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Phylogenetic characterization of tick-borne encephalitis virus from Bornholm, Denmark

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ABSTRACT

The Danish island of Bornholm in the Baltic Sea has been known as a tick-borne encephalitis (TBE) natural focus for more than 60 years. TBE in humans is diagnosed on a regular basis either in inhabitants or tourists of the island. Other areas in Denmark have been suggested as possible risk areas of TBE. Despite the long-known endemicity on Bornholm and the possibility of the virus circulating in other areas, no data on the prevalences of TBE virus (TBEV) in ticks, or adequate molecular characterization and phylogenetic studies are available for the circulating TBEV strains. This study aimed to detect TBEV in ticks collected on the island of Bornholm and other possible risk areas, with the attempt to isolate the circulating viruses for molecular and phylogenetic analysis and confirm the presence of virus in the predicted risk areas.

From 2014 to 2016, 9321 *I. ricinus* (nymphs, females, and males) were collected by flagging 31 locations in Denmark. The ticks were pooled and tested for TBEV by qPCR. The envelope gene of the detected TBE virus strains was amplified and sequenced by RT-PCR. After successful virus isolation, whole genome sequencing was performed. Phylogenetic analysis of the obtained sequences was done by the Maximum Likelihood method.

One pool of 11 females and one pool of eight males from a total of 34 tick pools collected from the north-western shore of lake Rubinsøen on Bornholm tested positive, resulting in a local estimated point prevalence of 0.6% [CI95% 0,1–1.85%] in this microfocus. We were not successful in confirming any other of the predicted TBEV-endemic areas. Alignment of the two complete E genes from Bornholm revealed identical sequences. Virus isolation and whole genome sequencing were succeeded from one of the positive samples. Phylogenetic analysis showed that the isolated virus had the closest phylogenetic relationship to TBEV sequences detected in Eastern and Central Europe.

1. Introduction

Tick-borne encephalitis virus (TBEV) is a flavivirus, which is typically transmitted to humans by ticks and can cause disease of the central nervous system of different severity, ranging from mild forms of meningitis to severe meningoencephaloradiculitis (Borde and Zajkowska, 2017; Gritsun et al., 2003; Haglund and Günther, 2003). The TBEV genome comprises a linear positive-stranded RNA molecule of approximately 11 kb. The viral RNA encodes three structural proteins: capsid (C), membrane (M) and envelope (E), and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These translate into a polyprotein from which structural and non-structural proteins are processed by cellular and viral proteases (Chambers et al., 1990; Růžek et al., 2017). When whole genome sequences are not available the E gene has been suggested as a critical target for nucleotide-based phylogenetic analysis because, despite being

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subjected to purifying selection, the encoded E protein is the most constrained protein within the TBEV genome (Uzcátegui et al., 2012; Weidmann et al., 2013).

TBEV has traditionally been divided into three subtypes - European (TBE-Eu) primarily transmitted by *Ixodes ricinus*, Siberian (TBE-S) and Far-Eastern (TBE-FE) primarily transmitted by *I. persulcatus* (Dobler and Tkachev, 2017). However, two recent studies propose that TBEV are reclassified into five genetic subtypes, adding the Baikalian subtype which is subdivided from TBE-S (Kovalev and Mukhacheva, 2017), and the Himalayan subtype forming an independent branch in a phylogenetic tree based on E gene sequences (Dai et al., 2018).

TBE-Eu is the only subtype occurring in central Europe. Genetic clades of the Siberian and Far-Eastern subtypes, however, have been detected in the Baltic countries, in Finland and the European part of Russia (Jääskeläinen et al., 2006; Lundkvist et al., 2001). An estimated one-third of the humans infected are asymptomatic; one-third show unspecific transient febrile symptoms, and the remaining will develop clinical signs of meningoencephalitis with possible persistent, debilitating neurological sequelae (Borde and Zajkowska, 2017; Haglund and Günther, 2003). Approximately 2000 cases of human tick-borne encephalitis (TBE) are reported annually to the European Center for Disease Control and Prevention (ECDC) (European Centre for Disease Prevention and Control, 2018), but as TBE is not a notifiable disease in all the European countries, this probably does not comprise all cases. Epidemiological studies have suggested the number of the European TBE cases to be in the range of 3000–4000 annually (Donoso Mantke et al., 2011; Gritsun et al., 2003).

The geographical distribution of TBEV is closely linked to the geographical range of their vector ticks (Kupča et al., 2010); whereas TBEV circulation is dependent on a complex enzootic cycle in which small mammals serve as reservoirs and amplifying hosts and ixodid ticks as both vectors and reservoirs. Non-systemic transmission between ticks co-feeding on small mammals is considered pivotal for the maintenance of TBEV circulation (Labuda and Randolph, 1999; Overzier et al., 2013). For this reason, TBEV natural foci can be limited to small areas (Dobler et al., 2011; Kupča et al., 2010).

