



Short communication

Field samplings of *Ixodes ricinus* ticks from a tick-borne encephalitis virus micro-focus in Northern Zealand, Denmark

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ABSTRACT

In 2008–2009 a tick-borne encephalitis virus (TBEV) micro-focus was detected in Northern Zealand, Denmark. No new cases of TBE with an epidemiological link to Northern Zealand has been reported since. Here we undertook to investigate *Ixodes ricinus* ticks from this endemic micro-focus in 2016 and 2017. In addition to TBEV, *I. ricinus* ticks may host other pathogens that include *Borrelia* spp., *Babesia* spp., *Rickettsia* spp. and *Neoehrlichia mikurensis*, together with various endosymbiont microorganisms. To detect multiple organisms we used a metagenomics PanVirus microarray and next-generation sequencing to examine the persistence and evolution of other emerging viruses, bacteria and parasites. Here we report the rise and fall of the Danish TBEV micro-focus in Northern Zealand. However, we identify for the first time in Danish *I. ricinus* ticks the presence of Uukuniemi virus in addition to a tick-borne phlebovirus and a range of bacteria.

1. Introduction

Tick-borne encephalitis (TBE) is a viral infectious disease that causes severe neurological sequelae in humans and has mortality rates of up to 3.0% (Lenhard et al., 2016). In contrast to the evenly distributed and widespread *Borrelia* bacteria, the tick-borne encephalitis virus (TBEV) tends to occur in micro-foci within endemic areas. TBE is a growing public health concern in Europe and the overall notification rate in EU/EEA rose from 0.4 cases per 100,000 population in 2015 to 0.6 cases per 100,000 population in 2016 (European Centre for Disease Prevention and Control (2018)). The incidence and geographical range of TBE is similarly increasing in Scandinavia (Jaenson et al., 2012; Sidorenko et al., 2018). In Denmark, TBE has been reported since the 1950s only from the Bornholm Island east of mainland Denmark in the Baltic Sea with an incidence of 3.8 cases per 100,000 persons per year (Ocias et al., 2017; Fomsgaard, 2018). In 2008 and 2009, two cases of TBE were reported from Tokkekøb Hegn in Northern Zealand, where after TBEV was identified in a pool of *Ixodes ricinus* nymphs collected from the edge of the forest and water surrounding a patient's property and a public recreational area (Fomsgaard et al., 2009). TBEV was detected at the same micro-focus in 2010 and 2011, which confirms the

establishment of an additional micro-focus of TBEV in Denmark (Fomsgaard et al., 2013). Since 2009 there has been no new cases of TBE in Denmark with an epidemiological link to Northern Zealand. We therefore undertook to investigate this endemic TBEV micro-focus again in 2016 and 2017. Since ticks may also host other pathogenic microorganisms including *Borrelia* spp., *Babesia* spp., *Neoehrlichia mikurensis* and *Rickettsia* spp. (Fertner et al., 2012), the aim of the study was not only to re-examine the area for persistence of TBEV but also to look for other viruses, bacteria and parasites.

2. Materials and methods

2.1. Study design and nucleic acids extraction

In total 4100 ticks were collected by flagging from Tokkekøb Hegn, Northern Zealand (Fomsgaard et al., 2009, 2013) at four sampling times in 2016 and 2017 (Table 1). All ticks were identified as *I. ricinus* on the basis of morphology and sorted according to stage (nymphs vs adults). The ticks were then divided into 71 pools of nymphs, 6 pools of adult males and 5 pools of adult females containing 50 ticks in each pool (Table 1) as previously described (Fomsgaard et al., 2009, 2013). Each

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Table 1
TBEV-specific RT-PCR and PanVirus microarray.

Sampling date	Tick Stage	No. of pools (n = 82)	TBEV RT-PCR	PanVirus microarray (n = 20)	UUKV RT-PCR
August 30th 2016	Nymphs	14	0/14	negative	0/14
	Adults	2	0/2	negative	0/2
September 27th 2016	Nymphs	10	0/10	negative	0/10
	Adults	4	0/4	negative	0/4
June 26th 2017	Nymphs	25	0/25	BTPV-1 (L), BTPV-2 (L)	0/25
	Adults	2	0/2	negative	0/2
August 8th 2017	Nymphs	22	0/22	UUKV (S, M, L), BTPV-1 (L), BTPV-2 (L)	2/22
	Adults	3	0/3	negative	0/3

TBEV; Tick borne encephalitis virus, UUKV; Uukuniemi virus, BTPV; Blacklegged tick phlebovirus, L; segment L, S; segment S, M; segment M. Each pool consisted of 50 ticks.

tick pool were homogenized in 1 ml of MagNA Pure Lysis/Binding Buffer (Roche Diagnostics A/S, Risch-Rotkreuz, Switzerland), incubated overnight at 5 °C before nucleic acid (NA) was extracted with the MagNA Pure Large Volume kit on a MagNA Pure 96 instrument (Roche Diagnostics A/S, Risch-Rotkreuz, Switzerland). The final elution volume was 50 µl.

