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Original article

Low risk of seroconversion or clinical disease in humans after a bite by an *Anaplasma phagocytophilum*-infected tick

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ABSTRACT

The risk of contracting human granulocytic anaplasmosis (HGA) after a tick bite is mainly unknown. In this study we investigated the clinical and serological response in 30 humans bitten by ticks positive for *Anaplasma phagocytophilum* (Group A), 30 humans bitten by *Borrelia burgdorferi* sensu lato (s.l.)-positive ticks (Group B), and 30 humans bitten by ticks negative for both *A. phagocytophilum* and *B. burgdorferi* s.l. (Group C). Ticks, blood samples and questionnaires were collected from tick-bitten humans at 34 primary healthcare centres in Sweden and in the Åland Islands, Finland, at the time of the tick bite and after three months. A total of 2553 ticks detached from humans in 2007–2009 were analyzed by polymerase chain reaction, and 31 (1.2%) were positive for *A. phagocytophilum*, 556 (21.8%) were positive for *B. burgdorferi* s.l., and eight (0.3%) were co-infected by *A. phagocytophilum* and *B. burgdorferi* s.l. The overall prevalence of *Anaplasma* IgG antibodies in the included participants ($n = 90$) was 17%, and there was no significant difference between the groups A-C. Only one of the participants (in Group C) showed a four-fold increase of IgG antibodies against *A. phagocytophilum* at the three-month follow-up, but reported no symptoms. The frequency of reported symptoms did not differ between groups A-C, and was unrelated to the findings of *A. phagocytophilum* and *B. burgdorferi* s.l. in the detached ticks. We conclude that the risk for HGA or asymptomatic seroconversion after a tick bite in Sweden or in the Åland Islands is low, even if the tick is infected by *A. phagocytophilum*.

1. Introduction

Tick borne fever is a well-recognised problem in veterinary medicine in Europe and in Scandinavia (Egenvall et al., 1997; Stuen et al., 2002; Stuen, 2007). The causative agent, *Anaplasma phagocytophilum*, is transmitted by *Ixodes* ticks, and known to occasionally also cause human disease (Chen et al., 1994). The first verified cases of human granulocytic anaplasmosis (HGA) in Europe were reported from Slovenia in 1997, followed by cases from Sweden, Norway and the Netherlands (Petrovec et al., 1997; Lotric-Furlan et al., 1998; Bjoersdorff et al., 1999a; van Dobbenburgh et al., 1999; Karlsson et al., 2001). HGA is typically a febrile illness with headache, myalgia and malaise accompanied by leukopenia, thrombocytopenia and elevated hepatic enzymes. The symptoms are generally mild to moderate, and subclinical infections occur. However, the disease course can be severe and even fatal in immunocompromised individuals (Bakken et al., 1996b; Dahlgren et al., 2011; Jereb et al., 2012; Schotthoefer et al., 2014). Since *A. phagocytophilum* shares vectors with *Borrelia burgdorferi* sensu lato (s.l.), co-infections occur (Bakken et al., 1996a; Nadelman et al., 1997; Bjoersdorff et al., 1999b). Data from animal studies suggest that such co-infections are more severe, possibly due to immunosuppression induced by *A. phagocytophilum* (Bakken et al., 1996b; Thomas et al., 2001).

The prevalence of *A. phagocytophilum* in field-collected ticks in Sweden was recently estimated to be 1.3–15% (Severinsson et al., 2010). The human seroprevalence in Sweden has previously been reported to be 8–21% (Dumler et al., 1997; Bjoersdorff et al., 1999b;

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Wittesjö et al., 2001), and the seroconversion rates 3.9–11.1% per year in a highly endemic area in the south-east of Sweden (Wittesjö et al., 2001). However, the importance of *A. phagocytophilum* as a human pathogen in Sweden is still uncertain, and little is known about co-infections with *B. burgdorferi* s.l. as well as the risk of contracting HGA after a single tick bite.