In Denmark, the island of Bornholm in the Baltic Sea has been known as an endemic area of TBEV for more than 60 years (Freundt, 1963). The inhabitants or tourists visiting the island are regularly diagnosed with TBE with an incidence of 4/100.000 (Freundt, 1963; Fomsgaard, 2017; Kristiansen, 2002; Laursen and Knudsen, 2003; Ocias et al., 2017)(Freundt, 1963; Kristiansen, 2002; Ocias et al., 2017). Despite this long-known endemicity, no molecular characterization and phylogenetic studies are available of circulating TBEV, apart from a 443 nucleotide long partial E gene (GenBank Acc. No EF565946) recovered from the cerebrospinal fluid (CSF) of a Norwegian tourist bitten by a tick while visiting Bornholm (Skarpaas et al., 2006) In 1999, Louping ill virus (LIV) was found to co-circulate with TBEV in *I. ricinus* on Bornholm, determined by a species-specific quantitative reverse transcription polymerase chain reaction (qRT-PCR). These viruses have not been further characterized, and the sequences have not been submitted to GenBank at the national center for biotechnology information (NCBI) (Jensen et al., 2004). LIV is genetically the closest relative to TBEV; both viruses are members of the TBE complex and share *I. ricinus* as a vector. LIV can primarily cause encephalitis in sheep, but infection in humans has been described (Davidson et al., 1991).

In 2009, two human cases of TBE from north Zealand (Tokkekøb Hegn) were diagnosed (Fomsgaard et al., 2009). TBEV from ticks collected in the area was isolated, and molecular characterization of the complete E gene was performed, which confirmed the existence of a new Danish microfocus outside of Bornholm (Fomsgaard et al., 2013).

Ixodes ricinus is abundant throughout Denmark. TBEV risk areas have been predicted in various parts of Denmark, either using sentinel animals or statistical climate-matching models based on the known spatial distribution of TBEV transmission cycles (Andersen et al., 2019; Lindhe et al., 2009; Randolph, 2001; Skarphédinsson et al., 2005). The most recent Danish study is a nationwide TBE seroprevalence study using roe deer (*Capreolus capreolus*) as sentinels by Andersen et al. (2019). Here 51 of the 736 roe deer blood samples were found to have neutralizing antibodies against TBEV. The positive roe deer samples were distributed across all major islands and mainland Denmark, including Bornholm.

This study aimed to detect, isolate, and characterize TBEV and/or LIV in ticks collected on the island of Bornholm and in selected areas where the TBE complex viruses are predicted to circulate, with the attempt to confirm the presence of these viruses in the predicted risk areas and to isolate the circulating TBE complex viruses for molecular and phylogenetic analysis.

2. Materials and method

2.1. Tick sampling

Questing ticks were collected in July and, August of 2014, 2015 and 2016 by flagging the low herbaceous vegetation. On the endemic island of Bornholm 12 locations were chosen based on previous studies showing TBEV neutralizing antibodies in roe deer or PCR-positive ticks as described by Andersen et al. (2019), Jensen et al. (2004) and Skarphédinsson et al. (2005) (Fig. 1). Outside of Bornholm, nine locations were chosen from the Andersen et al. (2019) study based on the existence of TBEV neutralizing antibodies in roe deer. Ten locations were selected as negative control areas. Here the roe deer had no neutralizing antibodies (Fig. 1).

In a TBE-endemic area, the TBEV prevalence in ticks is often less than 1%, and in Scandinavia (Denmark, Norway, Sweden, and Finland) the overall prevalence is 0.28% (Pettersson et al., 2014). For this reason, we tried to collect at least 350 ticks (nymphs and adults) per

