Close Geographic Association of Human Neoehrlichiosis and Tick Populations Carrying “Candidatus Neoehrlichia mikurensis” in Eastern Switzerland

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Neoehrlichiosis caused by “Candidatus Neoehrlichia mikurensis” is an emerging zoonotic disease. In total, six patients have been described in Europe, with the first case detected in 2007. In addition, seven patients from China were described in a report published in October 2012. In 2009, we diagnosed the first human case of “Ca. Neoehrlichia mikurensis” infection in the Zurich area (Switzerland). Here, we report two additional human cases from the same region, which were identified by broad-range 16S rRNA gene PCR. Both patients were immunocompromised and presented with similar clinical syndromes, including fever, malaise, and weight loss. A diagnostic multiplex real-time PCR was developed for specific detection of “Ca. Neoehrlichia mikurensis” infections. The assay is based on the signature sequence of a 280-bp fragment of the “Ca. Neoehrlichia mikurensis” 16S rRNA gene and incorporates a “Ca. Neoehrlichia mikurensis” species, a “Ca. Neoehrlichia” genus, and an Anaplasmataceae family probe for simultaneous screening. The analytical sensitivity was determined to be below five copies of the “Ca. Neoehrlichia mikurensis” 16S rRNA gene. Our results show that the assay is suitable for the direct detection of “Ca. Neoehrlichia mikurensis” DNA in clinical samples from, for example, blood and bone marrow. In addition, it allows for monitoring treatment response during antibiotic therapy. Using the same assay, DNA extracts from 1,916 ticks collected in four forests in close proximity to the patients’ residences (<3 km) were screened. At all sampling sites, the minimal prevalence of “Ca. Neoehrlichia mikurensis” was between 3.5 to 8% in pools of either nymphs, males, or females, showing a strong geographic association between the three patients and the assumed vector.

Detection of the “Candidatus Neoehrlichia mikurensis” 16S rRNA gene was first described in 1999 from a tick isolated in The Netherlands and initially named the “Schottii variant” (1). After further sequence detections in ticks from Italy (2, 3), “Ca. Neoehrlichia mikurensis” was validly described as a novel intracellular pathogen in 2004 by a Japanese group investigating infections caused by members of the Anaplasmataceae in rats on the small island of Mikura, 60 km east of Tokyo, Japan (4). Since then, several groups reported the discovery of highly similar DNA sequences in potential arthropod vectors such as Ixodes ovatus, Ixodes persulcatus, and Ixodes ricinus ticks in eastern Asia (5), the Netherlands (1, 6–8), Belgium (6), Germany (9), Denmark (10), the Czech Republic (11), Slovakia (12), Russia (13), Italy (3, 14, 15), and recently Switzerland (16). In addition, “Ca. Neoehrlichia mikurensis” was found in rodent populations in China (17), Japan (18), Sweden (19), and the Netherlands (6). Moreover, an infection was described in a dog in Germany with symptoms similar to ehrlichiosis (20).

By mid-2012, a total of six European human cases of “Ca. Neoehrlichia mikurensis” bacteremia had been described in Sweden, Germany, Switzerland, and the Czech Republic (9, 21–23). “Ca. Neoehrlichia mikurensis” has also been detected in several parts of Asia (4, 5, 17). Only very recently (October 2012) seven human infections in China were described (24). Specific serological tests have not been established, and cross-reactivity with serologies for Ehrlichia spp., Anaplasma spp., and Rickettsia spp. has not been observed (24; also unpublished data). So far, all human infections were detected by 16S rRNA broad-range PCR followed by sequencing of the amplicon. The infection was fatal in one case and characterized by an unspecific sepsis syndrome with fever, malaise, and weight loss in the other cases. Cure was achieved by antibiotic treatment covering intracellular pathogens (tetracyclines alone or in combination with rifampin). Ticks are considered the most probable vector although formal proof for transmission from ticks to humans has not yet been provided.

This study describes two new human cases of “Ca. Neoehrlichia mikurensis” infections in the Zurich area. In this context, we aimed at (i) the development of a diagnostic multiplex real-time PCR for the rapid and accurate detection of “Ca. Neoehrlichia mikurensis” in clinical and environmental samples and (ii) the detection of “Ca. Neoehrlichia mikurensis” in tick populations collected in the patients’ residential areas.

