

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

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

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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



Linköping University

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.

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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 Lövmär

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 Åland Islands. Here, the text indicates that the prevalence is HIGHER in the Åland 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 Åland 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 Genbank,”. 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.

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

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4 1 **TITLE PAGE**

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6 2 **Title**

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9 3 Clinical/serological outcome in humans bitten by *Babesia* species positive *Ixodes*
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11 4 *ricinus* ticks in Sweden and on the Åland Islands

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29

30

31 **ABSTRACT**

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

47

48 Keywords

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

51 INTRODUCTION

52 Human babesiosis is caused by parasites of the genus *Babesia* and transmitted by several tick
53 species (Vannier and Krause, 2012). There are more than 100 known *Babesia* spp. that infect
54 animals but only a few are known to infect humans. The first documented case of human
55 babesiosis in Europe was reported in 1957 from Yugoslavia (Skrabalo and Deanovic, 1957).
56 Other cases followed in Western Europe, including Scandinavia (Haapasalo et al., 2010;
57 Morch et al., 2015) and a travel-associated case in Denmark (Holler et al., 2013). In Sweden
58 there have been two reported cases of human babesiosis, both in splenectomized patients
59 (Bläckberg et al., 2018; Uhnoo et al., 1992). In North America, babesiosis is considered an
60 emerging health threat that is expanding into new geographical areas and may be overlooked
61 by clinicians in regions not previously considered endemic (Gray and Herwaldt, 2019). Most
62 cases in the United States have been reported in immunocompetent patients (Vannier et al.,
63 2015). The first case in the United States was reported in 1966 (Scholtens et al., 1968).

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

178
179
180 72 Hildebrandt et al., 2007; Moniuszko-Malinowska et al., 2016). Infection with *B. venatorum*,
181
182 73 giving mild to severe symptoms in splenectomized patients, has been reported (Haselbarth et
183
184 74 al., 2007; Herwaldt et al., 2003). In the United States, *B. microti* is the most common causative
185
186 75 agent of babesiosis; it causes mild to moderate symptoms and subclinical infections in
187
188 76 immunocompetent persons (Vannier et al., 2015; Vannier and Krause, 2012). However, severe
189
190 77 babesiosis may also occur, even in previously apparently healthy individuals (Gray and
191
192 78 Herwaldt, 2019; Hatcher et al., 2001).

195
196 79 In Sweden, the prevalence of *Babesia* spp. in questing *I. ricinus* ticks was
197
198 80 recently estimated to be 4.4% and included *B. microti* (3.2%), *B. venatorum* (1.0%) and *B.*
199
200 81 *divergens* (0.2%) (Karlsson and Andersson, 2015). *B. capreoli* has been reported in roe deer,
201
202 82 but this *Babesia* sp. is not known to cause human infections (Andersson et al., 2016;
203
204 83 Malandrin et al., 2010). In Italy, the prevalence of *B. venatorum* in ticks that have bitten
205
206 84 humans was estimated to be 0.6% (Otranto et al., 2014). To our knowledge, the clinical
207
208 85 outcome and the rate of seroconversion after a bite by a *Babesia* containing tick or a tick co-
209
210 86 infected with *Borrelia*, have not been investigated.

212
213 87 Co-infection with *Babesia* and *Borrelia* spp. has been documented several times
214
215 88 (Diuk-Wasser et al., 2016; Knapp and Rice., 2015). There are differing opinions on the
216
217 89 consequences of co-infection for severity of symptoms and prognosis in humans. According
218
219 90 to Diuk-Wasser et al., (2016), the severity and duration of symptoms are greater in co-
220
221 91 infected patients and there are indications that co-infection may result in altered or
222
223 92 suppressed immune response, which in turn leads to worse pathogenesis; more research is,
224
225 93 however, needed. The aims of the present study were to investigate the prevalence of *Babesia*
226
227 94 spp. in ticks that had bitten humans and to evaluate the concomitant risk of clinical babesiosis
228
229 95 or subclinical seroconversion against *Babesia* spp.

96 MATERIAL AND METHODS

97 TBD STING-study

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

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

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

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

116 A total of 2110 ticks detached from 1770 participants were delivered from PHCs during
117 2008–2009. In this study, cDNAs from 2098 ticks detached from 1769 participants were
118 analyzed whereas cDNAs from the remaining 12 ticks were not available for analysis.