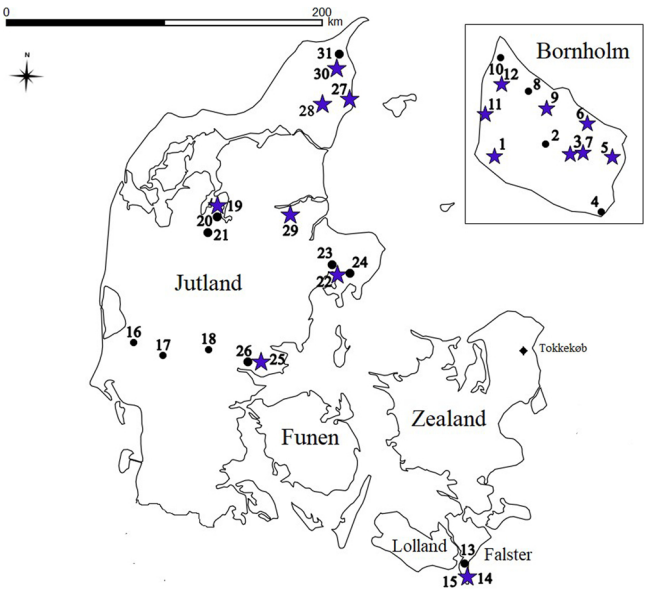

Fig. 1. Tick collection sites in Denmark.
The points and corresponding numbers refer to GPS-localities where questing *Ixodes ricinus* nymphs and adults were collected by flagging the low herbaceous vegetation (Hillyard, 1996). The blue stars equal sites with TBEV neutralizing antibodies in roe deer or TBE/LIV infection in ticks (LIV only on Bornholm) as described in studies by Jensen et al. (2004); Skarphédinsson et al. (2005) and Andersen et al. (2019). The black points are areas with no indication of the existence of the TBE complex (Andersen et al., 2019; Jensen et al., 2004; Skarphédinsson et al., 2005). Table 2 gives information on: the name of each numbered locality, GPS coordinates, and the number of the collected ticks. The map was created using the software QGIS Geographic Information System 2.18. QGIS Development Team (2018). Open Source Geospatial Foundation Project. OBS, Bornholm is not to scale and is displaced from its location in the Baltic sea.

location, if feasible. For each location, suitable vegetation was sought out preferably with a transition of forest to meadows, paths on the ground where people would walk, low herbaceous vegetation and mixed forests with oak (*Quercus* spp.), spruce (*Picea abies, Abies nordmanniana* etc.), pine (*Pinus sylvestris*) and beech (*Fagus sylvatica*). Date, GPS coordinates of each site, and the route taken through the area were registered. The ticks were identified using morphological characters according to Hillyard (1996) and Estrada-Peña et al. (2014). After identification the ticks were briefly washed in 70% alcohol, dried and stored at −80 °C until processing.

2.2. Nucleic acid extraction

Tick nymphs, females, and males were thawed and divided in respective pools of up to 13 ticks per pool. Each pool was homogenized in 1 ml Minimum Essential Medium (MEM, Invitrogen, Karlsruhe, Germany) containing an antibiotic-antimycotic solution (Invitrogen, Karlsruhe, Germany) using the Fast Prep Savant FP120 tissue lyser (Bio101, Vista, USA), with three rounds at speed 6.5, for 30 s each. Nucleic acid was extracted from each pool using the MagNA Pure LC RNA/DNA Kit (Roche) in a MagNA Pure LC instrument (Roche) according to the manufacturer's instructions.

2.3. TBEV screening by PCR

An RT-qPCR protocol described by Schwaiger and Cassinotti (2003), amplifying a 68-bp fragment of the 3′non-coding region of all three TBEV subtypes and LIV, were used for screening for TBEV- and LIV-specific sequences (Donoso Mantke et al., 2007). An MX 3000 PCR instrument (Stratagene, Heidelberg, Germany) was used to perform the PCR.

2.4. E gene sequencing

The nucleic acid of the positive pools was amplified using an E gene-specific RT-PCR as described by Kupča et al. (2010). The RT-PCR products were visualized in a 1% agarose gel and purified using the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing of the amplification products (approx. 1686 bp) was performed by GATC sequencing service (Konstanz, Germany). The final E gene sequence was generated using the de novo assembly tool from Geneious 9.1.5 (https://www.geneious.com).

2.5. Virus isolation

Cultivation of viruses was performed with a modified version of the Kupča et al. (2010) protocol in both A549 cells (MEM, 1x Non-Essential Amino Acids (NEAA), 10% Fetal Bovine Serum (FBS) (Gibco ™, Thermo Fisher Scientific, Inc.) and VeroB4 cells (MEM, 1x NEAA, 5% FBS (Gibco ™, Thermo Fisher Scientific, Inc.). T25 flasks were used for cultivation. The cells were inoculated for one hour with 300 µL of a 1:10 dilution of the homogenates from the PCR-positive tick pools and then incubated at 37 °C for 5 to 7 days (MEM, 1x NEAA, 2% FBS (Gibco ™, Thermo Fisher Scientific, Inc.). Cultivation success was tested using the RT-PCR by Schwaiger and Cassinotti (2003).

