



Serological reactivity to *Anaplasma phagocytophilum* in neehrlichiosis patients

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Abstract

The tick-borne bacterium *Candidatus* (Ca.) Neoehrlichia (N.) mikurensis is a cause of “fever of unknown origin” because this strict intracellular pathogen escapes detection by routine blood cultures. Case reports suggest that neoehrlichiosis patients may display serological reactivity to *Anaplasma* (*A.*) *phagocytophilum*. Since *Anaplasma* serology is part of the diagnostic work-up of undetermined fever in European tick-exposed patients, we wanted to investigate (1) the prevalence of *A. phagocytophilum* seropositivity among neoehrlichiosis patients, (2) the frequency of misdiagnosed neoehrlichiosis patients among *A. phagocytophilum* seropositive patients, and (3) the frequency of *A. phagocytophilum* and *Ca. N. mikurensis* co-infections. Neoehrlichiosis patients ($n = 18$) were analyzed for *A. phagocytophilum* IgM and IgG serum antibodies by indirect immunofluorescence assay. Serum samples from suspected anaplasmosis patients ($n = 101$) were analyzed for bacterial DNA contents by singleplex PCR specific for *A. phagocytophilum* and *Ca. N. mikurensis*, respectively. One fifth of the neoehrlichiosis patients (4/18) were seropositive for IgM and/or IgG to *A. phagocytophilum* at the time of diagnosis. Among the patients with suspected anaplasmosis, 2% (2/101) were positive for *Ca. N. mikurensis* by PCR whereas none (0/101) had detectable *A. phagocytophilum* DNA in the serum. To conclude, patients with suspected anaplasmosis may in fact have neoehrlichiosis. We found no evidence of *A. phagocytophilum* and *Ca. N. mikurensis* co-infections in humans with suspected anaplasmosis or confirmed neoehrlichiosis.

Introduction

Candidatus (Ca.) Neoehrlichia (N.) mikurensis is a tick-borne pathogen found in Europe and Asia [1], which was first reported to be a human pathogen in 2010 [2–4]. It can give rise to a severe infectious disease named neoehrlichiosis that features fever and vascular events in immunocompromised patients [5]. Immunocompetent individuals infected by *Ca. N. mikurensis* may present with fever and symptoms indicative

of systemic infection, isolated erythematous skin rashes, or no symptoms at all [2, 4, 6–9].

Like all members of the *Anaplasmataceae* family, *Ca. N. mikurensis* is a strict intracellular pathogen, and consequently does not grow in cell-free media, which is why it escapes detection by routine blood cultures [1]. At present, the only microbiological diagnostic option is PCR since there are no serological assays available. The restricted diagnostic alternatives, together with the novelty of this emerging pathogen, explain

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why many patients with severe neehrlichiosis remain undiagnosed and fall under the epithet of “fever of unknown origin” [5]. In central and northern Europe, *A. phagocytophilum* serology is part of the diagnostic work-up of (tick-exposed) patients with unexplained fever. There are at least three case reports of immunocompetent patients infected by *Ca. N. mikurensis* who were seropositive for *A. phagocytophilum* as determined by indirect immunofluorescence assay (IFA): two of the cases had de novo production of *Anaplasma*-reactive antibodies, whereas the third one had pre-existing antibodies [2, 6]. This might indicate that *Ca. N. mikurensis* infection can trigger the production of *Anaplasma* cross-reactive antibodies or the occurrence of double infections with *Ca. N. mikurensis* and *A. phagocytophilum*. Consequently, neehrlichiosis patients may be wrongly diagnosed with anaplasmosis. The aims of this study were to address these issues. Specifically, the goals were to investigate (1) the prevalence of *Anaplasma* seropositivity among patients diagnosed with neehrlichiosis, (2) whether neehrlichiosis patients are misdiagnosed as anaplasmosis patients based on serological findings, and (3) the existence of *A. phagocytophilum* and *Ca. N. mikurensis* co-infections.

