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Species identification of ticks collected in Skåne county and their role in the epidemiology of Tick-borne Encephalitis (TBEV)

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Artbestämning av fästingar från Skåne och deras roll i epidemiologin av fästingburen encefalit virus (Tick-Borne Encephalitis Virus, TBEV)

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Abstract

Skåne is the southernmost county of Sweden where new tick species could become established due to the topography with milder climatic conditions and the geographical bordering with northern continental Europe. Ticks also play an important role as vectors and transmitter of tick-borne encephalitis, a viral disease caused by tick-borne encephalitis virus (TBEV), for which the incidence of human cases increased twice during 2011-2013 and 2014-2017 in Skåne county. In this study ticks (n=1000), either collected from five different host species (domestic dog, domestic cat, roe deer, fallow deer and moose) or questing ticks collected by flagging method during year 2011-2016 were identified at species level using morphological keys. The result showed that all but one ticks (n=999) were identified as common hard tick species *Ixodes ricinus* (adult females; n=613, adult males; n=190 and nymphs; n=196) and one tick found on one individual domestic cat (*Felis catus*) was identified as an adult female of *Ixodes hexagonus*. In order to assess if TBEV has been circulating in Skåne during the years of tick collection, the study also investigated the occurrence of TBEV in the same ticks samples using Real-Time PCR. The result showed that no TBEV positive ticks were found in the analyzed specimens.

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Introduction

Ticks in Sweden

Ticks are hematophagous ectoparasites of most vertebrates that are widely distributed around the world. Ticks are currently regarded as the second most important arthropod vectors of several human and animal disease pathogens including bacteria, viruses and protozoa after mosquitoes (de la Fuente et al., 2017; Parola et al., 2013; Parola and Raoult, 2001). Around nine hundred tick species are presently identified and classified worldwide (Barker and Murrell, 2004). In northern Europe, the majority of tick species belongs to the *Ixodidae* or hard tick family, and the minority belong to *Argasidae* or soft tick family. The Swedish tick fauna have been investigated by several studies since 1952 (i.e., Arthur, 1952; Brinck et al., 1967; Nilsson, 1988) and about fourteen tick species have been recorded regarding their host relationships and geographic distribution in 1994 (Jaenson et al., 1994). In Sweden, several tick species were regarded as permanently present and classified following their host species. Firstly, the ornithophagous ticks are *Ixodes uriae*, *I. arboricola*, *I. caledonicus*, *I. lividus* and *I. unicavatus*. Secondly, the mammalophagous ticks are *I. canisuga*, *I. hexagonus, I. trianguliceps* and *Argas vespertilionis*. Finally, the tick species feeding on both mammals and birds are *I. ricinus* and *Haemaphysalis punctata*. Other tick species are considered as occasionally present in Sweden, as *Rhipicephalus sanguineus* are introduced by imported dogs, *Hyalomma marginatum* and *I. persulcatus* are introduced by birds during their spring migration. Moreover, several non-indigenous tick species can be transported to and present in Sweden from imported exotic zoo animals and pets (Jaenson et al., 1994).

The most common tick species present in southern-central Sweden and along the northern coastal area of Baltic Sea is *I. ricinus*. During the last three decades *I. ricinus* has become more abundant in southern and central Sweden, and has gradually dispersed its range expansion northwards due to various factors. Climate change is one of the factors, since milder winters, warmer climate and extended vegetation period increase the possibility of both the tick and its sustenance hosts survival, proliferation and distribution. Moreover, the higher population density of available tick sustenance hosts, i.e., roe deer (*Capreolus capreolus*), moose (*Alces alces*), fallow deer (*Dama dama*), also support the tick expansion and its incremental (T. G. Jaenson et al., 2012).

In Skåne area, around eight tick species are recorded; *I. ricinus* has been recorded from a wide range of host animals that seems to include nearly all mammals and more than fifty bird species living in the area. *I. arboricola* has been recorded from any bird species while *I. lividus* has been recorded only from the sand martin (*Riparia riparia*) or other bird species using the nests of the sand martin. *H. marginatum* was found on five bird species as the tree pipit (*Anthus trivlalis*), the white wagtail (*Motacilla alba*), the sedge warbler (*Acrocephalus schoenobaenus*), the Eurasian blackcap (*Sylvia atricapilla*) and the collared flycatcher (*Ficedula albicollis*). *I. trianguliceps* has been found on eleven rodent and shrew species. *A. vespertilionis* specific host are bats and it has been found on two bat species. Lastly, *I. canisuga* and *I. hexagonus* have been reported from a variety of wild and domestic mammals i.e., *Capreolus capreolus*, *Alces alces*, *Dama dama,* domestic dogs *(Canis lupus familiaris)* and domestic cats (*Felis catus*) (Hillyard, 1996; Jaenson et al., 1994).