296
297
298
299 119 The 2098 cDNA samples of the individually extracted ticks were grouped into
300 120 pools of five, i.e. one μ l of cDNA from each tick was used per well. Samples from the
301 120
302 121 positive pools were individually analyzed using 2 μ l cDNA. The PCR mixture contained 10
303 121
304 122 μ l SYBR-green (Thermo Scientific, Helsingborg, Sweden), 0.4 μ l of each primer (10 μ M),
305 122
306 123 BJ1: 5'-GTC TTG TAA TTG GAA TGA TGG-3' (Invitrogen™, Thermo Scientific) and
307 123
308 124 BN2: 5'-TAG TTT ATG GTT AGG ACT ACG-3' (Casati et al., 2006), 4.2 μ l RNase free
309 124
310 125 water. Primers BJ1 and BN2 were designed to target the *Babesia 18S* rRNA gene to amplify
311 125
312 126 a 411 to 452 bp long amplicon depending on the species of *Babesia*. For species
313 126
314 127 determination, sequencing of the amplicon was carried out (see below). As a positive control,
315 127
316 128 2 μ l *B. microti* DNA (~10 ng/ μ l), extracted from *I. ricinus* ticks collected in Slovakia (kindly
317 128
318 129 provided by Dr Bronislava Vichová, through Dr Martin Andersson), and 2 μ l of a synthetic
319 129
320 130 plasmid preparation was used. The plasmid contained the target sequence of the SYBR green
321 130
322 131 real-time PCR assay, spanning the nucleotides 467-955 of the *B. divergens 18S* rRNA gene
323 131
324 132 (acc. no AJ439713), synthesized and cloned in a pUC57 vector (Genscript USA Inc, NJ). The
325 132
326 133 non-template control consisted of 10.8 μ l PCR mixture and 9.2 μ l RNase free water. The
327 133
328 134 SYBR-green real-time PCR on *Babesia* were performed using C1000™ Thermal Cycler,
329 134
330 135 CFX96™ system (BioRad Laboratories, Inc., Hercules, CA). The PCR run was initiated by a
331 135
332 136 denaturation step at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 1 min,
333 136
334 137 annealing at 55°C for 1 min and elongation at 72°C for 2 min. Extension was completed by a
335 137
336 138 further step at 72°C for 5 min, and melt curve analysis was performed (Casati et al., 2006).
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344 140 ***Babesia* spp. identification by sequencing**

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346 141 Samples positive for *Babesia* spp. in the real-time PCR assay were sent to Macrogen Inc.
347 141
348 142 (Amsterdam, the Netherlands) for nucleotide sequencing. The reactions were based on
349 142
350 143 BigDye chemistry. Chromatograms were edited using BioEdit Sequence Alignment Editor
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357 144 version 7.2.5 (Tom Hall, Ibis Therapeutics, Carlsbad, CA) and sequences examined using the
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359 145 Basic Local Alignment Tool (BLAST). Sequences obtained have been deposited in GenBank
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361
362 146 with accession numbers ranging from MH351680 to MH351744.

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364 147 Species determination for *B. microti* and *B. venatorum* is possible by sequencing the
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366 148 amplicon from the real-time PCR assay. To fully distinguish between the two genetically
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368 149 similar *Babesia* spp., *B. divergens* and *B. capreoli*, three sets of primers were used to amplify
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370 150 and sequence the complete 18S rRNA gene from all samples positive for these species, as
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372
373 151 earlier described (Malandrin et al., 2010).

