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Tick-borne encephalitis virus, *Borrelia burgdorferi* sensu lato, *Borrelia miyamotoi*, *Anaplasma phagocytophilum* and *Candidatus* Neoehrlichia mikurensis in *Ixodes ricinus* ticks collected from recreational islands in southern Norway

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ABSTRACT

The aim of this study was to determine the occurrence of tick-borne pathogens of medical importance in questing ticks collected from five recreationally used islands along the Norwegian coastline. Furthermore, since coinfection may affect the disease severity, this study aimed to determine the extent of coinfection in individual ticks or co-localization of tick-borne pathogens. In all, 4158 questing *Ixodes ricinus* ticks were analyzed. For detection of tick-borne encephalitis virus (TBEV), nymphs (3690) were analyzed in pools of ten. To detect *Borrelia burgdorferi* sensu lato, *B. miyamotoi*, *Anaplasma phagocytophilum* and *Candidatus* Neoehrlichia mikurensis, 468 nymphs were analyzed individually. A total of five nymph pools was infected with TBEV, giving an overall prevalence of 0.14%. In the individually analyzed ticks, *B. burgdorferi* s. l. (15.6%), *Candidatus* N. mikurensis (11%), *A. phagocytophilum* (1.4%) and *B. miyamotoi* (0.9%) were detected. Coinfection was found in 3.3% of the ticks, and the only dual infection observed was with *B. afzelii* and *Candidatus* N. mikurensis. This association was significantly higher than what would occur by random chance.

1. Introduction

The main tick vector for human and animal disease in Europe is *Ixodes ricinus*. Norway is part of the northern border of the geographical range of *I. ricinus*, and ticks are mainly found along the coastal regions from Østfold County in the southeast to Brønnøysund in Nordland County in the north (Mehl, 1983; Hvidsten et al., 2015; Soleng et al., 2018).

Ixodes ricinus maintains a diverse array of pathogens in enzootic cycles. Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis (TBE), which is considered the most serious viral tick-borne human disease in Europe (Süss, 2011). The European TBEV subtype is present in Norway (Andreassen et al., 2012), however, the incidence of TBE is low; from 1994 to 2017, there have been 142 reported TBE cases infected in Norway according to Norwegian Surveillance System for Communicable Diseases (MSIS, 2018).

Ixodes ricinus may also transmit *B. burgdorferi* s. l., which is widely distributed throughout the tick infested areas of Norway (Kjelland et al., 2010a; Soleng and Kjelland, 2013). This group includes the causative agents of Lyme borreliosis (LB), the most important human tick-borne disease in Europe in terms of disease incidence and public attention (Franke et al., 2013). In 2016, 333 cases of disseminated LB infection were reported in Norway, a national incidence of 6.3/100.000 inhabitants (MSIS, 2018). In the southernmost parts where the tick

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population density is higher, the occurrence of *Borrelia* infection is higher. For instance, in Vest-Agder County, where one of the islands in the present study is located, the incidence is 18.5/100.000 inhabitants. However, early localized infection as erythema migrans skin lesion is not notifiable in Norway, hence the total frequency of infection is unknown. Recently, *B. miyamotoi*, the only tick-borne member of the relapsing fever borreliae detected in *I. ricinus*, was reported in Norway (Kjelland et al., 2015; Quarsten et al., 2015).

Tick-borne fever (TBF) is caused by *Anaplasma phagocytophilum*, and is a major scourge of the sheep industry in Norway. It has been estimated that more than 300 000 lambs, 15% of lambs on summer pasture, are infected annually (Stuen and Bergström, 2001). In humans, clinical manifestations range from a mild self-limited febrile illness, to a life-threatening collapse of the immune system (Bakken and Dumler, 2015). Human infection has been reported in Norway, but the epidemiological importance of *A. phagocytophilum* in this country is unknown (Kristiansen et al., 2001; Hjetland et al., 2015).

Candidatus Neoehrlichia mikurensis (*Candidatus* N. mikurensis) is an emerging tick-borne pathogen belonging to the Rickettsiales. The first case of human disease caused by the pathogen was reported in 2010 from Sweden (Welinder-Olsson et al., 2010). Recently, neoerlichiosis was also described in one patient from Norway (Frivik et al., 2017). Neoehrlichiosis is primarily a disease of immunosuppressed patients, who experience recurring fevers accompanied by a variety of other symptoms including musculoskeletal pain and deep-vein thrombosis (Grankvist et al., 2014).