This study was part of the Scandinavian Tick-Borne Diseases (TBD) STING study described below, and the aims were to investigate the prevalence of *A. phagocyophilum* in ticks that have bitten humans, and to evaluate the risk for humans of developing clinical HGA or subclinical seroconversion against *A. phagocytophilum* after a tick bite in Sweden and on the Åland Islands, Finland, including the risk of disease after a bite by a tick co-infected with *A. phagocytophilum* and *B. burgdorferi* s.l.

2. Material and methods

2.1. Study design

This study was part of the on-going TBD STING study (Wilhelmsson et al., 2010, 2013; Fryland et al., 2011; Lindblom et al., 2014), in which tick-bitten individuals are asked to donate the tick along with a blood sample at their local primary healthcare centre (PHC). Three months later the participants are asked to come back to the PHC and give a second blood sample. At both visits, the participants are also asked to fill in questionnaires concerning locality of the tick bite, symptoms, *etc.* If the participants should observe additional tick bites during the three months study period, they are asked to collect those ticks as well and donate them to the study (these ticks were not analyzed in the present study). Medical records are scrutinized if the participants seek medical care during the study period. Only tick-bitten individuals ≥18 years of age and with no immunosuppressive disease or treatment are included in the study. No antibiotic prophylaxis is given after the tick bite but the study participants are ordered to seek medical care in case they get symptoms. The ticks and blood samples from individuals included in this particular part of the TBD STING study were collected during 2007–2009 at 34 PHCs in Sweden and on the Åland Islands, Finland (Fig. 1).

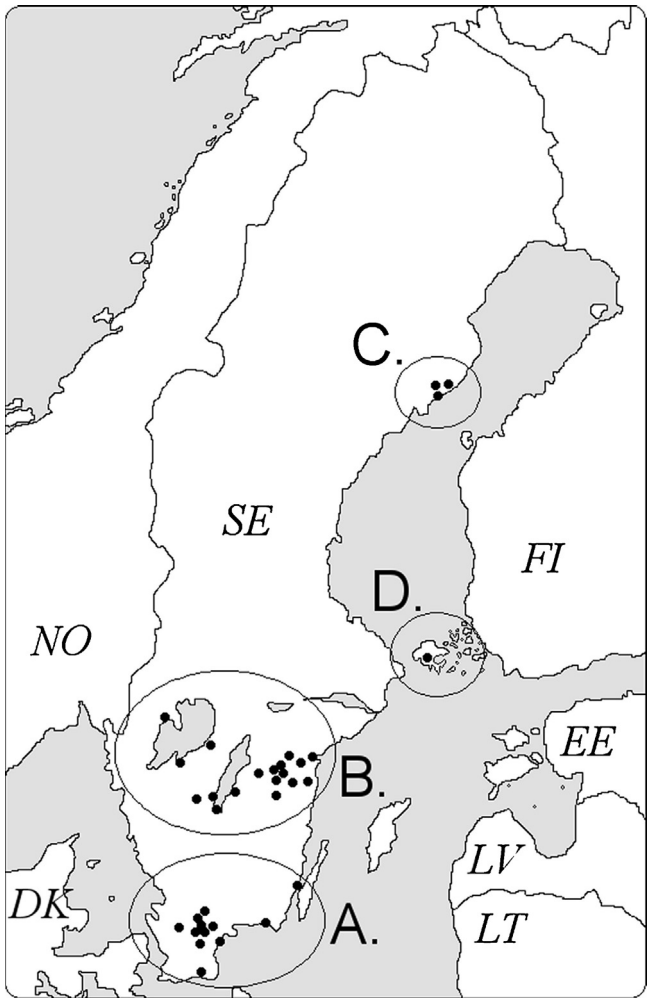

Fig. 1. Map showing the four regions where the 34 primary healthcare centres (PHCs) are located. (A) Southernmost Sweden (12 PHCs). (B) South-central Sweden (18 PHCs). (C) Northern Sweden (3 PHCs). (D) The Åland Islands (1 PHC). SE, Sweden; NO, Norway; FI, Finland; DK, Denmark; EE, Estonia; LV, Latvia; LT, Lithuania. Reprinted from Lindblom et al. (2014) with permission from Elsevier.