374 375 376 152 **Detection of *Babesia microti* IgG antibodies in human serum**

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378 153 The first and second serum samples (collected at recruitment of participants and three months
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380 154 later, respectively) from 53 of the 61 participants bitten by *Babesia*-positive ticks were
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382 155 analyzed for the presence of *B. microti* IgG antibodies, using an indirect immunofluorescence
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384 156 assay (IFA); (Focus Diagnostics, Cypress, CA). For the remaining eight participants, serum
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386 157 was not available for analysis, since it had been used for other analyses. Twice as many
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388 158 controls were selected; first sample sera from participants bitten by *Babesia*- and *Borrelia*-
389
390 159 negative ticks were matched geographically and used as controls (n=106) for analysis
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392 160 regarding IgG antibodies against *B. microti*. For *B. venatorum* there were no commercial kits
393
394 161 available at the time of the study. IFA titers $\geq 1:64$ were defined as positive. A cut-off value
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396 162 of 1:64 was used in accordance with previous research (Johnson et al., 2009). Samples were
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398 163 diluted to determine the highest positive titer. The IFA-slides were analyzed by two
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400 164 researchers independently, samples were defined as positive when both researchers found
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402 165 them positive. For the diagnosis of on-going or recent *Babesia* spp. infection, at least a four-
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404 166 fold rise of the IFA titer (Krause, 2003; Vannier and Krause, 2012) was required when first
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406 167 and second sera were tested simultaneously.
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416 **168 Self-reported symptoms in the questionnaires of the TBD STING-study and medical**
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418 **169 records**

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421 170 The questionnaires from participants with *Babesia* PCR-positive ticks were scrutinized for
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423 171 symptoms suggestive of babesiosis, i.e. chills, fever, headache, nausea, myalgia, malaise,
424
425 172 weight loss, arthralgia and lack of appetite (Vannier and Krause, 2012). Other symptoms
426
427 173 indicating TBD in the questionnaires were neck pain, vertigo, concentration difficulties,
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429 174 numbness, radiating pain. If participants sought medical care during the three-month study
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431 175 period, the medical records were obtained and scrutinized for symptoms of babesiosis and/or
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433 176 if they were diagnosed with babesiosis or another TBD.

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436 **177 Co-infection of tick-borne pathogens in ticks**

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439 178 *Borrelia*-data from the TBD STING-study were used to determine which ticks were co-
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441 179 infected with both *Babesia* spp. and *Borrelia* spp. (Wilhelmsson et al., 2013a).

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444 **180 Statistical analysis**

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447 181 The Chi square test was applied to compare prevalence of *Babesia* spp. between regions and
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449 182 between tick developmental stages, but when the expected frequency was < 5 in at least one
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451 183 of the cells of the contingency table, we used Fisher's exact test. Statistical analyses were
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453 184 performed using GraphPad Prism version 6.04 for Windows (GraphPad Software, San Diego,
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455 185 CA). P-values <0.05 were considered significant.

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458 **186 RESULTS**

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461 **187 *Babesia* species in ticks detached from humans**

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464 188 A total of 2098 ticks that had bitten humans were analyzed (Table 1). Sixty (2.9%) of the
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466 189 ticks were damaged to the extent that neither developmental stage nor the species could be
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468 190 determined. The remaining 2038 ticks were identified as *I. ricinus*; 86 (4.1%) larvae, 1466

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475 191 (70%) nymphs, and 486 (23%) adults. 65 of 2098 ticks (3.1%) were positive for *Babesia* spp.
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477 192 by real-time PCR assay. Based on nucleotide sequencing of the PCR products, three *Babesia*
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479 193 species were detected; *B. microti* (n=33), *B. venatorum* (n=27), and *B. capreoli* (n=5). Our
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481 194 analysis of the complete 18S rDNA sequences for the five samples initially determined as
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483 195 either *B. divergens* or *B. capreoli* revealed a signature typical of *B. capreoli* at positions 631,
484
485 196 663 and 1637 (GTT), as compared with *B. capreoli* sequence (GeneBank: AY26009). In our
486
487 197 study there was no significant difference in *Babesia* prevalence between adult ticks and
488
489 198 nymphs or between nymphs and larvae. However, the prevalence of *B. microti* was
490
491 199 significantly higher in adult ticks than in nymphs ($p<0.05$).
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494 200 The 65 PCR-positive ticks were collected from 61 participants and the duration
495
496 201 of tick feeding could be estimated for 58 of the 65 ticks (Appendix). 21 of these ticks had
497
498 202 been feeding for > 36 h and the remaining 37 ticks < 36 h. There were three participants
499
500 203 bitten by more than one infected tick. One participant from the Åland Islands was bitten by
501
502 204 three infected ticks, one from Southcentral Sweden was bitten by two infected ticks, and one
503
504 205 from Southernmost Sweden was bitten by two infected ticks (Appendix). Different ticks from
505
506 206 the same participant contained different *Babesia* spp. There was no significant difference in
507
508 207 prevalence of infected ticks between the geographic regions (Figure 1B). However, there was
509
510 208 a significant difference in species composition of *Babesia* between the geographic regions,
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512 209 with *B. venatorum* more prevalent in the Åland Islands than in Southcentral Sweden
513
514 210 ($p<0.03$). No other differences in species composition between the regions were significant.
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518 211 **Co-infection with *Babesia* spp. and *Borrelia* spp. in ticks**

