Prevalence of Neoehrlichia mikurensis in ticks and rodents from North-west Europe

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Abstract

Background: Neoehrlichia mikurensis is an emerging and vector-borne zoonosis. The first human disease cases were reported in 2010. Limited information is available about the prevalence and distribution of Neoehrlichia mikurensis in Europe, its natural life cycle and reservoir hosts. An Ehrlichia-like schotti variant has been described in questing Ixodes ricinus ticks, which could be identical to Neoehrlichia mikurensis.

Methods: Three genetic markers, 16S rDNA, gltA and GroEL, of Ehrlichia schotti-positive tick lysates were amplified, sequenced and compared to sequences from Neoehrlichia mikurensis. Based on these DNA sequences, a multiplex real-time PCR was developed to specifically detect Neoehrlichia mikurensis in combination with Anaplasma phagocytophilum in tick lysates. Various tick species from different life-stages, particularly Ixodes ricinus nymphs, were collected from the vegetation or wildlife. Tick lysates and DNA derived from organs of wild rodents were tested by PCR-based methods for the presence of Neoehrlichia mikurensis. Prevalence of Neoehrlichia mikurensis was calculated together with confidence intervals using Fisher’s exact test.

Results: The three genetic markers of Ehrlichia schotti-positive field isolates were similar or identical to Neoehrlichia mikurensis. Neoehrlichia mikurensis was found to be ubiquitously spread in the Netherlands and Belgium, but was not detected in the 401 tick samples from the UK. Neoehrlichia mikurensis was found in nymphs and adult Ixodes ricinus ticks, but neither in their larvae, nor in any other tick species tested. Neoehrlichia mikurensis was detected in diverse organs of some rodent species. Engorging ticks from red deer, European mouflon, wild boar and sheep were found positive for Neoehrlichia mikurensis.

Conclusions: Ehrlichia schotti is similar, if not identical, to Neoehrlichia mikurensis. Neoehrlichia mikurensis is present in questing Ixodes ricinus ticks throughout the Netherlands and Belgium. We propose that Ixodes ricinus can transstadially, but not transovarially, transmit this microorganism, and that different rodent species may act as reservoir hosts. These data further imply that wildlife and humans are frequently exposed to Neoehrlichia mikurensis-infected ticks through tick bites. Future studies should aim to investigate to what extent Neoehrlichia mikurensis poses a risk to public health.

Keywords: Vector-borne disease, Emerging zoonoses, Candidatus N. mikurensis, I. ricinus, Anaplasma phagocytophilum

Background

The most prevalent tick-borne infection of humans in the Northern hemisphere is Lyme [1] The same tick species transmitting the etiologic agents of Lyme disease also serve as the vector of pathogens causing tick-borne encephalitis and several forms of rickettsioses, anaplasmoses and ehrlichioses [2]. Members of the family Anaplasmataceae are obligatory intracellular bacteria that reside within membrane-enclosed vacuoles. Human ehrlichiosis and anaplasmosis are two closely related diseases caused by various members of the genera Ehrlichia and Anaplasma. A major difference between these two members is their cellular tropism. Ehrlichia chaffeensis, the etiologic agent of human monocytotropic ehrlichiosis (HME), is an emerging zoonosis that causes clinical manifestations ranging from a mild febrile illness to a fulminant disease characterized by multi-organ system failure [3]. Anaplasma phagocytophilum causes
human granulocytotropic anaplasmosis (HGA), previously known as human granulocytotropic ehrlichiosis [3]. Despite the presence of *Anaplasma phagocytophilum* in questing *Ixodes ricinus* ticks in the Netherlands [4], only one human case has been reported [5]. Seropositivity against anaplasmosis was observed in risk groups, such as foresters and suspected Lyme disease patients, but not in control groups [6]. Still, the incidence of these tick-borne diseases and the associated public health risks remain largely unknown.