2.2. Ticks

The ticks that participants provided were transported and handled as previously described (Wilhelmsson et al., 2013). All ticks were examined under a USB microscope (Dino-Lite Long AM4013TL, AnMo Electronics Corporation, Hsinchu, Taiwan) and photographed. Species and life stage of the ticks were determined, as well as the coxal and scutal indices in order to estimate the feeding duration according to Gray et al. (2005).

2.3. Tick homogenization, total nucleic acid extraction and reverse transcription of nucleic acid

Tick preparation, homogenization, process of total nucleic acid (NA) extraction and reverse transcribed NA (RTNA) synthesis have been described elsewhere; for ticks collected in 2007 (Wilhelmsson et al., 2010) and for ticks collected in 2008–2009 (Wilhelmsson et al., 2013). Briefly, total NA was extracted using a BioRobot M48 workstation (Qiagen, Hilden, Germany) and MagAttract® RNA Tissue Mini M48 Kit (Qiagen). The extractions were performed following the protocol from the manufacturer, except for not adding DNase to the RDD buffer, thus obtaining total NA including DNA. For the RTNA synthesis, 20 μL of the extracted total NA was used together with the Illustra™ Ready-to-Go RT-PCR Beads kit (GE Healthcare, Amersham Place, UK) according to the manufacturer's instructions.

2.4. Real-time PCR and conventional PCR assays

Detection of *A. phagocytophilum* DNA was based on a real-time PCR method targeting the *16S* rRNA gene. Each reaction consisted of 12.5 μL Maxima Probe qPCR Mastermix 2X (Fermentas St. Leon-Rot, Germany), 0.6 μL (10 μM) of each primer (AphGERfn 5′-TAGATCCTTCTTAACGGAAGGGCG-3′ and AphGERr 5′-AAGTGCCCGGCTTAACCCGCTGGC-3′) (Sigma–Aldrich Sweden AB, Stockholm, Sweden) (Goodman et al., 1996; Severinsson et al., 2010), 0.25 μL (10 μM) of the probe (AphGERp 5′-[6FAM]-CTGTCGTCAGCTCGTGTCGTGAGATGTTG-[BHQ-1]-3′) (Sigma–Aldrich Sweden AB), 6.05 μL RNase free water and 5.0 μL cDNA. The reaction was performed according to the following thermal cycle conditions: 95 °C for 10 min, 45 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s.

The real-time PCR-positive samples were further analyzed with a nested conventional PCR assay to amplify a longer sequence of the *16S* rRNA gene for species identification. The first reaction consisted of 5 μL Phusion HF buffer and 0.75 μL Phusion HF polymerase (2 U/μL) (Thermo Fisher Scientific Inc.), 2 μL (10 μM) of each primer (HGE1 5′-TCCTGGCTCAGAACGAAC-3′ and Aph16S5nR 5′-TAGTCTTGCGACCGTAGTC-3′) (Sigma–Aldrich Sweden AB, Stockholm, Sweden), 35.25 μl RNase free water and

Table 1
Number of collected ticks, their life stage and estimated feeding time.

	Total	Larvae	Nymphs	Adults	N.D.	Median feeding-time (h)
All collected ticks	2553	89	1806	612	46	31[b] (*n* = 1895)
Ticks positive for *A.p.*	31	0	23	8	0	29 (*n* = 20)
Ticks positive for *B.b.* s.l.	30[a]	0	21	9	0	26 (*n* = 27)
Ticks negative for *A.p.* and *B.b.*s.l.	30[a]	0	24	5	1	37 (*n* = 28)

N.D., life stage not determined; A.p., *Anaplasma phagocytophilum*; *B.b.*s.l., *Borrelia burgdorferi* sensu lato.
[a] Randomly selected from the collected ticks.
[b] Median duration of tick feeding is based on nymphs (*n* = 1451) and adult female ticks (*n* = 444).