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520
521 212 To find which ticks contained *Borrelia* spp., data from the TBD STING-study were used
522
523 213 (Wilhelmsson et al., 2013a). Thirty out of 65 (46%) *Babesia* spp. positive ticks contained
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525 214 *Borrelia* spp. (Appendix). There was a significant difference in the frequency of co-infections
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215 involving *B. microti* and *Borrelia* spp. (60%) and the frequency of co-infections involving *B.*
216 *venatorum* and *Borrelia* spp. (30%) ($p < 0.01$).

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

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

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

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

233 **DISCUSSION**

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

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592
593 239 (Karlsson and Andersson, 2015). Comparing prevalence of *Babesia* spp. in the different
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595 240 regions, *B. venatorum* was more prevalent on the Åland Islands than in Southcentral Sweden.
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597 241 One could speculate the difference is related to different *Babesia* reservoir host composition.
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599
600 242 We found that the most prevalent species in ticks collected from
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602 243 humans was *B. microti*, followed by *B. venatorum* and *B. capreoli*. In Sweden *B. capreoli* has
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604 244 been found in 44% of roe deer (Andersson et al., 2016), but this species is not known to cause
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606 245 human infections (Malandrin et al., 2010). We did not find any samples positive for *B.*
607
608 246 *divergens*. A study conducted in Norway found that 0.1% of field-collected ticks are infected
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610 247 with *B. divergens*, 0.1% with *B. capreoli* and 0.6% with *B. venatorum* (Øines et al., 2012). In
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612 248 Denmark, *B. divergens* was found in 1.9% of ticks and *B. venatorum* in 0.6% of ticks (Jensen
613
614 249 et al., 2017). Karlsson and Andersson (2015) found 0.2% questing ticks containing *B.*
615
616 250 *divergens* in Sweden and found no significant difference in prevalence of *Babesia* spp. in
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618 251 adult ticks compared to nymphs. They found a higher prevalence in nymphs than in adults
619
620 252 and they found no positive larvae among questing ticks, but we analyzed four times as many
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622 253 ticks and they were collected from humans. We found only one positive larva (Table 1) that
623
624 254 contained *B. capreoli*. *B. capreoli* has not previously been found in larvae, however, *B.*
625
626 255 *divergens* which is genetically similar to *B. capreoli* is known to be transovarially transmitted
627
628 256 (Bonnet et al., 2007).

