Clinical/serological outcome in humans bitten by Babesia species positive Ixodes ricinus ticks in Sweden and on the Åland Islands

Wilhelmsson, P.; Lövmar, M.; Krogfelt, Karen Angeliki; Nielsen, H.V.; Forsberg, Pia; Lindgren, P. E.

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Abstract

The risk of contracting babesiosis after a tick bite in Sweden and the Åland Islands, Finland, is unknown. We investigated clinical and serological outcomes in people bitten by Ixodes ricinus ticks positive for Babesia species. Ticks, blood and questionnaires were obtained from study participants in Sweden and on the Åland Islands. Sixty-five of 2098 (3.1%) ticks were positive by real-time PCR. Three Babesia species were detected, Babesia microti (n=33), B. venatorum (n=27) and B. capreoli (n=5), the latter species not known to cause human infection. Half (46%) of the Babesia PCR-positive ticks also contained Borrelia spp. Fifty-three participants bitten by a Babesia PCR-positive tick and a control group bitten by a Babesia PCR-negative tick were tested for B. microti IgG antibodies by IFA. The overall seroprevalence was 4.4%, but there was no significant difference between the groups. None of the participants seroconverted and no participant with a Babesia PCR-positive tick sought medical care or reported symptoms suggestive of babesiosis. Given the prevalence of Babesia in I. ricinus ticks in southern Sweden and on the Åland Islands, babesiosis should be considered a possible diagnosis in symptomatic residents who seek medical care following tick exposure.

Keywords	Babesia; Babesiosis; Human; Seroconversion; Ixodesricinus; Co-infection

Corresponding Author	Matilda Lövmar
Corresponding Author's Institution	Linköpings Universitet
Order of Authors	Peter Wilhemsson, Matilda Lövmar, Karen Krogfelt, Henrik Vedel Nielsen, Pia Forsberg, Per-Eric Lindgren

Submission Files Included in this PDF

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Figure2.jpg [Figure]

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Linköping University

27th August 2018

Dear Editor,

Please find enclosed a manuscript entitled "*Babesia* species present in *Ixodes ricinus* ticks and clinical/serological outcome in humans after an infected tick bite in Sweden and the Åland Islands".

I and my co-authors would be very pleased if you would consider this manuscript for publication as a Research article in the journal Ticks and Tick-borne Diseases.

Babesiosis is a tick-borne human infection in the temperate regions of North America and Eurasia. In many European countries, cases of babesiosis are not mandatorily notifiable by medical practitioners. Therefore, the number of babesiosis cases are unknown. The risk of developing a *Babesia* infection after a single tick bite is also unknown but depends likely on many factors such as developmental stage of the tick, duration of tick feeding, the *Babesia* species to be transmitted as well as the number of *Babesia* parasites in the tick and possible co-infection with other tick-borne pathogens.

In an effort to elucidate the incidence of babesiosis and to investigate how different factors influence the risk of developing a *Babesia* infection, we collected and analyzed ticks for the presence of *Babesia* spp. that had been found attached to people in Sweden and Åland Islands (Finland). At the time of the tick bite and three months later, we collected and analyzed blood samples for the presence of anti-*Babesia* antibodies from the tick-bitten people. In order to determine if participants were diagnosed with babesiosis within the three-month study period, medical records from participants that visited a health care provider were scrutinized. This study involved 1769 tick-bitten participants.

Our results indicate that the risk of contracting babesiosis after a tick bite is low, even if a *Babesia*-positive tick has been feeding for more than three days and contains up to 10^7 *Babesia* spp. genome copies per tick. Our findings of participants with positive serology indicate, however, that human infection with *Babesia* spp. with clinical symptoms occurs in Sweden and in the Åland Islands. Thus, babesiosis should not be neglected as a possible diagnosis in patients experiencing symptoms following a tick bite.

We are convinced that the results of this study should be of great interest to many readers of Ticks and Tick-borne Diseases. We decided to submit this manuscript to Ticks and Tick-borne

Diseases also because we consider this medical journal to be a highly appreciated scientific journal of excellent quality.

Being the corresponding author, I – and on behalf of all the authors – hereby certify that this paper is an original work. No part of this manuscript has been published previously. All authors have seen and approved the final version of the manuscript.

We very much appreciate your valuable work and look forward to your response.

Matilda Lövmar, MSc
Corresponding author

Linköping University
Department of Clinical and Experimental Medicine
Division of Medical Microbiology
SE-581 85 Linköping
Sweden

Fax: +46(0)10-1034789
E-mail address: matilda.lovmar@liu.se

Dear Editor,

We would yet again like to thank the reviewers for the valuable comments on our manuscript TTBDIS_2018_332, which have helped us to further substantially improve the quality of the manuscript. We have tried to fulfill all requests and answer all of the questions and give feed-back on the comments raised by the reviewers.

In this letter, point-by-point answers follow. The comments from the reviewers have been copied into this letter and formatted with the Calibri font and put in italics. Our answers follow after each comment in the Times New Roman font.
We hope that this will help with the final decision about the manuscript.

On behalf of all authors,

Matilda Lövmar

Enclosures:

1. The revised manuscript with figures
2. The two questionnaires used in the TBD-STING study, translated into English

Editor and Reviewer Comments:

- Section editor:

Accept after tidying up the last problems as indicated by reviewer 4!

-Managing ***Editor***

The questionable 2 paragraphs can stay in the paper.

Minor comments:

Abstract: Please, write out numbers at the beginning of sentences.

L109-110: These references need some copy editing.

L161 (similar cases also elsewhere in the text): Borrelia-negative

L205: 36 h [space missing]

L226: Twenty-nine

L227 (also elsewhere in the text): No double full stop.

L339: Nordström

L360-361: Please, use sentence case (no capitalisation of words) rather than title case.

L404: Bergström

L489: Nyström

All of the above mentioned comments have been corrected.

-***Reviewer 1***

- All of the issues I had raised were already addressed in the previous revision.

-***Reviewer 2***

-

-***Reviewer 3***

-

-***Reviewer 4***

-

Major Comments

Line 162: are the 106 controls made of ANY and ALL Babesia and Borrelia negative samples collected during the entire study period, and matched geographically? If not, the seroprevalence, as reported, would be meaningless.
We have changed the text slightly to clarify, see lines 157-160. The controls were chosen from all the *Babesia*- and *Borrelia*-negative ticks and geographically matched, twice as many controls were chosen as participants with positive ticks.

Line 212: Figure 2B appears to indicate that the prevalence of B. venatorum infected ticks is HIGHER in southcentral Sweden when compared with Aland Islands. Here, the text indicates that the prevalence is HIGHER in the Aland Islands. Which is correct? This comment also applies to the sentence on lines 243-244.
There is no figure 2B but we have assumed that the reviewers comment is regarding figure 1B. We have changed the figure text slightly to make this clearer, the statement in the manuscript is correct regarding higher prevalence in the Åland Islands. See the new figure text for Fig 1B.

Minor Comments

Lines 3-4: the following title "Clinical/serological outcome in humans bitten by Babesia spp. positive Ixodes ricinus ticks in Sweden and on the Aland Islands" may be more attractive to

the reader, in particular when browsing PubMed.
Thank you for this comment, we have changed the title accordingly.

Lines 36-37: change to "Sixty-five of 2098 ticks were positive (3.1%) by real-time PCR. Three Babesia species …"
Changed according to comment.

Line 39: change to "Half (46%) of the Babesia …."
Changed according to comment.

Line 39: change to "Fifty-three participants …"
Changed according to comment.

Lines 44-47: change to "Given the prevalence of Babesia in I. ricinus ticks in southern Sweden and on the Aland Islands, babesiosis should be considered a possible diagnosis in symptomatic residents who seek medical care following tick exposure."
Changed according to comment, we have also altered the conclusion where a similar phrasing was used, see lines 318-322.