5 μl cDNA. The reaction was performed according to the following thermal cycle conditions: 98 °C for 10 min, 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 30 s. This was followed by 72 °C for 7 min. An aliquot (5 μL) of the PCR product obtained in this assay was added to the second PCR mixture, which was prepared using the same volumes, concentrations, and amplification programme as those used for the first mixture, except with a different primer pair (Aph16SnF 5′-CAAGCTTAACACATGCAAGTCG-3′ and Aph16S6nR 5′-GAGTGCTTAACGCGTTAGCTAC-3′) (Sigma–Aldrich Sweden AB, Stockholm, Sweden), and the number of cycles was increased to 42. The obtained PCR products were sent to Macrogen Inc. (Seoul, South Korea), for nucleotide sequencing based on BigDye chemistry. Sequence chromatograms were initially edited using BioEdit Software (V7.0) (Tom Hall, Ibis Therapeutics, Carlsbad, CA) followed by a standard BLAST search against the GenBank database (http://blast.ncbi.nlm.nih.gov).

The extraction control consisted of a real-time PCR assay targeting the *Ixodes* 16S mitochondrial DNA, previously described by Schwaiger and Cassinotti (2003). Detection of *B. burgdorferi* s.l. was performed by real-time PCR targeting the *16S* rRNA gene as described previously (Wilhelmsson et al., 2013).

2.5. Serum samples

The acute and convalescent serum samples (collected at inclusion in the study and after three months) corresponding to the ticks positive for *A. phagocytophilum* (Group A) were analyzed for the presence of *A. phagocytophilum* IgG antibodies using a commercially available indirect immunofluorescent assay (IFA); (Focus Diagnostics, Cypress, CA, USA). Acute and convalescent sera from individuals bitten by *B. burgdorferi* s.l. positive ticks (Group B), and sera from individuals bitten by ticks negative for both *A. phagocytophilum* and *B. burgdorferi* s.l. (Group C) were randomly selected for analysis regarding IgG antibodies against *A. phagocytophilum*. All serum samples had been stored at −20 °C before analysis and processed according to the manufacturer's instructions, except that dilutions 1:80, 1:160, 1:320, *etc.* were used. End point titre was recorded as the reciprocal of the last serial dilution at which specific apple-green fluorescence of *Anaplasma* inclusion bodies were focally located in the cytoplasm of the infected cells. IFA titres ≥1:80 were interpreted as positive. This cut-off value was based on local Swedish serum samples used when validating the method (Bjoersdorff et al., 1999b). For the diagnosis of on-going or recent *A. phagocytophilum* infection, at least a four-fold rise of the IFA titre was required when acute and convalescent serum samples were tested simultaneously. All serum samples had been analyzed previously for the presence of antibodies against *B. burgdorferi* s.l. (Fryland et al., 2011).

2.6. Questionnaires

The questionnaires were scrutinized for symptoms suggestive of HGA; fever, headache, myalgia, arthralgia and malaise (Fryland et al., 2011).

2.7. Statistics

Comparison of clinical and serological data between groups were performed using the chi^2-test, and the Kruskal–Wallis test was applied for comparison of feeding-time (SPSS for Windows, version 15.0). *p*-Values <0.05 were considered as significant.

2.8. Ethics

The study was approved by the Regional Ethics Review Board in Linköping (M132-06), and by the local Ethics Committee of Åland Healthcare, 2008-05-23. Written consent was obtained from all participants.

3. Results

3.1. Ticks

In total, 2553 ticks that had bitten humans were collected during the years 2007–2009. All the collected ticks belonged to the *Ixodes ricinus* species; 89 (3.5%) larvae, 1806 (70.7%) nymphs, 612 (24.0%) adults. Forty-six (1.8%) of the ticks were so damaged that life stage could not be determined (Table 1).

