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Original article

Tick-borne encephalitis virus in ticks detached from humans and follow-up of serological and clinical response

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ABSTRACT

The risk of tick-borne encephalitis virus (TBEV) infection after a tick bite remains largely unknown. To address this, we investigated the presence of TBEV in ticks detached from humans in an attempt to relate viral copy number, TBEV subtype, and tick feeding time with the serological and clinical response of the tick-bitten participants. Ticks, blood samples, and questionnaires were collected from tick-bitten humans at 34 primary health care centers in Sweden and in the Åland Islands (Finland). A total of 2167 ticks was received from 1886 persons in 2008–2009. Using a multiplex quantitative real-time PCR, 5 TBEV-infected ticks were found (overall prevalence 0.23%, copy range $<4 \times 10^2$–7.7×10^6 per tick). One unvaccinated person bitten by a tick containing 7.7×10^6 TBEV copies experienced symptoms. Another unvaccinated person bitten by a tick containing 1.8×10^3 TBEV copies developed neither symptoms nor TBEV antibodies. The remaining 3 persons were protected by vaccination. In contrast, despite lack of TBEV in the detached ticks, 2 persons developed antibodies against TBEV, one of whom reported symptoms. Overall, a low risk of TBEV infection was observed, and too few persons got bitten by TBEV-infected ticks to draw certain conclusions regarding the clinical outcome in relation to the duration of the blood meal and virus copy number. However, this study indicates that an antibody response may develop without clinical symptoms, that a bite by an infected tick not always leads to an antibody response or clinical symptoms, and a possible correlation between virus load and tick feeding time.

Introduction

Tick-borne encephalitis (TBE) is a serious acute central nervous system (CNS) disease in Europe and Asia, which can lead to death or long-term morbidity. It has been found that as much as 46% of patients may suffer from postencephalitic symptoms (Haglund et al., 1996; Günther et al., 1997; Kaiser, 1999, 2011; Mickiene et al., 2002; Haglund and Günther, 2003). The TBE virus (TBEV) belonging to the genus Flavivirus is a positive-sense single-stranded RNA virus transmitted by *Ixodes* ticks. TBEV caused at least 7200 human cases of TBE in Europe during 2010, half of which occurred in Russia (Süss, 2011). The mean number of human TBE cases per year in Sweden increased from 63 in 1985–1999 to 184 in 2007–2010. In 2011, 284 TBE cases (3 per 100,000 inhabitants) were registered (Jaenson et al., 2012), and a few more (287 cases) in 2012. In contrast, in the Åland Islands (Finland), human TBE decreased from more than 20 annual cases in 2000–2002 (77 per 100,000 inhabitants) to 3–13 annual cases in 2006–2010 (11–50 per 100,000 inhabitants). A vaccination program initiated in 2006 covering the whole population from ≥7 years old in the Åland Islands is the probable explanation for this decrease (Jääskeläinen et al., 2011).

In Sweden and in the Åland Islands, only the European (Eu) subtype of TBEV, spread by the tick species *Ixodes ricinus*, has been found so far. Across the Baltic Sea, in Estonia, Latvia, and Lithuania,

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also the Far-Eastern (FE) and Siberian (Sib) TBEV subtypes, spread by the tick species *Ixodes persulcatus*, are present (Golovljova et al., 2004). Notably, a hotspot of TBEV-Sib was discovered in the Kokkola Archipelago (Finland) in 2004, 100 km east of the Swedish coast (Jääskeläinen et al., 2006).

Several studies from different European countries investigated the prevalence of TBEV in field-collected, unfed ticks, while only a few studies investigated the TBEV prevalence in ticks that have bitten humans (Supplementary Table S1). In 3 studies from Germany, a significantly higher prevalence of TBEV was found in ticks detached from humans than in field-collected ticks from the same area (Klaus et al., 2010; Süss et al., 2004, 2006). There is also a report from Karelia in Russia showing a similar pattern (Kotovich, 2012). Unlike the *Borrelia* spirochete, which requires 24–48 h for transmission to mice (Crippa et al., 2002), TBEV has been shown to be transmitted to mice directly after tick attachment due to the presence of virus in the tick salivary glands (Alekseev et al., 1996). However, regarding the risk of TBEV transmission to humans and the clinical outcome, the importance of factors such as the tick life stage, tick feeding-time, virus copy number, and virus subtype remains to be clarified.

To investigate the overall risk of contracting TBEV infection through a tick-bite, a comprehensive study, denoted the tick-borne diseases (TBD) STING study, was set up. The aim was to assess the prevalence of TBEV in ticks detached from humans and to evaluate if TBEV copy number, subtype, tick life stage, and tick feeding time influence the risk of virus transmission and the development of symptomatic or asymptomatic infections. In order to optimally determine the presence, amount, and subtype of TBEV in ticks, a multiplex quantitative real-time PCR assay based on 2 existing assays (Schwaiger and Cassinotti, 2003; Gäumann et al., 2010) was established.

