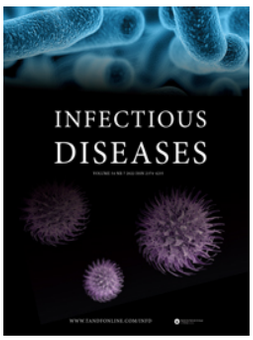

Infectious Diseases

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/infd20

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To cite this article: Lisa Labbé Sandelin, Jenny Olofsson, Conny Tolf, Louise Rohlén, Lars Brudin, Ivar Tjernberg, Per-Eric Lindgren, Björn Olsen & Jonas Waldenström (2022): Detection of *Neoehrlichia mikurensis* DNA in blood donors in southeastern Sweden, Infectious Diseases, DOI: 10.1080/23744235.2022.2087732

To link to this article: https://doi.org/10.1080/23744235.2022.2087732

Taylor & Francis
Taylor & Francis Group

INFECTIOUS DISEASES,
2022; VOL. 0,
NO. 0, 1–12

https://doi.org/10.1080/23744235.2022.2087732

ORIGINAL ARTICLE

Detection of *Neoehrlichia mikurensis* DNA in blood donors in southeastern Sweden

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ABSTRACT

Background: The tick-borne bacterium *Neoehrlichia mikurensis* can cause persistent asymptomatic bloodstream infections, but transfusion-mediated transmission has not been reported. This study aimed to investigate the prevalence of *N. mikurensis* in blood donors, and recipients of blood components from *N. mikurensis*-positive donors were traced.

Methods: In 2019 and 2021, 1007 blood donors were recruited. Participants completed a questionnaire and additional blood samples were collected during blood donation. Detection of *N. mikurensis* was performed by PCR followed by sequencing. Positive donors were interviewed and retested. Look-back was performed on positive donations and on all subsequent donations.

Results: *N. mikurensis* was detected in 7/1006 (0.7%) donors. A total of 380/1005 (38%) donors reported at least one noticed tick bite during the current season. The questionnaire could not detect any differences between negative and positive *N. mikurensis*-donors. Two of the positive donors were still positive on days 318 and 131 after the index donation, respectively. One donor with persistent *N. mikurensis* in blood experienced slight fatigue. All other had no symptoms attributable to neoehrlichiosis. Look-back included ten donations and 20 blood components. Eight components were discarded, and 12 recipients of *N. mikurensis*-positive donations were identified. PCR was negative in seven recipients. Five recipients had died, but their medical records gave no evidence for neoehrlichiosis.

Conclusions: Although *N. mikurensis* was found in 0.7% of blood donors, transfusion-mediated infection was not detected, despite several recipients being at high risk for severe neoehrlichiosis. The results warrant further studies as well as raised clinical awareness.

KEYWORDS
Neoehrlichia
tick-borne infections
transfusion-transmitted infections
blood donors
immunosuppression
emerging infectious diseases
blood safety

ARTICLE HISTORY
Received 24 February 2022
Revised 31 May 2022
Accepted 6 June 2022

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Supplemental data for this article is available online at https://doi.org/10.1080/23744235.2022.2087732.

Introduction

Blood-transfusion is an important treatment in many medical conditions but involves a risk of transmitting infections from donor to recipient [1,2]. Transfusion-transmitted infections (TTI) involve several pathogens, including bacteria, viruses, protozoa, and prions [2]. HIV and hepatitis B and C are well-known TTIs. The prerequisites for a TTI are that the pathogen is blood-borne, survives processing and storage of the blood product, remains infectious by the intravenous route, and causes disease in at least some of the transfusion recipients [1,3,4]. In addition, there must be an asymptomatic, infectious phase in the blood donor, since donors with infectious symptoms are excluded from donation [1,5].

Several emerging infectious agents that pose a real or theoretical threat to transfusion safety have been identified [4,6], and identification of new blood-borne pathogens unconditionally leads to questions about their potential to threaten the blood supply [7]. Transfusion-mediated transmission of infectious agents is of special concern in patients immunosuppressed by disease or treatment [8]. Due to combinations of climatic and environmental changes, socioeconomic factors and human behaviour, an increase in tick-borne infections is predicted in Europe [9,10]. The tick-borne bacterium *Candidatus Neoehrlichia mikurensis* was first described to cause disease in humans in 2010 [11]. Initially, the prefix *Candidatus* was used to denote that the bacterium had not yet been cultivated. Successful cultivation was achieved in 2019 and the bacterium is now referred to as *Neoehrlichia mikurensis* [12]. Since 2010, this pathogen has been shown to cause persistent asymptomatic infection in immunocompetent [13,14] as well as immunosuppressed [15] individuals, and it is assumed that the bacterium infects vascular endothelial cells [16]. Since the first case of neoehrlichiosis was identified, 95 cases have been diagnosed in Sweden (oral communication A. Grankvist 2022-06-07). Several patients had long-lasting symptoms and delayed diagnosis, mainly due to low clinical awareness [17–19]. There is concern about the ability of *N. mikurensis* to cause persistent asymptomatic bloodstream infections which can lead to transfusion-mediated transmission of neoehrlichiosis [14,15]. To our knowledge, transmission by blood transfusion has not yet been reported for *N. mikurensis*, but it has been suspected at least once [17]. The ability of *N. mikurensis* to survive and remain viable under blood component storage conditions is unknown.