A novel candidate species in the family of *Anaplasmataceae*, called *Candidatus* Neoehrlichia mikurensis (*N. mikurensis*), was first isolated from wild rats and was also found in *I. ovatus* in Japan [7]. *Neoehrlichia mikurensis* can be distinguished from other genera based on sequence analysis of 16S rDNA, citrate synthase (gltA) and heat shock protein GroEL genes [7]. This recently identified bacterium is detected in several tick species and rodents in different parts of the world under different names [7–11]. The *N. mikurensis* found in *I. ricinus* ticks in Italy has been referred to as *Candidatus Ehrlichia walkeri* [9] and the Ehrlichia species isolated from a rat in China was called “Rattus strain” [12]. Furthermore, a *N. mikurensis* has been described in *I. persulcatus* in Russia [13] and *I. ovatus* from China and Japan [12]. In the US, an Ehrlichia-like organism, closely related to *N. mikurensis*, was previously detected in raccoons. This variant is called *Candidatus Neoehrlichia lotoris* [14]. The Asian *N. mikurensis* isolates showed a 99% similarity based on the 16S rDNA to the *Ehrlichia schotti*. *Ehrlichia schotti* was first described in 1999 in *I. ricinus* in the Netherlands by Leo Schouls and was named after his technician [8]. Later this species was reported in *I. ricinus* in Russia [15] and subsequently in Germany and Slovakia [16]. These findings raised the question whether *Ehrlichia schotti* is the same as *N. mikurensis*.

It is unclear whether *N. mikurensis* poses a risk to public health. Until recently, there were no human infections reported. In 2010, the first case of human *N. mikurensis* infection was reported in a patient from Sweden [17]. In the same year, five other human infections were described in Germany, Switzerland and the Czech Republic [18]. More recently, a canine infection was reported in Germany [19]. The symptoms described in all of these cases were generally non-specific and usually seen in any other ordinary inflammation reaction (Table 1). These reported cases of human infections imply that re-evaluation is needed regarding the pathogenesis of this species. All but one case that have been described so far have occurred in patients who were immuno-compromised. The non-specificity of the reported symptoms, poor diagnostic tools and the lack of awareness of public health professionals could explain the absence of (reported) patients.

In this study we aim to investigate (i) whether *Ehrlichia schotti* is similar to the described *N. mikurensis* family, (ii) the distribution and prevalence of *N. mikurensis* in the Netherlands, Belgium and the UK, (iii) possible transmission routes of *N. mikurensis* in non-experimental settings and (vi) its putative mammalian hosts.

**Methods**

**Collection, identification and DNA extraction of ticks**

Questing *I. ricinus* from all stages and *Dermacentor reticulatus* adults were collected in 2009 and 2010 by flagging the vegetation at geographically different locations in the Netherlands and Belgium. Ticks collected in the UK and Vrouwenpolder (NL) have been described before [22]. For global geographic location, see Additional file 1: Figure S1. Questing *I. arboricola* were collected from bird nests in two different areas in Belgium. *Ixodes hexagonus* feeding on hedgehogs were collected in a hedgehog-shelter in 2010. *Ixodes ricinus* feeding on red deer (*Cervus elaphus*), European mouflon (*Ovis orientalis musimon*), wild boar (*Sus scrofa*) and sheep (*Ovis aries*) were collected. All the collected ticks were immersed in 70% alcohol and stored at −20°C until the DNA extraction. Based on morphological criteria, tick species and stages were identified to species level, with stage and sex recorded [23]. In doubtful cases, sequencing of tick mitochondrial 16S rDNA confirmed the tick-species [24]. DNA from questing ticks was extracted by alkaline lysis [4]. DNA from engorged ticks was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer’s manual (Qiagen, 2006, Hilden; Germany) following the manufacturer’s protocol for the purification of total DNA from ticks.

**Preparation of DNA lysates from wild rodents**

Longworth traps (Bolton Inc., UK), baited with hay, apple, carrot, oatmeal and mealworm were used to capture different species of rodents and insectivores at 7 different locations in the Netherlands between 2007 and 2010. Animals were anaesthetized with isoflurane and euthanized by cardiac puncture. Serum was collected and stored at −20°C. Spleen, liver, kidney, brain and other organs were collected and frozen at −80°C. DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit according to the manufacturer’s manual (Qiagen, 2006, Hilden; Germany). All animals were handled in compliance with Dutch laws on animal handling and welfare (RIVM/DEC permits).

