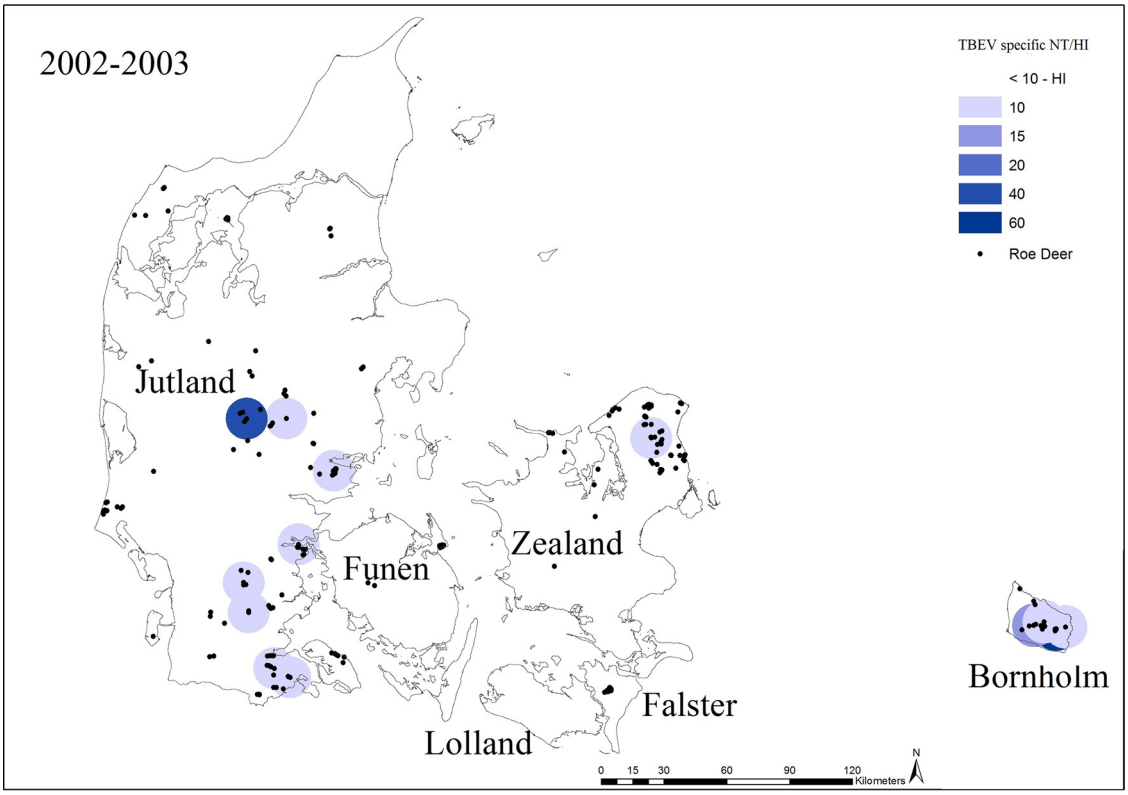

Fig. 2. The geographical distribution of Danish roe deer (*C. capreolus*) with neutralizing antibodies against tick-borne encephalitis virus, 2002.
Geographical distribution by GPS-coordinates of tick-borne encephalitis virus complex virus (TBEV/LIV) antibody positive and negative roe deer, in Denmark, based on virus neutralization test (NT). The results originate from the study by Skarphédinsson et al. (2005). The black dots represent the locations of the bagged roe deer. The blue shaded areas represent NT-seropositive roe deer and its corresponding titer with a 10 km buffer radius, reflecting the possible maximum migrating range of the roe deer (Strandgaard, 1972; Wahlström and Liberg, 1995). In this study, 229 roe deer samples were first screened for TBE complex antibodies by hemagglutinin inhibition test (HI) finding 20 HI-seropositive samples. The HI-seropositive samples were verified by TBEV-specific NT. Of these samples, 14 (6%) were NT-seropositive. The remaining six HI-seropositive samples could not undergo NT, either due to a toxic effect of the serum on the cells, or lack of additional material, and were therefore excluded from this illustration. The HI and NT were carried out at the Department of Virology, Medical University Vienna, Austria.