2.2. Virus-specific PCR

Five µl of extracted NA were used in a TBEV-specific real-time RT-PCR as previously described (Fomsgaard et al., 2009, 2013). A specific real-time RT-PCR assay for the M-segment (NC_005220) of Uukuniemi virus (UUKV) was designed: Uuk 3 M F: 5'-TCAAGCTGGAGTGTGTC AGG-3', Uuk 3 M R: 5'-GCCACACTGCTCAACACAGT-3', and Uuk 3 M P: 5'[HEX]-GAGCCTGCAAGGGAGAGGCC-[BHQ1]3'. The SensiFAST™ Probe No-ROX One-Step kit (Meridian Bioscience Inc., Cincinnati, OH, USA) was used according to the manufactures instructions with 5 µl of extracted NA. The reactions were performed on the MX3005 thermocycler (Stratagene, San Diego, CA, USA) using the following program: 30 min at 48 °C, 10 min at 95 °C and 45 cycles with 15 s at 95 °C and 1 min at 57 °C.

2.3. PanVirus microarray

Twenty-five µl of extracted NA from 20 randomly selected tick pools were treated with 4 U TURBO™ DNase (ThermoFisher, Waltham, MA, USA) and enriched for RNA virus using an unpublished *I. ricinus* tick-modified version of the published protocol (Matranga et al., 2016). Five µl of enriched RNA were used for random whole transcriptome amplification (WTA) using the Repli-g Cell WGA & WTA kit (Qiagen, Hilden, Germany) according to the manufactures instructions. Amplified cDNA was purified using the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) and 1.5 µg of purified cDNA was labelled with Cy5 using the SureTag DNA labelling kit (Agilent, Santa Clara, CA, USA). The labelled DNA was pooled according to the sampling date and tick life-cycle stage and hybridized to a PanVirus microarray (SurePrint G3 Custom Gene Expression Microarray (4 x 180 K) (Agilent, Santa Clara, CA, USA)) containing 60–160 probes for each sequenced virus described in GenBank (2016). Microarray data was analysed using a simple Excel-based data analysis method as described previously (Erlandsson et al., 2011; Rosenstjerne et al., 2014).

2.4. Microbiome analysis

The microbial content of pro- and eukaryotes was analysed using a modified version of the in-house custom build 16S/18S ribosomal ribonucleic acid (rRNA) amplicon-based approach, previously described by Ring et al., 2017 and Krogsgaard et al., 2018. In brief, 2 µl of extracted NA from the 20 randomly selected tick pools were PCR amplified using 4 different primer sets (the modified 341 F/806R primers targeting the prokaryotic V3-V4 16SrDNA region, and the G3F1/G3R1, G4F3/G4R3 and G6F1/G6R1 primers targeting the hypervariable regions V3-V5 of the eukaryotic 18SrDNA gene (Krogsgaard et al., 2018). Library preparation was performed as previously described (Krogsgaard et al., 2018). The resulting amplicons were quantified using the QuantITM dsDNA High Sensitive Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), pooled in equimolar amounts and sequenced on an Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA) using the 500-cycle MiSeq Reagent Kit V2 (Illumina Inc., San Diego, CA, USA). Sequences were taxonomically mapped using BION, a k-mer-based mapping software described previously (Krogsgaard et al., 2018). Query sequences originating from prokaryotes were compared with the 340–807bp rRNA positions derived from *Escherichia coli* in RDP 11.04 (<https://rdp.cme.msu.edu>), and eukaryotic query sequences compared against the same region in SILVA version 123 (www.arb-silva.de). A unique taxon was defined by denotation to a specific evolutionary taxonomic group.

3. Results and discussion

None of tick pools were positive for TBEV using either TBEV specific PCR or the PanVirus microarray (Table 1). We have previously shown the presence of TBEV using a TBEV-specific PCR in nymph pools containing 50–100 nymphs (Fomsgaard 2009; Fomsgaard 2013) indicating that the lack of detection of TBEV in the nymph pools was not due to assay performance. The negative TBEV finding and the absence of clinical cases of TBE with epidemiological link to Northern Zealand since 2009 (Ocias et al., 2017) could possibly indicate that the previously demonstrated TBEV micro-focus located in Tokkekøb Hegn in Northern Zealand has disappeared. It is not fully understood which factors support the transmission cycles of TBEV, but meteorological variations, fauna, vegetation as well as human behaviour play a role (Randolph and Rogers, 2000; Randolph et al., 2008; Knap et al., 2009). Even though the micro-focus may have been temporary, continued surveillance of the site will be performed and TBE should still be considered in Denmark outside Bornholm if clinically relevant symptoms are present. There are indications that TBEVs geographical range is increasing in Sweden, Norway and Denmark (Jaenson et al., 2012; Sidorenko et al., 2018; Andersen et al., 2019). It is thus important to be aware of the establishment of new micro-foci in Denmark outside Bornholm.