**Polymerase chain reactions**

Polymerase chain reaction (PCR) amplifications were performed in a *P×2* Thermal Cycler (Thermo Electron Corporation, Waltham, Massachusetts, USA). The presence of *Ehrlichia schotti* in questing *I. ricinus* was studied by Reverse Line Blotting as described [25]. Fragments of the 16S rDNA, citrate synthase gene gltA, and the chaperonin
GroEL of ehrlichial species were amplified from tick lysates and rodent tissue samples using novel primers and primers that were previously described (Table 2). Amplification of gltA and GroEL were both done in 50 μl reaction volumes containing 5 μl template DNA. GltA DNA was amplified using a final concentration of 800 nM of each primer, NMikfo-gltA and NMikre-gltA with the following PCR program, 15 min at 95°C, 40 cycles each consisting of 30 sec at 94°C, 25 sec at 53°C, and 10 min at 72°C. GroEL DNA was amplified using 500 nM of each primer NMik fo-groEL and NMik re-groEL. The PCR program used is as followed: 15 min at 95°C, 40 cycles each consisting of 30 sec at 94°C, 75 sec at 49°C, and 10 min at 72°C. The nested reaction was carried out at the same temperature as the first reactions; only 25 cycles were carried out with 1 μL of the first amplification product. The HotStarTaq Polymerase Kit (Qiagen)

Table 1 Reported human cases of N. mikurensis infection (until October 2011)

| Location       | Case                        | Symptoms and clinical signs                                                                 | Ref.  |
|----------------|-----------------------------|-----------------------------------------------------------------------------------------------|-------|
| Germany        | Male, 69yr                  | Episodes of fever, nonproductive cough, left thoracic pain, vein thrombosis, hypochromic anemia, reduced numbers of leukocytes, decreased percentage of lymphocytes and elevated levels of CRP, microbiological analysis were negative. | [18]  |
| Germany        | Male, 57yr                  | Headaches, fever, intracerebral and subarachnoid hemorrhage, aneuysm, elevated CRP, pulmonary infiltration, microbiological analyses were negative, elevated infection parameters. Patient died from septic multi-organ failure. | [18]  |
| Sweden         | Male, 77yr Chronic lymphocytic leukemia | Transitory ischemic attack, hemolytic anemia, fever, erysipelas-like rash, transitory weakness of the left side of face and arm, hemolytic anemia, thrombocytopenia, thrombosis, pulmonary infiltration, increased proportion of monocytes and elevated levels of CRP, blood and other cultures were negative. | [17]  |
| Switzerland    | Male, 61yr CABG surgery     | Malaise, fever, moderate dyspnea, elevated leukocytes/neutrophils, elevated CRP; microbiological analysis were negative | [20]  |
| Czech Republic | Female 55 yr Mantle Cell Lymphoma | Spiking fever, myalgias, arthralgias, ethrombosis, elevated CRP, blood-, urine culture and pharyngeal swab was negative. Antinuclear-, antinucleolar antigens and rheumatoid factor screens were negative. | [21]  |
| Czech Republic | Male, 58yr Liver transplantation and splenectomy | Spiking fever, extreme fatigue, joint pain, skin erythema, painful and stiffened subcutaneous veins, mild leukocytosis and elevated CPR, blood and urine cultures and pharyngeal swab was negative. | [21]  |

GroEL of ehrlichial species were amplified from tick lysates and rodent tissue samples using novel primers and primers that were previously described (Table 2). Amplification of gltA and GroEL were both done in 50 μl reaction volumes containing 5 μl template DNA. GltA DNA was amplified using a final concentration of 800 nM of each primer, NMikfo-gltA and NMikre-gltA with the following PCR program, 15 min at 95°C, 40 cycles each consisting of 30 sec at 94°C, 25 sec at 53°C, and 10 min at 72°C. GroEL DNA was amplified using 500 nM of each primer NMik fo-groEL and NMik re-groEL. The PCR program used is as followed: 15 min at 95°C, 40 cycles each consisting of 30 sec at 94°C, 75 sec at 49°C, and 10 min at 72°C. The nested reaction was carried out at the same temperature as the first reactions; only 25 cycles were carried out with 1 μL of the first amplification product. The HotStarTaq Polymerase Kit (Qiagen)

Table 2 Primers used for amplification and sequencing of gltA and GroEL genes of N. mikurensis, and the amplification of the Msp2 gene of A. phagocytophilum