As tick-borne pathogens often occur in the same area, wildlife and humans are frequently infected by multiple pathogens, or several genotypes of a single pathogen, simultaneously (Diuk-Wasser et al., 2016). The risk of coinfection with multiple pathogens after a tick bite differs by geographic location, depending on the prevalence of pathogens in the ticks and their host animals. However, the prevalence of coinfecting human pathogens among *Ixodes* ticks remains unknown in the majority of geographic locations. The aim of this study was to investigate the prevalence of multiple tick-borne pathogens in public-use recreational sites at five island locations in Norway.

2. Material and methods

2.1. Study area and collection of ticks

Questing *I. ricinus* nymphs (4158) were collected from five islands in the southern parts of Norway. All islands are frequently visited by the public throughout the spring, summer and autumn months, coinciding with the peak of the tick activity period in Norway. The ticks were collected during one single day of the year from each sampling site: Hille (Vest-Agder County), Tromøy (Aust-Agder County), Håøya and Brønnøya (Akershus County) and Spjærøy (Østfold County) (see Table 1 and Fig. 1). All ticks were collected by flagging as described by Andreassen et al. (2012). Larvae and adult ticks were omitted from the analyses as nymphs are responsible for the vast majority of tick bites on humans (Robertson et al., 2000). The collected nymphs were stored at 0 °C during transportation to the laboratory, then stored dry at −80 °C until analysis.

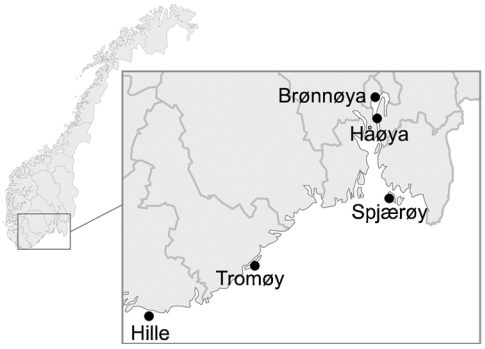

Fig. 1. Geographical location of the five islands located in southern Norway. Map created from Kartverket (Creative Commons Attribution ShareAlike 3.0).

2.2. RNA and DNA extraction

A total of 3690 *I. ricinus* nymphs in pools of ten were homogenized and total RNA was extracted as previously described (Andreassen et al., 2012). DNA was manually extracted from 468 individual nymphs by phenol-chloroform as previously described (Kjelland et al., 2010b), and stored at −20 °C until further analysis.

2.3. Detection of tick-borne pathogens

Total RNA extracts were reverse transcribed immediately after extraction and analyzed for TBEV by real-time PCR and pyrosequencing as previously described (Andreassen et al., 2012). Detection of *B. burgdorferi* and differentiation of the *B. burgdorferi* s. l. strains, as well as the detection of *B. miyamotoi* was also done as previously described (Kjelland et al., 2010a). *Candidatus* N. mikurensis was detected using primers Neo2F, GCA AAT GGA GAT AAA AAC ATA GGT AGT AAA A and Neo2R, CAT ACC GTC AGT TTT TTC AAC TTC TAA targeting the *groEL* gene (A. Jenkins, C. Raasok, K. Jensen, Å. Andreassen, A. Soleng, K. Skarsfjord Edgar, H. Heggen Lindstedt, V. Kjelland, S. Stuen, D. Hvidsten, B.-E. Kristiansen, unpublished). Applied Biosystems SYBR-green mastermix was used. The PCR program was 50 °C, 2 min; 95 °C, 10 min, (95 °C, 15 s; 60 °C, 1 min) × 45 cycles, followed by dissociation analysis (60 °C to 95 °C with 0.3 °C increments). *Anaplasma phagocytophilum* was detected as described by Henningsson et al. (2015), except that SYBR green was used instead of the TaqMan probe. After amplification, a melting curve was generated for verification of PCR positive samples. A Tm (melting temperature) between 71 °C and 74 °C was regarded as a positive result.

2.4. Calculations and statistical analysis

The required sample size for detection of TBEV was estimated by

Table 1
Sampling sites with global position coordinates, date of tick collection, description of sampling area and number of *I. ricinus* nymphs collected.