3.2. Prevalence of A. phagocytophilum and B. burgdorferi s.l. in the ticks

Thirty-one ticks (1.2%) were positive for *A. phagocytophilum*, and eight of them were co-infected with *B. burgdorferi* s.l. (0.3%). Five hundred-and-fifty-six ticks (21.8%) were positive for *B. burgdorferi* s.l. alone (Wilhelmsson et al., 2013). Of the *A. phagocytophilum*-positive ticks, 23 were nymphs and eight were adults. Feeding-time could be estimated in 20 of the *A. phagocytophilum*-positive ticks; median 29 h, range <15 h to >69 h. There were no significant differences regarding estimated tick feeding-time between the groups A-C (pp = 0.47) (Table 1).

3.3. Seroprevalence, seroconversion and reported symptoms of the tick-bitten subjects

Acute and convalescent serum samples from the thirty individuals bitten by ticks positive for *A. phagocytophilum* were obtained (one participant that had been bitten by an *A. phagocytophilum*- and *B. burgdorferi* s.l.-positive adult tick was lost to follow-up). The paired sera from the individuals bitten by *A. phagocytophilum*-positive ticks were analyzed for the presence of *A. phagocytophilum* IgG antibodies (Group A), as well as sera from thirty randomly chosen individuals bitten by ticks positive for *B. burgdorferi* s.l. (Group B), and thirty individuals bitten by ticks negative for both *A. phagocytophilum* and *B. burgdorferi* s.l. (Group C). The overall seroprevalence for *A. phagocytophilum* in all three groups was 17% (15/90) with no significant difference between the groups (Group A: 13%, Group B: 23%, Group C: 13%, *p* = 0.45). None of the participants reported having had confirmed HGA previously. The detected antibody titres were 1:80 (*n* = 8) and 1:160 (*n* = 7). Six individuals

in Group A reported symptoms (*e.g.* headache, neck pain, fatigue, vertigo, myalgia, arthralgia), but none of them displayed significant increase in *Anaplasma* antibody titre or seroconversion. Two of the seven individuals bitten by ticks co-infected by *A. phagocytophilum* and *B. burgdorferi* s.l. reported symptoms but showed no antibody response against any of the bacteria. Six individuals in Group B and five individuals in Group C reported symptoms (*e.g.* headache, neck pain, fatigue, vertigo, myalgia, arthralgia, paresthesia) but showed no seroconversion or increased antibody titres against *A. phagocytophilum* or *B. burgdorferi* s.l., except for one patient in Group C that was diagnosed with Lyme neuroborreliosis. Only one individual in the study (in Group C) showed seroconversion for *A. phagocytophilum* at the three-month follow-up, but reported no symptoms. Four participants showed seroconversion for *B. burgdorferi* s.l. during the study period (one in Group A, two in Group B and one in Group C), but reported no symptoms.

4. Discussion

Although increasingly detected, HGA is still a rather unknown illness in Europe (Blanco and Oteo, 2002; Strle, 2004), as opposed to the situation in the USA where HGA is a notifiable disease with increasing incidence (Centers for Disease Control and Prevention, 2011). Underreporting, differences in host range or reduced virulence in humans of the circulating *A. phagocytophilum* strains in Europe may be possible explanations, but precise knowledge is still lacking. There is a large amount of genetically diverse *A. phagocytophilum* lineages in Europe (Tveten, 2014), *e.g.* the mouse-associated lineages comprising variants that also have been isolated from human cases, and the deer-associated lineages that do not appear to be infectious for humans (Massung et al., 2002). This implies that there is possibly a European risk scenario similar to the one in the USA.

