Infections with Candidatus Neoehrlichia mikurensis and Cytokine Responses in 2 Persons Bitten by Ticks, Sweden

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The prevalence of Candidatus Neoehrlichia mikurensis infection was determined in 102 persons bitten by ticks in Sweden. Two infected women had erythematous rashes; 1 was co-infected with a Borrelia sp., and the other showed seroconversion for Anaplasmaphagocytophilum. Both patients had increased levels of Neoehrlichia DNA and serum cytokines for several months.

Candidatus Neoehrlichia mikurensis is a tick-borne pathogen found in Europe and Asia (1). It causes an infectious disease in immunocompromised persons that is characterized by fever and thromboembolic events (2). In contrast, Candidatus N. mikurensis infection in immunocompetent hosts has been linked to asymptomatic infection (3), systemic inflammation with various symptoms (4,5), and possibly lethal infection (6). Knowledge regarding the capacity of Candidatus N. mikurensis to cause disease in immunocompetent persons is still limited. The purpose of this study was to investigate the prevalence, rate of co-infections, clinical picture, and cytokine response to Candidatus N. mikurensis infection in immunocompetent patients participating in the Tick-Borne Diseases Study (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/8/15-0060-Techapp1.pdf).

The Study

The study was approved by the Ethics Committees of Linköping University (M132-06), and Åland Health Care (2008-05-23). DNA was robot-extracted (MagNA Pure Compact Extraction Robot; Roche, Basel, Switzerland) from 400 µL of EDTA-plasma (Nucleic Acid Isolation Kit I; Roche) and analyzed by using a real-time PCR specific for a 169-bp segment of the groEL gene of Candidatus N. mikurensis. Amplifications were performed in a 20-µL reaction mixture containing 1× FastStart Taqman Probe Master (Roche), 1 µmol/L of each primer (5′-CGG AAA TAA CAA AAG ATG GA-3′; 5′- ACC TTC TCG ATT ACT TTA G-3′), 100 nmol/L of probe (5′-6FAM- TTG GTG ATG ATG GA-3′), and 4 µL of DNA template. Real-time PCR was performed by using Rotorgene 6000 (QIAGEN, Hilden, Germany). Reaction conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, and a final cycle at 54°C for 1 min. A synthetic plasmid containing the 169-bp sequence cloned into a pUC57 vector (Genscript, Piscataway, NJ, USA) was used to estimate bacterial gene copy numbers. Positive samples were verified by using a pan-bacterial PCR specific for the 16S rRNA gene (online Technical Appendix). All PCR products were sequenced after electrophoresis on 2% agarose gels and analyzed by using an ABI PRISM 3130 Genetic Analyzer (Life Technologies Europe BV, Bleiswijk, the Netherlands). Obtained DNA sequences were edited and further analyzed by using the GenBank BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Ripsq mixed software (Isentio, Palo Alto, CA, USA).

Patient serum samples were analyzed for antibodies against Borrelia burgdorferi sensu lato by using the Recombead Borrelia IgM and IgG Kit (MikroBio Diagnostik, Neuried, Germany). Samples were analyzed for IgG against Anaplasmaphagocytophilum by using the A. phagocytophilum IFA IgG Kit (Focus Diagnostics, Cypress, CA, USA) and for 20 cytokines by using the Bio-Plex 200 System (Bio-Rad, Hercules, CA, USA).

A total of 102/3,248 study participants sought medical care during the 3-month study period and were further investigated. Their median age was 63 years (range 28–79 years) and 73 (72%) were women. All but 3 participants were immunocompetent (2 had cancer; 1 of them used methotrexate). Candidatus N. mikurensis DNA was detected in 2 (2.0%) of 102 patients, which is consistent with prevalences of 1.1% in China (5) and 1.6% in Poland (3).