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CASE REPORTS

Between October 2011 and January 2012, two cases of human neoehrlichiosis were diagnosed in our molecular diagnostic laboratory. Patient 1, a 68-year-old Swiss locksmith with a history of chronic lymphocytic leukemia (CLL), was referred to the University Hospital Zurich because of fever of unknown origin (FUO). He had a 4-week history of recurrent fever attended with chills, night sweats, weight loss, and pain in his left temporomandibular joint. The history of the patient was remarkable for clinically asymptomatic tick bites years ago and extensive travel all over the world. He was a dog owner and went for regular walks in the area surrounding his home. On admission, he was in reduced general condition, with a body temperature of 39.7°C. Laboratory findings showed normochromic anemia with a hemoglobin of 106 g/liter, leukocytosis of 19.1 × 10^9 cells/liter (94% neutrophils), an elevated C-reactive protein (CrP) level of 65 mg/liter (normal value, <5 mg/liter), and a procalcitonin level of 20.2 μg/liter (normal value, <0.5 μg/liter). A progression of CLL or therapy-associated myelodysplastic syndrome had been excluded by bone marrow biopsy specimen and splenectomy prior to hospitalization. The patient did not respond to empirical antibiotic therapy with meropenem. Repeated microbiological and immunological analyses as well as various imaging procedures (echocardiography, computed tomography [CT] scans of thorax and abdomen, and positron emission tomography [PET]) failed to reveal an infectious, malignant, or autoimmune cause of the fever.

Patient 2, a 58-year-old computer expert, was referred to the outpatient clinic at the Kantonsspital in Winterthur, Switzerland, in January 2012 because of progressive deterioration of his general condition for 3 months. He complained of pronounced fatigue, daily fever up to 40°C, chills, night sweats, and weight loss of 5 kg. He was under maintenance treatment with rituximab every 3 months for lymphoma and was taking a vitamin K antagonist (phenprocoumon) for recurrent deep vein thrombosis. Follicular lymphoma stage IV with involvement of the bone marrow and several lymph node regions on both sides of the diaphragm had been diagnosed 1 year earlier and was treated with six cycles of R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) chemotherapy until June 2011. He had reached complete clinical, biochemical, and radiological remission by August 2011 and thus had resumed his usual full-time work. He had no exposure to pets and did not recall any tick bites. Upon diagnosis, both patients received oral antibiotic treatment with doxycycline (100 mg twice daily [BD]) for 6 weeks. Patient 1 became afebrile within 2 days, and clinical symptoms improved rapidly. On day 28 after diagnosis, bacterial broad-range PCR of a blood sample was negative for the first time and has remained negative ever since (Fig. 1). In Patient 2, fever subsided within 5 days, and after 2 weeks, when the PCR was already negative, the patient had gained 6 kg. Antibiotic treatment was continued, and the patient made a full clinical recovery without any further signs of recurrence of neoehrlichiosis despite reintroduction of rituximab treatment.

MATERIALS AND METHODS

Clinical samples and DNA extraction. Blood and bone marrow samples collected from three patients suffering from symptomatic neoehrlichiosis were used to establish the real-time PCR. One of these patients has been described previously (21). In parallel, all samples were routinely tested by broad-range PCR targeting the 16S rRNA gene (25). DNA was extracted from 1 ml of blood or bone marrow aspirate (stored at −20°C) with an EZ1 DNA Tissue Kit (Qiagen, Hombrechtikon, Switzerland) following the manufacturer’s instructions. DNA extracts were eluted in 50 μl of PCR-grade water (Limulus amebocyte lysate [LAL] water; Lonza, Walkersville, MD) of which 5 μl was tested undiluted and in a 1:5 (broad-range PCR) or 1:1,000 dilution (species-specific assay) in order to reduce residual inhibitors of DNA polymerase activity.

All patients gave their written informed consent for the study.