| Gene      | Name                | Type      | Sequence                                      | Reference |
|-----------|---------------------|-----------|------------------------------------------------|-----------|
| gltA      | NMik fo-gltA        | Primer (forward) | 5'-aagtgctagcttgtgctatc-3'                   | This study |
| gltA      | NMik re-gltA        | Primer (reverse)  | 5'-tcagctgtctgtaaaataaat-3'                 | This study |
| GroEL     | NMikGroEL-F2a       | Primer (forward) | 5'-ccttgaaatatagcaagatcagtag-3'             | This study |
| GroEL     | NMikGroEL-R2b       | Primer (reverse)  | 5'-ccacaggtaacttaatgtactaag-3'              | This study |
| GroEL     | NMikGroEL-P2a       | Probe (RED)  | 5'-RED-cctctactaatagttgctgaagatgtaggaagac-BHQ2-3' | This study |
| GroEL     | NMik fo-groEL       | Primer (forward) | 5'-gaagtagctagtatttttgtc-3'                 | [18]      |
| GroEL     | NMik re-groEL       | Primer (reverse)  | 5'-taaaccctatacctgtagaaac-3'                | [18]      |
| GroEL     | NMik seq1groEL      | Primer (reverse)  | 5'-acctacggctctagaag-3'                     | [18]      |
| GroEL     | NMik seq2groEL      | Primer (forward) | 5'-aaggaattattagattatactctt-3'             | [18]      |
| GroEL     | NMik seq3groEL      | Primer (forward) | 5'-atataagctgtaagctagtagac-3'              | [18]      |
| GroEL     | NMik seq4groEL      | Primer (forward) | 5'-ctccccatttaactgtaaccc-3'                | [18]      |
| Msp2      | ApMSP2F             | Primer (forward) | 5'-ataggaaggatgttttgttgatgtg-3'            | [20]      |
| Msp2      | ApMSP2R             | Primer (forward) | 5'-ttgtctggatagcgcctgta-3'                 | [20]      |
| Msp2      | ApMSP2F             | Probe (FAM)  | 5'-FAM-tggtgctagggttagctagggtag-BHQ1-3'    | [20]      |

Primers were either identical to or slightly modified from the primers described in the reference papers.
was used for all PCR experiments. PCR products were detected by electrophoresis in a 1.5% agarose gel stained with SYBR gold (invitrogen).

Multiplex real-time PCR

Oligonucleotide primer and probe sequences were designed to be specific for the N. mikurensis GroEL gene using Visual OMP DNA (Software, Inc., Ann Arbor, USA). Primer sequences for the N. mikurensis GroEL gene were NMikGroEL-F2a and NMikGroEL-R2b and generated a 99-bp fragment which was detected with the NMikGroEL-P2a TaqMan probe (Table 2). Sequences were evaluated on the basis of the following criteria: predicted cross-reactivity with closely related organisms, internal primer binding properties for hairpin and primer-dimer potential, length of the desired amplicon, G-C content, and melting temperatures (Tm) of probes and primers. The specificity of the N. mikurensis GroEL primers for N. mikurensis in the multiplex real-time PCR assay was tested with DNA extracted from the following microorganisms: Rickettsia rickettsii, Anaplasma phagocytophilum, R. helvetica, Bartonella henselae, Ehrlichia canis, B. afzelii, B. garinii, B. sensu stricto, Babesia microti, Candidatus Midichloria mitochondrii and tick lysates containing Wolbachia species [22,25] None were amplified. Random samples of tick lysates which were N. mikurensis-positive

Table 3 Members of the N. mikurensis group are distinguished from other genera based on sequence analysis of 16S rDNA, citrate synthase (gltA) and heat shock protein GroEL genes

| Country (Ref.) | Species | Named gene | Accession | Similarity |
|---------------|---------|------------|-----------|------------|
| Netherlands [8] | I. ricinus | Ehrlichia-like 'schotti variant' | AF104680 | 100% |
| Russia [15] | I. ricinus | Ehrlichia-like 'schotti variant' | AF104680 | 100% |
| Germany [16] | I. ricinus | C. Ehrlichia walkenii | AY098730 | 100% |
| Italy [9] | I. ricinus | C. Ehrlichia walkenii | AY098729 | 100% |
| Italy [28] | I. ricinus | C. Ehrlichia walkenii | AY098730 | 100% |
| China [12] | Rattus norvegicus | Ehrlichia-like 'Rattus variant' | AF104680 | 100% |
| Japan [7] | Rattus norvegicus | E. ovatus | AB084582 | 99.1% |
| USA [14] | Procyon lotor | Ehrlichia-like organism | AY781777 | 99.8% |
| Japan [29] | A. argenteus | C. N. mikurensis (FIN686 and Nagano21) | AB196304 | 99.5% |
| Russia [13] | I. persulcatus | Ehrlichia-like 'schotti variant' | AF104680 | 100% |
| Italy [11] | C. glareolus | C. N. mikurensis | AY098730 | 99.6% |
| USA [31] | Procyon lotor | C. N. lotoris (RAC413) | EF633744 | 97.8% |
| Slovakia [32] | I. ricinus | C. N. mikurensis | AB196305 | 99.7% |
| Russia [30] | I. persulcatus | C. N. mikurensis | FJ966364 | 99.6% |
| Germany [18] | Human | C. N. mikurensis | EU810404 | 100% |
| Switzerland [20] | Human | C. N. mikurensis | GQ501089 | 100% |
| Germany [19] | Dog | C. N. mikurensis | EU432375 | 100% |

This strain has been reported in different parts of the world under diverse nominations. The similarity of these isolates with N. mikurensis isolates present in Dutch ticks isolates were calculated.
in the Q-PCR were routinely confirmed by conventional PCR using NMik fo-gltA and NMik re-gltA primers, followed by DNA sequencing.