631
632 257 Nearly one-half of the ticks positive for *Babesia* spp. were positive for *Borrelia*
633
634 258 spp. Sixty percent of ticks containing *B. microti* were co-infected with *Borrelia* spp.
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636 259 compared to 30% of ticks containing *B. venatorum*. This may reflect the genus ratio of
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638 260 *Babesia* spp. and *Borrelia* spp. in reservoir hosts. It has been observed in an experimental
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640 261 model that mice co-infected with a strain of *B. microti* and an invasive strain of *B.*
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642 262 *burgdorferi* s.s. increased *B. microti* frequency in *I. scapularis* that fed from them compared
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652 263 to mice infected with *B. microti* alone (Dunn et al., 2014). It remains to be confirmed if this
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654 264 finding applies to *I. ricinus* and one or several *Babesia* spp. it can transmit.
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657 265 Seven participants were positive for *B. microti* antibodies. Since
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659 266 none of them seroconverted and the antibody titers were low, we did not suspect an ongoing
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661 267 infection (Vannier and Krause, 2012). It is probable that they had a previous or subclinical
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663 268 infection and still carried antibodies. In a previous study, Lempereur et al. (2015) found no
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665 269 antibody cross-reactivity between *B. microti* and *B. venatorum* used as IFA antigens, but IgM
666
667 270 cross reactivity between *B. microti* and *B. divergens* has been observed in another study
668
669 271 (Haselbarth et al., 2007). Bläckberg et al. (2018) reported that a patient with *B. venatorum*-
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671 272 infection had *B. divergens* IgG antibodies. For the participants with a positive *B. microti*
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673 273 serology, only three had been bitten by *Babesia* PCR-positive ticks during the study period.
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675 274 Because they had the same antibody titers in the first serum sample as in the second, it is not
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677 275 likely that the tick-bite during the study period was the cause of the positive serology or that
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679 276 the participants had an ongoing infection. One study in southern Sweden has revealed a
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681 277 prevalence of 16.3% for *B. microti* and *B. divergens* antibodies in a geographically selected
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683 278 cohort of seropositive *Borrelia* s.l. patients; and a 2.5% prevalence in a healthy control group
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685 279 (Svensson et al., 2019). However, comparing this study to ours is complicated, since we have
686
687 280 used another serological assay for *Babesia* antibodies and the study populations differ from
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689 281 each other.
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694 282 In total, three participants were bitten by more than one *Babesia* PCR-positive
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696 283 tick, none of them developed antibodies against *B. microti* during the study period. One of
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698 284 these participants was bitten by ticks containing *B. capreoli*, not confirmed to be human
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700 285 pathogenic. Since we only analyzed for *B. microti* IgG we cannot draw any general
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702 286 conclusions regarding *Babesia* spp. antibodies. However, it is an interesting future
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704 287 prospective. None of the participants with more than one positive tick reported symptoms in
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711 288 their questionnaires or sought medical care during the study period. This suggests a low risk
712
713 289 of transmission despite being bitten by several positive ticks and different species.
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715 290 Furthermore, the efficacy of transmission has been shown to correlate with the duration of
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717 291 tick feeding (for *I. scapularis*) in hamsters and white-footed mice, with infection rates close
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719 292 to 100% if the tick is allowed to feed to repletion (Piesman and Spielman, 1980). However, it
720
721 293 is not known if this is true for *I. ricinus* and/or human hosts. In our study ticks were removed
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723 294 by the participant before repletion.
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726 295 Ten participants with *Babesia* PCR-positive ticks reported symptoms in their
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728 296 questionnaires. These symptoms were nonspecific and might indicate different conditions,
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730 297 babesiosis included. Only one participant bitten by a *Babesia* PCR-positive tick sought
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732 298 medical care during the study period. According to medical records, symptoms were
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734 299 unrelated to the tick bite. Thus, we conclude that none of the participants suffered from
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736 300 symptoms of babesiosis.
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739 301 Twenty-nine of 61 participants with *Babesia* PCR-positive ticks were bitten by
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741 302 ticks co-infected with *Borrelia*. Of the ten participants who reported symptoms, five had been
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743 303 bitten by ticks positive for both *Babesia* spp. and *Borrelia* spp. The symptoms reported could
744
745 304 be attributed either to babesiosis, borreliosis or other infections. None of the participants who
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747 305 reported symptoms, with the exception of the participant mentioned above, had sought
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749 306 medical care. This suggests that no participant suffered from severe illness.
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752 307 One of the potential limitations of this study is that we did not test the serum for
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754 308 *B. venatorum* antibodies since we did not have available commercial kits for these analyses.
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756 309 The conclusions that can be drawn from the serological analyses are further limited by the
757
758 310 lack of information regarding travel history, since this was not included in the questionnaires,
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760 311 designed for the TBD STING-study. Unfortunately, whole blood samples from the TBD
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762 312 STING-study were not available for real-time PCR analysis.
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313 Conclusions

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

323

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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 <i>Babesia</i> PCR-positive ticks (%)	No. of ticks PCR-positive for <i>B. microti</i>	No. of ticks PCR-positive for <i>B. venatorum</i>	No. of ticks PCR-positive for <i>B. capreoli</i>
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 1 st sample [‡]	Antibody titers in 2 nd sample [‡]	<i>Babesia</i> spp. in the tick [§]	Tick feeding duration (h)	Developmental stage of the tick [#]
Afa 89	1:128	1:128	<i>B. venatorum</i>	25	N
Vofa 15	1:256	1:256	<i>B. microti</i>	35	A
Vifa 25	1:64	1:64	<i>B. microti</i>	<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