Tick-borne encephalitis in Sweden and in Skåne county

One of the most important medical pathogens transmitted by *Ixodes* ticks is the TBE virus (TBEV) causing tick-borne encephalitis (TBE) in human. The virus is a single-stranded RNA virus belonging to the family *Flaviviridae* that is causing no clinical sign or mild illness symptom in its natural hosts. Humans serve as accidental hosts and can get a severe or lethal virus infection of the central nervous system (Belova et al., 2012). The TBEV infected ticks can be lifelong virus carriers. The virus enters all tick tissues and can be transovarially and transstadially transmitted within ticks (Dobler et al., 2018; Danielová et al., 2010; Nuttall et al., 1994; Nosek and Grulich, 1967). The virus transmission relies on the interactions between virus, ticks and the tick's vertebrate hosts. The infected ticks can transmit TBEV to non-infected ticks by co-feeding on the same host animal, the tick feeding site cells migration and cellular infiltration may provide a vehicle which support transmission that independent of the host viremia (Karbowiak and Biernat, 2016; Goodman et al., 2005; Nuttall and Labuda, 2003; Norman et al., 1999; Labuda et al., 1996). Ticks are both vectors and reservoirs of TBEV.

The ticks' egg production, cycle of development, distribution and population density are supported and accelerated by the increasing of temperature due to climate change in Europe (Semenza and Menne, 2009). In Europe, a minimum of 7,200 TBE cases in human has been reported during year 2010 (Süss, 2011). While in Sweden, the number of TBE cases in humans per year almost doubled from 63 to 184 cases from 1985-1999 to 2007-2010. Moreover, 284 TBE cases in human were registered in 2011 (Jaenson et al., 2012). There are three subtypes of TBEV (EU, Eastern, Far-Eastern), characterized by different geographical distribution, different tick vector species and different degrees of pathogenicity (Far-Eastern being the most pathogenic subtype (Dobler et al., 2018). In Sweden, *I. ricinus* has been found spreading the European (EU) subtype of TBEV (Golovljova et al., 2004).

Skåne is the most southern part of Sweden with approximately 1.2 million population. One TBE case had been diagnosed in 1998 and two cases in 2000. In 2001, three TBE cases of people living in northeastern Skåne was diagnosed and treated at the Central hospital in Kristianstad. After that, this area was identified as the first risk area for TBE in Skåne and reported in the Swedish magazine for physicians "Läkartidningen" in 2002 (Burenhult et al., 2002). One retrospective study investigated if TBE had been missed among 29 patients treated for meningoencephalitis of unknown etiology at Central hospital in Kristianstad during 1997-2000. Only one case turned out to be positive for TBE but had most probably been infected in Stockholm archipelago, a high risk area for TBE since the 1950:s (Haglund, 2003). Recent studies supported the hypothesis that TBE was introduced to Skåne at the beginning of the millennium and successively spread to new areas in Skåne (unpublish studies).

The first TBE case in southeastern part of Skåne had been diagnosed in 2006 and the second case in 2008 which makes this a risk area for TBE. The first TBE case in western part of Skåne had been diagnosed in 2010. Moreover, several TBE cases had been diagnosed in central part of Skåne in 2011 and 2012. Apparently, from 2000-2018 most of the cases have been in the eastern and specially in the northeastern part of Skåne. In 2018, two TBE cases had been diagnosed from the same area in western Skåne and two more cases had been diagnosed in eastern Skåne (Figure 1.). This makes these two areas, western and eastern Skåne, defined as new risk areas for TBE since at least two cases of TBE transmission had occurred.