**Optimized conditions for multiplex PCR**

PCR was performed in a multiplex format with a reaction volume of 20 μl, using the iQ Multiplex Powermix (Bio-Rad Laboratories, Hercules, USA), in the LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche, Basel, Switzerland). Final PCR reaction concentrations were 1x iQ Powermix, primers ApMSP2F and ApMSP2R at 250 nM each, probe ApMSP2P-FAM at 125 nM, primers NMikGroEL-F2a and NMikGroEL-R2b at 250 nM each, probe NMikGroEL-P2a-RED at 250 nM, and 3 μl of template DNA. Cycling conditions were: 95°C for 5 min, followed by 60 cycles of a 5 sec denaturation at 95°C followed by a 35 sec annealing-extension step at 60°C.

Ticks lysates were considered positive if the Ct-value of a proper sigmoid curve was maximally three cycles more than the highest dilution of the positive control sample.

For each PCR and real-time multiplex PCR, positive, negative controls and blank samples were included. A 10⁻³ to 10⁻⁵ dilution of a mixture of sequencing-confirmed *N. mikurensis*-positive tick lysates were used as positive controls. In order to minimize contamination, the reagent setup, the extraction and sample addition, and the real-time PCR as well as sample analysis were performed in three separate rooms, of which the first two rooms were kept at positive pressure and had airlocks.

**DNA sequencing and genetic analysis**

PCR amplicons were sequenced using the described primers (Table 2) and the BigDye Terminator Cycle sequencing Ready Reaction kit (Perkin Elmer, Applied Biosystem, Foster City, USA).
Table 4 The prevalence and distribution of *N. mikurensis* in questing *I. ricinus* in the Netherlands and Belgium

| Location                        | Tested (n) | Positive (n) | Prevalence (%) |
|---------------------------------|------------|--------------|----------------|
| Boswachterij Hardenberg         | 90         | 7            | 8% (3-15%)      |
| Dintelse Gorzen                 | 122        | 9            | 7% (3-14%)      |
| Drents-Friese Wold              | 29         | 1            | 3% (0-18%)      |
| Duin en Kruidenberg (2009)      | 320        | 52           | 16% (12-21%)    |
| Duin en Kruidenberg (2010)      | 137        | 11           | 8% (4-14%)      |
| Hoog Soeren                     | 217        | 3            | 1% (0-4%)       |
| Kop van Schouwen                | 238        | 23           | 10% (6-14%)     |
| Denekamp                        | 104        | 4            | 4% (1-10%)      |
| Pyramide van Austerlitz         | 270        | 32           | 12% (8-16%)     |
| Rijk van Nijmegen               | 53         | 3            | 2% (0-10%)      |
| Ulvenhoutse bos                 | 8          | 1            | 13% (0-53%)     |
| Vlijmerbos                      | 328        | 10           | 3% (2-5%)       |
| Vrouwenpolder                   | 86         | 6            | 7% (3-15%)      |
| Brussel-area, (Sonian forest),  | 153        | 0            | 0% (<2%)        |
| (Belgium)                       |            |              |                |
| Vlaanderen-area (Belgium)       | 114        | 3            | 3% (1-8%)       |
| Wallonie-area (Belgium)         | 106        | 3            | 3% (1-8%)       |
| **Total of all ticks**          | **2375**   | **166**      | **7% (6-8%)**   |
| **Average of all areas**        | **15**     | **14**       | **6%**          |

Confidence intervals (95%), which were calculated using Fisher’s exact test, are between brackets. The average of all areas was calculated by average of all prevalence’s excluding Duin en Kruidenberg 2009.