Island	County	Sampling site	Date of sampling	Description of sampling area	Number of nymphs collected
Hille	Vest-Agder	58°00′N; 07°21′E	12th June 2012	Southern steep hillside with small deciduous trees, grass and herbs. Rodent burrows and runways. Deer tramplings.	840
Tromøy	Aust-Agder	58°28′N; 08°54′E	13th June 2012	Mixed forest with grass, herbs, heather. Deer tramplings.	840
Håøya	Akershus	59°41′N; 10°34′E	31th May 2013	Deciduous trees with some pinetrees, grass, herbs, ferns and heather. Deer tramplings.	840
Brønnøya	Akershus	59°51′N; 10°32′E	6th June 2013	Forest edge with grass, herbs and heather. Deer tramplings.	828
Spjærøy	Østfold	59°05′N; 10°55′E	13th May 2012	Deciduous trees, grass, herbs, ferns and heather. Rodent burrows and runways, and deer tramplings.	810

Table 2
Co-localization of TBEV, *B. burgdorferi* s. l., *B. miyamotoi*, *A. phagocytophilum* and *Candidatus* N. mikurensis in nymphal *I. ricinus* ticks collected from five Norwegian islands.

Collection site	TBEV n/N (%)[a]	*B. burgdorferi s. l.* n/N (%)	*B. miyamotoi* n/N (%)	*A. phagocytophilum* n/N (%)	*Candidatus* N. mikurensis n/N (%)
Hille	4/740 (0.54)	25/100 (25)	0/100 (0)	0/80 (0)	15/80 (19)
Tromøy	0/740 (0)	10/100 (10)	0/100 (0)	0/95 (0)	9/95 (10)
Håøya	0/740 (0)	16/100 (16)	0/100 (0)	0/96 (0)	5/96 (5)
Brønnøya	0/730 (0)	10/98 (10)	4/98 (4)	1/92 (1)	11/92 (12)
Spjærøy	1/740 (0.14)[b]	12/70 (17)	0/70 (0)	5/66 (8)	7/65 (11)
Total	5/3690 (0.14)	73/468 (15.6)	4/468 (0.9)	6/429 (1.4)	47/428 (11.0)

[a] n: number of positive samples, N: number of analyzed ticks, %: The calculated prevalence for each tick-borne pathogen.
[b] TBEV results from Spjærøy is previously published by Larsen et al. (2014).

use of the "sample size calculation for fixed pool size and perfect tests" from EpiTools (Sergeant, 2015). It was estimated that it was necessary to analyze 730 nymphs from each location to be able to detect at least one positive pool of TBEV (Andreassen et al., 2012). The prevalence of TBEV was calculated as the minimum infection rate (MIR), as described by Andreassen et al. (2012).

The chi-squared test was used to test for under- or overprevalence of coinfections. In all cases a probability of $p \leq 0.05$ was regarded as statistically significant.

3. Results

3.1. Prevalence of TBEV

A total of 3690 pooled nymphs were analyzed for TBEV. Sequenced-confirmed TBEV was detected at two locations, with a prevalence of 0.54% and 0.14%, respectively (Table 2). One further real-time PCR positive pool was found at each of four locations, but these could not be confirmed with pyrosequencing.

3.2. Prevalence of Borrelia burgdorferi sensu lato, Borrelia miyamotoi, Candidatus Neoehrlichia mikurensis and Anaplasma phagocytophilum

Borrelia spp. was detected in 77/468 (16.5%) ticks. The prevalence ranged from 25% to 10% (Table 2). The most prevalent *B. burgdorferi* genospecies identified was *B. afzelii* (63/73, 86%), followed by *B. garinii* (9/73, 12%) and *B. valaisiana* (1/73, 1.4%). *Borrelia miyamotoi* was detected in 4 (0.9%) ticks, all collected from the same location. The overall prevalences of *Candidatus* N. mikurensis and *A. phagocytophilum* were 11% and 1.4%, respectively. *Candidatus* N. mikurensis was detected at all locations. The prevalence ranged from 18.8% to 5.2%. *Anaplasma phagocytophilum* was detected at two locations with prevalences of 7.6% and 1.1% respectively.

3.3. Coinfections and co-localization of tick-borne pathogens

428/468 ticks were analyzed for all four of the pathogens *B. burgdorferi* s. l., *B. miyamotoi*, *A. phagocytophilum* and *Candidatus* N. mikurensis. The only co-infection detected was *B. afzelii* and *Candidatus* N. mikurensis, which was found in 14/428 (3.3%) ticks. The prevalence of this coinfection was 3.3%, more than twice that expected by chance (1.5%, $p < 0.0005$). No other significant positive or negative correlations were detected. *Borrelia afzelii* and *Candidatus* N. mikurensis were present at all locations.