2.6. Whole genome sequencing

The whole genome was amplified in three DNA fragments of 5.2–5.6 kb, using primers covering large areas of the genomes (Table 1). The fragments were mechanically sheered using a Bioruptor UCD-200 (Diagenode Diagnostics) and prepared for sequencing using the TruSeq Nano DNA Low Throughput Library Prep Kit (Illumina, Inc.). The Illumina MiSeq platform was used for sequencing, and the sequencing chemistry used was the MiSeq reagent kit V3 (Illumina, Inc.), as described by the manufacturer. Assembly into a single contig was performed using the software Spades v.3.12 (Bankevich et al., 2012).

Table 1
Primers for the amplification and sequencing of the whole genome of TBEV.

Primers[a]	Sequence (5´–3´)	Size (nt)
TBEw-c11141	AGATTTTCTTGCACGTGCATGCG	23
TBEw-c5670	GGAGGTGGCAATCATGGATGA	21
TBEw-5451	GATCCAGTCAAACCCGTCAC	20
TBEw-1	AGCGGGTGTTTTTCCGAGTC	20

[a] Numbers correspond to nucleotide positions in the genome of TBEV-Eu strain Neudoerfl (GenBank accession. no. U27495).

2.7. Phylogenetic analysis

For the phylogenetic comparison TBEV-Eu whole genome sequences published in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), were chosen, as well as an LIV sequence which was used to root the tree. Phylogenetic and molecular evolutionary analyses were conducted using the software MEGA version 7 (Kumar et al., 2016). The sequence alignment was performed with the MUSCLE algorithm (Edgar, 2004). The evolutionary history was inferred by using the maximum likelihood approach based on the general time reversal model (Nei and Kumar, 2000). The bootstrap consensus tree was inferred from 1000 replicates. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates were collapsed (Felsenstein, 1985). Supplementary material S1 contains phylogenetic analyses based on 56 TBEV-Eu E gene sequences, using the maximum likelihood and neighbor-joining approaches.

2.8. Pooled prevalence analyses

The prevalence of TBEV or LIV infection in I. ricinus ticks was estimated by two methods; 1) Minimum Infection Rate (MIR) the most commonly used method for the estimation of pooled prevalence in ticks. This method assumes that only one infected tick is present in any positive pool. The MIR is considered an acceptable value for arboviruses occurring in their vector populations at low prevalence (Cowling et al., 1999; Ebert et al., 2010; Speybroeck et al., 2012). It has the disadvantage of not providing a measurement of the uncertainty of the estimation. Therefore, we also performed 2) Maximum likelihood estimates of individual prevalence (EP) in ticks by using Epitools, an online open-source epidemiological calculator available at http://www.ausvet.com.au/. This method is based on generalized linear modeling that takes in to account that the collected pools are of different sizes and assumes a perfect (100%) sensitivity and specificity of the tests (Williams and Moffitt, 2001). This tool and method have also been used in other tick studies to estimate pooled prevalence (Andreassen et al., 2012; Pilloux et al., 2015).

3. Results

3.1. TBEV in questing ticks

In total, 7673 nymphs and 1648 adult *I. ricinus* were collected from 31 locations in Denmark in the years 2014, 2015 and 2016. Of these, 1560 nymphs and 722 adults were collected from 12 sites on the TBE-endemic Bornholm island; 6113 nymphs and 926 adults were collected from 19 sites outside of Bornholm (Table 2). The area Bøtø Plantage was the only site, which was revisited a second time. During the second visit in 2016, an *I. Inopinatus* nymph was identified among the collected *I. ricinus*. This was the first identification of *I. inopinatus* in Denmark (Chitimia-Dobler et al., 2017). The *I. inopinatus* nymph was not examined for the presence of TBEV and is therefore not included in this study. No other tick species were identified. In total, two tick pools tested positive for TBEV, one pool of 11 females and one pool of 8

Table 2
Danish *Ixodes ricinus* collected in 2014, 2015 and 2016 analyzed for TBEV infection.