Results

Comparison of *Ehrlichia schotti* with *N. mikurensis*

Twenty-three tick lysates that were previously tested positive for the presence of *Ehrlichia schotti* by PCR and Reverse Line Blotting [5,9,27] were amplified by PCR on the three loci 16S rDNA, gltA and GroEL using primers specific for *N. mikurensis* (Table 2). Amplicons of all three partial genes were obtained in 21 cases. None of these three loci were successfully amplified in 15 *Ehrlichia schotti*-negative ticks. The PCR products of all three loci were sequenced and compared with each other and with *N. mikurensis* sequences available in Genbank. All the *Ehrlichia schotti* sequences were identical to each other on the three loci, 16S rDNA, gltA as well as the GroEL. The 1740 base pairs of the 16S rDNA sequences from the *Ehrlichia schotti* were 99.6% to 100% similar to the *N. mikurensis* sequences available in Genbank. All the *Ehrlichia schotti* sequences were identical to each other on the three loci, 16S rDNA, gltA as well as the GroEL. The 1740 base pairs of the 16S rDNA sequences from the *Ehrlichia schotti* were 99.6% to 100% similar to the *N. mikurensis* sequences and the *Candidatus Ehrlichia walkerii* sequence in Genbank (Table 3). The 233 base-pair fragment of the gltA sequences from the *Ehrlichia schotti* were identical to the *Candidatus Ehrlichia walkerii* gltA sequence (Table 3). The 1238 base-pairs of the GroEL isolates amplified from the tick lysates showed a 94.3% and 95.5%, 98.7% and 100% (AB084583 and AB074461, EF633745 and FJ966365) match with the *N. mikurensis* GroEL sequences in Genbank, respectively. Phylogenetic analyses of the gltA and GroEL sequences showed that the *Ehrlichia schotti* clustered with *N. mikurensis* isolates, but not with *A. phagocytophilum* or any of the Ehrlichia species present in Genbank (Figure 1).

Prevalence and distribution of *N. mikurensis*

In order to estimate the prevalence and distribution of *N. mikurensis* in North-West Europe, questing *I. ricinus* ticks were successfully tested positive for the presence of *Ehrlichia schotti* by PCR and Reverse Line Blotting [5,9,27] were amplified by PCR on the three loci 16S rDNA, gltA and GroEL using primers specific for *N. mikurensis* (Table 2). Amplicons of all three partial genes were obtained in 21 cases. None of these three loci were successfully amplified in 15 *Ehrlichia schotti*-negative ticks. The PCR products of all three loci were sequenced and compared with each other and with *N. mikurensis* sequences available in Genbank. All the *Ehrlichia schotti* sequences were identical to each other on the three loci, 16S rDNA, gltA as well as the GroEL. The 1740 base pairs of the 16S rDNA sequences from the *Ehrlichia schotti* were 99.6% to 100% similar to the *N. mikurensis* sequences and the *Candidatus Ehrlichia walkerii* sequence in Genbank (Table 3). The 233 base-pair fragment of the gltA sequences from the *Ehrlichia schotti* were identical to the *Candidatus Ehrlichia walkerii* gltA sequence (Table 3). The 1238 base-pairs of the GroEL isolates amplified from the tick lysates showed a 94.3% and 95.5%, 98.7% and 100% (AB084583 and AB074461, EF633745 and FJ966365) match with the *N. mikurensis* GroEL sequences in Genbank, respectively. Phylogenetic analyses of the gltA and GroEL sequences showed that the *Ehrlichia schotti* clustered with *N. mikurensis* isolates, but not with *A. phagocytophilum* or any of the Ehrlichia species present in Genbank (Figure 1).

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Prevalence and distribution of *N. mikurensis*

In order to estimate the prevalence and distribution of *N. mikurensis* in North-West Europe, questing *I. ricinus*

Table 5 Prevalence of *N. mikurensis* in questing *I. ricinus*, divided by lifecycle stage

| Stage   | Tested (n) | Positive (n) | Prevalence (%) |
|---------|------------|--------------|----------------|
| Larvae  | 55*        | 0            | 0% (<1%)       |
| Nymph   | 2003       | 137          | 7% (6-8%)      |
| Female  | 92         | 10           | 11% (5-20%)    |
| Male    | 173        | 19           | 11% (7-17%)    |

*Pools of 5 larvae. 95% Confidence intervals were calculated using Fisher’s exact test and are between brackets.

Table 6 *D. reticularis, I. hexagonus and I. arboricola* tested in the multiplex real-time PCR for the *N. mikurensis*

| Tick species | Tested (n) | Positive (n) | Prevalence (%) |
|--------------|------------|--------------|----------------|
| *I. arboricola* | 79         | 0            | 0% (<5%)       |
| *I. hexagonus* | 169        | 0            | 0% (<2%)       |
| *Dermacentor reticulatus* | 177        | 0            | 0% (<2%)       |

Confidence intervals (95%), which were calculated using Fisher’s exact test.