Patient 1 was a healthy 68-year-old woman who lived on the island of Tjurkö, southeast of Sweden. She sought medical care on day 77 of the study because of a rash on her right breast. She reported being bitten by a tick in the same

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Table 1. Evolution of Candidatus Neoehrlichia mikurensis gene copy numbers and antibody levels to Borrelia burgdorferi sensu lato complex and Anaplasma phagocytophilum for patient 1, Sweden

| Characteristic | Days after inclusion in study |
|---------------|-----------------------------|
|               | 0 | 77 | 169 |
| Clinical manifestations | None | Rash on right breast | None |
| Candidatus N. mikurensis DNA in plasma, gene copies/mL | 0 | 2,200 | None |
| Serum B. burgdorferi sensu lato IgM (points)* | Negative (4) | Positive (12) | Positive (8) |

B. burgdorferi sensu lato antigens

| Positive reactivity | Borderline reactivity |
|---------------------|-----------------------|
| None | OspC, p100 |

Serum B. burgdorferi sensu lato IgG (points)†

| Positive reactivity | Borderline reactivity |
|---------------------|-----------------------|
| None | OspC, p100 |

Serum A. phagocytophilum IgG (1:64)‡

| Serum A. phagocytophilum IgG (1:256)§ |
|--------------------------------------|
| ++ | ++ |

A. phagocytophilum OspC, p100

B. burgdorferi OspC, p58, VlsE, p58

B. burgdorferi OspC, p58, VlsE, p58, P100

P100, VlsE, p58, OspC

| Characteristic | Days after inclusion in study |
|---------------|-----------------------------|
|               | 0 | 98 | |
| Clinical manifestations | None | Rash on left breast | None |
| Candidatus N. mikurensis DNA in plasma, gene copies/mL | 0 | 260 | 1300 |
| Serum B. burgdorferi sensu lato IgM (points)* | Negative (0) | Negative (2) | Negative (1) |

B. burgdorferi sensu lato antigens

| Positive reactivity | Borderline reactivity |
|---------------------|-----------------------|
| None | None |

Serum B. burgdorferi sensu lato IgG (points)†

| Positive reactivity | Borderline reactivity |
|---------------------|-----------------------|
| None | None |

Serum A. phagocytophilum IgG (1:64)‡

| Serum A. phagocytophilum IgG (1:256)§ |
|--------------------------------------|
| ++ | ++ |

B. burgdorferi OspC, p100

B. burgdorferi OspC, p58, VlsE, p58

B. burgdorferi OspC, p58, VlsE, p58, P100

P100, VlsE, p58, OspC

Location 2 months earlier. The patient was given a diagnosis of erythema migrans, received phenoxymethylpenicillin (1 g, 3×/d for 10 days), and the rash disappeared.

Patient 2 was a 57-year-old woman who lived in Kalmar, Sweden. She had a history of allergy and was regularly taking aspirin. She had received treatment for Lyme borreliosis 8 years earlier. On day 65 of the study, she sought medical care because of a rash on her left breast. She reported being bitten by a tick in the same location 1.5 months earlier. The patient was also given a diagnosis of erythema migrans and received phenoxymethylpenicillin (1 g, 3×/d for 10 days).

Patient 1 had IgM against Borrelia outer surface protein C and pre-existing Borrelia-specific IgG titers that increased during the study (Table 1). This seroconversion may have resulted from cross-reactivity with Candidatus N. mikurensis, which was previously reported for an immunocompetent patient from Switzerland (4). Relatively high rates of seropositivity to A. phagocytophilum in Sweden (11,12) might be caused by cross-reactive antibodies because Candidatus N. mikurensis is common in ticks in Sweden, in contrast to A. phagocytophilum (13).

Both patients showed increased serum levels of cytokines, which appeared to mirror the numbers of Candidatus N. mikurensis gene copies (Figures 1, 2; online Technical Notes).
Appendix Figure). Cytokine levels for patient 1 were maximum on day 77 and returned to reference levels on day 167. All cytokines, except for interferon-γ-induced protein 10, reached maximum levels on day 98 for patient 2. The cytokines were selected because systemic inflammation (Figure 1) with neutrophilia (online Technical Appendix) is typical of Neoehrlichiosis in immunocompromised patients (2). In addition, a Th1-like immune response (Figure 2) is presumably required to eliminate an intracellular pathogen, such as Candidatus N. mikurensis. However, the cytokine response of patient 1 may in part have been caused by Borrelia spp. (14).