Although vector-borne infectious agents can be found in blood, they are generally not transmitted directly by blood contact, but by a vector, such as a tick or a mosquito [20]. As a result, vector-borne infections vary geographically depending on vector species distribution, competency, and available reservoirs [8,21]. Several tick-borne pathogens can potentially be transmitted through blood transfusion. Furthermore, many tick-borne microorganisms are located intracellularly, which is an excellent condition for transmission by transfusion [21]. Different tick-borne infections have different cell tropisms that affect prevalence and density in human blood, and thus the probability of transfusion-mediated transmission [22]. In the Northern Hemisphere, a limited number of tick-borne infections have been identified as TTIs [21,22]. The intraerythrocytic protozoan *Babesia* spp. is of greatest concern to recipient safety [1]. Of transfusion-transmitted tick-borne rickettsiae, *Anaplasma phagocytophilum,* which infects granulocytes and causes anaplasmosis, is most frequently reported [8,23].

Neoehrlichia mikurensis is widespread in the southern parts of Sweden with a prevalence of 6.0% in questing ticks [24], 2.1% in ticks collected from migrating birds [25] and between 8.8–22% in rodents [26,27]. In immunocompetent tick-bitten individuals from Sweden and the Åland Islands, Finland, the prevalence of *N. mikurensis* was 2.0% [13]. In 224 Swedish patients with persistent symptoms attributed to presumed tick bite exposure, the prevalence of *N. mikurensis* was 1.3% [28]. In a retrospective study of patients investigated for Lyme neuroborreliosis in southeastern Sweden during 2009–2013, only one of 600 (0.17%) study participants had detectable *N. mikurensis* DNA in blood. The seroprevalence of *N. mikurensis* in Sweden is unknown since no serological test is available. Albeit a rare disease in Sweden, neoehrlichiosis is of clinical importance since it can cause severe symptoms in immunocompromised individuals and can mimic non-infectious conditions in patients with B cell malignancies or autoimmune diseases [17]. In addition, medium and large vessel vasculitis has recently been reported in two immunocompetent individuals [16].

In this study, we wanted to investigate *N. mikurensis* as a putative emerging pathogen of concern for blood safety. There are several reasons to why this is relevant; (1) the prevalence of *N. mikurensis* in ticks biting humans is high in relation to the limited number of case reports, (2) the knowledge of the prevalence in immunocompetent individuals is sparse, (3) *N. mikurensis* has an ability to cause persistent asymptomatic bloodstream

infections in immunocompetent as well as immunosuppressed individuals, and (4) neoehrlichiosis can be severe in immunocompromised patients. In order to better assess the risk of transfusion-mediated transmission, the prevalence of *N. mikurensis* in blood donors in southeastern Sweden was studied, and recipients transfused with blood components from *N. mikurensis*-positive donors were traced and tested.

Materials and methods

Ethics statement

The study was approved by the Regional Ethics Review Board in Linköping (reg. no. 2017/227-31 and 2019-06034). Written informed consent was obtained from all blood donors. The ethical permit also included look-back and tracing of recipients.

Blood donors

A total of 1007 blood donors were recruited from blood banks at the three hospitals in Kalmar County (Kalmar, Oskarshamn and Västervik). Only donors who were eligible for donation (judged by a blood donor history questionnaire and an interview) were included. On a yearly basis, the number of registered blood donors in the county varies between 7200–8200, and the number of yearly donations is around 9000.

Recruitment of participants took place during 26 June–12 August 2019, and 8 February–18 November 2021. The study design allowed individuals to be sampled only once (Figure 1). Participants were selected to achieve an even gender and geographical distribution. At the time of sampling, participants gave oral and written informed consent to participate in the study and completed a questionnaire about tick exposure, tick