	Area	GPS-coordinates Latitude	Longitude	Collected (Mo, Year)	No. of collected ticks Nymphs	♀	♂	Total	Pools	Prevalence MIR	EP [CI95 %]	Previous studies TBEV/LIV In Ticks†	Roe deer: Pos/n (highest NT)‡
Bornholm													
1	Rønne Plantage	55.097	14.745	July, 2014	106	55	66	227	35	0.0	0.0	No/No	2/2 (120)
2	Chr. Høj	55.120	14.902	July, 2014	289	21	21	331	33	0.0	0.0	No/Yes	0/1 (<10)
3	Årker Plantage	55.102	14.978	July, 2014	140	28	24	192	25	0.0	0.0	-	1/1 (30)
4	Due Odde	54.990	15.073	July, 2014	6	1	2	9	3	0.0	0.0	No/No	-
5	Paradisbakkerne	55.096	15.108	July, 2014	228	48	33	309	31	0.0	0.0	Yes/Yes	-
6	Glappe	55.155	15.030	July, 2014	30	8	10	48	7	0.0	0.0	-	1/2 (40)
7	Østermarie Plantage	55.104	15.017	July, 2014	62	61	60	183	30	0.0	0.0	-	1/1 (160)
8	Krakken	55.213	14.850	July, 2014	79	29	29	137	20	0.0	0.0	-	0/2 (<10)
9	Rø Plantage	55.182	14.906	July, 2014	135	26	26	187	20	0.0	0.0	No/Yes	0/2 (<10)
10	Hammershus	55.272	14.763	July, 2014	204	55	49	308	43	0.0	0.0	No/No	0/1 (<10)
11	**Rubinsøen**	55.171	14.716	July, 2014	279	33	28	340	34	**0.6***	**0.6 [0.1-1.85]**	Yes/Yes	-
12	Knarre Mose	55.224	14.766	July, 2014	2	1	8	11	3	0.0	0.0	-	1/3 (120)
	All of Bornholm				**1560**	**366**	**356**	**2282**	**284**	**0.08**	**0.09 [0.04-0.27]**		
Rest of Denmark													
13	Bøtø Nor	54.684	11.922	July, 2015	326	15	34	375	52	0.0	0.0	-	0/3 (<10)
14	Bøtø Plantage	54.610	11.955	July, 2015	387	24	22	433	55	0.0	0.0	-	8/9 (940)
15	Bøtø Plantage	54.622	11.955	July, 2016	1558	109	116	1783	204	0.0	0.0	-	8/9 (940)
16	Lyne Plantage	55.808	8.490	Aug, 2015	48	13	18	79	11	0.0	0.0	-	0/7 (<10)
17	Eg Plantage	55.750	8.798	Aug, 2015	296	24	42	362	47	0.0	0.0	-	0/3 (<10)
18	Farre	55.807	9.249	July, 2014	33	2	5	40	5	0.0	0.0	-	0/1 (<10)
19	Hejlskov	56.623	9.214	Aug, 2015	382	16	14	412	51	0.0	0.0	-	2/5 (40)
20	Ørslev Kloster	56.596	9.215	Aug, 2015	219	15	7	241	34	0.0	0.0	-	-
21	Stoholm	56.512	9.131	Aug, 2015	60	4	6	70	11	0.0	0.0	-	1/1 (<10)
22	Kalø	56.280	10.499	Aug, 2015	386	14	15	415	51	0.0	0.0	-	1/5 (40)
23	Rostved	56.311	10.514	Aug, 2015	62	1	2	65	8	0.0	0.0	-	0/5 (<10)
24	Aarhus Airport	56.296	10.619	Aug, 2015	423	31	27	481	62	0.0	0.0	-	0/1 (<10)
25	Borchminde Skov	55.756	9.783	Aug, 2015	364	24	21	409	54	0.0	0.0	-	2/4 (40)
26	Daugaard	55.756	9.738	Aug, 2015	343	17	21	381	49	0.0	0.0	-	0/3 (<10)
27	Professorens Plantage	57.281	10.538	July, 2015	223	43	39	305	44	0.0	0.0	-	1/4 (15)
28	Dronninglund Storskov	57.242	10.223	July, 2015	285	37	39	361	48	0.0	0.0	-	1/4 (60)
29	Brokhede Plantage	56.601	9.975	Aug, 2015	106	3	7	116	15	0.0	0.0	-	1/1 (40)
30	Katsig bakker	57.449	10.344	July, 2015	282	21	31	334	46	0.0	0.0	-	1/1 (40)
31	Kragskovhede	57.517	10.365	July, 2015	330	22	25	377	49	0.0	0.0	-	0/4 (<10)
All locations incl. Bornholm					**7673**	**801**	**356**	**9321**	**1180**	**0.02**	**0.02 [0-0.07]**		

*One pool containing adult female ticks and one pool containing adult male ticks tested positive.
†Reference Jensen et al., 2004Jensen et al. (2004).
‡Reference Andersen et al., 2019Andersen et al. (2019).

males, with Ct values of 32.42 and 30.03 respectively. Both positive pools were collected on the 30th of July 2014 from a small area on the northwestern shore of the artificial lake Rubinsøen on Bornholm (Fig. 2). The local estimated prevalence of TBEV in all collected ticks from lake Rubinsøen was 0.6% [CI 95% 0.1–1.85%] Table 2. When only looking at adult ticks this prevalence increased to 3.85% [CI 95% 0.65–11.56 %].

3.2. E gene sequencing

Further confirmation of the two positive pools was obtained by sequencing the complete E-genes. Fragments of 1582 (TBEV-DK2014-Bornholm1) and 1597 (TBEV-DK2014-Bornholm2) nucleotides (nt) were amplified from the tick homogenates. Alignment revealed that the two sequences were identical.