Two phleboviruses were detected with the PanVirus microarray in the collected *I. ricinus* ticks (Table 1). No other virus were detected. In particular Uukuniemi virus (UUKV) was identified in a pool of nymphs from August 8th 2017. The PanVirus microarray detected all three UUKV genome segments (segment S, M, L) (data not shown). The presence of UUKV was verified using a UUKV specific real-time RT-PCR assay for the M-segment. All 82 collected tick pools were analysed and two tick pools from nymphs collected from August 8th 2017 were PCR positive (Table 1). This is the first report of UUKV in Danish *I. ricinus* ticks. UUKV has been identified in *I. ricinus* ticks in Finland (Saikku and Brummer-Korvenkontio, 1973), Norway (Traavik and Mehl, 1977), the Czech Republic (Kolman, 1970) and Sweden and the Netherlands (Gondard et al., 2018). To date, UUKV has not been associated with disease in humans. However, other tick-borne phleboviruses very similar to UUKV, such as severe fever with thrombocytopenia syndrome virus and Heartland virus (Palacios et al., 2013) has been associated with severe and often fatal disease in humans (Yu et al., 2011;

Table 2

Taxons identified in the microbiome analysis with number of positive pools detected from each flagging date. Only taxons with > 500 reads are listed.

Taxon	Sampling date							
	August 30 TH 2016		September 27 TH 2016		June 26 TH 2017		August 8 TH 2017	
	Pools analysed		Pools analysed		Pools analysed		Pools analysed	
	Nymphs (n = 3)	Adults (n = 2)	Nymphs (n = 3)	Adults (n = 2)	Nymphs (n = 3)	Adults (n = 2)	Nymphs (n = 3)	Adults (n = 2)
	Number of positive pools							
<i>Anaplasma phagocytophilum</i>	1							
<i>Borrelia afzelii</i>	2		2		1		3	
<i>B. miyamotoi</i>	1			1				
<i>B. burgdorferi</i>		2						
<i>B. garinii</i>					1			
<i>Buchnera aphidicola</i>					1			
<i>Ceratoppia bipilis</i>				1				1
<i>Cronobacter sakazakii</i>			1					
<i>Diplorickettsia (massiliensis)</i>		2		2	1	1	1	2
<i>Eupelops plicatus</i>			1					
<i>Exobasidium rhododendri</i>				1				1
<i>Ixodes ricinus</i>	3	2	3	2	3	2	3	2
<i>Janthinobacterium agaricidamnosum</i>					1			
<i>Massilia timonae</i>	1							
<i>Methylobacterium cerastii</i>	3		1		1		2	
<i>Methylorosula (polaris)</i>							1	
<i>Mucilaginibacter dorajii</i>							1	
<i>Mycobacterium madagascariense</i>	3		1		2		1	
<i>M. rhodesiae</i>	2		1				1	
<i>M. chelonae</i>			3					
<i>M. pyrenivorans</i>	1							
<i>Pantoea ananatis</i>			2					
<i>Patulibacter minatonensis</i>	1							
<i>Pedobacter agri</i>							1	
<i>P. daejeonensis</i>							1	
<i>Phyllobacterium ifriqiense</i>			1					
<i>Pseudomonas tolaasii</i>		1		1				
<i>P. graminis</i>	1							
<i>P. baetica</i>			1					
<i>Rhizobacter (fulvus)</i>					1			
<i>Rhodopseudomonas palustris</i>	2							
<i>Rhogostoma schuessleri</i>								1
<i>Rickettsia spp.</i>	3	2	3	2	3	2	3	2
<i>Serratia proteamaculans</i>		1						
<i>Sphingomonas desiccabilis</i>			1				1	
<i>S. dokdonensis</i>	3	1	3	1	3	1	3	1
<i>S. glacialis</i>	3		3	1	1	1	3	0
<i>S. melonis</i>			1					
<i>S. oligoaromaticivorans</i>							1	
<i>S. oligophenolica</i>	2		1		1			
<i>S. polyaromaticivorans</i>	1		1				1	
<i>S. sanxanigenens</i>			1					
<i>Variovorax ginsengisoli</i>	1							
<i>Williamsia deligens</i>	3				1			
<i>W. serinedens</i>		2	3	1			3	
<i>Wolbachia spp.</i>	3		1		2		2	

McMullan et al., 2012). A possible explanation for the lack of UUKV-associated disease in humans is that the non-structural protein is a weak interferon antagonist that is incapable of counteracting the interferon response (Rezelj et al., 2015).