Methods

Study subjects Eighteen patients diagnosed with neehrlichiosis based on PCR-positive blood samples were investigated for *A. phagocytophilum*-reactive antibodies in serum (Table 1). Clinical data pertaining to some of these patients have been published previously [3, 5, 10–12]. Serum samples derived from a total of 101 anonymous patients queried for *A. phagocytophilum* antibodies were analyzed by PCR for the presence of bacterial DNA corresponding to *Ca. N. mikurensis* and *A. phagocytophilum*, respectively. The sera were obtained from three clinical microbiology laboratories, two in Sweden (Sahlgrenska University Hospital in Göteborg, $n = 68$, and Ryhov County Hospital in Jönköping, $n = 22$), and one in Denmark (Statens Serum Institut in Copenhagen, $n = 11$). These laboratories are the only ones that perform serological analyses of human antibodies to *A. phagocytophilum* in Sweden and Denmark, respectively. Over half of the blood samples submitted to Sahlgrenska University Hospital (38/68) for analysis of *A. phagocytophilum* antibodies and *Ca. N. mikurensis* DNA were from a prospective study on human tick-borne infections conducted at the Center of Vector-borne Infections, Uppsala University Hospital, Sweden. The study was approved by the local Ethical Review Boards of Göteborg and Uppsala, Sweden. All analyses were performed on thawed blood samples that had been stored frozen at either $-20\text{ }^{\circ}\text{C}$ (sera tested for *A. phagocytophilum* antibodies) or $-120\text{ }^{\circ}\text{C}$ (plasma from neehrlichiosis patients).

***A. phagocytophilum* serology** A commercial IFA assay for analysis of IgG and IgM antibodies to a human isolate of *A. phagocytophilum* (Focus Diagnostics, Cypress, CA, USA) was used according to the manufacturer’s recommendations. Semi-quantitative endpoint antibody titers were obtained by serial twofold dilutions of reactive serum or EDTA-plasma samples. IgM titers $\geq 1:20$ were regarded as positive. IgG titers of $\geq 1:64$ were regarded as positive at Sahlgrenska University Hospital and Statens Serum Institut in Copenhagen, whereas a cutoff of $\geq 1:80$ was employed at Ryhov Hospital in Jönköping using the same assay.

***Ca. N. mikurensis* PCR** Bacterial DNA was robot extracted (MagNA Pure Compact Extraction Robot, Roche, Basel, Switzerland) from 400 μL of serum or EDTA-plasma (Nucleic Acid Isolation Kit I, Roche) and analyzed by using a real-time TaqMan PCR specific for a 169-bp segment of the *groEL* gene of *Ca. N. mikurensis* as previously described [6]. A synthetic plasmid containing the 169-bp sequence cloned into a pUC57 vector (Genscript, Piscataway, NJ, USA) was used to establish a standard curve and the limit of detection, which was 1×10^3 copies/mL. All positive isolates were confirmed by 16S rRNA-PCR and sequenced [6].

***A. phagocytophilum* PCR** Bacterial DNA was extracted as described above and analyzed by using two different TaqMan real-time PCR assays: the first targeted the *msp2* gene of *A. phagocytophilum* and was adapted from a duplex to a simplex assay [13]. The forward primer (5'-TTGG TCTTGAAGCGCTCGTA) and reverse primer (5'-AATA CCATAACCAACTGCCTTCCAT) generated a 77-bp fragment, which was detected with a TaqMan probe labeled with FAM (5'-CAATCTCAAGCTCAACCCTGGCACCA-MGB). The second PCR targeted the *groEL* gene of *A. phagocytophilum* [14]. The probe was modified to a TaqMan probe (5'-FAM-TAACACACTGTGCAATCTTACT-MGB) and the PCR reaction generated a 61-bp-long fragment. Both PCR-reactions contained $1 \times$ FastStart Taqman Probe Master (Roche, Basel, Switzerland), 600 nM of each primer, 150 nM of probe, and 5 μL of DNA template. Real-time PCR was performed by using Rotorgene 6000 (QIAGEN, Hilden, Germany). Reaction conditions were $95\text{ }^{\circ}\text{C}$ for 10 min, followed by 45 cycles at $95\text{ }^{\circ}\text{C}$ for 15 s, and $56\text{ }^{\circ}\text{C}$ ($53\text{ }^{\circ}\text{C}$ for *groEL*) for 1 min. The limit of detection for both assays was 1×10^4 copies/mL and was established using a synthetic plasmid (Genscript).