3.4. Prevalence of *A. phagocytophilum*

Of the 736 samples successfully analyzed for TBE complex virus by NT, 184 samples (25%) were chosen for *A. phagocytophilum* PCR. Samples from 39 TBEV NT-seropositive roe deer and 145 TBEV NT-seronegative roe deer from areas surrounding one of the positive roe deer samples were selected (Fig. 3). Overall 94.0% (173/184) of the roe deer samples were *A. phagocytophilum* positive by PCR. Of the TBEV NT-seropositive roe deer samples, 97% (38/39) was *A. phagocytophilum* PCR-positive. Only 93.1% (135/145) of the TBEV NT-seronegative roe deer samples were *A. phagocytophilum* PCR-positive. However, we did not find statistically significant difference between TBE complex NT-results and *A. phagocytophilum* prevalence, χ^2 (1, n = 184) = 1.03, p = 0.31. A negative association was found between *A. phagocytophilum,* PCR-positive roe deer, and the age of the roe deer, χ^2 (3, n = 184) = 19.65, p < 0.001 (Pearson's Chi-square of independence) (Table 1). No association was found between PCR results and roe deer sex χ^2 (1, n = 184) = 2.36, p = 0.12, the hunting season in which the roe deer were bagged χ^2 (2, n = 184) = 2.85, p = 0.092, or tick infestation level χ^2 (2, n = 184) = 3.70, p = 0.16.

4. Discussion

4.1. The TBE complex

Our study assessed the seroprevalence of virus neutralizing antibodies against TBE complex viruses in a cross-section of Danish roe deer to be 6.9% during the hunting seasons between 2013 and 2014. In a similar study by Skarphédinsson et al. (2005), the seroprevalence was 6.1% during the hunting season of 2002, and hence the TBE complex seroprevalence in roe deer over the last decade appears to have been stable. The sample size in this study is superior to any previous Danish study and so is the geographical distribution of the roe deer (Freundt, 1963; Skarphédinsson et al., 2005). Given the noted geographical variation in TBEV distribution, this study also appears to be a more suitable reference for the distribution of the TBE complex viruses in Denmark. Our study confirms known TBEV and LIV endemic areas on Bornholm and to a lesser degree north Zealand and agrees with TBE complex risk areas previously suggested by sentinel models on the eastern and southern coast of Jutland (Skarphédinsson et al., 2005). We show an expansion in the geographical range with new possible foci in northern-, central- and western Jutland, the south-eastern part of Funen, south- and central Zealand and Lolland and Falster (Fig. 1). All these locations correspond well to the TBEV areas predicted by Randolph (2001) using remotely sensed environmental variables and spatial statistics.

We found TBE complex neutralizing antibodies in 39% (7/18) of the roe deer on the TBE endemic island of Bornholm (area 1, Fig. 1) compared to 28% (5/18) in the year 2002/03 and 83% (24/29) between 1958 and 1962 (Freundt, 1963; Skarphédinsson et al., 2005). This indicates a small increase during the last decade, while at the same time it could be considered that the present seroprevalences in roe deer may be lower than 60 years ago. However, we consider the sample size too small for any definitive analysis. NT-techniques were used in all three studies but with different protocols (Skarphédinsson et al., 2005; Freundt, 1963). Whether this is in part the reason for the differences in