Tick populations. A total of 1,916 questing ticks were collected in 2009 at four collection sites in the greater Zurich area (Switzerland) by flagging low vegetation (see Fig. 4). The collection sites were Winterthur (+47°36′51″, +8°43′44″; altitude, 480 m), Bassersdorf (+47°26′20″, +8°36′40″; 470 m), Rümlang (+47°26′20″, 480 m), and Rüti ZH (+47°15′40″, +8°52′60″; 600 m). Ticks were identified based on morphological characteristics and immediately stored at −80°C. Subsequently, ticks were washed once in 75% ethanol and twice in deionized water, dried on paper towels, and sorted into pools of 10 nymphs or of 5 adult male or female ticks. DNA extracts from these pools were prepared by adding 600 μl of phosphate-buffered saline (PBS) and one 3-mm tungsten carbide bead (Qiagen) to each frozen tick pool, followed by homogenization for 4 min at 30 Hz using a TissueLyser system (Qiagen). After a short centrifugation step (5 s at 3,220 × g), the supernatants were collected in separate collection microtubes for further use. Nucleic acid extraction was performed using a QIAasympyhm Virus/Bacteria MIDI Kit (Qiagen) and a specially adapted protocol (CP Complex 920 F1X, version 1; Qiagen). DNA was eluted in a final volume of 60 μl and either directly used for downstream applications or stored at −80°C for further use.

Minimal prevalence of “Ca. Neoehrlichiia mikurensis” was determined for pools of nymphs and female and male ticks based on the assumption that at least one tick in each positive pool was infected with “Ca. Neoehr-
lence rates for males, females, and nymphs.

Positive-control plasmid. The positive-control plasmid pCNM1 containing a 400-bp segment of the 5′ end of the 16S rRNA gene (Escherichia coli 16S rRNA gene positions 14 to 414) was constructed using in silico design and de novo synthesis and subcloning (Genscript, CA). Plasmid DNA was purified from transformed Invitrogen Escherichia coli XL-1 blue (Life Technologies) using Wizard Plus Midiprep (Promega, Basel, Switzerland) and quantified by spectrophotometric analysis on the basis of plasmid size and the corresponding DNA mass using both a NanoDrop 2000 instrument (Thermo Fisher Scientific, Wohlen, Switzerland) and Invitrogen Quant-iT PicoGreen chemistry (Life Technologies).

Multiplex real-time PCR. Real-time PCR was performed on an Applied Biosystems 7500 fast instrument with 7500 System software (version 2.0.4). Each 25.5-μl PCR mixture contained 12.5 μl of 2X PCR Mastermix (Roche Diagnostics, Rotkreuz, Switzerland), 2.5 μl of 10× exogenous internal positive-control primer and probe mix (VIC-labeled), 0.5 μl of 50× exogenous internal positive-control target DNA (both, Life Technologies), 1.0 μl of each primer (stock concentration, 10 μM) and probe (stock concentration, 2.5 μM), and a 5.0-μl sample of DNA extract. The exogenous internal positive-control reagents were added to distinguish truly negative from falsely negative results due to PCR inhibition. PCR conditions were 120 s at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

The analytical sensitivity of the assay and reproducibility of the test results were determined by repeated testing of 10-fold dilutions of the plasmid positive control ranging from 5 × 10^8 to 5 × 10^3 copies in 10 independent runs. In addition, PCR-grade water (LAL water) was used as a negative control (Fig. 3). Specificity was evaluated by testing DNA ex-

**FIG 1 Detection of “Ca. Neoehrlichia mikurensis” in clinical samples of two patients with symptomatic neoehrlichiosis diagnosed in November 2011 and January 2012, respectively.** Shown are polyacrylamide gel electrophoresis analyses of broad-range 16S rRNA gene PCR products (insets) and the amplification plots for the “Ca. Neoehrlichia mikurensis” TaqMan species probe (Neo_spec) obtained from the same samples. Day 0 represents the start of antibiotic therapy.

(A) Patient 1, blood and bone marrow samples taken on days 28, and 49. (B) Patient 2, blood and bone marrow samples obtained on days 1, 15, 29, and 58. The C_{\text{t}} values for samples taken on days 12 and 1 correspond to 1.05 × 10^6 16S rRNA gene copies/ml bone marrow and 7.01 × 10^6 16S rRNA gene copies/ml blood, respectively. Samples were tested undiluted and in a 1:5 (16S rRNA gene broad-range PCR) or 1:1,000 (multiplex real-time PCR) dilution to reduce PCR inhibition. M, molecular mass standard; FAM, normalized reporter dye fluorescence; d, day. E. coli chromosomal DNA was used as a positive control for the broad-range 16S rRNA PCR assay. Dilutions were tested to detect possible PCR inhibitions.