Conclusions
Candidatus N. mikurensis DNA was detected in the blood of both patients for ≥1 and 3 months, respectively. Similarly, a healthy person in Poland showed a positive result for Candidatus N. mikurensis twice in a 4-month period (3). This finding suggests that Candidatus N. mikurensis infections persist for a long time or that frequent reinfections occur. Prolonged carriage seems more probable in view of the common occurrence of neoehrlichiosis during winter among immunocompromised patients (2); immunosuppressive therapy might reactivate such infections. An analogous finding was reported in a dog, which was believed to have been a chronic carrier of Candidatus N. mikurensis; infection became symptomatic when immune defenses were compromised by surgery (15).

In conclusion, an erythematous rash in a person bitten by a tick can be caused by Candidatus N. mikurensis, rather than by Borrelia spp. Moreover, immunocompetent persons may be infected by Candidatus N. mikurensis for...
unexpectedly long periods, even after symptoms have disappeared. Patients scheduled to receive immunosuppressive treatment, and who live in Candidatus N. mikurensis–endemic areas should be screened for this pathogen before beginning therapy.

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Technical Appendix

**Tick-Borne Diseases Study Design**

The ongoing Tick-Borne Diseases Study was initiated in 2007 to investigate the prevalence of pathogens in ticks that have fed on humans and the risk for developing tick-borne disease after a bite by an infected tick. Blood samples from persons residing in Sweden or the Åland Islands, Finland, who were bitten by ticks; health questionnaires; and ticks that had bitten the persons were collected at the 34 primary health care centers that participated in the study (1–3). At the time of inclusion, the participant completed a questionnaire containing questions about past and current medical history and data about the current tick bite. Tick(s) that had bitten participants were provided, and blood samples (plasma and serum stored in EDTA) were obtained.

At a follow-up visit 3 months later, the participant completed a second questionnaire containing questions about new tick bites during the study period, general health conditions, any signs or symptoms possibly associated with tick-borne disease; donated any additional ticks that had bitten the participant since the first visit; and provided blood samples. Study participants were asked to obtain health care when needed during the 3-month study period. Additional blood samples were collected at these visits and treatment with antimicrobial drugs was initiated at the discretion of the physician if tick-borne disease was suspected. During 2008–2012, a total of 3,248 persons participated in the Tick-Borne Diseases Study. In the present study, 102 participants who sought medical care at their health care center during the study were investigated.

**Pan-bacterial PCR**

For amplification of a segment of the bacterial 16S rRNA gene, the forward primer SSU1 5’-CGG CAG GCC TAA CAC ATG CAA GTC G-3’ and the reverse primer 806R, 5’-GGA CTA CCA GGG TAT CTA AT-3’ were used, which are complementary to conserved regions in the 5’ half of the 16S rRNA gene and yield a fragment of 766 bp between
nucleotide positions 41 and 806 for *Escherichia coli*. The amplification procedure was performed as described (4). For sequencing, PCR products of the expected size (766 bp) were cut out of the gel, purified by using the Qiaquick Gel Extraction Kit (QIAGEN, Göteborg, Sweden), and cycle-sequenced by using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Version 1.1 (Life Technologies Europe BV, Bleiswijk, the Netherlands) in 1 direction way with the reverse primer (806R).

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Technical Appendix Figure. Cytokines essential for recruitment or maturation of neutrophils in 2 patients infected with *Candidatus Neoehrlichia mikurensis*, Sweden. Concentrations of cytokines A) interleukin-8 (IL-8), B) interleukin-17A (IL-17A), C) granulocyte colony-stimulating factor (G-CSF), and D) granulocyte macrophage–colony-stimulating factor (GM-CSF) were measured in serum of patient 1 on days 0, 77, and 169 and in serum of patient 2 on days 0, 65, and 98. Levels of *Neoehrlichia* DNA in plasma are shown for both patients. Dotted lines indicate detection limit for each cytokine.