3.3. Virus isolation

Virus isolation was succeeded for the DK2014-Bornholm2 strain.

3.4. Whole genome sequencing

The whole genome of the isolated TBEV-DK2014-Bornholm2 was successfully sequenced to a length of 10,256 nt. The genomic ends have so far not been verified. Therefore the primer sequences were trimmed off prior to analysis.

3.5. Phylogenetic analysis

The TBEV-DK14-Bornholm2 strain is placed in the TBE-Eu subtype in the phylogenetic tree of 35 whole TBEV-Eu genome sequences (Fig. 3). The closest genetic relatives (bootstrap support = 90%) are a cluster of sequences from central European strains from Wagnitz, Austria; Bavaria, Germany, Joutseno; Finland, central Bohemia and Czech Republic and several strains from Slovakia (Formanová et al., 2015; Frey et al., 2014, 2013; Kupča et al., 2010; Uzcátegui et al., 2012). Phylogenetic analyses of 56 TBEV-Eu E genes are available in Supplementary material S1. Alignment of all Danish E genes shows that the Tokkekøb Hegn, Nort Zealand strains (DEN11-T2, DEN11-T2), the historic Bornholm strain (Denmark1 - GenBank EF565946) and the sequences presented in this study (DK2014-Bornholm1, DK2014-Bornholm2) are different from each other. Alignment data are available in Supplementary material S2. Alignment and BLAST against the NCBI Genbank database of the small 443 nt E gene fragment from the historic TBEV-Eu strain presumably from Bornholm show that this fragment is identical to the Russian Absettarov strain (data not shown).

4. Discussion

Tick-borne encephalitis has been known from clinically diagnosed human patients on Bornholm since the 1950s (Freundt, 1963). However, so far, no virus strains have been isolated, and only one short sequence of TBEV has been published (Skarpaas et al., 2006). TBEV and LIV in ticks have previously been described in multiple sites (Jensen et al., 2004), all these sites were revisited in the current study, and additional sites were chosen. We present the first isolated virus and whole genome sequence from ticks on the Danish Bornholm island, from an area on the northwestern shore of lake Rubinsøen (Fig. 2).

We only found one area on Bornholm with TBEV positive ticks, while no positive ticks could be detected in 11 other locations. In a previous study, 738 nymphs, 37 male, 41 female ticks were collected and analyzed at three suspected TBEV sites on Bornholm. There the authors also did not manage to find positive ticks (Fomsgaard et al., 2013). The absence of positive ticks in the former positive foci from the

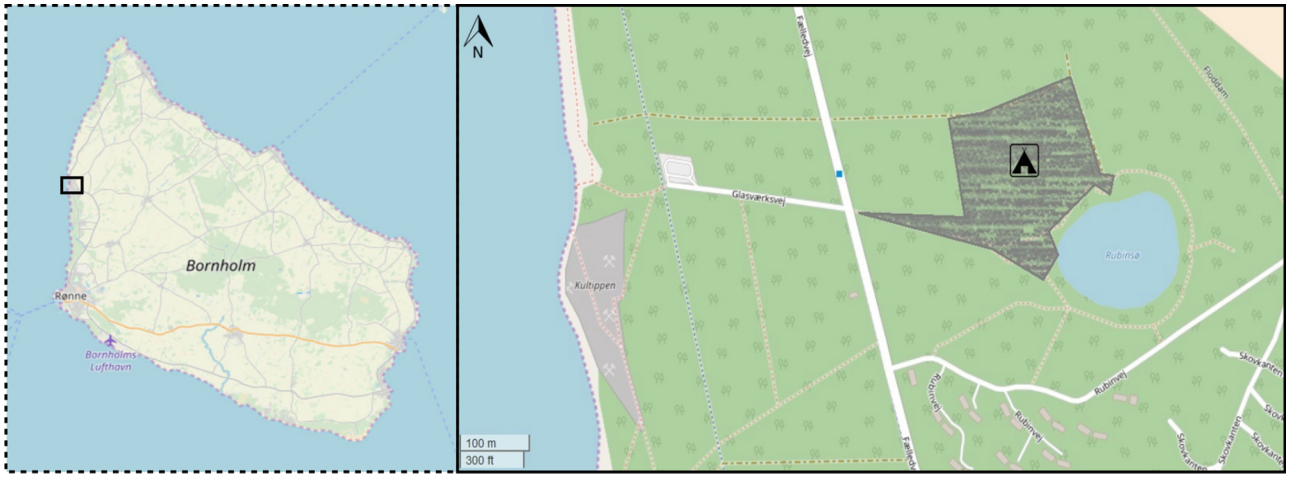

Fig. 2. TBEV microfocus at lake Rubinsøen, Bornholm, Denmark.
The grey scaled area illustrates the TBEV PCR-positive tick collection site at lake Rubinsøen, Bornholm, Denmark. In the center of the collectionsite, is a public camping ground with multiple shelters. Map data © OpenStreetMap contributors, Open Database License, "ODbL" 1.0.