**Primer design and TaqMan hydrolysis probes.** 16S rRNA sequences available from the GenBank database and representing all known genera of the *Anaplasmataceae* family were aligned using Lasergene MegAlign software (DNASTAR, Madison, WI) (Fig. 2). Real-time PCR primers and TaqMan hydrolysis probes were chosen using PrimerExpress software, version 3.0 (Life Technologies, Zug, Switzerland) following visual inspection of the aligned target sequences: Ana_for (5′-ATC CTG GCT CAG AAC GAA CG-3′), Neo_rev (5′-TGA TCG TCC TCT CAG ACC AGC-3′), Neo_spec (5′-6FAM-ACC CAT AGT AAA CTA CAG CTA CA-MGB-3′), Neo_genus (5′-NED-TAA CAC ATG CAA GTC GAA C-MGB-3′), where FAM is 6-carboxyfluorescein and MGB is minor groove binder), Neo_family (5′-Cy5-CTA GTA GTA TGG AAT AGC TGT TAG A-BBQ-3′), and Ana_family (5′-NED-2,7,8-trichloro-5-carboxyfluorescein). The forward primer Ana_for was designed by modification of the previously published broad-range *Anaplasmataceae* family amplification primer EE1 (26) in order to optimize its melting temperature for the reaction conditions of the “Ca. Neoehrlichia mikurensis” real-time PCR. For the same purpose, the Neo_genus probe was modified with four locked nucleic acid (LNA) bases (Exiqon, Vedbaek, Denmark) at the 5′ end. The *Anaplasmataceae* family probe (Ana_family) was used as previously published except for MGB modification (27). All primers and probes were checked for cross-reactivity with other published DNA sequences using the NCBI BLASTN algorithm.
tracts of four closely related members of the Anaplasmataceae, two Rickettsia spp., Francisella tularensis, Borrelia burgdorferi, and human DNA. To further assess the assay specificity, amplification products from nine tick pools tested either positive or negative with the "Ca. Neoehrlichia mikurensis" probe were sequenced and compared to known sequences using the NCBI BLAST tool.

RESULTS


A TaqMan-based real-time PCR allowing for highly specific and sensitive detection of both "Ca. Neoehrlichia mikurensis" and other bacteria belonging to the Anaplasmataceae family in clinical and environmental samples was developed based on homology analysis of the 16S rRNA genes present in public databases. A 280-bp fragment was selected at the 5’ end of the 16S rRNA gene, which allowed for the design of a "Ca. Neoehrlichia mikurensis"-specific probe (Neo_spec), a "Ca. Neoehrlichia" genus probe (Neo_genus), and an Anaplasmataceae family probe (Ana_family). Using different fluorescent labels with emission maxima in the blue (FAM), yellow (NED), or far-red (Cy5) part of the visible spectrum, all three targets can simultaneously be detected in one reaction (Fig. 2).

The specificity of the probes was analyzed by testing chromosomal DNA of Ehrlichia canis, Ehrlichia ruminantium, Anaplasma phagocytophilum, Wolbachia pipientis, Rickettsia helvetica, Rickettsia monacensis, Borrelia burgdorferi, and Francisella tularensis. No cross-reactions were observed for the "Ca. Neoehrlichia mikurensis" probe. The "Ca. Neoehrlichia" genus probe showed only cross-reactivity with Ehrlichia ruminantium. The Anaplasmataceae family probe detected all Anaplasmataceae family members tested and in addition members of the closely related Rickettsiaceae family, as shown for Rickettsia helvetica and Rickettsia monacensis. None of the probes reacted with Borrelia burgdorferi or Francisella tularensis.

The analytical sensitivity of the assay was determined using plasmid pCNM1, which contains a partial 16S rRNA gene of "Ca. Neoehrlichia mikurensis" including the 280-bp region targeted by the real-time PCR. Repeated testing of 10-fold serial dilutions of purified pCNM1 DNA in 10 independent runs consistently showed that the limit of detection for "Ca. Neoehrlichia mikurensis" by either the species or the genus probe was below 5 copies of the 16S rRNA gene. No amplification was detected for a template dilution of 5^(-10) copies and the negative control (Table 1 and Fig. 3).

Evaluation of the real-time PCR for detection of "Ca. Neoehrlichia mikurensis" DNA in clinical samples.