Results

Participants and study material There were 18 neehrlichiosis patients included in the study, all of whom were diagnosed by PCR analysis of blood samples (Table 1). The majority of the

Table 1 Clinical characteristics of neoehrlichiosis patients

| Patient ID | Age | Sex | Disease | Immune suppression | Fever | Ref. |
|-------------------|-----|-----|--|--------------------|-------|------------|
| SE01 | 77 | M | B-chronic lymphocytic leukemia | Splen | Yes | [3] |
| SE02 | 75 | M | B-chronic lymphocytic leukemia | Splen, Rtx, CH, Co | Yes | [5] |
| SE03 | 67 | F | Systemic lupus erythematosus | Splen, Co | Yes | [5] |
| SE05 | 54 | M | Psoriasis, hereditary gout | CH, Co | Yes | [5] |
| SE06 | 59 | M | Diffuse large B cell lymphoma | Splen, Rtx, CH | Yes | [5] |
| SE09 | 78 | M | Rheumatoid arthritis | Rtx, CH | Yes | [10] |
| SE10 | 55 | M | Granulomatosis with polyangiitis | Rtx CH, Co | Yes | [10] |
| SE12 | 57 | M | Pre-B-acute lymphocytic leukemia | CH, Co | Yes | [10] |
| SE13 | 65 | F | Autoimmune hemolytic anemia, Crohn's disease | Splen, Co, Azt | Yes | This study |
| SE15 ^a | 57 | F | Multiple sclerosis | Rtx | Yes | [11] |
| SE16 | 23 | M | Healthy | – | No | This study |
| SE17 | 58 | M | Follicular lymphoma | Rtx, CH | Yes | This study |
| SE18 ^a | 69 | M | B-chronic lymphocytic leukemia | Splen, Rtx, Co | Yes | This study |
| SE19 | 81 | M | Polymyalgia rheumatica | Co | Yes | This study |
| SE20 | 68 | F | B-chronic lymphocytic leukemia | Ibr, CH | Yes | This study |
| SE21 | 63 | F | Primary hypogammaglobulinemia | Splen | Yes | This study |
| SE22 | 60 | F | Focal segmental glomerulosclerosis | Co, CyA | Yes | This study |
| NO01 | 63 | M | Cured Hodgkin lymphoma | Splen | Yes | [12] |

SE Sweden, NO Norway, M male, F female, Splen splenectomy, Rtx rituximab, CH chemotherapy, Co systemic corticosteroids, Azt azathioprine, Ibr ibrutinib

^a Diagnosed in retrospect based on clinical data

patients (17/18) was immunocompromised and had typical risk factors for severe neoehrlichiosis, e.g. an underlying hematologic or systemic autoimmune disease, splenectomy, rituximab treatment, chemotherapy, and/or systemic corticosteroids. Every one of the immunocompromised patients presented with febrile disease, but for one who suffered from nightly sweats and headache (SE16) (Table 1). All of the patients responded completely to treatment with doxycycline and cleared the infection with one exception (SE18), who was given 100 mg doxycycline for 10 days as empiric treatment for unexplained fever instead of the recommended dose of 200 mg doxycycline for 2–3 weeks [1]; the patient in question had PCR-positive and symptomatic recurrence of the infection about 40 days later.

In addition, clinical serum samples originating from 101 anonymous patients submitted to the three clinical laboratories that currently perform *A. phagocytophilum* serology in Sweden and Denmark were analyzed. Whereas the serum samples from County Hospital Ryhov ($n = 22$) and Statens Serum Institut ($n = 11$) were selected based on being positive for *Anaplasma* antibodies, those from Sahlgrenska University Hospital were mostly consecutive samples ($n = 68$) submitted to the laboratory for analysis of *Anaplasma* serology, of which 21 (31%) turned out to have an IgG titer of 1:64 or higher. The serum samples from Sahlgrenska University Hospital were obtained from patients aged between 19 and 80 with a median age of 58, with an even sex distribution (53% women), similar

to the serum samples from County Hospital Ryhov (Jönköping), which were from patients 32 to 76 years old (median age 56 years; 45% women). The majority of the serum samples from Statens Serum Institut (Copenhagen) was from women (73%) having an age range of 27 to 67 years and a median age of 53 years.