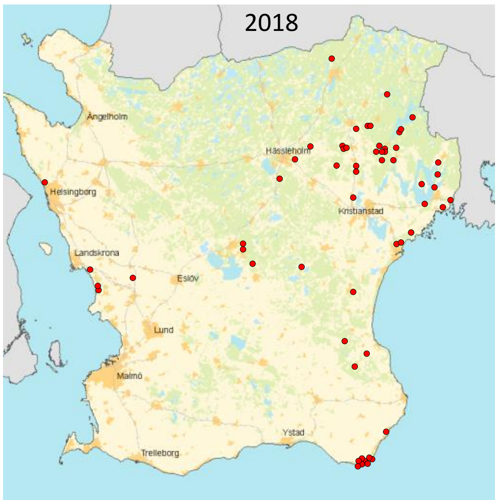

Figure 1. The accumulated number of TBE cases infected map in Skåne (2000-2018). Each dot representing the most probable place where the disease was contracted. The red-areas are considered as TBE risk areas and vaccination are recommended for residents and frequent visitors in these areas. (source: Mattias Waldeck)

In Skåne, the incidence of TBE is around 0.5-0.7 cases per 100,000 inhabitants. Concerning the whole population (1.2 millions), the incident is rather low but considerably higher while concerning a single municipality where a case has occurred. There was a rising trend of TBE infected cases incidences from 1998 to 2018 in Skåne county (Figure 2). In 2010, around 500 questing ticks were field collected from the southeastern hotspot and 200 from north eastern part of Skåne but no positive sample for TBEV was found (unpublished data) but the prevalence of TBEV in tick species in Sweden is rather low, in *I. ricinus*: 0.23%-4.48% (Pettersson et al., 2014). However, the climate change is not a conclusive factor that can explain the increasing incidence of TBE cases in Sweden, but ticks had been increasing in numbers and spread progressively during these climate change scenarios (Semenza and Menne, 2009).

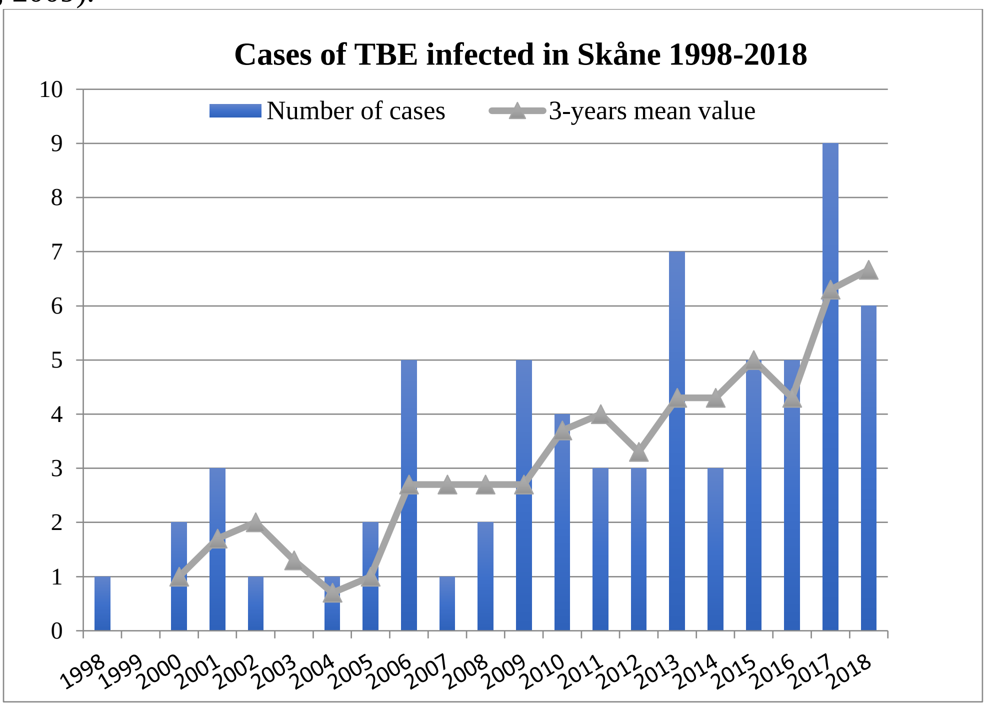

Figure 2. Number of TBE infected cases in Skåne 1998-2018 (source: Mattias Waldeck)

Climate change has already affected several natural systems because of the increasing world surface temperature. The terrestrial ecosystems are included which would affect the distribution of plant and animal species (Dantas-Torres, 2015). Skåne county is a geographic area bordering with northern continental Europe where new species could become established due to the milder climatic conditions. This study aims to provide updated information on tick populations infesting wild and domestic animals in Skåne county both in terms of species composition (using morphological taxonomic keys) and in terms of their role of reservoirs for TBEV in this geographic region. This latter information can be used for the improvement of TBEV risk maps in Skåne county.

