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Original article

Tick-borne bacteria in *Ixodes ricinus* collected in southern Norway evaluated by a commercial kit and established real-time PCR protocols

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ABSTRACT

Ticks are important vectors of human pathogens. The knowledge of disease causing agents harboured by ticks in Norway is limited. The focus of this study was (a) to detect the bacteria of medical importance in ticks collected from the vegetation at locations in the southern part of the country and (b) to evaluate a novel commercially available multiplex PCR based method by comparing results with conventional established real-time PCR protocols.

Borrelia burgdorferi sensu lato was confirmed to be the most prevalent pathogen detected (31%) among one hundred individually analysed adult ticks. *Borrelia miyamotoi*, a spirochete associated with relapsing fever, was detected in one sample. *Anaplasma phagocytophilum* was found in 4% of the ticks, followed by *Rickettsia helvetica* which was detected in one sample. Similar pathogen prevalence was also detected in 500 ticks analysed in pools. This is the first report of the spotted fever group *Rickettsia* in Norway. *Francisella tularensis*, *Bartonella* species or *Coxiella burnetti* was not detected. However, due to the low number of ticks analysed, the possible presence of these pathogens in the region cannot be ruled out.

All isolates were screened by at least two different molecular methods for each bacterial target; one commercially available multiplex PCR based tick-borne bacteria flow chip system (Master Diagnostica) and corresponding real-time PCR protocols. The comparison of methods verified that most findings were detected by both methods (71 *Borrelia*, 15 *Anaplasma* and 2 *Rickettsia*), whereas two additional *Borrelia* and *Anaplasma* infected samples were detected by the real-time protocols.

Introduction

Ticks are important vectors for human pathogens all over the world, including bacteria causing Lyme borreliosis (LB), anaplasmosis, spotted fever group (SFG) rickettsiosis, Q fever, tularemia, trench fever, relapsing fever (RF) and cat scratch disease. Some of these tick-borne diseases are also commonly reported in Europe (Heyman et al., 2010; Parola and Raoult, 2001a).

Borrelia species are the causative agents for LB and RF. Some of the genospecies in the *Borrelia burgdorferi* sensu lato (s.l.) group, mainly transmitted by *Ixodes* ticks, may cause LB, the most common human tick-borne disease in Europe with a major impact on public health. Another distinct group of *Borrelia* may cause RF, a disease reported in other parts of the world, but not in the northern parts of Europe despite the detection of the pathogen in questing ticks (Fraenkel et al., 2002; Wilhelmsson et al., 2010, 2013). Whether this is due to lack of diagnosing/reporting of the disease or less pathogenic subspecies is uncertain.

Tick-borne rickettsiosis is caused by bacteria belonging to the SFG of the rickettsiaceae family. *Rickettsia* infections are prevalent in the south-eastern part of Europe (Oteo and Portillo, 2012). There are more than ten known *Rickettsia* subspecies causing spotted fever syndrome, harboured by different tick species. There is an emerging number of rickettsioses in the central and even northern part of Europe, where newly recognised *Rickettsia* subspecies are identified as the causative agents (Oteo and Portillo, 2012). One of them, *R. helvetica* usually cause a mild self-limiting disease, but a few reports of patients with severe disease have been published (Nilsson et al., 1999, 2010).

Human anaplasmosis is an acute infection caused by *Anaplasma phagocytophilum*, a bacterium detected in *Ixodes* species from most European countries (Stuen et al., 2013). Anaplasmosis is a

Abbreviations: LB, lyme borreliosis; RF, relapsing fever; SFG, spotted fever group; PCR-FCS, multiplex PCR bacteria flow chip system.

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mild self-limiting, but potentially severe, febrile illness (Dumler et al., 2007). A limited number of confirmed cases of human anaplasmosis in Europe have been reported, however, serological evidence of human exposure exists in many countries all over Europe (Brouqui et al., 2004). A plausible explanation for this may be that human infections in Europe usually result in minimal or no clinical manifestation.

European ticks may also be infected with *Francisella tularensis* (causing tularemia), *Coxiella burnetii* (causing Q fever) and *Bartonella* species (causing trench fever and cat scratch disease) (Angelakis et al., 2010; Parola and Raoult, 2001b). However, *Bartonella* is most commonly transmitted by fleas, whereas *F. tularensis* and *C. burnetii* mainly cause disease after exposure to infected animals or contaminated biological material. The role of ticks in human infections caused by *F. tularensis, C. burnetii* and *Bartonella* is largely unknown (Angelakis et al., 2010; Parola and Raoult, 2001b).

The incidence of tick-borne diseases varies by geographical locations and causative agents. Different tick species may act as vectors for different bacterial species (Parola and Raoult, 2001b). The various tick species have different requirements for environmental conditions and biotopes, which determine their geographical distribution and the risk-area for the various tick-borne diseases. The diversity of prevalent tick species is higher in the southern parts of Europe than in the northern parts, resulting in a higher burden of many tick-borne diseases in the south (Parola and Raoult, 2001b). In Norway, the incidence of human tick-borne disease is restricted by *Ixodes ricinus* being the only significant vector of human pathogens (Mehl et al., 1987). The bacteria transmitted by *I. ricinus* include *Borrelia* species, *A. phagocytophilum*, *Rickettsia* species, *F. tularensis*, *C. burnetti* and *Bartonella* species (Parola and Raoult, 2001b).