Line 53: change to "… transmitted by several tick …"
Changed according to comment.

Line 55: change to "The first documented case of …"
Changed according to comment.

Lines 56-57: change to "… was reported in 1957 from Yugoslavia (Skrabalo and Deanovic, 1957). Other cases followed in Western Europe …"
Changed according to comment.

Line 65: change to "… include fever, malaise, chills, sweats, headache and myalgia …"
Changed according to comment.

Lines 71-73: Add to the citations the report by Moniusko-Malinowska et al. in Infectious Diseases vol 48, pp.537-543, 2016.
This reference has been added to the citations.

Line 81: delete "In Sweden,"
Changed according to comment.

Line 84: delete "However,"
Changed according to comment.

Line 86: delete "previously"
Changed according to comment.

Line 87: modify to "Co-infection with Babesia and Borrelia spp. has been documented …"
Changed according to comment.

Line 90: delete the number 3, unless it means something.
Deleted according to comment.

Line 92: replace "intensified" with "worse".
Changed according to comment.

Lines 96: delete this part of the sentence "relative to the ..... were used", and use the edited sentence as the last sentence of the previous paragraph.'
We deleted the last part of one sentence according to this comment and moved the sentence to the previous paragraph. The last sentence of the paragraph was moved to the Material and Methods section. See lines 93-101.

Line 104: rephrase as "(PHCs) in the three regions of ..."
Changed according to comment.

Line 114: delete "sera from".
Changed according to comment.

Line 115: delete "serologically".
Changed according to comment.

Line 116: delete "and analyzed for the presence of Babesia spp. using real-time PCR" because the current version of the manuscript no longer includes data on Babesia spp. detected by PCR in human blood samples.
Changed according to comment.

Lines 119-120: rephrase to "In this study, cDNAs from 2098 ticks detached from 1769 participants were analyzed whereas cDNAs from the remaining 12 ticks were not available for analysis."
Changed according to comment.

Lines 123-124: delete ", in total 5 uL cDNA per well."
Changed according to comment.

Line 141: replace "Amplification" with "Extension".
Changed according to comment.

Line 157: rephrase as "... from 53 of the 61 participants bitten by Babesia positive ticks were ..."
Changed according to comment.

Line 165: start sentence as "Samples were ...".
Changed according to comment.

Line 182: replace with "Statistical Analysis"
Changed according to comment.

Lines 198-199: delete ", as compared with B. capreoli sequence (AY26009) deposited in Genebank,". This info should be moved to the Methods section.

We have left some of this information because it is a result from our study and as such should be presented in the results. Because of this comment we have rephrased it slightly, see lines 193-196.

Lines 206-207: move the sentence "Different ticks …Babesia spp." to after the next sentence.
Changed according to comment.

Lines 213-214: rephrase to "No other differences in species composition between regions were significant".
Changed according to comment.

Line 224: rephrase to "Seven participants …".
Changed according to comment.

Line 252: rephrase to "… in 0.6% of ticks (Jensen et al. 2017)".
Changed according to comment.

Line 256: change to "…positive larvae among questing ticks, …".
Changed according to comment.

Line 259: change to "… B. capreoli is known to be …".
Changed according to comment.

Line 280: delete "on seroprevalence"
Changed according to comment.

Line 282: remove "antibodies".
Changed according to comment.

STING-study Code …… Date ……

To participants of the STING-study

Please answer all questions!

When did you notice that you had been tick-bitten?

Year-Month-Day: _____ _____ _____ Time _____

When do you think you were tick-bitten?

Year-Month-Day: _____ _____ _____ Time _____

Where do you think you were when you were tick-bitten? Please state the name of the municipality.

_____

What kind of habitat (vegetation type) had you visited?

Lake/Sea ☐ Forest ☐ Garden ☐ Lawn ☐

Other: _____

When was the tick removed?

Year-Month-Day: _____ _____ _____ Time _____

Where on the body was the tick attached?

_____

Did you remove the whole tick? Yes ☐ No ☐ Do not know ☐

Have you had any other tick bites this season? Yes ☐ No ☐ Do not know ☐

If Yes, how many? 1-4 ☐ 5-9 ☐ >10 ☐

Have you ever been treated for the tick-borne infection Borrelia?

Yes ☐ No ☐ Do not know ☐ If Yes; Year–Month–Day _____ _____ _____

Did you receive any medicine?

Yes ☐ No ☐ Do not know ☐ If Yes; what kind of medicine did you get?
_____

Have you ever been treated for "Erythema migrans"?
(Erythema migrans = red ring-like or homogenous expanding rash.)

Yes ☐ No ☐ Do not know ☐ If Yes; Year–Month–Day _____ _____ _____

Did you then receive any medicine to treat the infection?

Yes ☐ No ☐ Do not know ☐ If Yes; what kind?_____

Have you ever been treated for the tick-borne infection "Ehrlichia"
(= Ehrlichiosis, also called "Anaplasma" or anaplasmosis)?

Yes ☐ No ☐ Do not know ☐ If Yes; Year–Month–Day _____ _____ _____

Did you receive any medicine to cure the Ehrlichia (Anaplasma) infection?

Yes ☐ No ☐ Do not know ☐ If Yes; what kind?_____

Have you ever been treated for the tick-borne infection TBE?
(TBE is a viral disease which sometimes causes disease in the central nervous system.)

Yes ☐ No ☐ Do not know ☐ If Yes; Year–Month–Day_____ _____ _____

Did you receive any medicine?

Yes ☐ No ☐ Do not know ☐ If Yes; what kind?_____

Do you have any of the following diseases?

Asthma	Yes ☐	No ☐	Do not know ☐
Allergy	Yes ☐	No ☐	Do not know ☐
Diabetes	Yes ☐	No ☐	Do not know ☐
Tumour-related	Yes ☐	No ☐	Do not know ☐

Are you on medication? Yes ☐ No ☐
If Yes; what kind of medicine?

_____

Do you smoke? Yes ☐ No ☐ Stopped smoking ☐ Year _____

If Yes, how many cigarettes per week? _____

How many years have you smoked? _____

Do you have any pets? Yes ☐ No ☐

Dog Yes ☐ No ☐

Cat Yes ☐ No ☐

Bunny (rabbit) Yes ☐ No ☐

Other: _____

Have you been vaccinated against TBE? Yes ☐ No ☐ Do not know ☐

If Yes; Year-Month-Day_____ _____ _____

Have you been vaccinated against Yellow fever? Yes ☐ No ☐ Do not know ☐

If Yes; Year-Month-Day_____ _____ _____

Have you been vaccinated against Japanese encephalitis? Yes ☐ No ☐ Do not know ☐

If Yes; Year-Month-Day_____ _____ _____

Thank you for your answers!

Dear STING participant!

Three months have passed since you initiated your participation in the Tick-Borne Diseases STING-study. We previously received blood samples from you and a filled in questionnaire. Now, we need a follow-up blood sample. Therefore, you are requested to visit your primary health care centre at _____, week ___, Monday, Tuesday, Wednesday, or Thursday, between _____ and _____ a clock.

If you had any additional tick-bites since your study initiation and if you have collected the ticks in the tube with yellow cork, the please take that tube with you to the blood-sampling.

We would also like to know if you have had any symptoms related to tick-borne diseases during the study period. Please answer the following three questions and write your name, birth date and telephone number on the next page. We might contact you if you reported symptoms. Take this paper to your primary health care centre when you go for the sample-taking.

1) Have you had any additional tick-bites since the first sample-taking?