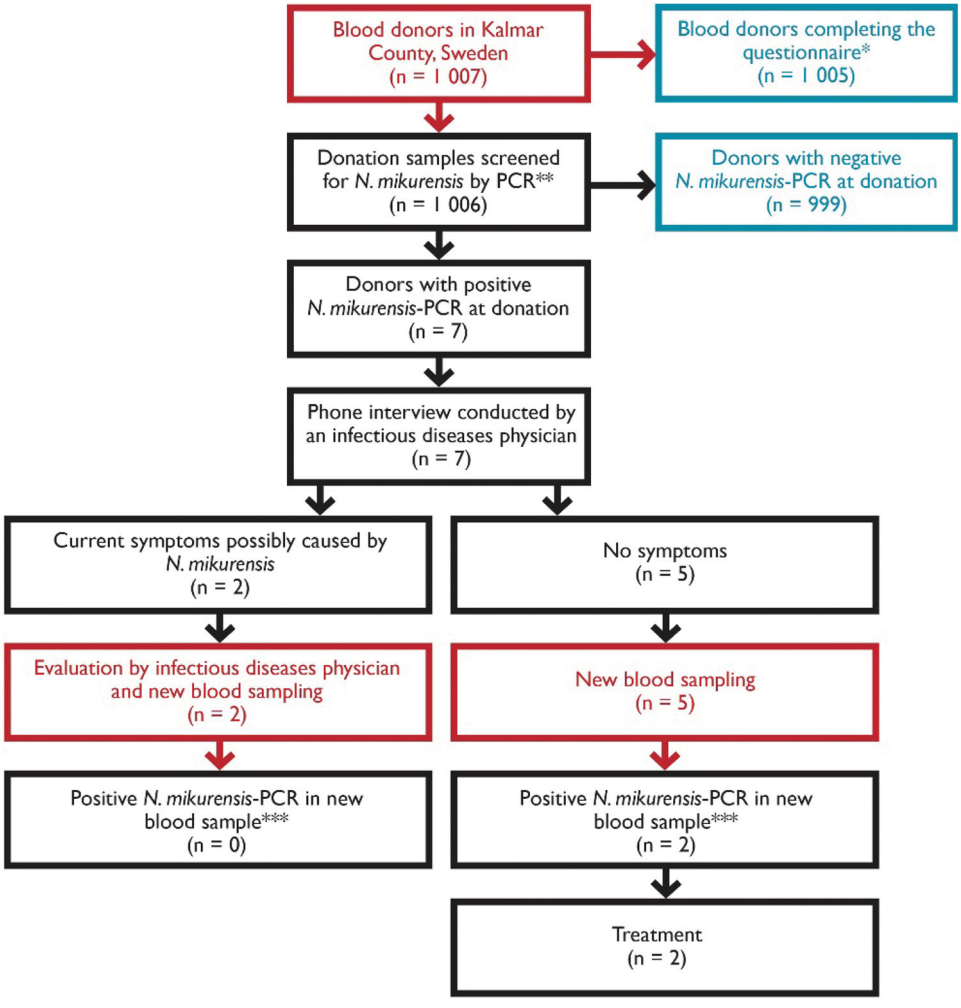

Figure 1. Flow-chart showing the study design and *N. mikurensis*-positive blood donors. *Two participants did not complete the questionnaire. **One sample was lost during transport. ***Two of the seven *N. mikurensis*-positive donors were still positive at follow-up, both without current symptoms.

bites, history of previous tick-borne infections, current medication, household pets and whether they had been splenectomised or received blood transfusions. After completion of the questionnaire, each blood donor performed a routine blood donation (450 mL) including the required standard infectious screening and haemoglobin sampling. In addition, 7.5 mL EDTA whole blood was collected from each blood donor for study purposes. The additional blood was stored in −80 °C until analysed. PCR analyses were performed several months after sampling.

Molecular detection and sequencing of N. mikurensis

Extraction

Total DNA was extracted from 100 µL whole blood with the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, except for using 100 µL Buffer AE instead of 200 µL for the final elution step.

PCR analyses

Detection of *N. mikurensis* was performed using an SYBR green real-time PCR assay, as previously described [24]. The primers NeogroELQ_F and NeogroELQ_r3 are designed to amplify a 129 bp long fragment of the *N. mikurensis groEL* gene (Table 1). For analyses, 20 µL reactions consisting of 10 µL 2x iQ™ SYBR® Green Supermix (Bio-Rad), 400 nM of forward and reverse primer, 6.4 µL water and 2 µL DNA template were prepared. Analyses were performed using a LightCycler® 480 II instrument (Roche Diagnostics). Thermal conditions include initial denaturation at 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and a final melting curve analysis where fluorescence was continuously measured while raising the temperature from 60 to 95 °C. In order to verify validity of the analysis, two negative and one positive control were included in each assay.

Real-time PCR positive samples were further analysed with a nested PCR [25]. A 1259 bp of the *N. mikurensis 16S* rRNA gene was sequenced, employing a nested PCR method including specific primers Neo_16S_95_F and Neo_16S_1393_R for PCR 1 and Neo_16S_127_F and Neo_16S_1363_R for PCR 2 (Table 1). Amplifications were performed using a Phusion Mastermix containing a Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer's protocol. Thermal conditions included incubation at 98 °C for 30s, followed by 35 cycles at 98 °C for 10s, 66 °C (PCR1) or 63 °C (PCR2) for 30s and 72 °C for 45s, and a final 5 min elongation at 72 °C. Five µL DNA extracted from blood samples were used as a template in the first PCR and 0.5 µL of the PCR product from PCR 1 was used as template in the second PCR. The amplified product was purified and sequenced by the Sanger method (Macrogen Europe, The Netherlands).

Table 1. Primers used for real-time PCR screening and sequencing.

Primer name	Target gene	Sequence	Ref.
NeogroELQ_f	*groEL*	5′-ACAGCCAATACTACCTATCCTTGA-3′	[24]
NeogroELQ_3r		5′-ACATGYAATCCACCACGYAACT-3′	
Neo_16S_95_F	*16S* rRNA	5′-TTAGTGGCAGACGGGTGAGTAATG-3′	[25]
Neo_16S_127_F		5′-TCTGCCTAGTAGTATGGAATAGCTG-3′	
Neo_16S_1363_R		5′-AAACCAATTTCCAGGGCATGACGG-3′	
Neo_16S_1393_R		5′-TCCTTACGGTTAGCTCACCAGCTT-3′	

Follow-up and treatment of positive donors

Blood donors with *N. mikurensis* DNA in blood were followed up by a phone interview conducted by an infectious diseases specialist (LLS) and were offered further blood sampling to rule out persistent infection (Figure 1). Blood analyses included haematological and biochemical analyses and a new real-time PCR for *N. mikurensis*. The latter was performed as part of routine diagnostics at Sahlgrenska University Hospital Laboratory, Gothenburg, Sweden [13]. Donors with symptoms possibly caused by neoehrlichiosis were offered a consultation with an infectious diseases physician for clinical and laboratory evaluation. Donors with the persistence of *N. mikurensis* DNA in blood at follow-up were treated with doxycycline 100 mg bid for 3 weeks, and treatment was followed by a new blood sample with subsequent PCR analysis. Donors with detectable *N. mikurensis* DNA in blood were deferred from donating blood until PCR analysis was negative at follow-up, either by spontaneous clearance or by treatment.

