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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.

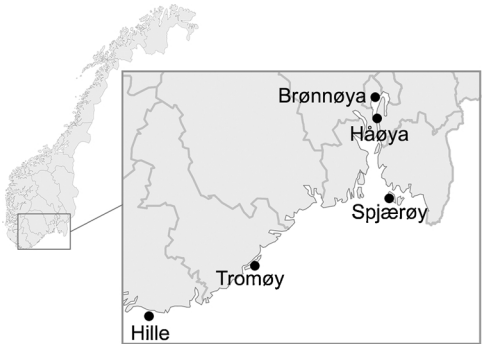

Fig. 1. Geographical location of the five islands located in southern Norway. Map created from Kartverket (Creative Commons Attribution ShareAlike 3.0).

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