To evaluate the suitability of the "Ca. Neoehrlichia mikurensis" assay in the routine diagnostic laboratory, patient specimens of the two new cases described above were tested and compared with the results of the broad-range 16S rRNA PCR assays. Testing of DNA extracts of all blood and bone marrow samples obtained from the two patients showed a complete concordance between TaqMan-based and...
broad-range 16S rRNA gene PCR results (Fig. 1). By generating a standard curve from 10-fold serial dilutions of positive-control plasmid pCNM1, an estimate was made of the number of 16S rRNA gene copies per ml of patient sample. Bacterial DNA loads for patient 1, diagnosed in November 2011 using bone marrow and blood specimens obtained at days 30, 8, 1, 6, 14, 28, and 49 (as calculated from the initiation of antibiotic therapy) were determined based on the obtained threshold cycle (C_T) values. Bacterial loads were estimated to be $1.48 \times 10^4$ 16S rRNA gene copies/ml bone marrow at day 30, $3.53 \times 10^7$ 16S rRNA gene copies/ml bone marrow at day 8, $1.32 \times 10^6$ 16S rRNA gene copies/ml blood at day 6, and $4.53 \times 10^5$ 16S rRNA gene copies/ml blood at day 14. Blood samples obtained at 28 and 49 days after initiation of antibiotic treatment were negative using both tests. Similar calculations were made for patient 2, showing bacterial loads of $1.05 \times 10^5$ copies/ml....
TABLE 1 Analytical sensitivity of the three TaqMan hydrolysis probes used in the “Ca. Neoehrlichia mikurensis” multiplex real-time PCR

<table>
<thead>
<tr>
<th>Probe</th>
<th>Plasmid copy no. tested or control</th>
<th>Avg C&lt;sub&gt;T&lt;/sub&gt; value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Neo_spec</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>22.43 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>24.86 ± 0.25</td>
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<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>27.01 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>29.87 ± 0.33</td>
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<td></td>
<td>10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>33.14 ± 0.31</td>
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<td></td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>35.27 ± 1.04</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Neo_genus</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>24.35 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>26.66 ± 0.17</td>
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<tr>
<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>28.72 ± 0.22</td>
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<td></td>
<td>10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>31.37 ± 0.42</td>
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<tr>
<td></td>
<td>10&lt;sup&gt;1&lt;/sup&gt;</td>
<td>33.86 ± 0.17</td>
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<tr>
<td></td>
<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>35.11 ± 0.80</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Ana_family</td>
<td>10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>25.51 ± 0.56</td>
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<td></td>
<td>10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>27.95 ± 0.37</td>
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<td></td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>30.11 ± 0.36</td>
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<td>32.22 ± 0.44</td>
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<td>10&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>ND</td>
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</table>

<sup>a</sup> Shown are the average C<sub>T</sub> values and standard deviations for serial dilutions of the positive-control plasmid pCNM1, which contains a partial 16S rRNA gene of “Ca. Neoehrlichia mikurensis.” Each data point was measured in 10 separate PCRs. ND, not detected.

10<sup>6</sup> 16S rRNA gene copies/ml bone marrow at day −12 and 7.01 × 10<sup>6</sup> 16S rRNA gene copies/ml blood at day 1. Blood samples obtained on days 15, 29, and 58 after the start of antibiotic therapy were negative using both tests.

Detection of “Ca. Neoehrlichia mikurensis” in local I. rici-nus tick populations. Since all three patients pursued regular outdoor activities and since ticks are considered to be the main zoonotic vector of “Ca. Neoehrlichia mikurensis,” four forest sites in close proximity to the patients’ residential areas (<3-km distance) were analyzed for the presence of questing ticks carrying “Ca. Neoehrlichia mikurensis” (Fig. 4). For this purpose, DNA from 1,916 ticks initially collected in 2009 for viral pathogen screening (28) were tested using the “Ca. Neoehrlichia mikurensis” real-time PCR. To facilitate fast screening of large populations, ticks were separated into groups of nymphs, males, and females and pooled (5 to 10 per pool) before DNA extraction.

The weighted means for the prevalence of “Ca. Neoehrlichia mikurensis” were 0.9% (Winterthur), 2.4% (Bassersdorf), 1.9% (Rümlang), and 4.7% (Rüti). This calculation was made based upon the assumption that at least one tick per positive pool was infected with “Ca. Neoehrlichia mikurensis.” Furthermore, test results showed that at every sampling site, the prevalence was between 3.5% and 8% in at least one of the three subpopulations (nymphs, females, or males) (Fig. 4).