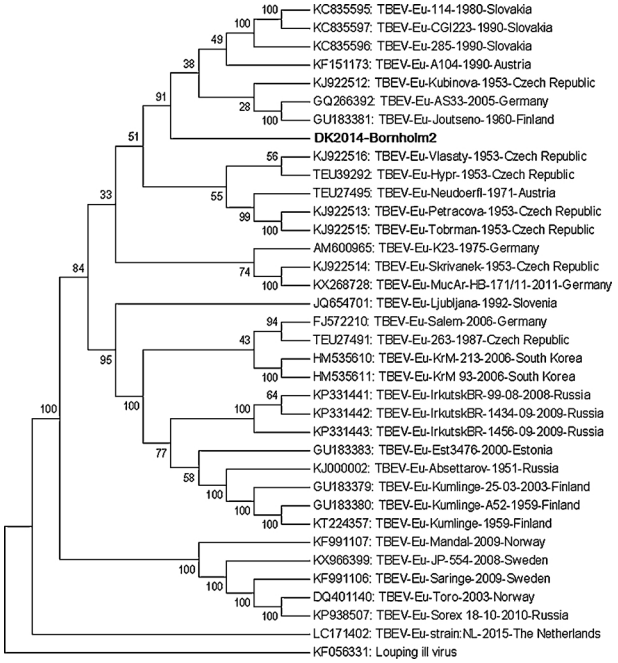

Fig. 3. Molecular phylogenetic analysis of TBEV whole genome sequences by the maximum likelihood approach.
The tree was generated using 35 TBE-Eu whole genome sequences. The NCBI GenBank accession numbers are available in the figure, so are the year of isolation and country of origin. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of TBEV-Eu. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree was rooted using Louping ill virus (LIV). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 10,242 positions in the final dataset. Evolutionary analyses were conducted using the software MEGA 7 (Kumar et al., 2016).

1999 study is unusual, as the TBE incidence in humans of 4/100,000 on the island seems to be stable and this indicates stable transmission cycles (Laursen and Knudsen, 2003; Ocias et al., 2017). However, due to the small size of TBEV natural foci maybe the foci were missed during the flagging activities. Also, the number of ticks from the respective foci were not high enough to detect the virus. It could be that TBEV foci are established after an introduction and then disappear after some time. This has been seen in the microfocus on north Zealand Denmark; here no new TBE patients has been found since the first two patients were diagnosed in 2009. Also, ticks collected in this microfocus, previously testing TBEV-positive by PCR, have tested negative since 2016 (Fomsgaard, 2017). However, Finish and German groups found that TBE natural foci are stable for decades and identical virus strains have been detected there for more than 50 years (Jääskeläinen et al., 2010). Therefore, further analysis of whole genome sequences from Bornholm is needed to confirm this hypothesis.

In total, we collected and analyzed 2282 ticks from Bornholm. The estimated prevalence of TBEV was calculated by both MIR and EP (Table 2), albeit using EP as the method of choice. We estimated the TBEV prevalence in ticks on the island to be 0.09% [CI 95% 0.04–0.27%]. Focusing on the area on the northwestern shore of lake Rubinsøen, the estimated prevalence was 0.6% [CI 95% 0.1–1.85%], which is entirely within the range found in other natural foci in central Europe (Pettersson et al., 2014; Stefanoff et al., 2013). These data are also consistent with a TBE microfocus, as described in central Europe, and Denmark (Dobler et al., 2011; Fomsgaard, 2017).

The phylogenetic analysis demonstrated that the closest related sequences to the whole genome TBEV strain from Bornholm (DK2014-Bornholm2) were TBEV sequences detected from Austria; Germany, Finland, Slovakia, and the Czech Republic (Fig. 3). The bootstrap consensus phylogenetic trees of TBEV-Eu E genes, unfortunately, do not contribute with substantial additional information due to the week bootstrap values (Supplementary S1). However, when looking at the tree made by the neighbour joining approach it does seem that the Danish strains from Tokkekøb Hegn, north Zealand (DEN11-T2, DEN11-T3) cluster in a genetic clade with Scandinavian strains from Sweden, Norway and Finland, whereas the Bornholm strains presented in this study (DK2014-Bornholm1, DK2014-Bornholm2) cluster in a different clade with strains from Finland, Sweden, Slovenia, Germany, Czech Republic and Austria. This finding is further supported by the phylogenetic analysis of the whole genome sequences, because many of the strains in the analysis of the E genes are identical to the strains clustering with the DK2014-Bornholm2 strain in the whole genome sequence analysis (Fig. 3 and Supplementary S1).