Yes ☐ No ☐ Do not know ☐

If Yes; when? Year-Month-Day: _____ ____ ____

2) How have you been feeling in general since the first sample-taking?
Have you been feeling good/as usual?

Yes ☐ No ☐ Do not know ☐

If No; please report if you had any of the following symptoms:

Headache	Yes ☐	No ☐
Fatigue	Yes ☐	No ☐
Fever, 38° or higher	Yes ☐	No ☐
Neck pain	Yes ☐	No ☐
Loss of appetite	Yes ☐	No ☐
Nausea	Yes ☐	No ☐
Weight loss	Yes ☐	No ☐
Vertigo	Yes ☐	No ☐
Concentration difficulties	Yes ☐	No ☐
Radiating pain	Yes ☐	No ☐
Muscle or joint pain	Yes ☐	No ☐

Numbness Yes ☐ No ☐

3) If you reported any symptoms in question 2, did the symptoms appear before or after any additional tick-bites?

Before additional tick-bite Yes ☐ No ☐ Do not know ☐

After additional tick-bite Yes ☐ No ☐ Do not know ☐

4) If you reported any symptoms in question 2, did you visit your primary health care centre due to the symptoms?

Yes ☐ No ☐

5) If you reported any symptoms in question 2, how many days did the symptoms last?

_____

Thanks for your answers!

Please make sure you answered every question!
Bring this paper to your new sample-taking!

Name: _____

Date of birth: _____

Telephone number:
Home _____
Work _____
Mobile _____

Best regards
Lotta Lindvall
Forskningssköterska
Infektionskliniken
Universitetssjukhuset
581 85 Linköpings
Tfn xxx/xxx xxxx

2020-04-16

TITLE PAGE

Title

Clinical/serological outcome in humans bitten by *Babesia* species positive *Ixodes ricinus* ticks in Sweden and on the Åland Islands

AUTHORS

Wilhelmsson P[1,2]*

Lövmar M [1]*

Krogfelt KA[3,4]

Nielsen HV[3]

Forsberg P[5]

Lindgren PE[1,2]

[1]Division of Medical Microbiology, Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

matilda.lovmar@liu.se

[2] Division of Medical Services, Department of Clinical Microbiology, Ryhov County Hospital, Jönköping, Sweden

per-eric.lindgren@liu.se

[3]Department of Bacteria Parasites and Fungi, Statens Serum Institute, Copenhagen, Denmark

kak@ssi.dk, hvn@ssi.dk

[4] Department of Science and Environmental, Roskilde University, Denmark

[5]Division of Infectious Diseases, Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

pia.forsberg@liu.se

* these authors contributed equally to this article

*Corresponding author: Matilda Lövmar, Email: matilda.lovmar@liu.se

Linköping University, Department of Biomedical and Clinical Sciences, Division of Medical Microbiology, 581 85 Linköping Phone: (+46) 101032054

ABSTRACT

The risk of contracting babesiosis after a tick bite in Sweden and the Åland Islands, Finland, is unknown. We investigated clinical and serological outcomes in people bitten by *Ixodes ricinus* ticks positive for *Babesia* species. Ticks, blood and questionnaires were obtained from study participants in Sweden and on the Åland Islands. Sixty-five of 2098 (3.1%) ticks were positive by real-time PCR. Three *Babesia* species were detected, *Babesia microti* (n=33), *B. venatorum* (n=27) and *B. capreoli* (n=5), the latter species not known to cause human infection. Half (46%) of the *Babesia* PCR-positive ticks also contained *Borrelia* spp. Fifty-three participants bitten by a *Babesia* PCR-positive tick and a control group bitten by a *Babesia* PCR-negative tick were tested for *B. microti* IgG antibodies by IFA. The overall seroprevalence was 4.4%, but there was no significant difference between the groups. None of the participants seroconverted and no participant with a *Babesia* PCR-positive tick sought medical care or reported symptoms suggestive of babesiosis. Given the prevalence of *Babesia* in *I. ricinus* ticks in southern Sweden and on the Åland Islands, babesiosis should be considered a possible diagnosis in symptomatic residents who seek medical care following tick exposure.

Keywords

Babesia; Babesiosis; Human; Seroconversion; *Ixodes ricinus*; Co-infection

INTRODUCTION

Human babesiosis is caused by parasites of the genus *Babesia* and transmitted by several tick species (Vannier and Krause, 2012). There are more than 100 known *Babesia* spp. that infect animals but only a few are known to infect humans. The first documented case of human babesiosis in Europe was reported in 1957 from Yugoslavia (Skrabalo and Deanovic, 1957). Other cases followed in Western Europe, including Scandinavia (Haapasalo et al., 2010; Morch et al., 2015) and a travel-associated case in Denmark (Holler et al., 2013). In Sweden there have been two reported cases of human babesiosis, both in splenectomized patients (Bläckberg et al., 2018; Uhnoo et al., 1992). In North America, babesiosis is considered an emerging health threat that is expanding into new geographical areas and may be overlooked by clinicians in regions not previously considered endemic (Gray and Herwaldt, 2019). Most cases in the United States have been reported in immunocompetent patients (Vannier et al., 2015). The first case in the United States was reported in 1966 (Scholtens et al., 1968).

Symptoms of babesiosis include fever, malaise, chills, sweats, headache and myalgia accompanied by anemia, leukopenia or leukocytosis, thrombocytopenia and elevated hepatic enzymes (Vannier and Krause, 2012). In Europe the most common cause of human babesiosis is *Babesia divergens*, which typically is diagnosed in immunocompromised individuals and gives rise to a severe illness (Vannier et al., 2015; Vannier and Krause, 2012). Infection with *B. divergens* has also been reported in immunocompetent patients (Martinot et al., 2011). A few cases of *B. microti* and *B. venatorum* infection have been reported in Europe (Bläckberg et al., 2018; Blum et al., 2011; Haselbarth et al., 2007; Herwaldt et al., 2003;

Hildebrandt et al., 2007; Moniuszko-Malinowska et al., 2016). Infection with *B. venatorum*, giving mild to severe symptoms in splenectomized patients, has been reported (Haselbarth et al., 2007; Herwaldt et al., 2003). In the United States, *B. microti* is the most common causative agent of babesiosis; it causes mild to moderate symptoms and subclinical infections in immunocompetent persons (Vannier et al., 2015; Vannier and Krause, 2012). However, severe babesiosis may also occur, even in previously apparently healthy individuals (Gray and Herwaldt, 2019; Hatcher et al., 2001).

In Sweden, the prevalence of *Babesia* spp. in questing *I. ricinus* ticks was recently estimated to be 4.4% and included *B. microti* (3.2%), *B. venatorum* (1.0%) and *B. divergens* (0.2%) (Karlsson and Andersson, 2015). *B. capreoli* has been reported in roe deer, but this *Babesia* sp. is not known to cause human infections (Andersson et al., 2016; Malandrin et al., 2010). In Italy, the prevalence of *B. venatorum* in ticks that have bitten humans was estimated to be 0.6% (Otranto et al., 2014). To our knowledge, the clinical outcome and the rate of seroconversion after a bite by a *Babesia* containing tick or a tick co-infected with *Borrelia*, have not been investigated.

Co-infection with *Babesia* and *Borrelia* spp. has been documented several times (Diuk-Wasser et al., 2016; Knapp and Rice., 2015). There are differing opinions on the consequences of co-infection for severity of symptoms and prognosis in humans. According to Diuk-Wasser et al., (2016), the severity and duration of symptoms are greater in co-infected patients and there are indications that co-infection may result in altered or suppressed immune response, which in turn leads to worse pathogenesis; more research is, however, needed. The aims of the present study were to investigate the prevalence of *Babesia* spp. in ticks that had bitten humans and to evaluate the concomitant risk of clinical babesiosis or subclinical seroconversion against *Babesia* spp.