In this study, we found an *A. phagocytophilum* prevalence of 1.2% in ticks that have bitten humans in Sweden or on the Åland Islands, which is within the same range as the prevalence found in field-collected ticks from Sweden (Severinsson et al., 2010). Only 0.3% of the analyzed ticks were co-infected with *A. phagocytophilum* and *B. burgdorferi* s.l., similar to the co-infection rates found in previous studies on field-collected ticks and ticks collected from birds in Europe (Schicht et al., 2011; Coipan et al., 2013; Soleng and Kjelland, 2013; Capligina et al., 2014). To our knowledge, no previous data on prevalence of *A. phagocytophilum* and co-infections with other pathogens in ticks detached from humans has been reported from northern Europe.

The seroprevalence of 17% that we found among the TBD STING study participants is in accordance with previous studies on other tick-exposed populations in Sweden, *i.e.* patients with Lyme borreliosis or people living in tick-endemic areas (Dumler et al., 1997; Bjoersdorff et al., 1999b; Wittesjö et al., 2001). The seroprevalence among healthy blood donors (probably a population less exposed to tick bites than the participants in the TBD STING study) from Jönköping county, using the same IFA and the same cut-off level as in the present study, has been found to be 8.3% (unpublished data). None of the subjects in this study had been diagnosed with HGA previously, which may indicate that the disease is generally mild or even asymptomatic, but it may also indicate that physicians rarely suspect HGA in their patients. In this study, subjects receiving immunosuppressive treatments were not included, and it is plausible that the course of HGA is more severe in the immunocompromised, as has been shown previously (Bakken et al., 1996b; Dahlgren et al., 2011; Jereb et al., 2012; Schotthoefer et al., 2014). This has also been shown for infections caused by *Candidatus* Neoehrlichia mikurensis, another member of the family *Anaplasmataceae* (Welinder-Olsson et al., 2010; Grankvist et al., 2014).

We conclude from our data that the risk of contracting HGA after a tick bite is low even if the tick is infected by *A. phagocytophilum* and has been feeding for ≥29 h. This corroborates previous findings from central Europe (Tijsse-Klasen et al., 2011). There is possibly a delay in the transmission of *A. phagocytophilum* from the tick to the host, but since feeding-time could be estimated in only 20 of the infected ticks and none of the study participants seroconverted, no firm conclusions could be drawn from our data regarding this. Delayed transmission has been shown for *B. burgdorferi* s.l. (Piesman et al., 1987), and attachment and partial feeding of a *B. burgdorferi* s.l.-infected tick does not necessarily lead to infection or disease (Fryland et al., 2011) which also seems to be the case for *A. phagocytophilum* according to experimental studies on mice by Katavolos et al. (1998). The overall seroprevalence of 17% in this tick-bitten population that, however, did not report previously diagnosed HGA, may also indicate a low risk for clinical disease after exposure to *A. phagocytophilum*. There have been previous reports of verified symptomatic HGA cases from Sweden, however the incidence is low (Bjoersdorff et al., 1999a, 2002). In European countries the number of clinically diagnosed cases of HGA is low, while the seroprevalence of antibodies against *A. phagocytophilum* is 2–28% (Strle, 2004). Such high levels of seropositivity in regions with low numbers of clinical cases may be explained by the circulation of strains that are non-pathogenic to humans or by serologic cross-reactivity with other bacteria (Rar and Golovljova, 2001). Evidence from cross-infection experiments have indicated that *A. phagocytophilum* isolates of distinct host origin are not uniformly infectious for other heterologous hosts (Rar and Golovljova, 2001; Jin et al., 2012). The proportion of *A. phagocytophilum* strains pathogenic to humans that occur in *I. ricinus* ticks in Europe is still uncertain, and results from different molecular strain characterization studies are not easily comparable, since different gene regions and fragment lengths have been investigated. A recent analysis of the population structure of *A. phagocytophilum* based on multilocus sequence typing revealed that all strains isolated from humans in Europe belonged to the same dominating clonal complex (Huhn et al., 2014).