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

1181							
1182							
1183	Southcentral Sweden	Bafa 6	A	<24	<i>B. microti</i>	<i>B. afzelii</i>	3.4 x 10 ²
1184							
1185		Bafa 73A	A	<24	<i>B. microti</i>		
1186		Bafa 86	A	<24	<i>B. capreoli</i>	<i>B. burgdorferi</i> sensu stricto	3.1 x 10 ⁴
1187							
1188		Ekfa 84	A	-	<i>B. microti</i>	<i>B. afzelii</i>	2.7 x 10 ⁴
1189		Grfa 28	N	56	<i>B. microti</i>	<i>B. garinii</i>	1.3 x 10 ³
1190		Hafa 8	N	<24	<i>B. venatorum</i>		
1191		Hafa 16	N	25	<i>B. microti</i>	<i>B. afzelii</i>	3.1 x 10 ⁴
1192		Hafa 44B	A	<24	<i>B. venatorum</i>	<i>B. miyamotoi</i>	1.9 x 10 ⁶
1193		Hafa 115	A	61	<i>B. venatorum</i>	<i>B. afzelii</i>	1.7 x 10 ¹
1194		Jofa 7	N	47	<i>B. microti</i>	<i>B. afzelii</i>	6.3 x 10 ²
1195		Lidfa 14	N	55	<i>B. venatorum</i>	<i>B. garinii</i>	1.4x 10 ³
1196		Lidfa 30	A	<24	<i>B. microti</i>	ND	1.1 x 10 ²
1197		Lidfa 39A	L	-	<i>B. capreoli</i>		
1198		Lidfa 39B	N	52	<i>B. capreoli</i>		
1199		Lidfa 46	N	26	<i>B. venatorum</i>		
1200		Lidfa 92B	N	<24	<i>B. microti</i>	<i>B. afzelii</i>	3.9 x 10 ⁴
1201		Mekfa 16	A	-	<i>B. venatorum</i>		
1202		Sofa 76	A	166	<i>B. microti</i>	ND	1.1 x 10 ²
1203		Vvfa 66	A	-	<i>B. microti</i>		
1204		Vifa 20	N	70	<i>B. microti</i>		
1205		Vifa 25	A	<24	<i>B. microti</i>	<i>B. afzelii</i>	1.9 x 10 ⁵
1206	Southernmost Sweden	Blefa 13	N	<24	<i>B. microti</i>	<i>B. afzelii</i>	7.9 x 10 ³
1207		Blefa 19	N	37	<i>B. microti</i>		
1208		Blefa 38B	N	<24	<i>B. microti</i>	<i>B. afzelii</i>	8.8 x 10 ³
1209		Blefa 38D	N	<24	<i>B. venatorum</i>	<i>B. afzelii</i>	9.5 x 10 ²
1210		Kafa 7	N	57	<i>B. venatorum</i>		
1211		Kafa 34	N	59	<i>B. venatorum</i>		
1212		Kafa 36	N	<24	<i>B. microti</i>	<i>B. afzelii</i>	3.5 x 10 ³
1213		Kafa 75	N	49	<i>B. microti</i>		
1214		Kafa 84A	N	60	<i>B. microti</i>	<i>B. afzelii</i>	7.0 x 10 ¹
1215		Kafa 100A	N	25	<i>B. microti</i>	<i>B. afzelii</i>	1.8 x 10 ⁴
1216		Kfa 9	N	<24	<i>B. venatorum</i>		
1217		Kfa 18	N	-	<i>B. venatorum</i>		
1218		Kfa 25	N	37	<i>B. capreoli</i>		
1219		Lafa 13	A	51	<i>B. venatorum</i>		
1220		Ofa 15C	N	35	<i>B. microti</i>	<i>B. afzelii</i>	1.4 x 10 ⁴
1221		Vofa 15	A	35	<i>B. microti</i>		
1222		Ysfa 9	A	38	<i>B. microti</i>	<i>B. afzelii</i>	1.5 x 10 ¹

545 *Letter after Id. Code for participants who turned in more than one tick to the PHC, the first tick XXfaA, the second XXfaB etc.