MATERIAL AND METHODS

TBD STING-study

Ticks, serum and questionnaires from the Tick-Borne Diseases (TBD) STING-study were used (Fryland et al., 2011; Grankvist et al., 2015; Henningsson et al., 2015; Henningsson et al., 2016; Lindblom et al., 2014; Wilhelmsson et al., 2010; Wilhelmsson et al., 2013a; Wilhelmsson et al., 2013b).

Ticks and blood samples were collected during 2008–2009 at 34 primary healthcare centers (PHCs) in the three regions of Sweden (Northern Sweden, South Central Sweden and Southernmost Sweden) and on the Åland Islands, Finland (Figure 1A). Only immunocompetent tick-bitten individuals ≥18 years were included. Questionnaires, ethical approval, collection, transport and storage of ticks and blood samples, determination of developmental stage and feeding time of ticks, and extraction and treatment of nucleic acids have been described previously (Andersson et al., 2016; Wilhelmsson et al., 2013a; Wilhelmsson et al., 2013b). *Babesia* spp. were detected using real-time PCR-based amplification of reverse-transcribed total nucleic acids.

Blood samples were collected from participants within three days of the tick bite and three months later (Figure 2). Serum samples from participants with *Babesia* PCR-positive ticks and a negative control group, consisting of participants bitten by a *Babesia* PCR-negative tick, geographically matched, were analyzed by IFA.

Detection of *Babesia* spp. in ticks using SYBR-green real-time PCR assay

A total of 2110 ticks detached from 1770 participants were delivered from PHCs during 2008–2009. In this study, cDNAs from 2098 ticks detached from 1769 participants were analyzed whereas cDNAs from the remaining 12 ticks were not available for analysis.

The 2098 cDNA samples of the individually extracted ticks were grouped into pools of five, i.e. one µl of cDNA from each tick was used per well. Samples from the positive pools were individually analyzed using 2 µl cDNA. The PCR mixture contained 10 µl SYBR-green (Thermo Scientific, Helsingborg, Sweden), 0.4 µl of each primer (10 µM), BJ1: 5´-GTC TTG TAA TTG GAA TGA TGG-3´ (Invitrogen™, Thermo Scientific) and BN2: 5´-TAG TTT ATG GTT AGG ACT ACG-3´ (Casati et al., 2006), 4.2 µl RNase free water. Primers BJ1 and BN2 were designed to target the *Babesia 18S* rRNA gene to amplify a 411 to 452 bp long amplicon depending on the species of *Babesia*. For species determination, sequencing of the amplicon was carried out (see below). As a positive control, 2 µl *B. microti* DNA (~10 ng/µl), extracted from *I. ricinus* ticks collected in Slovakia (kindly provided by Dr Bronislava Víchová, through Dr Martin Andersson), and 2 µl of a synthetic plasmid preparation was used. The plasmid contained the target sequence of the SYBR green real-time PCR assay, spanning the nucleotides 467-955 of the *B. divergens 18S* rRNA gene (acc. no AJ439713), synthesized and cloned in a pUC57 vector (Genscript USA Inc, NJ). The non-template control consisted of 10.8 µl PCR mixture and 9.2 µl RNase free water. The SYBR-green real-time PCR on *Babesia* were performed using C1000™ Thermal Cycler, CFX96™ system (BioRad Laboratories, Inc., Hercules, CA). The PCR run was initiated by a denaturation step at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min. Extension was completed by a further step at 72°C for 5 min, and melt curve analysis was performed (Casati et al., 2006).

Babesia spp. identification by sequencing

Samples positive for *Babesia* spp. in the real-time PCR assay were sent to Macrogen Inc. (Amsterdam, the Netherlands) for nucleotide sequencing. The reactions were based on BigDye chemistry. Chromatograms were edited using BioEdit Sequence Alignment Editor

version 7.2.5 (Tom Hall, Ibis Therapeutics, Carlsbad, CA) and sequences examined using the Basic Local Alignment Tool (BLAST). Sequences obtained have been deposited in GenBank with accession numbers ranging from MH351680 to MH351744.

Species determination for *B. microti* and *B. venatorum* is possible by sequencing the amplicon from the real-time PCR assay. To fully distinguish between the two genetically similar *Babesia* spp., *B. divergens* and *B. capreoli*, three sets of primers were used to amplify and sequence the complete *18S* rRNA gene from all samples positive for these species, as earlier described (Malandrin et al., 2010).

Detection of *Babesia microti* IgG antibodies in human serum

The first and second serum samples (collected at recruitment of participants and three months later, respectively) from 53 of the 61 participants bitten by *Babesia*-positive ticks were analyzed for the presence of *B. microti* IgG antibodies, using an indirect immunofluorescence assay (IFA); (Focus Diagnostics, Cypress, CA). For the remaining eight participants, serum was not available for analysis, since it had been used for other analyses. Twice as many controls were selected; first sample sera from participants bitten by *Babesia*- and *Borrelia*-negative ticks were matched geographically and used as controls (n=106) for analysis regarding IgG antibodies against *B. microti*. For *B. venatorum* there were no commercial kits available at the time of the study. IFA titers ≥1:64 were defined as positive. A cut-off value of 1:64 was used in accordance with previous research (Johnson et al., 2009). Samples were diluted to determine the highest positive titer. The IFA-slides were analyzed by two researchers independently, samples were defined as positive when both researchers found them positive. For the diagnosis of on-going or recent *Babesia* spp. infection, at least a four-fold rise of the IFA titer (Krause, 2003; Vannier and Krause, 2012) was required when first and second sera were tested simultaneously.

Self-reported symptoms in the questionnaires of the TBD STING-study and medical records

The questionnaires from participants with *Babesia* PCR-positive ticks were scrutinized for symptoms suggestive of babesiosis, i.e. chills, fever, headache, nausea, myalgia, malaise, weight loss, arthralgia and lack of appetite (Vannier and Krause, 2012). Other symptoms indicating TBD in the questionnaires were neck pain, vertigo, concentration difficulties, numbness, radiating pain. If participants sought medical care during the three-month study period, the medical records were obtained and scrutinized for symptoms of babesiosis and/or if they were diagnosed with babesiosis or another TBD.

Co-infection of tick-borne pathogens in ticks

Borrelia-data from the TBD STING-study were used to determine which ticks were co-infected with both *Babesia* spp. and *Borrelia* spp. (Wilhelmsson et al., 2013a).

Statistical analysis

The Chi square test was applied to compare prevalence of *Babesia* spp. between regions and between tick developmental stages, but when the expected frequency was < 5 in at least one of the cells of the contingency table, we used Fisher's exact test. Statistical analyses were performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego, CA). P-values <0.05 were considered significant.

RESULTS

Babesia species in ticks detached from humans

A total of 2098 ticks that had bitten humans were analyzed (Table 1). Sixty (2.9%) of the ticks were damaged to the extent that neither developmental stage nor the species could be determined. The remaining 2038 ticks were identified as *I. ricinus*; 86 (4.1%) larvae, 1466

(70%) nymphs, and 486 (23%) adults. 65 of 2098 ticks (3.1%) were positive for *Babesia* spp. by real-time PCR assay. Based on nucleotide sequencing of the PCR products, three *Babesia* species were detected; *B. microti* (n=33), *B. venatorum* (n=27), and *B. capreoli* (n=5). Our analysis of the complete 18S rDNA sequences for the five samples initially determined as either *B. divergens* or *B. capreoli* revealed a signature typical of *B. capreoli* at positions 631, 663 and 1637 (GTT), as compared with *B. capreoli* sequence (GeneBank: AY26009). In our study there was no significant difference in *Babesia* prevalence between adult ticks and nymphs or between nymphs and larvae. However, the prevalence of *B. microti* was significantly higher in adult ticks than in nymphs ($p<0.05$).