Materials and methods

Tick collection

A thousand tick samples were collected between 2011 to 2016 in Skåne area, the southernmost part of Sweden. Fifty-tree ticks were detached and collected from privately owned dogs and cats in Kristianstad area while the majority of them (669 ticks) were detached and collected from hunted roe deer (plus other wild ruminants, i.e. fallow deer and moose) at slaughterhouses, the sample distribution and collecting area of the sample described in detail in supplementary material (*Table S1., Figure S1., Figure S2.*). Two-hundred-seventy-eight ticks were collected by flagging in areas where endemic cases of TBEV had been recorded in 2011-2012.

Table 1. Summary of ticks collected in Skåne

Collecting year	Host species/ flagged	Females (n=614)	Males (n=190)	Nymphs (n=196)
August 2014	Roe deer (*Capreolus capreolus*)	458	123	-
November 2011	Fallow deer (*Dama dama*)	64	16	-
November 2011	Moose (*Alces alces*)	5	3	-
August 2014- May 2016	Dog *(Canis lupus familiaris)*	42	-	-
May 2015	Cat (*Felis catus*)	7	4	-
May 2011- May 2012	Flagged	38	44	196

Tick morphological identification

All ticks were collected and preserved individually in 2 ml tube, -80°C until morphological identification started. The identification was performed under a stereomicroscope (Leica MZ16, Leica Microsystems, Stockholm, Sweden) with up to x200 of magnifications together with illustrations and morphological taxonomic keys (Arthur, 1963; Estrada-Peña et al., 2018; Filippova, 1977; Hillyard, 1996; Manilla, 1998) which provided morphological characteristics and descriptions of all the species potentially occurring in the study area. Tick identification was performed by the student and was confirmed by the supervisor afterwards.

Tick photo and measurement

All female ticks' estimated feeding duration were calculated from the scutal index (SI) and/or the coxal index (CI) following Gray et al., 2005. The measurements were performed using DinoCapture® program with accurate adjustment of specific magnification calibration with a USB-digital microscope (Dino-Lite pro AM413TL, AnMoElectronics Corp., Taiwan) under a magnification of up to 40x; at the same time the photos were captured from dorsal and ventral view. The maximal scutal width, maximal idiosoma length and inter 4th coxal gap length were measured (Figure 3) and recorded to a Microsoft Excel file.

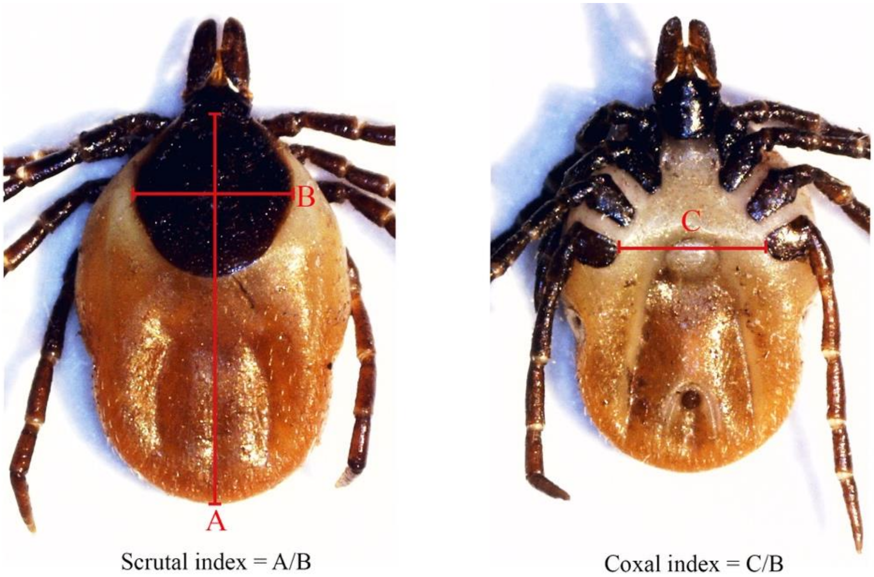

Figure 3. Tick measuring model for calculating scrutal index (SI) and coxal index (CI) in result of estimating feeding time.

Estimated feeding time analyzation

The estimated feeding time of adult female ticks was calculated from scutal index (SI) and coxal index (CI) using regressing equations following Gray et al., 2005 (Table 2.).