Table 7 Spleens of wild rodent and insectivore species were tested by PCR and sequencing using *N. mikurensis* specific primers

| Rodent species | Tested (n) | Positive (n) |
|----------------|------------|--------------|
| *Apodemus flavicollis* | 2          | 0            |
| *A. sylvaticus* | 23         | 5            |
| *Crocidura russula* | 5          | 0            |
| *Microtus arvalis* | 8          | 2            |
| *Myodes glareolus* | 35         | 4            |
| *Sorex araneus* | 6          | 0            |

**Total** | 79 | 11 |
Ticks from Ticks

Table 8 I. ricinus adults feeding on animals living in nature reserve areas in the Netherlands were tested by multiplex real-time PCR for the presence of N. mikurensis

| Ticks from | Ticks tested (n) | Ticks Positive (n) | Prevalence in positive ticks (%) | Animals tested (n) | Animals with positive ticks (n) |
|------------|------------------|--------------------|----------------------------------|-------------------|--------------------------------|
| Cervus elaphus | 409 26 | 6% (4-9%) | 17 10 |
| Sus scrofa | 48 4 | 8% (2-20%) | 8 2 |
| Ovis aries | 264 33 | 13% (9-17%) | 24 13 |
| Ovis orientalis musimon | 233 10 | 4% (2%-8%) | 18 4 |

Role of ticks in the transmission of N. mikurensis

Transovarial (vertical) transmission has been implicated for Rickettsia [33] and Anaplasma [34], but not for Ehrlichia species [35]. Whether N. mikurensis is transmitted transovarily in I. ricinus has not been investigated so far. The prevalence of N. mikurensis was determined in 55 pools of 5 questing I. ricinus larvae from Vrouwenpolder, where nymphal and adult ticks were found to be positive for N. mikurensis (Table 4). None of the 55 pools were N. mikurensis-positive (Table 5). Some of the pools were positive for A. phagocytophilum, approving the used methodology. The prevalence of N. mikurensis in questing I. ricinus nymphs was ~7%, whereas the prevalence in adult ticks was ~11% (Table 5). No significant differences were observed in the prevalence between questing male and female I. ricinus ticks. To investigate the role of other tick species in the transmission of N. mikurensis: Dermacentor reticulatus, I. hexagonus and I. arboricola were analysed for the presence of N. mikurensis (in the multiplex real-time PCR). None were found positive (Table 6). Again, some were found positive for the A. phagocytophilum msp2 gene (data not shown), indicating that there is no significant inhibition within these samples.

Potential reservoir hosts of N. mikurensis

To investigate the possible mammalian hosts for N. mikurensis, 79 spleen samples of different wild small mammals were tested by (nested)-PCR for the presence of gltA and GroEL (Table 7). PCR-positive samples were sequenced to confirm the presence of N. mikurensis. Both the GroEL and gltA sequences isolated from spleen were identical to the N. mikurensis sequences found in the questing ticks in the Netherlands (Figure 1). Spleen samples from Apodemus sylvaticus, Microtus arvalis and Myodes glareolus were N. mikurensis-positive. After the spleen was found positive, other organs (kidney, liver and brain) were also tested for N. mikurensis. All the tested organs were positive.

Whether other mammals in the Netherlands are reservoir hosts is difficult to address, due to the protective status of these animals. An animal can be considered a potential reservoir host when the prevalence of N. mikurensis in ticks feeding on this animal is significantly higher than the prevalence in questing ticks. This is for example the case for Anaplasma phagocytophilum [36-40]. I. ricinus feeding on red deer (Cervus elaphus), European mouflon (Ovis orientalis musimon), wild boar (Sus scrofa) and sheep (Ovis aries) were tested by multiplex real-time PCR. The prevalence of N. mikurensis in feeding ticks was comparable to the prevalence in questing ticks (Table 8).