The 65 PCR-positive ticks were collected from 61 participants and the duration of tick feeding could be estimated for 58 of the 65 ticks (Appendix). 21 of these ticks had been feeding for > 36 h and the remaining 37 ticks < 36 h. There were three participants bitten by more than one infected tick. One participant from the Åland Islands was bitten by three infected ticks, one from Southcentral Sweden was bitten by two infected ticks, and one from Southernmost Sweden was bitten by two infected ticks (Appendix). Different ticks from the same participant contained different *Babesia* spp. There was no significant difference in prevalence of infected ticks between the geographic regions (Figure 1B). However, there was a significant difference in species composition of *Babesia* between the geographic regions, with *B. venatorum* more prevalent in the Åland Islands than in Southcentral Sweden ($p<0.03$). No other differences in species composition between the regions were significant.

Co-infection with *Babesia* spp. and *Borrelia* spp. in ticks

To find which ticks contained *Borrelia* spp., data from the TBD STING-study were used (Wilhelmsson et al., 2013a). Thirty out of 65 (46%) *Babesia* spp. positive ticks contained *Borrelia* spp. (Appendix). There was a significant difference in the frequency of co-infections

involving *B. microti* and *Borrelia* spp. (60%) and the frequency of co-infections involving *B. venatorum* and *Borrelia* spp. (30%) ($p<0.01$).

Seroprevalence, seroconversion and reported symptoms of the tick-bitten participants

The overall seroprevalence for *B. microti* was 7 out of 159 (4.4%) with no significant difference between the participants bitten by *Babesia* PCR-positive ticks and participants bitten by *Babesia* PCR-negative ticks. Seven participants were found seropositive both in first and second sample sera (Table 2).

Twenty-nine participants were bitten by ticks containing both *Babesia* spp. and *Borrelia* spp. One of these participants was bitten by two ticks positive for *Babesia* spp. and *Borrelia* spp. Data regarding seroconversion for *Borrelia* spp. showed that one of the participants who seroconverted to *Borrelia* had a *Babesia* PCR-positive tick. However, this participant had a negative *B. microti* serology.

There were ten participants with *Babesia* PCR-positive ticks who reported symptoms in their questionnaires. The symptoms included headache, muscle pain, fatigue, neck pain, dizziness, concentration difficulties, numbness, radiating pain, joint pain and nausea. Only one of these participants had *B. microti* IgG antibodies. This participant sought medical care at the PHC during the study period but according to notes found in the medical records, symptoms were deemed not relevant to the tick bite.

DISCUSSION

To our knowledge this is the first time *Babesia* has been found in ticks that have bitten humans in Sweden. In total, 3.1% of ticks collected from four regions in Sweden and in the Åland Islands contained *Babesia* spp. *Babesia* infected ticks were found in three of the four regions studied (Fig. 1B). A previous study on the prevalence of *Babesia* spp., in questing ticks in southern Sweden, found a prevalence of 4.4% which is in line with our findings

(Karlsson and Andersson, 2015). Comparing prevalence of *Babesia* spp. in the different regions, *B. venatorum* was more prevalent on the Åland Islands than in Southcentral Sweden. One could speculate the difference is related to different *Babesia* reservoir host composition.

We found that the most prevalent species in ticks collected from humans was *B. microti*, followed by *B. venatorum* and *B. capreoli.* In Sweden *B. capreoli* has been found in 44% of roe deer (Andersson et al., 2016), but this species is not known to cause human infections (Malandrin et al., 2010). We did not find any samples positive for *B. divergens*. A study conducted in Norway found that 0.1% of field-collected ticks are infected with *B. divergens*, 0.1% with *B. capreoli* and 0.6% with *B. venatorum* (Øines et al., 2012). In Denmark, *B. divergens* was found in 1.9% of ticks and *B. venatorum* in 0.6% of ticks (Jensen et al., 2017). Karlsson and Andersson (2015) found 0.2% questing ticks containing *B. divergens* in Sweden and found no significant difference in prevalence of *Babesia* spp. in adult ticks compared to nymphs. They found a higher prevalence in nymphs than in adults and they found no positive larvae among questing ticks, but we analyzed four times as many ticks and they were collected from humans. We found only one positive larva (Table 1) that contained *B. capreoli*. *B. capreoli* has not previously been found in larvae, however, *B. divergens* which is genetically similar to *B. capreoli* is known to be transovarially transmitted (Bonnet et al., 2007).

Nearly one-half of the ticks positive for *Babesia* spp. were positive for *Borrelia* spp. Sixty percent of ticks containing *B. microti* were co-infected with *Borrelia* spp. compared to 30% of ticks containing *B. venatorum*. This may reflect the genus ratio of *Babesia* spp. and *Borrelia* spp. in reservoir hosts. It has been observed in an experimental model that mice co-infected with a strain of *B. microti* and an invasive strain of *B. burgdorferi* s.s. increased *B. microti* frequency in *I. scapularis* that fed from them compared

to mice infected with *B. microti* alone (Dunn et al., 2014). It remains to be confirmed if this finding applies to *I. ricinus* and one or several *Babesia* spp. it can transmit.

Seven participants were positive for *B. microti* antibodies. Since none of them seroconverted and the antibody titers were low, we did not suspect an ongoing infection (Vannier and Krause, 2012). It is probable that they had a previous or subclinical infection and still carried antibodies. In a previous study, Lempereur et al. (2015) found no antibody cross-reactivity between *B. microti* and *B. venatorum* used as IFA antigens, but IgM cross reactivity between *B. microti* and *B. divergens* has been observed in another study (Haselbarth et al., 2007). Bläckberg et al. (2018) reported that a patient with *B. venatorum*-infection had *B. divergens* IgG antibodies. For the participants with a positive *B. microti* serology, only three had been bitten by *Babesia* PCR-positive ticks during the study period. Because they had the same antibody titers in the first serum sample as in the second, it is not likely that the tick-bite during the study period was the cause of the positive serology or that the participants had an ongoing infection. One study in southern Sweden has revealed a prevalence of 16.3% for *B. microti* and *B. divergens* antibodies in a geographically selected cohort of seropositive *Borrelia* s.l. patients; and a 2.5% prevalence in a healthy control group (Svensson et al., 2019). However, comparing this study to ours is complicated, since we have used another serological assay for *Babesia* antibodies and the study populations differ from each other.

In total, three participants were bitten by more than one *Babesia* PCR-positive tick, none of them developed antibodies against *B. microti* during the study period. One of these participants was bitten by ticks containing *B. capreoli*, not confirmed to be human pathogenic. Since we only analyzed for *B. microti* IgG we cannot draw any general conclusions regarding *Babesia* spp. antibodies. However, it is an interesting future prospective. None of the participants with more than one positive tick reported symptoms in

their questionnaires or sought medical care during the study period. This suggests a low risk of transmission despite being bitten by several positive ticks and different species. Furthermore, the efficacy of transmission has been shown to correlate with the duration of tick feeding (for *I. scapularis*) in hamsters and white-footed mice, with infection rates close to 100% if the tick is allowed to feed to repletion (Piesman and Spielman, 1980). However, it is not known if this is true for *I. ricinus* and/or human hosts. In our study ticks were removed by the participant before repletion.

Ten participants with *Babesia* PCR-positive ticks reported symptoms in their questionnaires. These symptoms were nonspecific and might indicate different conditions, babesiosis included. Only one participant bitten by a *Babesia* PCR-positive tick sought medical care during the study period. According to medical records, symptoms were unrelated to the tick bite. Thus, we conclude that none of the participants suffered from symptoms of babesiosis.