Table 2. Regression equations for feeding time estimation

Index	Estimated feeding time (*t*)
Female scutal index	$t = 74.49\left(\frac{SI-1.875}{4.175-SI}\right)^{0.235}$
Female coxal index	$t = 109.96\left(\frac{CI-0.409}{2.909-CI}\right)^{0.515}$

SI = measured scutal index, CI = measured coxal index.

The coxal index gives a more accurate estimate of feeding time for ticks that had duration of feeding time up to 24 hours and the scutal index for ticks that had duration of feeding time longer than 24 hours.

Tick homogenization

Tick homogenization was performed individually in 2 ml screw-lock micro tubes (Sarstedt AG, Nümbrecht, Germany) which contained a five-millimeter diameter sterile stainless-steel bead (Qiagen, Hilden, Germany) and 450 microliters of mixed lysis buffer solution, 441μl of RNeasy Lysis Buffer (Qiagen, Hilden, Germany) and 9 μl of 2M Dithiothreitol (DTT). All the prepared tubes with a tick were lysed with a 'TissueLyser' machine (Qiagen) twice at 30 times per second frequency for 1 minute per round and the tube position was rotated 180 degrees before starting the second round. Each batch contains 48 samples including one negative control. The finished homogenized ticks were centrifuged at 20,000 ×g for 3 minutes to be prepared for the extraction.

Tick extraction

Ninety microliter of tick supernatant comprising the genetic material were transferred manually and individually by pipetting to the 96 wells extraction plate with 10 μl of Proteinase K from Tritirachium: buffer aqueous glycerol solution (Sigma life science, Germany). Tick RNA extraction was conducted by an extraction robot (Magnatrix 8000+) using a commercial extraction kit (Vet Viral NA kit, NorDiag, Sweden). RNA was extracted from 88 samples (85 tick samples, 2 negative controls, 1 positive control) at the same time. The positive control contained 5 μl of inactivated TBEV strain K23 (Encepur®, Chiron Vaccines, Marburg, Germany) and 5 μl of *Borrelia burgdorferi* sensu stricto B31 ATCC 35210 (10^8 cells/ml).

cDNA Synthesis

The complementary DNA (cDNA) was synthesized by reverse-transcription of most of the RNA from the genetic material previously extracted using Illustra Ready-To-Go RT-PCR Beads (GE Healthcare, Amersham Place, UK). Twenty microliters of 85 RNA samples were manually pipetted to 96 wells PCR plate and 10 μl of pd(N)6: random hexamer primers (0.25 μg/μl) were added to each sample and incubated for 5 minutes at 97°C using a PTC-100 thermal cycler (MJ Research, MA, USA). After that the mixture of RT-PCR bead dissolved in 20 μl RNAse-free water were added to each sample and incubated for 30 minutes at 42°C, followed by 5 minutes at 97°C. The end product was 50 μl cDNA.

Real-time PCR for TBEV detection

To evaluate the occurrence of TBEV in the examined ticks, the molecular detection of the virus was performed using a combined 2 published TaqMan™ real-time PCR assays (Gäumann et al., 2010; Schwaiger and Cassinotti, 2003) as a multiplex TaqMan™ assays following Lindblom et al., 2014 . The probes of two assays were labelled differently with

FAM (6-carboxy-fluorescine) and HEX (6-carboxy-hexachlorofluorescein): fluorescent reporters. This combination made possible the detection of the different and potentially occurring TBE-strains and subtypes in one reaction. The real-time PCR reactions were manually set up by hand pipetting. Each reaction volume was 20 μl consisted of 200 nM of each primers and probes (Table 3.), 10 μl Maxima®Probe qPCR MasterMix (Fermentas, St. Leon-Rot, Germany), 2 μl pooled cDNA sample (four samples in each pool) and 5.6 μl RNAse-free water. The PCR reactions were proceeded in a 96-well plate. The amplification and detection were performed on a Bio-Rad CFX96 Real-Time system (Bio-Rad Laboratories, Inc.). The protocol started with activation step at 95◦C for 5 minutes, and 45 cycles of 10 seconds at 95◦C and 60 seconds at 60◦C.