To the best of our knowledge, the only TBEV sequence available from Bornholm is the partial E gene Denmark1 (GenBank EF565946) from the CSF of the Norwegian TBE patient (Skarpaas et al., 2006). Although not a complete E gene sequence, even the analysis of the fragment clearly shows that the historical sequence is different from the sequences found in the current study. The strain also differs from the

TBEV strains identified from ticks from the microfocus of Tokkekøb Hegn, north Zealand (DEN11-T2, DEN11-T3) (Supplementary S2). However, the geographical origin of the Denmark1 strain is more uncertain. TBEV is endemic in local areas in Norway and transmission from a Norwegian tick is therefore a possibility.

These results imply multiple importations of TBEV to Denmark, and even to Bornholm and corresponds well with the theory that TBEV is spread by migrating birds carrying infected ticks (Suzuki, 2007; Waldenström et al., 2007; Weidmann et al., 2013). However, further studies are warranted to obtain additional whole genome sequences from Danish TBEV strains for further phylogenetic and phylogeographic comparison, in order to answer the question of the virus introduction to different geographical regions.

Outside of Bornholm, tick collection areas were chosen, where neutralizing antibodies against TBEV were detected in roe deer bagged during hunting, as a surrogate marker for the existence of TBEV transmission. We had a particular interest in the area Bøtø Plantage which is forest area of more than 1,500,000 m^2, on the southern tip of the Danish island of Falster, primarily comprising of pine wood and an important first Danish landing point for migrating or breeding birds. In this site, 89% (8/9) roe deer were observed to have neutralizing antibodies against TBEV (Andersen et al., 2019). This area was sampled in two consecutive years (area 14 in 2015 and area 15 in 2016, see, Table 2) and 2216 ticks were collected and tested, however all with negative results. We only covered a small proportion of Bøtø Plantage. The small spatial distribution of natural TBE foci might be the reason why we did not find positive ticks, we could have easily chosen the wrong sampling site without natural TBEV circulation, within the large forested area. The failure to find positive ticks indicates that roe deer might not be the best way to detect TBEV natural foci on a small geographical scale. Roe deer usually only disperse 2–3 km from their birthplace (Strandgaard, 1972; Wahlström and Liberg, 1995), but it is not feasible to flag ticks in the entirety of a roe deer's home range. Nevertheless, the detection of circulating TBEV in ticks is essential for valid identification of TBE-endemic areas, the same goes for characterization of the circulating TBEV strains and its pathogenicity and hence the potential risk of disease in humans (Dobler et al., 2012; Süss, 2011).

We did not manage to rediscover LIV on Bornholm nor did we find TBEV or LIV in the predicted TBE complex risk areas outside of Bornholm. One reason for this could be that we did not collect a sufficient amount of ticks, in a given risk area, to be sure to find infected ticks. In many areas, i.e., 4, 12, 16, 18, 21 and 23 (Fig. 1) it was not feasible to collect the desired minimum of 350 ticks. Due to a low tick density, two areas with positive roe deer, near area 16 and 17 (Fig. 1) were abandoned entirely due to lack of ticks (data not shown). Even on Bornholm, known for a high tick density, it was difficult to reach the desired number. The summer of 2014, when the ticks were collected on Bornholm, was arid with high temperatures, probably making most ticks hide in the vegetation (Jensen et al., 2004, 2000). Precisely for this reason, screening of ticks by PCR is not a recommended method for assessment of human TBE risk (Stefanoff et al., 2013). This can, however, not be the explanation in areas; 2, 14, 15, 22 and 28 (Fig. 1 and Table 2), where the tick density was high, and we reached more than the desired number of ticks and still did not find any positive ticks.

5. Conclusion

To the best of our knowledge, we present the first Danish TBEV whole genome. The virus originates from the endemic island of Bornholm. Our results show that TBEV is still endemic on Bornholm and the genetic relationship to central and eastern European TBEV strains is an additional indication of transport and spread of TBEV via migrating birds. It must be further elucidated if the TBEV strain found on Bornholm is the only strain circulating there, or whether different strains and other tick-borne viruses (Louping ill virus) are circulating in ticks on the island. We did not manage to confirm any of the predicted TBE complex areas outside of Bornholm.

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Declarations of interest

The author declares no conflict of interest.

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.2018.12.008.

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