A. phagocytophilum seropositivity among neoehrlichiosis patients First, we investigated the blood samples from the 18 patients diagnosed with neoehrlichiosis regarding serologic reactivity to *A. phagocytophilum*. Four of the 18 patients (22%) were *Anaplasma* seropositive at the time of diagnosis (Table 2): the only immunocompetent patient included in the study and 3 out of the 17 who were immunocompromised. One patient had both IgM and IgG antibodies to *Anaplasma* and three had only IgG antibodies. All of the IgG titers were below 1:256.

Ca. N. mikurensis and A. phagocytophilum DNA in the blood of patients with suspected anaplasmosis Two out of the 101 serum samples queried for *Anaplasma* antibodies were positive for *Ca. N. mikurensis* DNA by PCR (SE15 and SE18; Table 2). Both of these patients had typical symptoms of neoehrlichiosis and a characteristic risk profile (an underlying systemic autoimmune or hematologic disease, rituximab therapy, splenectomy) (Table 1). Neither of these two patients developed antibodies to *A. phagocytophilum* (Table 1). Moreover, not a single one of the 54 patients seropositive for

Table 2 Serum antibody titers to *A. phagocytophilum* in neoehrlichiosis patients

| Patient ID | IgM | IgG |
|------------|------|-------|
| SE01 | 1:80 | 1:160 |
| SE02 | N | N |
| SE03 | N | N |
| SE05 | N | N |
| SE06 | N | N |
| SE09 | N | N |
| SE10 | N | 1:320 |
| SE12 | N | N |
| SE13 | N | N |
| SE15 | N | N |
| SE16 | N | 1:80 |
| SE17 | N | N |
| SE18 | N | N |
| SE19 | N | N |
| SE20 | N | N |
| SE21 | N | 1:160 |
| SE22 | N | N |
| NO01 | N | N |

N negative

A. phagocytophilum was positive in either of the two *A. phagocytophilum* PCRs (data not shown).

Kinetics of antibody levels to *A. phagocytophilum* in neoehrlichiosis patients Repeated (paired) serum samples were only available from five of the neoehrlichiosis patients. Three of these patients (SE02, SE13, SE18) were seronegative for *A. phagocytophilum* on both sampling occasions. One patient developed IgM antibodies 1 week after diagnosis (NO01) that decreased 4-fold over a 5-week period (Fig. 1). Another

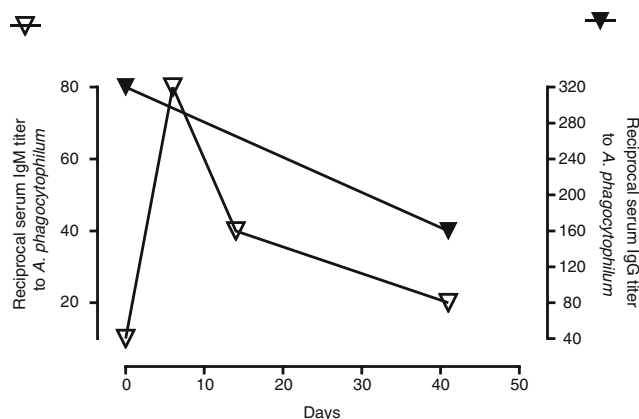


Fig. 1 Consecutive *A. phagocytophilum* antibody titers displayed by two neoehrlichiosis patients. One patient (NO01) presented with IgM antibodies (white triangles) and another (SE10) with IgG antibodies (black triangles). Day 0 indicates the day of diagnosis of the *Ca. N. mikurensis* infection

patient (SE10) had IgG antibodies with a titer of 1:320 that was halved over a period of 41 days (Fig. 1).

Discussion

The main finding of this study is that every fifth of the neoehrlichiosis patients had low titers of *A. phagocytophilum* antibodies in the blood at the time of diagnosis. This is a surprisingly high figure in view of the fact that the majority of the patients were immunocompromised. This seroreactivity to *A. phagocytophilum* might represent (1) previous exposure to or infection with *A. phagocytophilum*, (2) co-infection with *Ca. N. mikurensis* and *A. phagocytophilum*, or (3) *Anaplasma*-crossreactive antibodies elicited or boosted by *Ca. N. mikurensis* infection.