The only frequently diagnosed bacterial tick-borne disease in Norway is LB. However, also a few cases of anaplasmosis have been reported (Kristiansen et al., 2001). Other bacterial tick-borne infections have so far not been reported. It has been speculated that the *I. ricinus* pathogens emerge from the southern parts of Europe to previously non-endemic areas further north when circumstances favourable to their maintenance and transmission arise (Vorou et al., 2007). Hence, increased monitoring on the various infectious bacterial agents present in ticks in Norway, part of the northern distribution limit for *I. ricinus*, is important. Increased knowledge may help to understand the possible spreading patterns of tick pathogens, as well as provide important information for clinical diagnostics. The aim of this study was to narrow the gap of knowledge on the presence of potential human pathogenic bacteria in *I. ricinus* ticks in Norway. Furthermore, a new commercial molecular method developed for simultaneous detection of several tick-borne pathogens was evaluated by comparison of the results with established real-time PCR protocols.

Material and methods

Tick collection and DNA extraction

In total, 600 questing *I. ricinus* ticks were collected by the flagging method from the vegetation at three localities in the Agder counties in southern Norway (Lillesand, Søgne and Farsund). The ticks were either kept alive in humid environment or stored in 70% ethanol at +4 to +8 °C until preparation.

Pooled samples, containing ten ticks, were prepared from 330 nymphs (33 pools) and 170 adults (17 mixed pools; 51 females and 119 males). One hundred female ticks from one location (Søgne) were prepared individually. The ethanol preserved ticks were washed in phosphate buffered saline, rinsed in sterile water and lightly dried on filter paper prior to DNA extraction. The ticks were thoroughly chopped by sterile scalpel in ATL lysis buffer of the Qiagen DNA Mini kit (Qiagen, Venlo, Netherlands) supplemented with proteinase K and incubated overnight at 56 °C. Further processing for extraction of DNA was performed according to the manufacturers' instruction. The isolated DNA samples were stored at −70 °C for later analysis.

Tick-borne bacteria flow chip kit

A tick-borne bacteria flow chip kit from Master Diagnostica (Granada, Spain) was used for detection of DNA from tick-borne bacteria by multiplex PCR, followed by automated reverse dot blot hybridisation with specific probes for each bacteria. The kit detects pathogenic bacteria belonging to the genera *Borrelia, A. phagocytophilum*, *Rickettsia* typhus group *(TG)* and SFG, *F. tularensis, Bartonella*, *C. burnetii* and *Ehrlichia* (*E. chaffeensis* and *E. ewingii*). Due to lack of control material and clinical relevance in Europe the results of the *Ehrlichia* species (no findings of either of the two variants) are not reported in this study. The samples were analysed according to the manufacturers' instruction.

Real-time PCR

To verify correct detection by the flow chip kit, samples were also tested by corresponding real-time PCR protocols detecting *B. burgdorferi* s.l., *B. miyamotoi*, *A. phagocytophilum*, *Rickettsia* TG and SFG, *F. tularensis, Bartonella*, and *C. burnetii* (Fujita et al., 2006; Gooskens et al., 2006; Haschke-Becher et al., 2010; Klee et al., 2006; Stenos et al., 2005; Tsao et al., 2004; Ehrenborg et al., 2008). The sequences and final concentrations of all primers and probes used in the real-time PCR protocols are listed in Table 1.

All protocols, apart from the *B. miyamotoi* specific PCR, were performed using 5 μl of DNA in a 15 μl reaction mixture consisting of 5 mM $MgCl_2$, 0.5 units Uracil DNA-glycosylase (Eurogentec S.A. Seraing, Belgium), LightCycler FastStart DNA Master HybProbe (Roche) with primers and probe(s) (Table 1). *B. burgdorferi* s.l. (either targeting ospA or 16s rDNA), *Rickettsia, Bartonella* and *C. burnetii* real-time assays were run on a LightCycler (LC) 480 with the following thermocycling parameters; 2 min at 40 °C followed by 10 min at 95 °C and 47 cycles of 15 s at 95 °C, 30 s at 60 °C and 20 s at 72 °C. The following PCR program was used for running the protocol detecting *A. phagocytophilum* on a LC 2.0; 2 min at 40 °C followed by 10 min at 95 °C and 55 cycles of 5 s at 95 °C, 10 s at 55 °C and 5 s at 72 °C. The program for analytical melting was 5 s at 95 °C, 2 min at 40 °C and an increase to 80 °C at a 0.2 °C/s ramp rate. The amplification parameters used for the protocol detecting *F. tularensis* on LC 2.0 was; 2 min at 40 °C followed by 10 min at 95 °C and 45 cycles of 10 s at 95 °C, 15 s at 60 °C and 10 s at 72 °C. The program for analytical melting was 20 s at 95 °C, 30 s at 45 °C and an increase to 85 °C at a 0.1 °C/s ramp rate.

B. miyamotoi specific real-time PCR (modified from Tsao et al., 2004, Kjelland et al., 2015) was performed using StepOnePlus (Applied Biosystems Inc. (ABI)). Briefly, 4.5 μl of template DNA and primers and probe (Table 1) were added to 20 μl PCR mixture which included 1X ready-to-use reaction mixture (TaqMan Universal PCR Master Mix, ABI) containing reaction buffer, Taq DNA polymerase, deoxynucleoside triphosphate and $MgCl_2$. The PCR conditions were as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 55 cycles of 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 30 s.

Species identification and confirmation

Differentiation of the *B. burgdorferi* s.l. strains was done by a species specific, single run, real-time PCR based on the protein HBb (*hbb*) gene sequence as previously described (Kjelland et al., 2010a). Briefly, protocols were performed using iCycler/MyIQ™ (Bio-Rad). The 25 μl PCR mixture included 1X ready-to-use reaction mixture (TaqMan Universal PCR Master Mix) and primers and probe at the