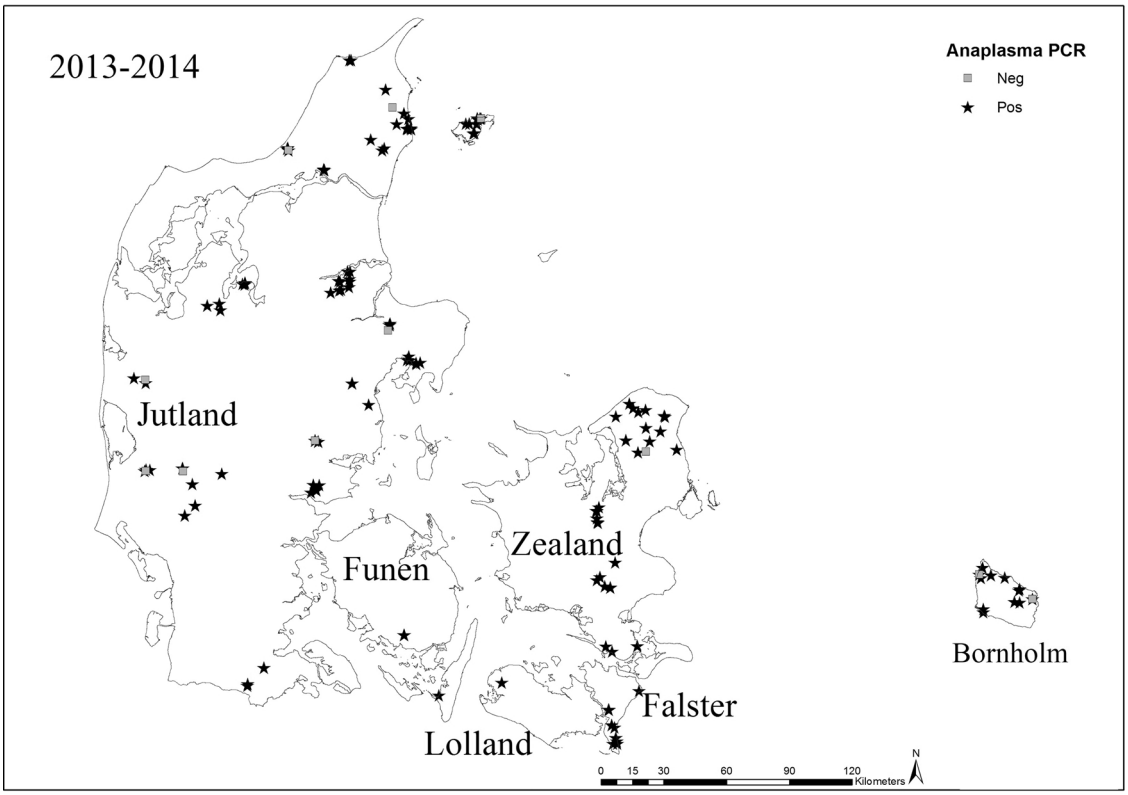

Fig. 3. Geographical distribution of *A. phagocytophilum* in Danish roe deer (*C. capreolus*), 2013–2014 Geographical distribution by GPS-coordinates of roe deer tested for the presence of *A. phagocytophilum* by real-time PCR as described by Courtney et al. (2004). Black stars represent roe deer where real-time PCR could detect *A. phagocytophilum* in the blood and grey squares represent roe deer tested negative.

prevalence is unknown. Finally, it should be noted that the human TBE incidence on Bornholm has been stable around 4/100,000 in recent decades (Fomsgaard et al., 2013; Ocias et al., 2017). It was not possible to obtain samples from the small TBE-endemic area of Tokkekøb Hegn, North Zealand. The whole of North Zealand was represented by only 17 roe deer, of which only one was found positive. The positive sample was retrieved approximately 35 km from Tokkekøb Hegn.

Our results are also in good agreement with similar European sentinel studies from TBE endemic and non-endemic areas. In Hesse, Germany, the TBE incidence in humans is between 0,5-2,5/100.000 inhabitants. Kiffer et al. (2012) reported the overall seroprevalence of TBEV antibodies in roe deer from this area to be 22.9% (24/105), with considerable variations between collection sites (15–50 %). In a cross-sectional Austrian study by Duscher et al. (2015), 2.4% (22/945) of roe deer had antibodies against TBEV, whereas 1.8% were borderline positive. In both studies, a majority of the positive and borderline positive roe deer were from areas known to be TBE endemic, whereas areas with seropositive roe deer, where TBE has not been described in humans, were considered possible risk areas (Duscher et al., 2015; Kiffner et al., 2012). The same consideration is appropriate for our study, as we have considerably more NT-seropositive roe deer on the island of Bornholm compared to the rest of Denmark.

Areas containing more than one seropositive roe deer and areas overlapping previously described risk areas are of particular interest because they are more likely to represent a true endemic area.

In our study, Bøtø plantation (area 2, Fig. 1) was the only area outside of the island of Bornholm with a large group of TBEV NT-seropositive roe deer. The seroprevalence was 89% (8/9), with NT-titers exceeding the ones obtained for NT-seropositive roe deer on Bornholm, the highest titer being 960. These findings increase the probability of this area being a true risk area. TBE endemic areas exist on the opposite shores of this site; to the north-east: Sweden and Bornholm, and to the South: Germany (Fomsgaard et al., 2013; Frimmel et al., 2014; Jaenson et al., 2012). Area 3 (Mariagerfjord), area 4 (Højslev) and area 5 (Daugård) (Fig. 1) had more than one NT-seropositive roe deer within a distinct smaller area and are therefore also more likely to represent a true risk area.

The possible TBE complex risk areas identified in this study are located near the sea, along fjords or on islands. Except for one NT-seropositive roe deer found in the central part of Jutland, there does not seem to be any other NT-seropositive roe deer when traveling approximately 30–50 km inland. This is especially noticeable on the central ridge of Jutland running inland from north to south. We do not have an exact explanation for this, but it conforms with the hypothesis that the long distance spreading of TBEV is driven by migrating birds, since these coastal areas may serve as a resting area and winter location for migrating birds (Weidmann et al., 2013).

This being a sentinel serosurvey, there were limitations. The antibodies within the TBE complex are expected to last a lifetime (Gerth et al., 1995), providing a stable account of past events. However, it is possible that young roe deer become infected in one area, then migrate and settle in a different area (Strandgaard, 1972). This could give a false indication of a possible focus, especially in areas with just one positive roe deer sample (Klaus et al., 2014). In contrast to this interpretational challenge, other methodological challenges are less relevant. Viruses of different serocomplexes, such as the Japanese encephalitis complex, usually do not cross-neutralize members of the TBE complex (Calisher et al., 1989), whereas cross-reactivity is measurable in enzyme-linked immunosorbent assay (ELISA) and hemagglutination inhibition assay (HI) (Holzmann et al., 1996; Stiasny et al., 2013). However, there is some cross-neutralization within the TBE complex (Calisher et al., 1989; Klaus et al., 2014; Orlinger et al., 2011). It is not known to what extent LIV is present in ticks outside of the island of Bornholm, and hence we must acknowledge that the serological findings might reflect both TBEV and LIV. It follows that the interpretation of possible human risk areas requires further critical review. It is,

therefore, necessary to identify the specific species of TBE complex viruses circulating in the possible risk areas given in this study, e.g., in ticks or small mammals to assess the viruses' pathogenicity for humans. Identification of patients, outside of the island of Bornholm, with symptoms of TBE and antibodies against TBE complex viruses, is also an essential next step in future studies of the TBE complex in Denmark.