At no location were all four bacterial pathogens plus sequence-confirmed TBEV found, however multiple pathogens were detected at all locations (Table 2).

4. Discussion

In the present study, we assessed the prevalence of TBEV, *B. burgdorferi* s. l., *B. miyamotoi*, *A. phagocytophilum* and *Candidatus* N. mikurensis in questing *I. ricinus* ticks collected at five island locations in southern Norway popular for recreational purposes. The local tick populations are supported by diverse animal species which are supplemented by animals that cross from the mainland when the sea freezes in cold winters. Ticks may be transported to the islands by residents, their pets and domestic animals, and birds. Especially ground feeding avian species are considered important in the dispersal of ticks and tick-borne pathogens, and migratory birds have been shown to carry large amounts of ticks to Norway (Kjelland et al., 2010b; Hasle et al., 2011). Our results indicate that visitors to these islands risk exposure to ticks that carry multiple pathogens.

Borrelia burgdorferi s. l. was the most prevalent pathogen detected in 10–25% of the nymphs, depending on location. The prevalence of *B. burgdorferi* s. l. in questing ticks in Norway varies between locations and through the season, however typically ranging from 20 to 30% (Jenkins et al., 2001; Kjelland et al., 2010a; Soleng and Kjelland, 2013). The most prevalent *B. burgdorferi* genospecies identified was *B. afzelii* (86%), followed by *B. garinii* (12%) and *B. valaisiana* (1.4%). This is similar to what is reported previously in southern Norway (Kjelland et al., 2010a), as well as in Europe in general (Strnad et al., 2017). The genospecies composition may be of clinical importance, as different genospecies are associated with different clinical manifestations of Lyme borreliosis (Strle and Stanek, 2009). Previously, the pathogenic species *B. garinii*, *B. afzelii* and *B. burgdorferi* sensu stricto have all been detected in Norway, in addition to the less pathogenic *B. valaisiana*, *B. finlandensis* and *B. turdi* (Kjelland et al., 2010a; Hasle et al., 2011; Kjelland et al., 2011). *Borrelia miyamotoi*, a spirochete associated with relapsing fever, was found in 4 ticks, all collected from the island Brønnøya (total and local prevalence was 0.9% and 4.1%, respectively). The real-time PCR probe used in the method applied in present study (Kjelland et al., 2010a) targets *B. burgdorferi* s. l., and *B. miyamotoi* was detected by chance. Although the primers do detect *Borrelia* spp., the probe is not designed to detect *B. miyamotoi*, and it is not unlikely that the infection prevalence found in this study is underestimated. Although a previous study (Kjelland et al., 2015) detected *B. miyamotoi* at all the investigated locations, in view of the low prevalences observed this is consistent with the present findings. Human cases of tick-borne relapsing fever are reported in other parts of Europe, but none have so far been reported in Scandinavia (Kjelland et al., 2015).

Candidatus N. mikurensis was detected in 11% of the ticks. The pathogen was detected in questing ticks from Norway for the first time in 2001 (Jenkins et al., 2001), and has later been found to be widespread in Norway, with an overall prevalence of 6.7% (A. Jenkins, C. Raasok, K. Jensen, Å. Andreassen, A. Soleng, K. Skarsfjord Edgar, H. Heggen Lindstedt, V. Kjelland, S. Stuen, D. Hvidsten, B.-E. Kristiansen, unpublished). We cannot exclude the possibility that other *Neoehrlichia* species are represented; the current PCR test is expected to cross-react with *Candidatus* N. lotoris and no relevant sequence data is available for the remaining species. However, all PCR products so far sequenced were identical to a European variant of *Candidatus* N. mikurensis (A. Jenkins, C. Raasok, K. Jensen, Å. Andreassen, A. Soleng, K. Skarsfjord Edgar, H. Heggen Lindstedt, V. Kjelland, S. Stuen, D. Hvidsten, B.-E.

Kristiansen, unpublished). Recently, the first human cases of *Candidatus* N. mikurensis infection in Norway was reported after detecting the pathogen in 7/70 blood samples collected from symptomatic adults recently bitten by ticks (Quarsten et al., 2017). Shortly after this report, the first case of human neoerlichiosis was confirmed (Frivik et al., 2017). This, combined with the relatively high prevalence of *Candidatus* N. mikurensis suggests that undiagnosed human infections may be frequent.