Look-back investigation and recipient testing

Upon recognition that there may have been a risk of transmitting an infectious agent from a donor to a recipient, a look-back investigation is initiated [29]. The blood donation in which *N. mikurensis* DNA was detected was considered the index donation and a look-back investigation was performed on the index donation and on all consecutive donations following the index donation date. Cellular components were traced and, if possible, retrieved. Health care facilities/physician in charge were notified that patients might have received an infectious blood product and testing of the recipient was recommended. Recipient testing with *N. mikurensis*-

PCR was conducted at Sahlgrenska University Laboratory [13].

Statistical analysis

Differences between donors with and without *N. mikurensis* DNA in blood regarding results from the questionnaire were analysed using Mann–Whitney U-test for age and Fisher's exact test for categorical variables (Table 2). Differences between inclusion years regarding number of tick-bites and spending time outdoors were analysed using Chi-square test. A p-value $<.05$ was considered to indicate statistical significance.

Table 2. Questionnaire-reported characteristics of *N. mikurensis*-positive and *N. mikurensis*-negative blood donors.

	N. mikurensis (PCR)			
	Positive	Negative	*p*-Values	Total
Variables				
N	7	998		1005
Age (yrs)				
Mean (SD)	53.7 (7.6)	45.8 (11.9)		45.9 (11.9)
Median (range)	50 (45–67)	47 (18–69)	.100	47 (18–69)
Age category (yrs)				
≤47	1 (14.3)	516 (51.7)		517 (51.4)
>47	6 (85.7)	482 (48.3)	.063	488 (48.6)
Sex n (%)				
Females	3 (42.9)	484 (48.5)		487 (48.5)
Males	4 (57.1)	514 (51.5)	>.90	518 (51.5)
Blood bank				
Kalmar	1 (14.3)	334 (33.5)		335 (33.3)
Oskarshamn	1 (14.3)	319 (32.0)		320 (31.8)
Västervik	5 (71.4)	345 (34.6)	.054a	350 (34.8)
Inclusion year				
2019	2 (28.6)	398 (39.9)		400 (39.8)
2021	5 (71.4)	600 (60.1)	.709	605 (60.2)
Spending time outdoors				
No	0 (0.0)	152 (15.2)		152 (15.1)
Yes	7 (100.0)	845 (84.7)	.603	852 (84.8)
Tick bites ever				
No	0 (0.0)	96 (9.6)		96 (9.6)
Yes	7 (100.0)	901 (90.3)	.710	908 (90.3)
Tick bites this season				
0	4 (57.1)	595 (59.6)		599 (59.6)
1–4	3 (42.9)	337 (33.8)		340 (33.8)
5–9	0 (0.0)	40 (4.0)	>.90b	40 (4.0)
Last tick bite outside of county				
No	5 (71.4)	732 (73.3)		737 (73.3)
Yes	1 (14.3)	53 (5.3)	.347	54 (5.4)
Previous TBD				
No	6 (85.7)	826 (82.8)		832 (82.8)
Yes	1 (14.3)	166 (16.6)	>.90	167 (16.6)
Pets				
No	2 (28.6)	473 (47.4)		475 (47.3)
Yes	5 (71.4)	524 (52.5)	.456	529 (52.6)
Medication				
No	4 (57.1)	723 (72.4)		727 (72.3)
Yes	3 (42.9)	274 (27.5)	.402	277 (27.6)
Splenectomised				
No	7 (100.0)	996 (99.8)		1003 (99.8)
Yes	0 (0.0)	2 (0.2)	>.90	2 (0.2)
Blood transfusion				
No	7 (100.0)	937 (93.9)		944 (93.9)
Yes	0 (0.0)	43 (4.3)	>.90	43 (4.3)
Tick season (Apr–Oct)				
No	0 (0.0)	184 (18.4)		184 (18.3)
Yes	7 (100.0)	814 (81.6)	.361	821 (81.7)

Differences analysed using Mann–Whitney U-test for age and Fisher's exact test for categorical variables (p-values). Notes: (a) Västervik plus Oskarshamn vs Kalmar, (b) Never vs more than once.

Results

Study population

A total of 1007 blood donors were included in the study. Two did not complete the questionnaire, resulting in 1005 questionnaires available for analyses (Figure 1). One blood sample was lost during transport and 1006 blood samples were available for further analyses. Participant details are shown in Table 2. A total of 487 (49%) women and 518 (52%) men were included. The mean age was 46 years and the median age 47 years (range 18–69). A total of 277 donors (28%) were on medication, primarily for hypertension, asthma, allergies, menopausal discomfort, or oral contraceptives. In accordance with the eligibility criteria for blood donation, no participant received immunosuppressive treatment. Two participants reported to be splenectomised, but none of them provided information on when or why this was performed. Forty-three (4.3%) blood donors had previously received a blood transfusion.

Tick exposure and tick bites

A total of 852 (85%) donors stated that they often spent time outdoors (Table 2). Ninety-six (10%) donors had never been bitten by a tick. No tick bites during the current season were reported by 599/1005 (60%) while 340/1005 (34%) reported 1–4 tick bites and 40/1005 (4.0%) reported 5–9 tick bites. Information on the geographical location of the last tick bite was included in 791 questionnaires and 737/791 (93%) were in Kalmar County.

Previous tick-borne disease (TBD) was reported by 167/1005 (17%) participants, and Lyme borreliosis was by far the most frequently reported TBD. One participant reported tick-borne encephalitis (TBE), one reported suspected TBE, and one was previously diagnosed with ehrlichiosis. A total of 529 (53%) participants had pets, most commonly cats or dogs (Table 2). When comparing the pre-pandemic year 2019 with the COVID-19 pandemic year 2021, there were no significant differences in spending time outdoors (Chi-2 = 0.19; df = 1; $p = .67$) or tick bites (Chi-2 = 1.56; df = 1; $p = .21$).