A positive signal for Blacklegged tick phlebovirus (BTPV) was also detected by the PanVirus microarray in the pool of nymphs from August 8th and June 26th 2017 (Table 1). In contrast to the microarray signal for UUKV, the microarray signal for BTPV was only positive for a partial region of the BTPV L-segment (data not shown) which indicated cross-hybridization of probes to an unidentified phlebovirus. The presence of BTPV was not verified in this study. BTPV was first identified in *I. scapularis* in the United States (Tokarz et al., 2014); however new tick-borne phleboviruses are continuously being identified all over Europe which cluster to BTPV (Tokarz et al., 2014; Papa et al., 2016; Prinz et al., 2017). The exact identification and molecular characterization of

the phlebovirus in Danish *I. ricinus* ticks from Northern Zealand remains to be performed.

The 20 randomly selected tick pools were also analysed for the presence of other microorganisms using 16S/18S rRNA amplicon-based metagenomics. Only species detected with at least 500 sequence reads in the 16S/18S rRNA amplicon-based metagenomics are presented (Table 2). *Rickettsia* spp. was the most prevalent bacteria species detected in all 20 pools with the consistent highest number of reads (Table 2, Supplementary Table). *Rickettsia* may be present in *I. ricinus* ticks either as an endosymbiont (mutualistic relationship) or as a pathogen, including *R. helvetica* and *R. monacensis*. The metagenomics analysis was not able to differentiate the *Rickettsia* species as they are almost identical in the amplified area of 16S. Other prevalent bacteria were *Borrelia* spp., including, *B. afzelii* (8 pools), *B. miyamotoi* (2 pools), *B. burgdorferi* (2 pools) and *B. garinii* (1 pool), *Mycobacterium* spp.,

including *M. madagascariense* (7 pools), *M. rhodesiae* (4 pools), *M. cheilonae* (3 pools) and *M. pyrenivorans* (1 pool) (Table 2). No parasites, including protozoan, were detected in any of the tested pools. Some fungi were detected, but not analysed further in this study.

We detected two human pathogens in our tested tick pools: *Borrelia* spp. and *Anaplasma phagocytophilum*. *B. miyamotoi* was also detected, although we are not aware that *B. miyamotoi* disease has been diagnosed in Denmark. Other well-known tick-borne pathogens such as *Babesia*, *Francisella*, *Bartonella*, *Ehrlichia*, and *Candidatus Neoehrlichia mikurensis* were not detected in this study. This is in agreement with the previous studies from Denmark where only *A. phagocytophilum* and *Borrelia* spp. were detected in *I. ricinus* ticks from Jutland, Funen and Bornholm (Andersen et al., 2019; Skarphédinsson et al., 2007). However, two *Babesia* species (*B. divergens* and *B. venatorum*) were demonstrated in 2012 in *I. ricinus* ticks from Zealand (Michelet et al., 2014) and *Candidatus Neoehrlichia mikurensis* were found at three locations in Denmark in 2011, including Tokkekøb Hegn (Fertner et al., 2012).

We did not report any *Midichloria mitochondrii*, an endosymbiont frequently associated to *I. ricinus* (Cafiso et al., 2019). The RDP 11.04 database only contains sequences from *Candidatus Midichloria*, taxonomically defined at subtype level without any genus and species names and were therefore ignored by BION. However, when mapping raw sequence data to the entire RDP database, including taxonomically undefined references, reads from 18 of 20 samples mapped to *Candidatus Midichloria* sp. Ixholo1. Endosymbiont microorganisms exhibit both negative and beneficial effects on tick fitness (de la Fuente et al., 2017d; Bonnet et al., 2017), in addition to providing protection against other bacteria, viruses and eukaryotic parasites, which indicates that they can interact with other microorganisms sharing the same host environment (de la Fuente et al., 2017d; Bonnet et al., 2017).

In conclusion, we find that a previously identified TBEV micro-focus in Northern Zealand, Denmark seems to have disappeared for undefined reasons. We propose continued surveillance of known TBEV micro-foci. We also demonstrated for the first time UUKV in Danish *I. ricinus* ticks and the possible presence of a new phlebovirus. We confirm that *I. ricinus* ticks are vectors of pathogens of public health concern.

Conflict of interests

The authors declare that there is no conflict of interest in this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.ttbdis.2019.05.005>.

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