Twenty-nine of 61 participants with *Babesia* PCR-positive ticks were bitten by ticks co-infected with *Borrelia*. Of the ten participants who reported symptoms, five had been bitten by ticks positive for both *Babesia* spp. and *Borrelia* spp. The symptoms reported could be attributed either to babesiosis, borreliosis or other infections. None of the participants who reported symptoms, with the exception of the participant mentioned above, had sought medical care. This suggests that no participant suffered from severe illness.

One of the potential limitations of this study is that we did not test the serum for *B. venatorum* antibodies since we did not have available commercial kits for these analyses. The conclusions that can be drawn from the serological analyses are further limited by the lack of information regarding travel history, since this was not included in the questionnaires, designed for the TBD STING-study. Unfortunately, whole blood samples from the TBD STING-study were not available for real-time PCR analysis.

Conclusions

In conclusion our results indicate that immunocompetent individuals have a low risk of developing severe babesiosis after an *I. ricinus* tick bite in Sweden and on the Åland Islands, particularly when the tick has been feeding for less than 36 hours. Our findings of participants with positive serology suggests that human infection with *B. microti* occurs in Sweden, although we do not know about the travel history of these participants. Given the prevalence of *Babesia* in *I. ricinus* ticks as well as the seroprevalence of *Babesia* antibodies among residents in southern Sweden and on the Åland Islands, babesiosis should be considered a possible diagnosis in symptomatic residents who seek medical care following tick exposure.

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Table 1. Analyzed ticks and distribution of developmental stages.

Developmental stage of the tick	Total no. of ticks analyzed	No. of *Babesia* PCR-positive ticks (%)	No. of ticks PCR-positive for *B. microti*	No. of ticks PCR-positive for *B. venatorum*	No. of ticks PCR-positive for *B. capreoli*
Adult females	478	20 (4.2)	13	6	1
Adult males	8	1 (12.5)	0	1	0
Nymphs	1466	43 (2.9)	20	20	3
Larvae	86	1 (1.2)	0	0	1
ND*	60	0 (0)	0	0	0
Total	2098	65 (3.1)	33	27	5

*Developmental stage could not be determined due to damaged tick

Table 2. *B. microti* IgG antibody titers for samples positive in the serological analysis (n=7).

Participant Id. code. *	Antibody titers in 1st sample‡	Antibody titers in 2nd sample‡	*Babesia* spp. in the tick§	Tick feeding duration (h)	Developmental stage of the tick#
Afa 89	1:128	1:128	*B. venatorum*	25	N
Vofa 15	1:256	1:256	*B. microti*	35	A
Vifa 25	1:64	1:64	*B. microti*	<24	A
†Kafa 6	1:256	1:256	Neg.	<24	N
†Kafa 52	1:64	1:64	Neg.	25	N
†Kfa 11	1:128	1:64	Neg.	ND¶	N
†Kfa 13	1:64	1:64	Neg.	<24	A

*Participant Id. code. Letters representing primary healthcare center where tick was collected followed by serial number.

†Sample from control group

‡1st sample collected at inclusion, 2nd sample after three months

§ *Babesia* spp. found in the tick collected at inclusion

¶ ND = not determined due to deformed tick, making scutal and coxal indices impossible to determine

A = Adult female, N = nymph

Figure 1. A. Map, showing the four regions (Northern Sweden, South Central Sweden, Southernmost Sweden, Åland Islands) where the 34 primary health care centers (PHCs, black dots) are located. **B.** Map showing PHCs where ticks positive for different *Babesia* species were collected, *Babesia microti* (red circles), *Babesia venatorum* (black crosses) and *Babesia capreoli* (filled green circles). Numbers (X/Y) next to region showing number of positive ticks in each region (X) with total number of ticks collected (Y). Maps modified from Wilhelmsson et al. 2013b.

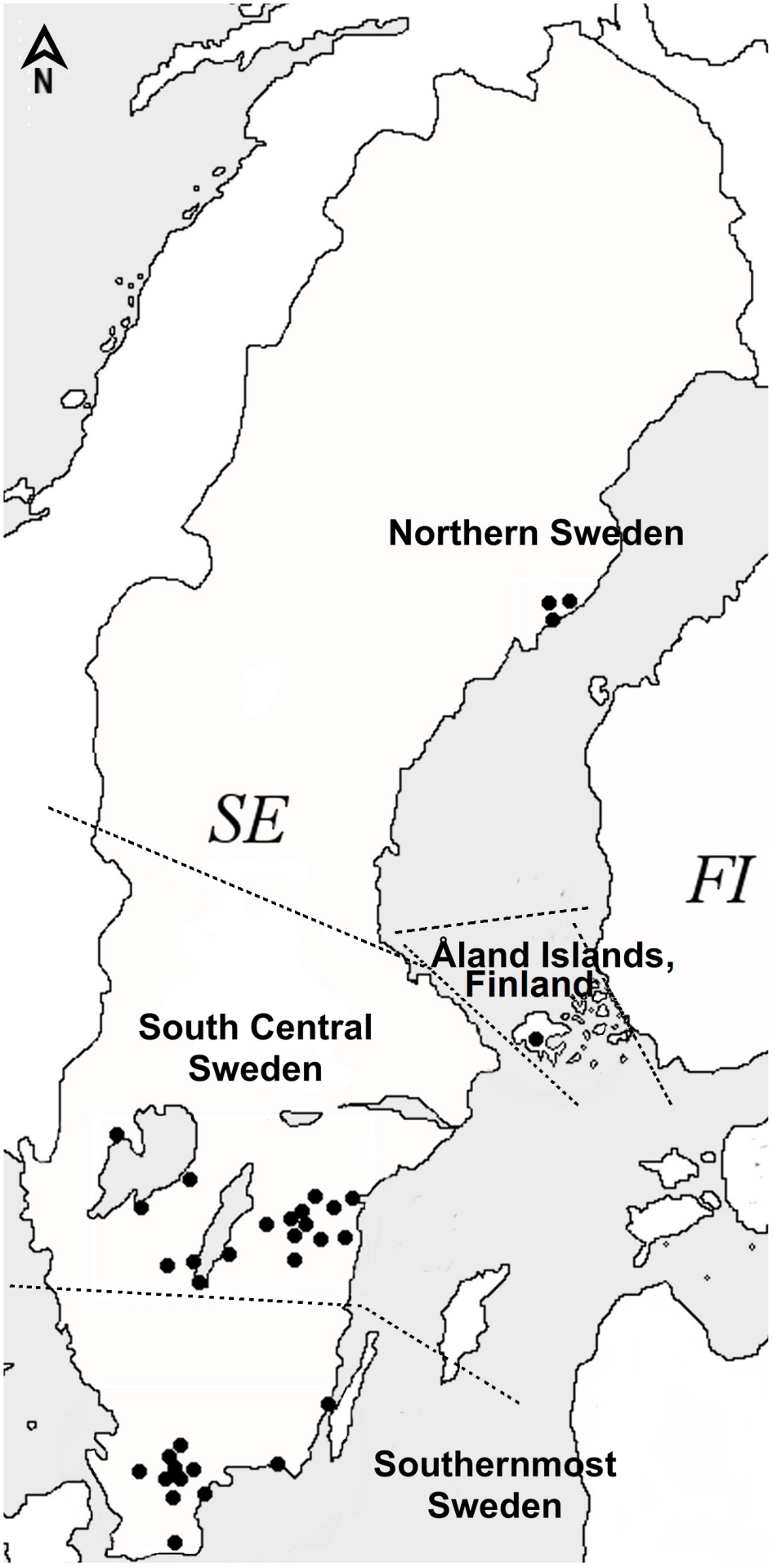
Figure 2. Flow-chart showing the study design and methods. Green for information regarding ticks, red for blood samples and blue for questionnaires.
*Excluded because no samples were available, they had been used for previous analyses.
† Excluded since sera were not available, it had been used for previous analyses.
‡ Denoted as the negative control group.