PCR products from three tick pools which tested positive in the “Ca. Neoehrlichia mikurensis” TaqMan assay were sequenced. Furthermore, DNA from the same pools was separately analyzed by broad-range PCR targeting the 16S rRNA gene. Along the 226-bp section of the 16S rRNA gene that could be compared, the electropherograms obtained by either method showed complete identity to the “Ca. Neoehrlichia mikurensis” sequences obtained from the clinical specimens. In addition, several “Ca. Neoehrlichia mikurensis”-negative, but Anaplasmataceae family probe-positive pools showed 16S rRNA sequences of Rickettsia helvetica, Wolbachia pipientis, and “Candidatus Hamiltonella defensa.”

DISCUSSION

“Ca. Neoehrlichia mikurensis” is an emerging human pathogen causing septicemia and clinical symptoms such as relapsing fever, malaise, and weight loss (21). So far, six human case reports have been published, in 2010 and 2011, from four different European countries, including Germany (n = 2), Sweden (n = 1), Switzerland (n = 1), and the Czech Republic (n = 2), showing that “Ca. Neoehrlichia mikurensis” is widespread over Europe (9, 21–23). This is supported by several reports of the detection of “Ca. Neoehrlichia mikurensis” in questing tick populations in different European countries, with an average prevalence between 5 and 8% (29). In this study, we describe two new human cases of neoehrlichiosis in immunocompromised patients. Both were identified in

FIG 4 Geographic association between three patients diagnosed with neoehrlichiosis in 2009 (Kloten area), 2011 (Rüti area), and 2012 (Winterthur area) and infection rates of “Ca. Neoehrlichia mikurensis” in nymphal and adult Ixodes ricinus ticks (total n = 1,916) collected in four forests in the greater Zurich area in September 2009 (black arrowheads). The gray circles depict areas (radius, 3 km) that include both tick collection sites and the patients’ homes. Of note, only C<sub>T</sub> values of <35.0 were counted as positive for the Anaplasmataceae family probe (12 pools showed C<sub>T</sub> values of >35.0). The minimal percentage of positive ticks was calculated based on the fact that at least 1 tick per pool must have carried “Ca. Neoehrlichia mikurensis” DNA. The map in the figure is used with permission from swisstopo.
close geographic proximity to each other as well as to the first patient we diagnosed in 2009 (Fig. 4) (21). This indicates that the greater Zurich region is a high-risk area for “Ca. Neoehrlichia mikurensis” infections, especially for immunocompromised individuals. All three patients have in common that they frequently went for outdoor activities, e.g., gardening, hiking, and playing golf. Two patients were dog owners, but blood samples from both dogs tested negative for “Ca. Neoehrlichia mikurensis” DNA (data not shown). Although both patients did not recall any tick bites at least during the last 2 years, this is still the most likely mode of transmission given that (i) I. ricinus and other tick species are highly prevalent in the area, (ii) a significant percentage of the investigated ticks tested positive for “Ca. Neoehrlichia mikurensis” DNA (Fig. 4), and (iii) only 50 to 70% of patients with Lyme disease remember a tick bite (30). In addition, none of the patients reported having come into contact with rodents, another potential source of infection.

Until now, in all patients infected with “Ca. Neoehrlichia mikurensis,” the pathogen has been detected by rather laborious broad-range 16S rRNA gene analysis followed by sequencing. However, human infections with “Ca. Neoehrlichia mikurensis” may have been underdiagnosed in the past due to a lack of diagnostic techniques such as serology or species-specific PCR. In this context, the apparent increase of neoehrlichiosis may also be explained by increasing awareness among physicians.

Recently, a “Ca. Neoehrlichia mikurensis” reverse line blot has been developed and used to test ticks, but it has not been applied to the testing of human samples (16). In order to extend diagnostic tools for specific, sensitive, fast, and cost-efficient testing and screening purposes, we developed a TaqMan-based real-time PCR targeting the 16S rRNA gene sequence of “Ca. Neoehrlichia mikurensis” (Fig. 2). The amplification product of this assay is 280 bp in size, which allows for sequencing and specificity control. Results show that the limit of detection is approximately five copies per reaction (Fig. 3). Since chromosomes of members of the Anaplasmataceae family contain a single 16S rRNA gene (31, 32), we made the same assumption for “Ca. Neoehrlichia mikurensis” and made the 16S rRNA gene count equal to the number of bacterial cells.