Fourteen nymphs (3.3%) were coinfected with *Candidatus* N. mikurensis and *B. burgdorferi* s. l., all of them belonging to the *B. afzelii* genospecies. This is significantly higher than what would be expected under random co-occurrence. Coinfections with *Candidatus* N. mikurensis and *B. afzelii* have also previously been reported in European tick populations at prevalence ranging from 0.8 to 2.3% (Andersson et al., 2013; Glatz et al., 2014). This association between *Candidatus* N. mikurensis and *B. afzelii* is thought to be due to a common reservoir host; wild rodents (Andersson and Raberg, 2011). Human coinfections are likely to occur; although few reports have been published so far, a recent Swedish study indicated coinfection of *Borrelia* spp. and *Candidatus* N. mikurensis in serum from a patient with erythema migrans (EM) (Grankvist et al., 2015). The clinical consequences of such coinfections deserve further scrutiny.

Anaplasma phagocytophilum was found in 1.4% of the ticks. This is lower than in previous Norwegian studies, although variations between locations have been reported (Jenkins et al., 2001). Although found at a low prevalence in most locations, the pathogen has a profound negative impact on the sheep industry (Stuen et al., 2003). Human anaplasmosis occurs in areas of the United States that are endemic for Lyme disease (Sanchez et al., 2016), and is reported from several European countries (Strle, 2004), including Norway (Kristiansen et al., 2001)., and antibodies have been detected in 16.2% of blood donors (Hjetland et al., 2015). The number of confirmed clinical cases is, however, small; whether this is due to underreporting, underdiagnosis or low pathogenicity of local *A. phagocytophilum* strains remains unclear (Stuen et al., 2013).

Sequence-confirmed tick-borne encephalitis virus (TBEV) was detected at 2/5 locations, in 5/369 tick pools (overall minimum infection rate (MIR) = 0.14). However, real-time PCR results suggested the presence of TBEV in tick pools from all locations. As most samples are close to the detection limit of the assays, it is not possible to conclusively determine whether these results are false positives in real-time PCR or if they are true positives but below the detection limit of the pyrosequencing assay. Circumstantial evidence favors the latter interpretation; the island of Tromøy, where TBEV was detected only by real-time PCR, is located in the region with the highest number of reported human TBE cases, and the virus has been detected in nymphal ticks collected at this island previously (Skarpaas et al., 2004; Skarpaas et al., 2006; Andreassen et al., 2012). Seasonal variation and focal distribution of tick-borne pathogens, in particular TBEV, may also have influenced the results (Randolph and Rogers, 2000; Andreassen et al., 2012).

Of the pathogens investigated here, transovarial transmission has only been demonstrated for *B. miyamotoi* and TBEV (Danielova and Holubova, 1991; Richter et al., 2012), and for *A. phagocytophilum* only in the tick *Dermacentor albipictus* (Baldridge et al., 2009). Even for these pathogens, transmission rates are low, and the main route of infection is by feeding on infected host animals. *Ixodes ricinus* may take its blood meals from a wide variety of animals and is exposed to pathogens the host may carry (Parola and Raoult, 2001). Adult ticks, which have fed twice (as larva and as nymph) may acquire multiple infections in a serial fashion from two different hosts. Nymphs have only fed once, and any multiple infections must have been acquired during a single blood meal, either from a multiply-infected host or by co-feeding, unless a transovarially-transmitted pathogen is involved; the frequency of multiple infection is thus lower in nymphs. As expected, the frequency of multiple infections in this study, which includes only nymphs, was low. However, their presence confirms that multiple infections can be acquired during a single blood meal.

The study demonstrates the presence of several tick-borne pathogens circulating in the same localities, indicating a risk of infection by multiple pathogens after tick bite(s). The medical consequences of coinfection are still mostly unknown; however, some reports indicate an altered clinical picture (Diuk-Wasser et al., 2016). In the present study, tick-borne pathogens were detected on islands frequently used for outdoor sports and leisure-time activities in the southeastern regions, where there traditionally has been little attention to the risk of tick-bite. To our knowledge, this is the first study in Scandinavia to investigate the prevalence of multiple tick-borne pathogens in *I. ricinus* collected in such areas. To increase knowledge and to prevent human disease, future studies should include a broader geographic area and include other tick-borne pathogens. Risk communication campaigns aimed at implementing preventive measures against infectious tick bites in these recreational habitats deserve particular public health efforts.

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