Table 3. Primers and probes used for detection of TBEV

Reagens	**Sequence (5’→ 3’)**
Forward: F-TBE[a]	GGG CGG TTC TTG TTC TCC
Reverse: R-TBE[a]	ACA CAT CAC CTC C TT GTC AGA CT
Probe: TBE-probe-WT[a]	TGA GCC ACC ATC ACC CAG ACA CA(BHQ1)
Fluorescent reporters:	FAM
Forward: TBEE-F6[b]	GGC TTG TGA GGC AAA AAA GAA
Reverse: TBEE-R2[b]	TCC CGT GTG TGG TTC GAC TT
Probe: TBEE-P4[b]	AAG CCA CAG GAC ATG TGT ACG ACG CC(BHQ1)
Fluorescent reporters:	HEX

FAM, 6-carboxy-fluorescine; HEX, 6-carboxy-hexachlorofluorescein; BHQ, Black Hole Quencher.
a Schwaiger and Cassinotti (2003)
b Gäumann et al. (2010)

Results

Tick species identification

Nine-hundred-ninety-nine ticks of infesting ticks on five different host species (wild animals: roe deer, fallow deer, moose; domestic animals: dog and cat) and questing ticks were identified belonging to the species *Ixodes ricinus* as follows: six-hundred-thirteen adult females, one-hundred-ninety adult male and one-hundred-ninety-six nymphs. One adult female tick from one cat (*Felis catus*) was identified as *Ixodes hexagonus*.

Tick estimated feeding time

Estimated feeding time were calculated following Gray et al. (2005) from 434 adult female ticks feeding on roe deer, 56 adult female ticks feeding on fallow deer, 5 adult female ticks feeding on moose, 42 adult female ticks feeding on dog and 7 adult female ticks feeding on cat. The results in *Table 4* show the amount tick from each species classified by feeding time length as up to 24-hour and more than 24 hour of estimated feeding time.

Table 4. Amount of ticks classified by duration of estimated feeding time.

	Number of female ticks following estimated feeding time	
Host species	**<24 hr**	**>24 hr**
Roe deer	33 (7.6%)	401 (92.4%)
Fallow deer	2 (3.6%)	54 (96.4%)
Moose	-	5 (100%)
Dog	5 (11.9%)	37 (88.1%)
Cat	1 (14.3%)	6 (85.7%)

Around 92.4% of ticks collecting from roe deer had been feeding on their host longer than 24 hours, as well as the majority of the adult female ticks collected from other animal species.

Real-time PCR for TBE detection

A total of two-hundred-fifty pools with four samples in each pool (corresponding to 1,000 individual samples) were analyzed and the result showed that no TBEV-positive tick was detected in the tick samples collected from different regions of Skåne county.

Discussion

The world surface temperature increased due to climate change, which in turn increased the northern Europe climate suitability and possibility of tick species to be able to spread and to establish in new area. The most common hard tick specie in Europe (*Ixodes ricinus*, the main vector of TBEV) had already increased its population densities and even distributed northwards in Sweden since 1980s (Tälleklint and Jaenson, 1998). In 2006, a potential vector of TBEV, *Ixodes persulcatus* (taiga tick) has been found northwestwards from recorded area in 2004 in Finland (Jääskeläinen et al., 2006) and had been reported as present and established for the first time in northern Sweden in 2015 (Jaenson et al., 2016). Besides, *Dermacentor reticulatus*, another hard tick species was first time found in Netherlands in 2006 (Nijhof et al., 2007) and was recently found on a jackal in Denmark (Klitgaard et al., 2017). Another tick species, *Hyalomma marginatum*, is usually introduced as nymph into central and northern Europe by migratory birds but due to its temperature condition requirements it could not reach the adult stage and survive in these geographical areas. Anyhow in southern Germany, an adult female of this tick species was found in 2006 (Kampen et al., 2007; Emelyanova, 2005; Papadopoulos et al., 2002). These cases uphold the hypothesis that there is the possibility of new tick species establishment in Sweden. Skåne is the southernmost county of Sweden with boundary contact with the northern continental Europe.

The results of the present study aimed at investigating the possibility of new tick species establishment in this area from ticks collected during 2011-2016 showed that no new species were found in the specimens collected and examined from Skåne county. The result is in accordance with the recent information on tick species that could be found on wild animal and domestic animals in Sweden (Jaenson et al., 1994). However, in 2018, adult ticks belonging to the species *Hyalomma marginatum* and *Hyalomma rufipes* were found in the middle and southern part of Sweden (SVA, 2018a, 2018b). These recent reports showed the possibility for this tick species to develop to adult stage of its life cycle under the warmer climatic condition experienced during the summer of 2018 in Sweden some years after the sample collecting period of the present study. It must be noted that the majority of *Hyalomma* adult ticks are usually found on horses and cattle (ECDC, 2019; Spengler and Estrada-Peña, 2018), and therefore it is not known if they might be found also on wild ruminants as the ones from which tick specimens were collected in the present study. Also, a tick species originally described as present only in northern Africa and southern Europe (*I. inopinatus*) has been recently described in several regions in Germany (Chitimia-Dobler et al., 2018; Petney et al., 2015; Estrada-Peña et al., 2014). For this reason, this species was included in our differential species identification (Estrada Pena 2018).