Homology analysis showed a certain degree of variability in the 16S rRNA gene of different “Ca. Neoehrlichia mikurensis” strains (Fig. 2). To overcome this problem, which may lead to decreased assay sensitivity, two TaqMan probes were constructed. The first probe (Neo_spec) was optimized for the detection of the “Ca. Neoehrlichia mikurensis” sequence which is typically found in Switzerland (GenBank accession number GQ501090). All sequences obtained from the three patients and various ticks from Switzerland showed identical 16S rRNA sequences. Cross-reactivity of this probe was not observed. The second probe (Neo_genus) was designed to hybridize with all currently published sequence variants of “Ca. Neoehrlichia spp.,” including “Candidatus Neoehrlichia lorisotus” (33) and some of the most closely related species such as Ehrlichia ruminantium. In case of negative Neo_spec and positive Neo_genus signals, sequencing of the PCR product may reveal new sequences. In these cases, it may also be useful to perform an additional 16S rRNA gene broad-range PCR. In this study, no samples with negative Neo_spec probe and positive Neo_genus probe signals were detected. The assay contains an additional third probe, which detects members of the Rickettsiales order (Fig. 2). This probe is considered an additional control and can also detect other infectious agents of the Anaplasmataceae and Rickettsiales families. Using this probe, we found that almost all tick pools analyzed were positive for DNA of Anaplasmataceae, for example, Anaplasma phagocytophilum and Rickettsia helvetica, which were identified by sequencing of the PCR amplicon.

The assay we describe here serves as a valuable complementation of current diagnostic protocols since it facilitates both semi-quantitative detection of “Ca. Neoehrlichia mikurensis” DNA in human samples (Fig. 1 and 3) and rapid screening of tick populations for epidemiological purposes (Fig. 4). Data show that results of the real-time PCR were fully consistent with the results obtained by the broad-range 16S rRNA gene PCR (Fig. 1). By comparing Ct values with a standard curve generated from serial dilutions of the positive-control plasmid pCNM1, we were able to estimate for the first time the bacterial DNA load in clinical samples before and during antibiotic treatment. In both patients presented in this study, initial DNA concentrations were relatively high (Fig. 1). However, bacterial DNA was completely cleared from the bloodstream at most by 4 weeks after initiation of antibiotic treatment with doxycycline. In patient 2, both broad-range and real-time PCR were already negative after 2 weeks of antibiotic treatment. In addition to the rapid improvement of clinical symptoms, these data prove the excellent therapeutic efficacy of doxycycline against “Ca. Neoehrlichia mikurensis.”

Some DNA extracts from bone marrow and blood specimens showed inhibitory effects in the PCR (Fig. 1). This is a well-known phenomenon, which can be overcome by parallel testing of (up to 1,000-fold) dilutions of the DNA extracts although sensitivity will decrease as a consequence. Further improvement of DNA extraction methods from blood is therefore desirable.

After establishing the real-time PCR, we screened questing I. ricinus tick populations collected in forests located less than 3 km from the patients’ residences. The assay proved to be a powerful tool for high-throughput screening of close to 2,000 ticks using 96-well microtiter plates. The minimal prevalence rates for “Ca. Neoehrlichia mikurensis” DNA in I. ricinus ticks at the four sampling sites located in the Zurich area (eastern Switzerland) were slightly lower than the prevalence data recently published for western Switzerland (6.4%) (16). However, since it was assumed that only one tick of each positive pool carried DNA from “Ca. Neoehrlichia mikurensis,” the true prevalence in the tick populations of the Zurich area may be higher. Together, these data support the finding that ticks in Switzerland are commonly infected with “Ca. Neoehrlichia mikurensis” without a clear predominance for nymphs or female or male ticks (Fig. 4).

In conclusion, the real-time multiplex PCR described here provides a highly sensitive and specific tool that can assist clinical microbiologists and researchers in detection of “Ca. Neoehrlichia mikurensis” as an emerging pathogen in both human and animal samples. The close geographic association of tick collection sites with the affected patients’ homes strongly supports the paradigm that neoehrlichiosis is a vector-borne disease transmitted by ticks.

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REFERENCES