Table 3. Clinical data of *N. mikurensis*-positive blood donors.

Positive donor	I	II	III	IV	V	VI	VII
Age and sex	50 M	50 F	61 F	68 M	46 M	53 M	50 M
Blood bank	Västervik	Västervik	Kalmar	Oskarshamn	Västervik	Västervik	Västervik
IS or splenectomy Y/N	N	N	N	N	N	N	N
Spending time outdoors	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Tick bites* ever	Yes (seldom bitten)	Yes	Yes (seldom bitten)	Yes (seldom bitten)	Yes (seldom bitten)	Yes	Yes (seldom bitten)
Tick bites* this season	1–4 times	1–4 times	0 times	0 times	0 times	1–4 times	0 times
Last known tick bite*	June 2019	June 2019	2020	2020	2019	6 weeks prior	Many years ago
Pets	Cat	No	Two outdoor cats	Dogs	Dog	Dog	No
Index donation date	22 July 2019	30 July 2019	13 July 2021	2 June 2021	10 May 2021	29 July 2021	18 August 2021
Follow-up (date)	4 June 2020	30 April 2020	1 November 2021	3 November 2021	6 December 2021	6 December 2021	27 December 2021
Time to follow-up (days)	318	275	111	154	210	130	131
PCR at follow-up	Pos	Neg	Neg	Neg	Neg	Neg	Pos
Symptoms	Slight fatigue	None	Headache	Pericarditis Oct 2021	None	None	None
Doxycycline before PCR	No	No	No	No	No	No	No
Antibiotic treatment	DX 100 mg bid 3w	No	No	No	No	No	DX 100 mg bid 3w
PCR after treatment	Neg (5 Oct 2020)						N/A
Current medication	Atorvastatin		Antihypertensive			Zolmitriptan	
Other comments	Donated blood on two additional occasions	Donated blood on one additional occasion	LP: normal cell count. *Borrelia*-serology neg. in plasma + CSF		All components form the index donation were discarded	Forestry worker	

Information gathered from questionnaire and oral interview. *: 'tick bites' refers to recalled tick bites; IS: immunosuppression; DX: doxycycline; 3w: three weeks; LP: lumbar puncture; CSF: cerebrospinal fluid; N/A: not available.

PCR-positive donors

The molecular screening detected *N. mikurensis* DNA in 7/1006 blood samples, corresponding to a prevalence of 0.7% (95% CI 0.3 − 1.4) (Figure 1; Table 3). Ct-values had a mean of 34, ranging from 33 to 35. All seven sequences were identical to each other as well as to three human isolates from Sweden [30] and to sequences recovered from ticks collected from migrating birds at Ottenby Bird Observatory [25] which is situated on the island of Öland in Kalmar County. Positive donations occurred from May to August (Table 3). None of the *N. mikurensis*-positive donors was immunocompromised, splenectomised or had received blood transfusions. Five of the positive donors were recruited at the blood bank in Västervik, and one each in Kalmar and Oskarshamn (Figure 3).

All *N. mikurensis*-positive donors were followed up with new blood samples (Figure 1; Table 3). The mean time between the index donation and retesting at the first follow-up was 190 days (range 111–318). Two of the seven (28%) positive donors (donors I and VII; Table 3) were still positive at follow-up 318 and 131 days after the index donation, respectively. Ct-values at follow-up were 33 in donor I and 38 in donor VII. None of the seven positive donors had received antibiotic treatment between the index donation and the retesting. Donor I had experienced slight fatigue during the months preceding follow-up (Table 3). Donor III suffered from headache, which started around the time of the index donation and was still present at follow-up. At follow-up, her blood PCR was negative for *N. mikurensis,* lumbar puncture showed a normal cell count, and serology for Lyme borreliosis was negative in both plasma and cerebrospinal fluid. Further investigations after follow-up revealed a tooth infection, which may have been the cause of her headache. Donor IV was diagnosed with non-specified pericarditis between the index donation and follow-up. No microbiological analyses were performed, and no antibiotic treatment was initiated. Donors II, V, VI and VII did not report any symptoms compatible with neoehrlichiosis around and after the time of the index donation, despite detectable *N. mikurensis* DNA in blood at follow-up of donor VII.

All seven *N. mikurensis*-positive donors spent time outdoors and five had pets (Table 3). All positive donors had previously been bitten by ticks, but 5/7 reported that tick bites seldom occurred. Donor VI was a forestry worker, thus with a high level of occupational tick exposure. Four positive donors had not noticed any tick