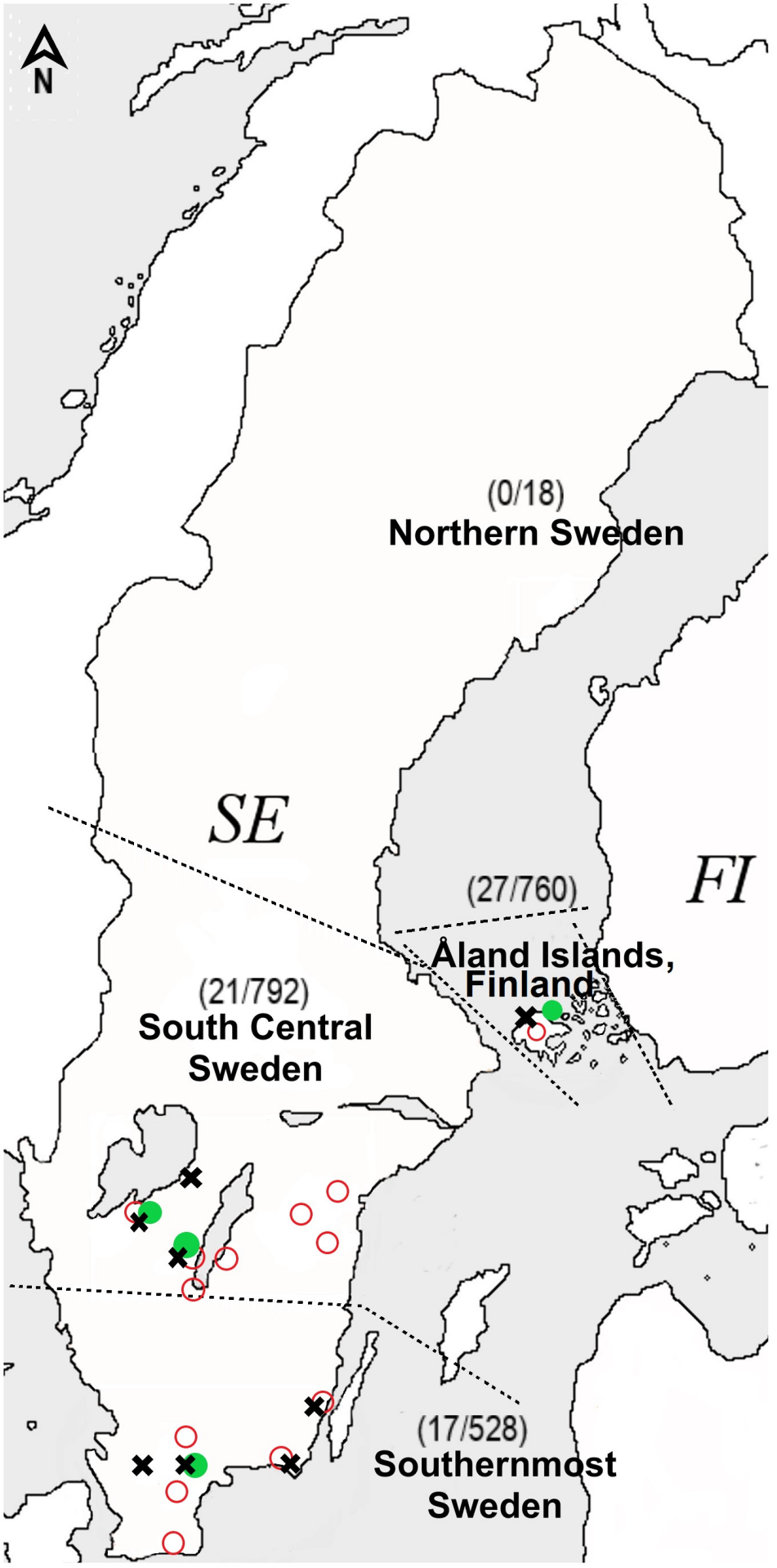
Appendix. 65 ticks positive for *Babesia* in real-time PCR

Region	Id. Number	Developmental stage of the tick§	Feeding time (h)	*Babesia* spp. in the tick	*Borrelia* spp. in the tick	Estimated no. of spirochetes
Åland Islands	Afa 22	A	<24	*B. microti*	*B. afzelii*	3.3×10^2
	Afa 89	N	25	*B. venatorum*	*B. afzelii*	3.1×10^3
	Afa 115B*	N	<24	*B. venatorum*		
	Afa 132B	N	46	*B. venatorum*	ND‡	1
	Afa 132C	N	<24	*B. microti*		
	Afa 132D	A	30	*B. venatorum*		
	Afa 144	N	50	*B. venatorum*		
	Afa 156A	A(male)	-†	*B. venatorum*		
	Afa 178	N	42	*B. venatorum*		
	Afa 185	A	-	*B. microti*		
	Afa 190	N	<24	*B. venatorum*	*B. afzelii*	2.3×10^4
	Afa 217	A	<24	*B. microti*	*B. afzelii*	1.6×10^4
	Afa 237	A	34	*B. venatorum*		
	Afa 338	A	<24	*B. microti*		
	Afa 350	N	<24	*B. microti*	*B. afzelii*	1.1×10^4
	Afa 380	N	51	*B. capreoli*		
	Afa 381	N	30	*B. microti*		
	Afa 412	N	45	*B. venatorum*		
	Afa 465	N	<24	*B. venatorum*		
	Afa 466	N	42	*B. venatorum*	*B. afzelii*	6.7×10^4
	Afa 476	N	<24	*B. microti*	*B. afzelii*	5.3×10^4
	Afa 498D	N	<24	*B. venatorum*		
	Afa 499	N	<24	*B. venatorum*		
	Afa 518A	N	<24	*B. microti*		
	Afa 537	N	<24	*B. venatorum*		
	Afa 560	N	<24	*B. microti*		
	Afa 615B	N	33	*B. microti*	*B. valaisiana*	3

Southcentral Sweden	Bafa 6	A	<24	*B. microti*	*B. afzelii*	3.4 x 10^2
	Bafa 73A	A	<24	*B. microti*		
	Bafa 86	A	<24	*B. capreoli*	*B. burgdorferi* sensu stricto	3.1 x 10^4
	Ekfa 84	A	-	*B. microti*	*B. afzelii*	2.7 x 10^4
	Grfa 28	N	56	*B. microti*	*B. garinii*	1.3 x 10^3
	Hafa 8	N	<24	*B. venatorum*		
	Hafa 16	N	25	*B. microti*	*B. afzelii*	3.1 x 10^4
	Hafa 44B	A	<24	*B. venatorum*	*B. miyamotoi*	1.9 x 10^6
	Hafa 115	A	61	*B. venatorum*	*B. afzelii*	1.7 x 10^1
	Jofa 7	N	47	*B. microti*	*B. afzelii*	6.3 x 10^2
	Lidfa 14	N	55	*B. venatorum*	*B. garinii*	1.4x 10^3
	Lidfa 30	A	<24	*B. microti*	ND	1.1 x 10^2
	Lidfa 39A	L	-	*B. capreoli*		
	Lidfa 39B	N	52	*B. capreoli*		
	Lidfa 46	N	26	*B. venatorum*		
	Lidfa 92B	N	<24	*B. microti*	*B. afzelii*	3.9 x 10^4
	Mekfa 16	A	-	*B. venatorum*		
	Sofa 76	A	166	*B. microti*	ND	1.1 x 10^2
	Vvfa 66	A	-	*B. microti*		
	Vifa 20	N	70	*B. microti*		
	Vifa 25	A	<24	*B. microti*	*B. afzelii*	1.9 x 10^5
Southernmost Sweden	Blefa 13	N	<24	*B. microti*	*B. afzelii*	7.9 x 10^3
	Blefa 19	N	37	*B. microti*		
	Blefa 38B	N	<24	*B. microti*	*B. afzelii*	8.8 x 10^3
	Blefa 38D	N	<24	*B. venatorum*	*B. afzelii*	9.5 x 10^2
	Kafa 7	N	57	*B. venatorum*		
	Kafa 34	N	59	*B. venatorum*		
	Kafa 36	N	<24	*B. microti*	*B. afzelii*	3.5 x 10^3
	Kafa 75	N	49	*B. microti*		
	Kafa 84A	N	60	*B. microti*	*B. afzelii*	7.0 x 10^1
	Kafa 100A	N	25	*B. microti*	*B. afzelii*	1.8 x 10^4
	Kfa 9	N	<24	*B. venatorum*		
	Kfa 18	N	-	*B. venatorum*		
	Kfa 25	N	37	*B. capreoli*		
	Lafa 13	A	51	*B. venatorum*		
	Ofa 15C	N	35	*B. microti*	*B. afzelii*	1.4 x 10^4
	Vofa 15	A	35	*B. microti*		
	Ysfa 9	A	38	*B. microti*	*B. afzelii*	1.5 x 10^1