4.2. *Anaplasma phagocytophilum*

Our study also assessed the prevalence of *A. phagocytophilum* in a selected group of roe deer (n = 184). In this group, 39 samples were TBEV NT-seropositive; the remaining 145 samples were chosen from the area surrounding these 39 positive roe deer samples (Fig. 3). We found an overall *A. phagocytophilum* prevalence of 94%. This represents approximately twice the rate observed a decade ago (Skarphédinsson et al., 2005). It does, however, correspond well with a recent study from central Germany by Kauffmann et al. (2017), finding an overall prevalence of 96.1% (Kauffmann et al., 2017). We did not find a significant seasonal variation in the prevalence of *A. phagocytophilum* as previously described in Denmark (Skarphédinsson et al., 2005). This might be due to the rise in prevalence among roe deer. We found a negative association between roe deer age and *A. phagocytophilum* prevalence ($p < 0.001$). When considering the high prevalence, it is possible that adult roe deer that tested negative in this study included animals that had successfully recovered from previous infections. The strain variation within *A. phagocytophilum* is considerable, and evidence points to a difference in host tropism and virulence between strains e.g., strains circulating in deer do not seem to be the same as those pathogenic for humans. For this reason, the presence of *A. phagocytophilum* in roe deer cannot be translated directly into a potential risk of human HGA infection (Al-Khedery et al., 2012; Barbet et al., 2013; Jahfari et al., 2014; Stuen et al., 2013). Phylogenetic characterization of the circulating *A. phagocytophilum* from different hosts are necessary to elucidate the risk of HGA in humans in Denmark.

Finally, we did not find a statistically significant association between roe deer TBE complex seroprevalence and roe deer infected with *A. phagocytophilum*. This can be explained by the fact that almost all roe deer in our analysis were infected with *A. phagocytophilum*.

5. Conclusions

Previous studies have found a change in the distribution of TBE complex infections in northern Europe with new risk areas emerging. In this study, we found antibodies against TBE complex viruses in roe deer from the known Danish TBE foci of Bornholm, verified areas previously suggested as foci of TBE complex viruses and identified new potential risk areas, thus documenting the geographical expansion of TBE complex viruses in Denmark. Of particular interest is one new area on the island of Falster where 8/9 roe deer samples within the same small geographical area had TBEV-specific neutralizing antibodies. All the above indicate the existence of Danish TBE complex risk areas outside of Bornholm and rationalize the use of sentinel models to monitor the distribution and expansion of risk areas. To confirm whether TBEV or LIV is circulating in the areas found in our study and to distinguish between the two, it is pivotal that future studies isolate TBEV and/or LIV from ticks or small rodents and identify human TBEV or LIV infection from the afflicted areas.

We assessed the prevalence of *A. phagocytophilum* to be 94.0% in roe deer. This is a doubling since 2002, which documents the expansion of the bacteria within its reservoir population. This, however, cannot provide information on the risk of human HGA infection, as different strains infect roe deer than those causing HGA in humans. Still, we encourage local healthcare providers to be more attentive of tick-borne illnesses especially TBEV infection, when treating patients with compatible illnesses, not only in previously identified risk areas but also in the new risk areas identified in this study.

Declaration of interests

The authors declare that they have no competing interests.

Funding

This study received support from the following Danish organizations: 15. Juni Fonden [grant number 2014-B-93], Læge Else Poulsens Mindelegat, the Region of Southern Denmark [grant number 11/6324], Odense University Hospital and the University of Southern Denmark. The sponsors did not have any role in the study design, collection, analysis, and interpretation of data, in the writing process or in the decision to submit the article for publication.

Acknowledgments

We are grateful to the Danish Hunters' Association and Danske Herregaardsjægere (the Danish manor house hunters) as well as all participating hunters for delivering roe deer samples. We acknowledge F.X. Heinz from the Center for Virology, Medical University Vienna, Austria, for facilitating the NT analysis. S. Stuen from the Section for Small Ruminant Research at the Norwegian University of Life Sciences for positive control material in the testing of *A. phagocytophilum*. We are thankful to S. Behnke from the Danish Hunters Association for his help with the GIS-map.

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