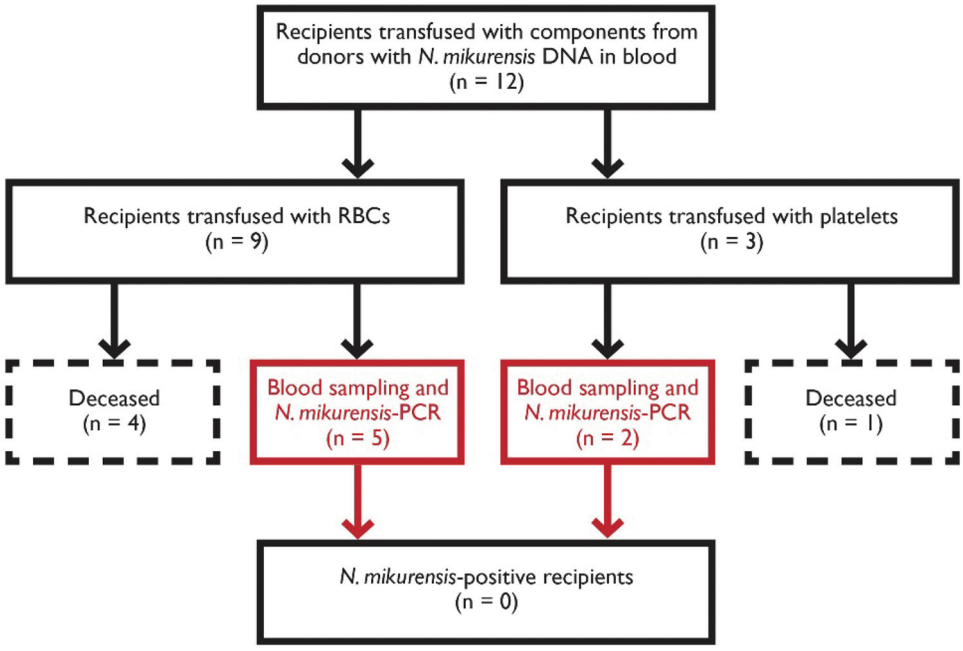

Figure 2. Flow-chart showing recipients transfused with components from *N. mikurensis*-positive donors. RBC: Red blood cells.

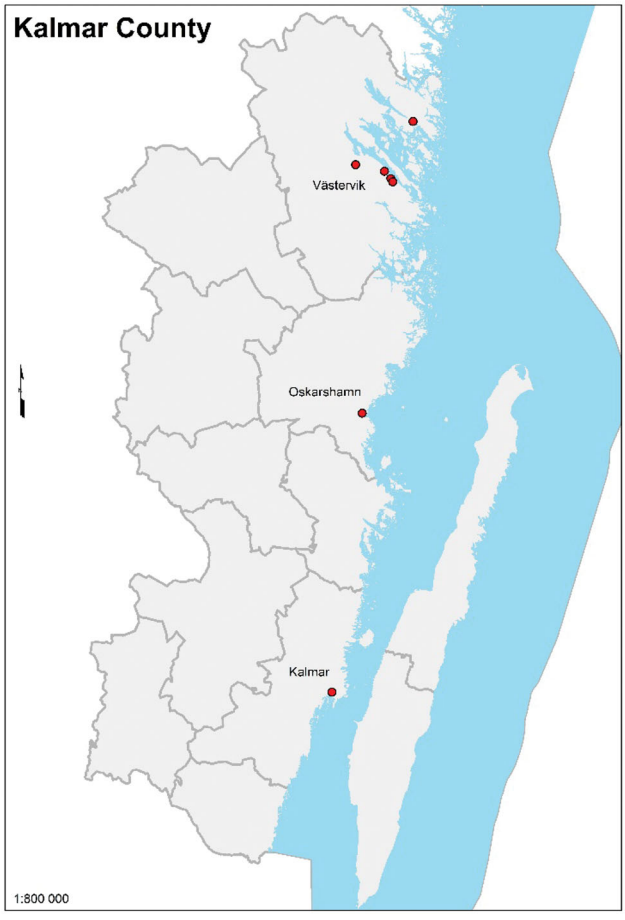

Figure 3. Place of residence of *N. mikurensis*-positive blood donors.

bite during the current season and 3/7 reported 1–4 tick-bites during the current season. Six donors remembered the geographical location of the last tick bite and five were in Kalmar County. There were no significant differences between *N. mikurensis*-positive and negative blood donors regarding age, sex, blood bank location, inclusion year, spending time outdoors, tick bites ever, tick bites this season, last tick bite outside of county, previous TBD, pets, medication, splenectomy, blood transfusion or tick season (Table 3).

Look-back investigation and recipient tracing

Ten donations from seven *N. mikurensis*-positive donors were traced (Table 3; Supplementary Table 1). Look-back included the index donation and all subsequent donations until a negative PCR. Since donor testing was performed several months after the index donation, the donated routine blood components had already been transfused and could not be discarded or further investigated. Twelve recipients who received *N. mikurensis*-positive donations were identified (Supplementary Table 1). Nine units of RBCs and three units of platelets were transfused. The mean age of transfused RBCs was 14 days, and that of transfused platelets 10 days. Four RBC components and four platelet components from *N. mikurensis*-positive donors (including all donated components from donor V) were discarded according to the blood banks' standard procedure. Donor II had donated blood on one occasion after the index donation, and both donations were included in the look-back. Two donors (donors I and VII; Table 3) were still PCR positive at follow-up several months after the index donation.

Donor I donated blood on two additional occasions between the index duration and follow-up. Donor VII did not donate blood during the follow-up period.

Seven of the 12 recipients transfused with positive index donations provided samples for PCR and all were negative (Figure 2; Supplementary Table 1). None had received antibiotic treatment between the index donation and blood sampling. The mean time from index donation to PCR sampling of recipients was 201 days (range 73–381 days). Five recipients were dead at the time of identification of the *N. mikurensis*-positive donation, and they could not be tested for *N. mikurensis*. Their medical records were retrieved and carefully evaluated by an infectious diseases specialist. In all five cases, the cause of death was more likely due to other diagnoses than from symptomatic neoehrlichiosis. The mean age of recipients was 72 years and median age 77 years (range 20–94). All 12 recipients had considerable morbidity with chronic illnesses, cancer, haematological or rheumatological diseases (Supplementary Table 1). At least one recipient was treated with rituximab, and one was scheduled for a stem cell transplantation. According to the medical records, no recipient was splenectomised.