546 †Feeding time could not be determined

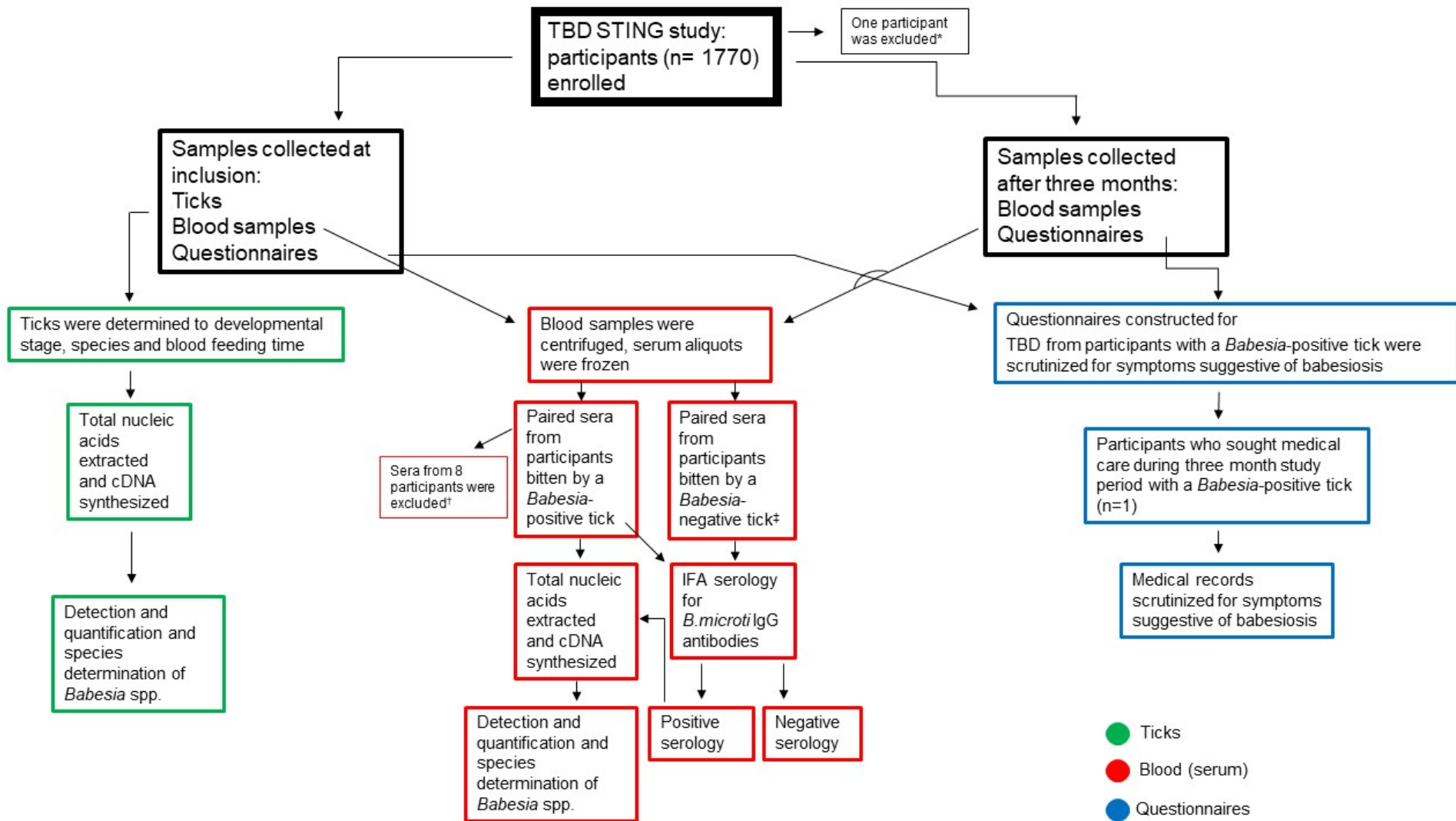
547 *ND = Species could not be determined

548 §A = Adult female, N = nymph

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