*Letter after Id. Code for participants who turned in more than one tick to the PHC, the first tick XXfa**A**, the second XXfa**B** etc.
†Feeding time could not be determined
‡ND = Species could not be determined
§A = Adult female, N = nymph


N
Northern Sweden
SE
FI
Åland Islands,
Finland
South Central
Sweden
Southernmost
Sweden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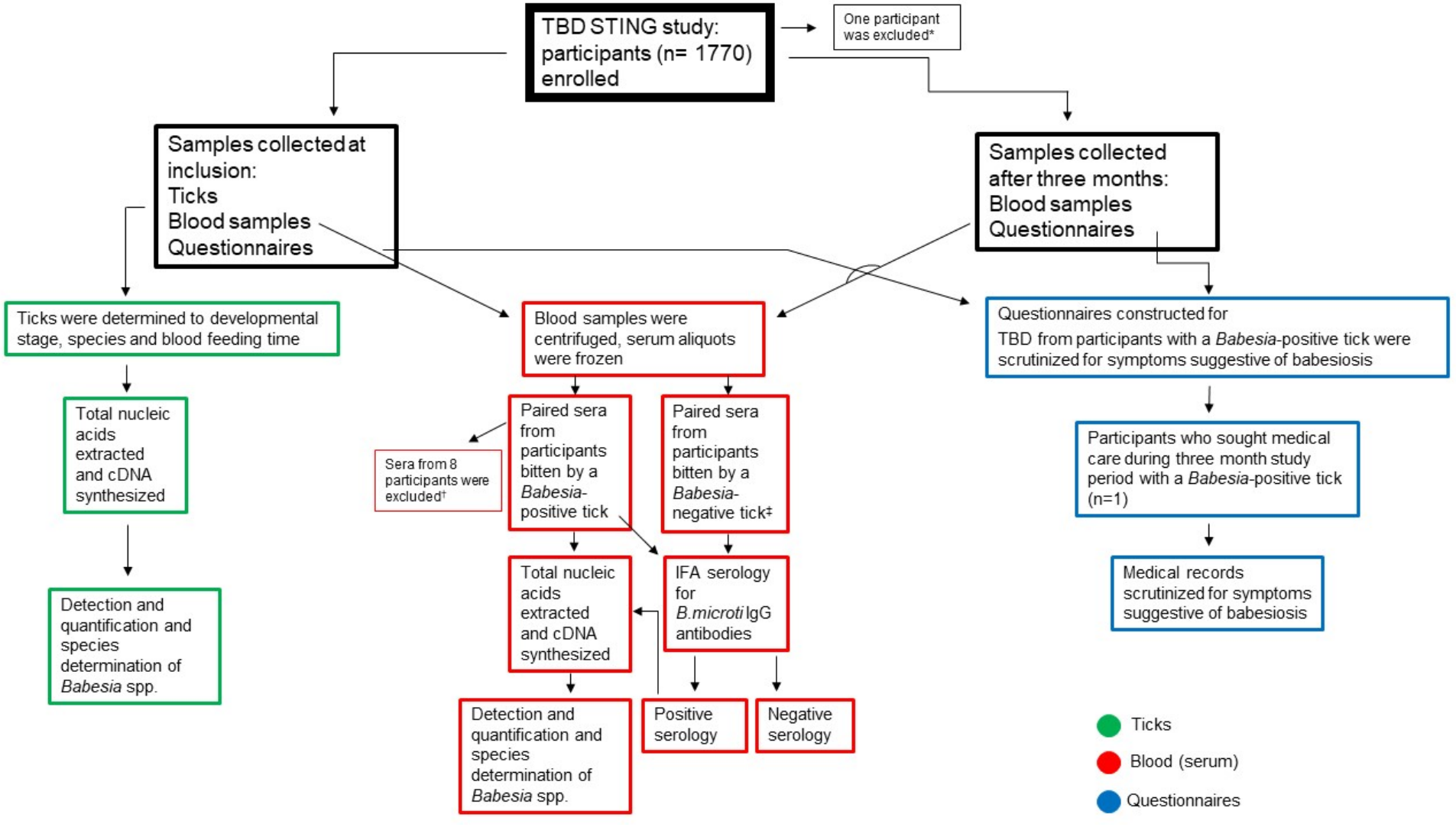
Northern Sweden
SE
FI
(27/760)
Åland Islands,
Finland
(21/792)
South Central
Sweden
(17/528)
Southernmost
Sweden


TBD STING study: participants (n= 1770) enrolled
One participant was excluded*
Samples collected at inclusion:
Ticks
Blood samples
Questionnaires
Samples collected after three months:
Blood samples
Questionnaires
Ticks were determined to developmental stage, species and blood feeding time
Total nucleic acids extracted and cDNA synthesized
Detection and quantification and species determination of Babesia spp.
Blood samples were centrifuged, serum aliquots were frozen
Paired sera from participants bitten by a Babesia-positive tick
Sera from 8 participants were excluded†
Paired sera from participants bitten by a Babesia-negative tick‡
Total nucleic acids extracted and cDNA synthesized
IFA serology for B.microti IgG antibodies
Detection and quantification and species determination of Babesia spp.
Positive serology
Negative serology
Questionnaires constructed for TBD from participants with a Babesia-positive tick were scrutinized for symptoms suggestive of babesiosis
Participants who sought medical care during three month study period with a Babesia-positive tick (n=1)
Medical records scrutinized for symptoms suggestive of babesiosis
Ticks
Blood (serum)
Questionnaires

CREDIT AUTHOR STATEMENT

Wilhelmsson, Peter:

Conceptualization
Formal Analysis
Investigation
Writing – Review & Editing
Supervision

Lövmar, Matilda:

Investigation
Data Curation
Writing – Original Draft
Writing – Review & Editing
Visualization

Krogfelt, Karen Angeliki:

Conceptualization
Project Administration
Writing – Review & Editing

Nielsen, Henrik Vedel:

Investigation
Resources
Writing – Review & Editing

Forsberg, Pia:

Conceptualization
Resources
Writing – Review & Editing
Supervision
Project Administration
Funding Acquisition

Lindgren, Per-Eric:

Conceptualization
Writing – Review & Editing
Supervision

Project Administration
Funding Acquisition