Estimates of the seroprevalence of *A. phagocytophilum* antibodies in the general population in Scandinavia vary greatly. Two older studies report seroprevalence figures of 2.0 and 2.5% among Danish and Norwegian blood donors [15, 16], but a newer Norwegian study gives a figure of 16% [17]. The estimates of the *A. phagocytophilum* seroprevalence in populations that are heavily tick-exposed range from 10% in Norway [15], 11–17% in Sweden [18, 19], to 21% in Denmark [16]. It should be noted that the more recent seroepidemiologic surveys have utilized the same IFA as in the present study, which is based on a human isolate of *A. phagocytophilum* [16, 17, 19], whereas the older studies have used an equine *A. phagocytophilum* isolate [15, 18]. In contrast to the relatively high seroprevalence of *A. phagocytophilum* antibodies, there is a scarcity of case reports of anaplasmosis from the Scandinavian countries [20, 21]. The main explanation for this disparity is that the European variant of this infectious disease is relatively mild in humans, at least compared to human anaplasmosis in North America [22]. Presumably, the human-tropic European *A. phagocytophilum* strains are less virulent than the American ones and give rise to discrete symptoms or only subclinical infections in the majority of cases. Thus, symptomatic anaplasmosis appears to be a rare disease in Scandinavia. This may account for our inability to detect *A. phagocytophilum* DNA in the serum samples derived from the *Anaplasma*-reactive anonymous patient samples, even when using two different PCRs targeting different *A. phagocytophilum* genes. However, we cannot exclude that our use of serum or plasma may have given a poorer DNA yield compared to if we had used buffy coat or whole blood in view of the intracellular nature of *A. phagocytophilum*, which resides within granulocytes.

It is possible that the *Anaplasma* seroreactivity we have detected among neoehrlichiosis patients reflects previous exposure to *A. phagocytophilum*. We have no evidence to suggest that the neoehrlichiosis patients were doubly infected with *A. phagocytophilum* and *Ca. N. mikurensis*

since all patients were negative for *A. phagocytophilum* by PCR and confirmatory sequencing of the *Ca. N. mikurensis* PCR amplicons was in no case ambiguous.

The third possible explanation for why up to every fifth of the neehrlichiosis patients presented with *Anaplasma* antibodies is that these antibodies were in fact directed against *Ca. N. mikurensis* and cross-reactive with *A. phagocytophilum* antigens. One indication that this might be the case is the semblance of an antibody response among the few neehrlichiosis patients from whom it was possible to obtain repeated blood samples in the present study. Two published studies have also implied that neehrlichiosis patients may respond with *Anaplasma*-reactive antibodies, indicative of cross-reactivity [2, 6]. However, the issue of cross-reactivity will only be addressable once *Ca. N. mikurensis* antigens are available, which will require its cultivation.

Irrespective of the underlying mechanisms behind the *A. phagocytophilum* seroreactivity demonstrated by some neehrlichiosis patients, the main significance of this finding is that a certain degree of vigilance is warranted: patients believed to have anaplasmosis may in fact have neehrlichiosis. Moreover, patients queried for *A. phagocytophilum* antibodies that turn out to be seronegative may have neehrlichiosis. Two of the neehrlichiosis patients described in this study were discovered among patient samples submitted for *A. phagocytophilum* serology thanks to relevant clinical data, both of whom were seronegative for *Anaplasma*. A correct diagnosis is of utmost importance since these two infectious diseases differ with regard to one vital aspect: neehrlichiosis patients have a substantial risk of contracting vascular events such as deep vein thrombosis, arterial aneurysms or transitory ischemic attacks, which are not recognized to be part of an infectious process [5]. If neehrlichiosis patients with vascular complications are correctly diagnosed and adequately treated with antibiotics, they do not incur new vascular events [5].

To conclude, patients with fever of uncertain origin or with suspected anaplasmosis may in fact have neehrlichiosis. Misdiagnosed or undiagnosed cases of neehrlichiosis may be identified either among *Anaplasma* seropositive patients or among patient samples queried for *Anaplasma* antibodies that are negative by *A. phagocytophilum* IFA but have typical risk factors for severe neehrlichiosis. We recommend that such patients be assayed for the presence of *Ca. N. mikurensis* DNA by PCR performed on EDTA blood or plasma to determine if they have contracted neehrlichiosis.

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Compliance with ethical standards

The study was approved by the local Ethical Review Boards of Göteborg and Uppsala, Sweden.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in the studies involving human participants were in accordance with the ethical standards of the regional research committees and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants in the study.

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