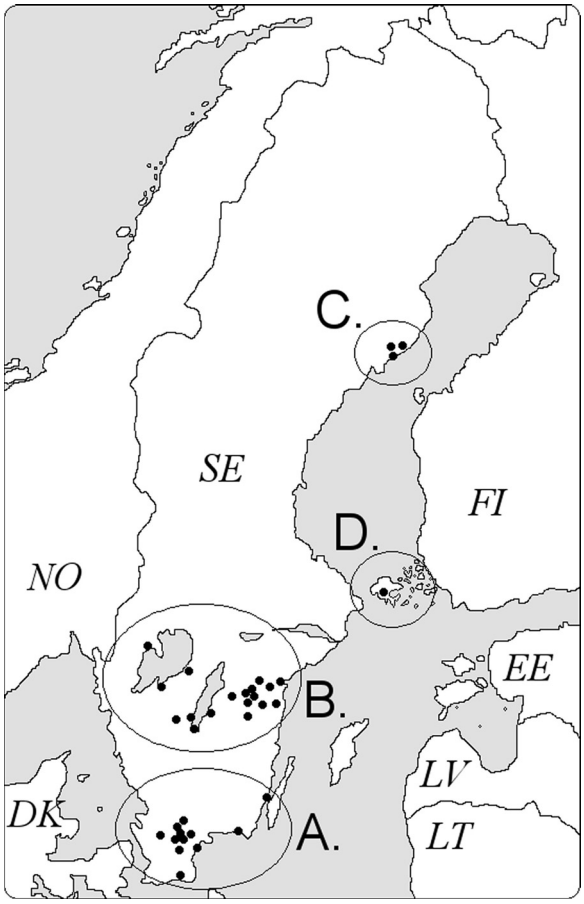

Fig. 1. Location of the 34 primary health care centers (PHCs), from where tick-bitten persons were recruited, divided into 4 geographical regions. (A) Southernmost Sweden (10 PHCs); (B) south-central Sweden (20 PHCs); (C) northern Sweden (3 PHCs); and (D) the Åland Islands (1 PHC). SE, Sweden; NO, Norway; FI, Finland; DK, Denmark; EE, Estonia; LV, Latvia; LT, Lithuania.

Materials and methods

Study design

Collection of ticks, blood samples, and questionnaires from tick-bitten humans were done in collaboration with 34 primary health care centers (PHCs) during 2008 and 2009. The PHCs were located in the Åland Islands (Finland) and in 3 regions of Sweden (Fig. 1). All the included regions are TBE endemic except northern Sweden, but with a patchy distribution. At each PHC, local advertisements through different media were used to publicize the study in order to recruit tick-bitten volunteers, 18 years and older. At inclusion, after the participant had provided informed consent, the tick/ticks that had bitten the participant were collected, blood samples were taken, and a questionnaire was completed. Follow-up blood samples and questionnaires were collected from each participant 3 months after the tick bite(s). During the 3-month study period, the participants were also asked to collect any additional ticks that had bitten them. Ticks and blood samples were sent within 3 days to Linköping University, where they were frozen at −70 °C until analysis. If a participant developed clinical symptoms, he/she was asked to visit the PHC where additional samples were taken. Ethical approval for the TBD STING study was granted by the Regional Ethics Committee in Linköping (M132-06) and by the local Ethics Committee of the Åland Health Care, 2008-05-23.

Tick photo and measurement

Each tick was photographed and measured dorsally and ventrally, using a USB microscope (Dino-Lite Long AM4013TL, AnMo Electronics Corp., Taiwan) to determine species, life stage, and making an estimate about feeding duration using coxal and scutal indices (Gray et al., 2005).

RNA extraction and cDNA synthesis

Ticks were homogenized individually by bead-beating in 2 ml safe-lock microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) with a 5-mm stainless steel bead (Qiagen, Hilden, Germany) in 450 μl RLT buffer (Qiagen), supplemented with 1% β-mercaptoethanol (Qiagen), using a TissueLyser (Qiagen), 2 min at 25 Hz. After centrifugation at 20,000 × *g* for 3 min, 400 μl supernatant were transferred to new microcentrifuge tubes for RNA extraction, using MagAttract® RNA Tissue Mini M48 kit (Qiagen) in a Biorobot M48 workstation (Qiagen), using a 50-μl elution volume. Each batch of 48 samples consisted of 46 ticks, one positive control [5 μl inactivated TBEV strain K23, Encepur®, Chiron Vaccines, Marburg, Germany, and 5 μl of *Borrelia burgdorferi* sensu stricto B31 ATCC 35210 (108 cells/ml)] and one negative control (H_2O) extracted simultaneously. The RNA was reverse-transcribed to cDNA using illustra Ready-To-Go RT-PCR Beads (GE Healthcare, Amersham Place, UK). Twenty microliters RNA and 10 μl pd(N)6 random hexamer primers (0.25 μg/μl) were incubated for 5 min at 97 °C and then mixed with one RT-PCR bead dissolved in 20 μl RNAse-free water. The mixture was incubated for 30 min at 42 °C, followed by 5 min at 97 °C, producing 50 μl cDNA. Pipetting was done using a CAS-1200 pipetting robot (Corbett robotics Pty Ltd.,