TBEV is endemic in western and central Europe including Scandinavia where the hard tick species *Ixodes ricinus* serves as the main vector. In Europe, the incidences of TBE cases had increased over the past 30 years (Dobler et al., 2018; Süss, 2005). In 2012, TBE became noticeable disease in European Union (EU). In 2014 instead, the European Centers for Disease Control and Prevention (ECDC) had received a report of 1986 confirmed cases (out of 2057 TBEV infected suspected cases) following the ECDC diagnostic criteria; these figures led to the calculation of around 0.42 cases per 100,000 population (ECDC, 2012). In Sweden, the mortality rate associated to TBEV infection is reaching 14% (Haglund et al., 1996) and up to 238 cases of TBEV-infection had been reported annually during years 2007-2016 (Dobler et al., 2018). The incidence of TBE human cases increased twice from 2011-2013 and from 2014-2017 in Skåne area (Figure 2). The incidence rate of TBE in Europe (including Sweden) is noticeably similar to our study area (Skåne county), but concerning the incidence rate in single municipalities in Skåne which is higher than the average incidence in Skåne, this makes a topic of interest the investigation of the role of *Ixodes ricinus* and of other competent TBEV vector species in the epidemiology of this disease. The TBEV natural transmission cycle is complex with a variety of tick vectors and various reservoir mammal hosts involved, while humans are just accidentally entering this transmission cycle. The central role of TBEV natural transmission cycle is covered by small mammals and ticks, but large vertebrates and birds are also play important role in spreading and maintaining of TBEV. Roe deer, fallow deer and other wild animals also have a role in the distribution of TBEV as tick maintenance hosts but they should not act as a source of virus for ticks (Korenberg and Kovalevsky, 1997).

There are several studies on TBEV prevalence in ticks, mostly focusing on *Ixodes ricinus* in TBEV endemic foci. In northern Europe (i.e., Sweden, Norway, Denmark, Finland), the mean minimum infection rate (MIR) of TBEV in *Ixodes ricinus* (nymphs and adult) was 0.28% (Pettersson et al., 2014). The prevalence of TBEV ticks in known endemic regions ranged from 0.04 to 0.44%: 0.04% (n=13,885) in Italy, 0.19% (n=3,234) in Lithuania, 0.43% (n=589) in Finland, 0.41% (n=18,360) in Germany and 0.44% (n=3,404) in Austria (Han et al., 2005, 2002; Hudson et al., 2001; Labuda et al., 1993; Süss et al., 2004). In Norway, neighboring country, Andreassen et al. (2012) studied the prevalence of TBEV in tick nymphs in the endemic foci on the southern coast and the prevalence was ranging between 0.35-0.75% (n=5630). One study in western Gotland, Sweden, showed that the prevalence of TBEV in *I. ricinus* was rather low, 0.10-0.42%, but comparable to the prevalence from more established TBEV endemic regions. In this study, 1,000 tick samples collected both from 5 different host species and from questing ticks collected by flagging from several areas in Skåne, characterized by an increased incidence of TBEV infected patients within the sampling time frame.

As the female tick is regarded to have a major role in the transmission of the virus, male ticks might also be involved in virus transmission even if don't feed, by participating in the virus circulation in the environment, since ticks can be infected with TBEV in every active life stage (Karbowiak and Biernat, 2016). In this study both nymphs, females and male ticks were examined but none of the samples were containing TBEV as assessed with PCR assay. The combined PCR assay that was performed in the present study following Lindblom et al. (2014) could lead to an effective detection of several TBEV strains in one reaction. Since the prevalence of TBEV in tick is rather low even in the established endemic regions the result of this study is not surprising as the virus might not circulating in those sampling areas during that period of time. Anyway, the prevalence in the tick sampled population did not correlate to the increasing number of TBEV infected cases in this region. It might be needed to sample and analyze a higher amount of specimens from more regions and from more recent years to be able to trace and identify potential sources of risk for of TBEV infection in humans in Skåne county.