Discussion

In this study, we found the prevalence of *N. mikurensis*-DNA in blood donors to be 0.7% (95% CI 0.3 – 1.4). No previous data is available on the prevalence of *N. mikurensis* DNA in blood donors, but the results in this study are comparable to prevalences of 1.3% (5/316) in Polish forestry workers [14] and 2.0% (2/102) in tick-bitten individuals in Sweden and in the Åland Islands, Finland [13]. Sequence analysis showed that all *N. mikurensis* sequences retrieved from blood donors were identical to each other and to sequences from Swedish human isolates as well as from ticks collected from migrating birds in the region.

Neoehrlichia mikurensis was identified in seven donors, but the true prevalence might be underestimated. Pre-analytical conditions, such as choice of sampling tube and sampling material (serum, plasma, whole blood) may affect sensitivity. Furthermore, the DNA extraction method and freeze-thaw cycles may also influence the detection sensitivity of *N. mikurensis*. Thus, a negative PCR does not rule out neoehrlichiosis. On the other hand, a positive result proves the presence of DNA from *N. mikurensis* but does not necessarily prove an ongoing active infection [31]. Two similar PCR methods were used in this study: (1) when screening donated blood and (2) when sampling positive donors (at follow-up) and transfused recipients. Apart from slight interlaboratory and PCR protocol differences, the main difference between the two procedures was the use of whole blood in (1) and plasma in (2). According to a Norwegian study, *N. mikurensis* DNA is more frequently recovered from the pellet/plasma than from whole blood [32].

In Sweden, ticks are most active from April to October, and all positive donors were sampled during this interval and 5/7 acquired their last tick bite in the county. Since *N. mikurensis* can persist in blood for months, it cannot be ruled out that *N. mikurensis*-positive donors can present at blood banks outside of the tick season. Although based on a limited sample, the homes of *N. mikurensis*-positive donors were clustered around Västervik (Figure 3). In accordance with the eligibility requirements for blood donors, none of the positive donors were immunosuppressed (Table 3). There were no significant differences between *N. mikurensis*-positive and -negative blood donors regarding risk factors, tick exposure and tick bites. Since symptoms usually are absent or non-specific, it is difficult to identify neoehrlichiosis in blood donors based on clinical questions. All positive donors were asymptomatic, except donor I who experienced slight fatigue during the months after the index donation.

Follow-up of positive donors was conducted several months after the index donation. Despite this, two donors (donors I and VII; Table 3) were still positive at follow-up, at 318 and 131 days, respectively. This finding could indicate asymptomatic carriage or reinfection. At present, available molecular methods cannot distinguish between these conditions, as the reduced genome size and presumed clonal population structure of the bacterium have hampered the development of discriminative typing methods. However, the presence of *N. mikurensis* DNA in blood over time is in accordance with earlier findings of asymptomatic carriage [13–15], which seems to be the most plausible explanation. Donor I was positive on retesting 318 days after the index donation, the longest reported persistence of *N. mikurensis* in blood to date. To our knowledge, there are no previous reports of pericarditis in patients with neoehrlichiosis, but the possibility that *N. mikurensis* may cause pericarditis, as was observed in donor IV, warrants further observation. Altogether, only one positive donor complained of symptoms that could be considered to be caused by

neoehrlichiosis, and this symptom (slight fatigue) was unspecific.

Twelve recipients who received blood components from *N. mikurensis*-positive donors were traced, but no case of transfusion-transmitted neoehrlichiosis was detected (Figure 2; Supplementary Table 1). The medical records gave no suspicion of neoehrlichiosis as cause of death in the five deceased recipients. Nevertheless, since no testing was performed, it cannot be ruled out with certainty that transmission by transfusion of *N. mikurensis* did not occur, or that this contributed to the death of the patient. Furthermore, look-back was only performed from the index donation and forward. Current guidelines regarding proper look-back time for neoehrlichiosis are lacking. The Association for the Advancement of Blood & Biotherapies, AABB, has published a review and fact sheets on emerging infectious agents and the potential threat to transfusion safety [4]. The fact sheets are continuously updated online [33] and new fact sheets on emerging threats are added, but *N. mikurensis* is not yet included. The priority level regarding blood safety for the closely related pathogen *A. phagocytophilum* is assessed as moderate according to scientific and epidemiologic evidence; low regarding public perception and/or regulatory concern and low to moderate in focal endemic areas for the public concern [34]. There are two important differences between *N. mikurensis* and *A. phagocytophilum,* for the latter, transmission by transfusion is well established but chronic carriage in humans is not documented. The ability of *N. mikurensis* to cause a chronic asymptomatic carrier state would theoretically pose a greater risk to blood supply than *A. phagocytophilum*. The seroprevalence of *N. mikurensis* is not known. The seroprevalence of *A. phagocytophilum* in residents in tick-endemic areas in Sweden varies from 11% to 28% and rates fluctuate over time [35,36]. Some neoehrlichiosis patients show serological reactivity to *A. phagocytophilum* antibodies [37].

The length of the asymptomatic carriage in blood, the frequency of blood donation during this period and the immune status of recipients are important factors influencing the rate at which an infection is transmitted to blood recipients [4]. Since *N. mikurensis* can cause severe disease in immunocompromised individuals, it is inferred that transfusion-transmitted neoehrlichiosis can potentially be severe in these patients. Many recipients in our study had multiple morbidities and several had risk factors severe neoehrlichiosis [16], i.e. haematological malignancies, and treatment with rituximab and/or corticosteroids (Supplementary Table 1).