The reported symptoms in any of the study groups are probably not related to HGA or Lyme borreliosis, since no corresponding antibody responses could be detected in the reporting subjects. Only one participant showed seroconversion against *A. phagocytophilum* at follow-up, but reported no subjective symptoms. This participant had donated an *A. phagocytophilum*-, as well as *B. burgdorferi* s.l.,-negative tick, but had probably, in conjunction with the study period, had additional tick bites that passed unnoticed. No increased risk for HGA or Lyme borreliosis after a bite by a co-infected tick could be detected in this study, however the number of individuals bitten by a co-infected tick was low ($n = 7$). The number of individuals that reported symptoms during the study period did not differ between the groups A-C, and seemed to be unrelated to whether the biting tick contained *A. phagocytophilum*, *B. burgdorferi* s.l., both or none of these two bacteria. Therefore, analysis of the tick after a tick bite seems to be of little or no value for predicting disease or making treatment decisions in humans.

We used a cut-off in the IFA of 1:80, as 1:64 and 1:80 have been widely used for epidemiological purposes before. However, it has previously been discussed by others whether these cut-offs may be set too low (Aguero-Rosenfeld et al., 2002; Walder et al., 2003), since a significant proportion of the population without clinical evidence of HGA will then test positive for *A. phagocytophilum* antibodies. Furthermore, serological cross-reactions may complicate the interpretation of IFA results. False positive results may be due to *e.g.* Epstein–Barr virus infection, Lyme borreliosis or autoimmune disorders (Dumler et al., 2007), and this, together with the uncertainty of the proper cut-off, should be kept in mind when

interpreting the results and may be an explanation to the discrepancy between the rather high seroprevalence found in our study population and the low prevalence of *A. phagocytophilum* found in the analyzed ticks. However, Dumler et al. (1997) found a seroprevalence of 11.4% among inhabitants on the Koster island off the western cost of Sweden (cut-off 1:80), Wittesjö et al. (2001) found a seroprevalence of 28% in inhabitants on the Aspö island located off the southeast coast of Sweden (cut-off 1:80), and Skarphedinsson et al. (2001) found a seroprevalence of 21% among Danish patients with confirmed or suspected Lyme borreliosis (cut-off 1:64), so the seroprevalence of 17% found in our study seems reasonable. As discussed above, it may represent an over-estimate, but the selected cut-off allows for the comparison.

Another possible limitation to the study is that the second serum samples were collected after three months, and it cannot be completely ruled out that a few of the subjects bitten by an *A. phagocytophilum*-infected tick actually did seroconvert but the antibody titre had dropped again at follow-up. However, this seems rather unlikely since antibodies usually remain for several months or longer (Bakken et al., 2002; Aguero-Rosenfeld, 2003).

In conclusion, the prevalence of *A. phagocytophilum* in ticks that have bitten humans do not differ from the prevalence found in field-collected ticks in Sweden and on the Åland Islands. The seroprevalence of 17% among the study participants indicate that the population in the study area is exposed to *A. phagocytophilum*, but the infection is seemingly often unnoticed or at least undiagnosed since none of the seropositive individuals reported a previous HGA infection. The risk of contracting HGA after a single tick-bite is however low, even if the tick is infected with *A. phagocytophilum* and has been feeding for several days. Thus, analysis of the tick after a tick bite is not clinically useful and should be avoided since it may lead to unnecessary anxiety for the tick-bitten individual as well as unfounded use of antibiotics. Any firm conclusions whether the risk of disease is increased or not after a bite by a tick co-infected with *A. phagocytophilum* and *B. burgdorferi* s.l. could not be drawn from this study due to the low number of co-infected ticks. Lyme borreliosis and HGA co-infections need to be further evaluated, since previous data is ambiguous (Belongia et al., 1999; Krause et al., 2002; Steere et al., 2003; Horowitz et al., 2013), and animal studies suggest an aggravated course of such infections (Bakken et al., 1996b; Thomas et al., 2001). The continuing TBD STING study provides possibilities to bring new insights into the interplay between ticks, tick-borne pathogens and humans, and permits improved risk assessments for human disease following a tick bite which is important from a public health perspective.

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