Discussion

Recently, six human and one canine case of N. mikurensis infection were reported in different locations in Europe. These reports advocate a re-assessment of the occurrence of this microorganism in questing ticks. Schouls and colleagues described an Ehrlichia-like organism (Ehrlichia schotti) in Dutch ticks [8]. In our study, the three genetic markers 16S rDNA, gltA and GroEL of Ehrlichia schotti-positive field isolates turned out to be similar and identical to DNA sequences available from N. mikurensis. Thus, Ehrlichia schotti and N. mikurensis are most likely one and the same species. Previous findings on E. schotti can be interpreted as findings on N. mikurensis. Thus, N. mikurensis has already been present in the Netherlands in 1999 [25,41]. Furthermore, 11% of 289 engorged I. ricinus removed from humans were N. mikurensis-positive, indicating that the Dutch population is being exposed to ticks infected with N. mikurensis [42]. Remarkably, human and animal cases of N. mikurensis infection in the Netherlands have not yet been described.
The development of a Q-PCR specific for *N. mikurensis* allowed us to test significant numbers of ticks without having to perform the labour-intensive Reverse-Line blotting. These analyses showed that the *N. mikurensis* is present in vegetation ticks throughout the Netherlands and Belgium. No *N. mikurensis*-positive ticks were found in one location in Belgium. One possible explanation is that this location in the Brussels-area is exceptional due to its reduced fauna and flora caused by human interference. This forest in the Brussels-area is also highly fragmented because of a railroad and several major motorways that run through the forest. Several parts of it can be ecologically considered ‘islands’, which could -through isolation of mammal and tick populations- explain the absence of the pathogen in this forest. More ticks of this unique area need to be tested in order to address this hypothesis. *Neoehrlichia mikurensis* was also not detected in ticks from the UK. This could indicate that these species have not (yet) been established on this island.

The overall prevalence of *N. mikurensis* in questing nymphs and adults is approximately 7%. From the public health point of view, it indicates that a significant proportion of people contracting a tick bite are exposed to *N. mikurensis*. Transmission of the *N. mikurensis* in ticks appears to occur horizontally rather than vertically. None of the tested larvae were found positive, even though the prevalence of nymphs is approximately 7% and 11% for adults. Other tick species, with more restricted host preference than *I. ricinus*, were also tested for the presence of *N. mikurensis*. *Dermacentor reticulatus, I. hexagonus* and questing *I. arboricola* were found negative. The data indicate that these tick species probably play insignificant roles in the transmission of *N. mikurensis*. In contrast, *I. ricinus* can be considered as its main vector in the Netherlands and Belgium.

A potential group of reservoir hosts for *N. mikurensis* are wild rodents. Indeed, spleen samples and other organs (kidney, liver and brain) of some rodent species turned out to be *N. mikurensis*-positive, which indicates a systemic infection of these rodents with *N. mikurensis*. The *N. mikurensis* isolates from ticks and wild rodents (Table 7) were genetically identical, indicating that rodents are potential reservoir hosts [43]. However, the reservoir potential of rodents can only be by xenodiagnosis or experimental infection. The prevalence of *N. mikurensis* in *I. ricinus* ticks feeding on red deer, European mouflon, wild boar and sheep were comparable to the prevalence in questing ticks. From these prevalence data, it was not possible to infer the role of these animals in the transmission of *N. mikurensis*. However, it is clear that these animals are being exposed to the *N. mikurensis* through tick bites. Further experiments are necessary to determine whether there are other mammalian reservoirs than wild rodents.

Conclusions
Although human infection in the Netherlands has not been reported in the Netherlands, it is unclear to what extent *N. mikurensis* poses risks to public health. The symptoms described in all of the *N. mikurensis* infection cases were generally non-specific and usually seen in any other ordinary inflammatory reaction. What’s more, most of the *Ehrlichia* infections are known to be either asymptomatic or mild, self-limiting diseases [3]. In other words, infection can occur without causing disease. So far, diagnosis has relied only on PCR amplification of the *N. mikurensis*. The lack of serological tests makes diagnosis particularly difficult. Against these backdrops, the actual incidence of human infection with *Ehrlichia* is likely to be much higher than currently reported in Europe. Thorough surveillance and improvement of diagnostic tools will probably increase the number of identified human cases, and consequently provide more insight in the public health relevance of *N. mikurensis*.

Additional file

Additional file 1: Figure S1. Geographical distribution of locations (rounds) or global areas (stars) of questing *I. ricinus* tested positive (red) or negative (green) for *N. mikurensis* in The Netherlands and Belgium. Exact coordinates of geographical locations are available upon request.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
CR, EJS, WT, PH, DH, JK and JM organized and participated in the fieldwork for the collection and determination of wildlife samples and ectoparasites. SJ and MF developed the laboratory tests for the simultaneous detection of *N. mikurensis*. 

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