The majority of female tick samples had fed on their host longer than 24 hours; it might be that smaller ticks have been missed at the collection, especially at slaughterhouse. Anyhow, this is not particularly relevant regarding TBEV transmission, that starts promptly when the tick start feeding by secreting the viral particles in the saliva and in turn into host tissues (Okulova et al., 1989). In accidental hosts as humans, the transmission of TBEV occurred within 1 hour after the attachment and the viral particles amount in salivary gland of the tick seems to increase with the tick feeding duration (Alekseev et al., 1996; Belova et al., 2012). However, *Ixodes ricinus* is not only a vector for TBEV but also a primary vector for Gram-negative Spirochaetae bacteria belonging to Borreliaceae: *Borrelia burgdorferi* sensu lato (s.l.) complex causing Lyme borreliosis in human (Mannelli et al., 2012). Since the tick feeding duration is directly correlated to the transmission risk of this pathogen (Gray et al., 2005), the results of this study can be used for further investigation of *Borrelia burgdorferi* sensu lato in this tick population.

The tick species are spreading its distribution northwards due to many factor including climate changes and there is possibility of new tick species that could be establish in Skåne area and the TBEV prevalence information in tick in this TBE risk area is scarce. Even if results from the present study confirm that despite its position no new risks have been introduced in Skåne during the study period, there is need for future studies in this field. These should possibly take in account larger number of specimens and new sample collections from this geographical area, in order to keep the community prepared to face new potential risks related to the introduction of new tick species or new pathogens potentially threatening human and animal health.

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Supplementary material

Supplementary Table S1. summary of ticks collected in Skåne with collecting area

Collecting year	Host species/ flagged	Collecting area	Females (n=614)	Males (n=190)	Nymphs (n=196)
August 2014	Roe deer	Alleholm, Arkelstorp, Farstorp, Finja, Fjälkinge, Färingtofta, Färlöv, Huaröd, Hässleholm, Hästveda (Norra), Höllviken, Oppmanna, Osby, Råbelöv, Röke, Rörum, Röstånga, Sjunkaröd, Skepparslöv, Sösdala, Tjörnarp, Tyringe, Vankiva, Vinslöv, Vånga, Öllsjö, Önnestad	458	123	-
November 2011	Fallow deer	Skåne area	64	16	-
November 2011	Moose	Skåne area	5	3	-
August 2014-May 2016	Dog	Degeberga, Espet åhus, Mosslunda	42	-	-
May 2015	Cat	Gumlösa	7	4	-
May 2011-May 2012	Flagged	Hörröd, Lunkeskog, Svinstallet, Övarp	38	44	196

Supplementary Figure S1. The map showed the distribution of tick collecting area of ticks collected from roe deer and other wild animals.

Supplementary Figure S2. The map showed the distribution of tick collecting area of ticks collected from dog and cat.


Båstad
Rånneslöv
Markaryd
Killeberg
Grevie
Västorp
Hishult
Emmaljunga
Osby
Lönsboda
Förslöv
Hallandsås
Vilshult
Kyrkhult
Vejbystrand
Vittsjö
Örkelljunga
Bjärnum
Glimåkra
Olofström
Ängelholm
Munka Ljungby
Röke
Mala
Broby
Jämshög
Tånga
Ö Ljungby
Knislinge
Näsum
Mörrum
Åstorp
Klippan
Hässleholm
Hanaskog
Viken
Hyllinge
Pappersbruket
Tormestorp
Östanå
Norje
Hornbæk
Helsingborg
Kristianstad
Gualöv
Bromölla
Helsingør
Bårslöv
Ekeby
Ljungbyhed
Sölvesborg
Restanga
N Rörum
Oveshölm
Viby
Vallåkra
Billinge
Nivå
Tågarp
Svalöv
Höör
Tollarp
Åhus
Stehag
Everöd
Hørsholm
Landskrona
Eslöv
Osbyholm
Hörby
Huaröd
Vittskövle
Trørød
Hanöbukten
Kävlinge
Önnekop
Furulund
Löberöd
Östraby
Hellerup
Bjärred
S Sandby
Vollsjö
Brösarp
havn
Lund
Arlöv
Staffanstorp
Lövestad
Hvidovre
Malmö
Sjöbo
Onslunda
Vik
Genarp
Dragør
Bara
Blentarp
Bunkeflostrand
Oxie
Tomelilla
Simrishamn
Svedala
Gessie villastad
Hököpinge
Sövestad
Hammenhög
Rydsgård
Vellinge
Borrby
Skillinge
Höllviken
Klagstorp
Skivarp
Ystad