The incubation period for transfusion or transplant transmitted anaplasmosis, ehrlichiosis and babesiosis is longer than the typical incubation period for a tick transmitted infection [23,31]. Inoculum size and the site of inoculation might affect the time to onset of symptom [23]. For *N. mikurensis*, the minimum infectious dose, the incubation period after a tick bite as well as the duration of infectiousness are unknown. Long incubation periods after blood transfusions pose a challenge, since a blood transfusion given several months before symptoms appear is not always noted.

Recipient tracing did not identify any transfusion-transmitted cases of neoehrlichiosis. Time from index transfusion to PCR sampling in recipients varied between 73–381 days. A long interval could lower the odds for diagnosing a transfusion-transmitted infection. On the other hand, no recipient showed clinical signs of neoehrlichiosis during this time, and none were treated with doxycycline. If transmission did occur, either infection was not established, or the bacterial concentration was below the limit of detection.

The importance of donor risk mitigation strategies to prevent transfusion-transmitted infections has been stressed repeatedly [7,31]. Strategies to prevent transfusion-transmitted tick-borne infections include questioning donors about history of tick exposure, tick bites and previous tick-borne infections (e.g. babesiosis), deferral of individuals with acute illness or fever and, for *Babesia*, screening of donors in endemic areas [7]. Questioning donors lacks both sensitivity and specificity [4] and recollection of tick bites is not a good marker for tick-borne disease [7,38,39]. This is corroborated by our study, where no statistically significant differences were observed regarding tick bite exposure in blood donors with or without *N. mikurensis* DNA in blood (Table 2. The frequency of reported tick exposure was high (39% of donors reported one or more tick bites the current season) and there was a low correlation between self-reported tick exposure/tick bites versus tick-borne disease. Therefore, deferral based on anamnestic data is not a viable strategy in the study region since this would exclude too many donors. Furthermore, it has been suggested that donors who report tick bites are more inclined to look for ticks and to take additional precautions to avoid bites [39].

In this study, the prevalence of *N. mikurensis* in blood donors was low, and transmission by transfusion could not be demonstrated even though many exposed recipients had one or several risk factors for severe neoehrlichiosis. Although no cases of transfusion-transmitted

neoehrlichiosis were identified, the possibility that *N. mikurensis* may be transmitted by transfusion cannot be excluded. In order to make better informed decisions on optimal screening strategies, further studies are needed on the ability of *N. mikurensis* to survive the processing and storage of blood components as well as on prevalence in blood component recipients, especially those immunocompromised. Since the impact on public health is low, general Nucleic Acid Testing (NAT) for *N. mikurensis* in blood donors is most likely not cost-effective, but the professional and public opinion on the possibility of transmission by transfusion has to be considered [4]. The public reaction may be disproportional to the severity of the infection, but neoehrlichiosis is easily treated. The first and most important step is to increase clinical awareness of the risk of transfusion-transmitted tick-borne diseases in general and neoehrlichiosis in particular. Transfusion-transmitted neoehrlichiosis is an important differential diagnosis should symptoms like fever and vascular events appear after transfusion of blood components, especially in immunocompromised recipients. Suspicion should be raised regardless of time of year. Conversely, when diagnosing diseases caused by microorganisms that can be transmitted by transfusion, patients should be interviewed about recent blood donations. Guidelines regarding prudent look-back time for recipient tracing and donor deferral duration are also needed.

Effective surveillance systems for transfusion-transmitted diseases are important to keep the blood supply safe [2,21]. Currently, there are no guidelines for prevention and no coordinated national surveillance of transfusion-transmitted tick-borne infections in Sweden. Early reporting of suspected transfusion-associated transmission is essential and should lead to timely tracing and recall of blood components as well as donor and recipient tracing [8].

In conclusion, although *N. mikurensis* was found in 0.7% (7/1006) of blood donors, no case of transfusion-transmitted infection was identified, although several recipients were at high risk for severe neoehrlichiosis. To achieve better informed decisions on optimal screening strategies, further studies on transfusion-mediated transmission of *N. mikurensis* are needed as well as raised clinical awareness.

Acknowledgments

The authors would like to express our sincerest gratitude to the staff at the Blood banks in Kalmar, Oskarshamn and Västervik for recruiting study participants. A special thanks goes to Hanna Carlsson, Mira Österman and Kim Hägerström for helping with recipient and component tracing and retrieving blood samples. We also thank the Department of Infectious Diseases, Kalmar, for assisting with the follow-up of some of the participants. We further thank Anna Grankvist and Christine Wennerås, Sahlgrenska University Hospital Laboratory, Gothenburg for help with PCR analyses performed on recipients. We thank Malgorzata Postula-Gorecka for providing the map over positive donors.

Disclosure statement

L Labbé Sandelin has been awarded a scholarship from the Infectious Diseases Society of Sweden and Pfizer AB. PE Lindgren is an external senior scientific expert to Valneva Austria GmbH, Vienna, Austria and Pfizer Inc, US. I Tjernberg has served at advisory board for Pfizer Inc.

Funding

This work was supported by FORSS [FORSS-932183]; Pfizer; Svenska Infektionsläkarföreningen and Kalmar County Council. PE Lindgren was supported by the European Union through the European Regional Development Fund and the Interreg North Sea Region Programme 2014–2020 as part of the NorthTick project [reference number J-No.: 38-2-7-19].

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