Close geographic association of human neoehrlichiosis and tick populations carrying *Candidatus* Neoehrlichia mikurensis in Eastern Switzerland.

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Abstract
Neoehrlichiosis caused by Candidatus Neoehrlichia mikurensis is an emerging zoonotic disease. In total, 6 patients have been described in Europe with the first case detected in 2008 and 7 patients in China published in October 2012. In 2009, we diagnosed the first human case of C. 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 a similar clinical syndrome including fever, malaise and weight loss. A diagnostic multiplex real-time PCR was developed for specific detection of C. Neoehrlichia mikurensis infections. The assay is based on the signature sequence of a 282-bp fragment of the C. Neoehrlichia mikurensis 16S rRNA gene and incorporates a C. Neoehrlichia mikurensis species, a Neoehrlichia genus and an Anaplasmataceae family probe for simultaneous screening. The analytical sensitivity of was determined to be below 5 copies of the C. Neoehrlichia mikurensis 16S rRNA gene. Our results show that the assay is suitable for the direct detection of C. Neoehrlichia mikurensis DNA in clinical samples like blood and bone marrow. In addition, it allows for monitoring treatment response during antibiotic therapy. Using the same assay, DNA extracts from 1916 ticks collected in four forests in close proximity to the patients’ residences (<3 km) were screened. At all sampling sites, the minimal prevalence of C. Neoehrlichia mikurensis was between 3.5-8% in pools of either nymphs, males or females, showing a strong geographic association between the three patients and the assumed vector.
Introduction

Detection of Candidatus Neoehrlichia mikurensis 16S rRNA gene DNA was first described in 1999 from a tick isolated in The Netherlands and initially named the "Schottii variant" (26). After further sequence detections in ticks from Italy (4, 25), C. Neoehrlichia mikurensis was validly described as a novel intracellular pathogen in 2004 by a Japanese group investigating Anaplasmataceae infections in rats on the small island of Mikura, 60 km east of Tokyo (14). Since then, several groups reported the discovery of highly similar DNA sequences in potential arthropod vectors such as Ixodes ovatus, I. persulcatus and I. ricinus ticks in eastern Asia (29), The Netherlands (13, 19, 26, 30), Belgium (13), Germany (31), Denmark (10), the Czech Republic (21), Slovakia (28), Russia (23), Italy (5, 6, 25), and recently Switzerland (16). In addition, C. Neoehrlichia mikurensis was found in rodent populations in China (20), Japan (18), Sweden (1) and the Netherlands (13). Moreover, an infection was described in a dog in Germany with symptoms similar to ehrlichiosis (7).

By mid-2012, a total of 6 European human cases of C. Neoehrlichia mikurensis bacteraemia have been described in Sweden, Germany, Switzerland and the Czech Republic (9, 22, 31, 32). C. Neoehrlichia mikurensis has also been detected in several parts of Asia (14, 20, 29). Only very recently (October 2012) seven human infections in China were described (15). Specific serological tests have not been established and cross reactivity with serologies for Ehrlichia spp., Anaplasma spp. and Rickettsia spp. has not been observed (unpublished data; 15). 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 characterised 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 *C. 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 *C. Neoehrlichia mikurensis* in clinical and environmental samples and (ii) the detection of *C. Neoehrlichia mikurensis* in tick populations collected in the patients’ residential areas.

**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 leukaemia (CLL), was referred to the University Hospital Zurich because of fever of unknown origin (FUO). He had a four-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 travelling 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 anaemia with a haemoglobin of 106 g/L, leucocytosis of 19.1 Gpt/L (94% neutrophils), an elevated C-reactive protein (CrP) level of 65 mg/L (normal value: <5 mg/L) and a procalcitonin level of 20.2 μg/L (normal value: <0.5 μg/L). A progression of CLL or therapy-associated myelodysplastic syndrome had been excluded by bone marrow biopsy and splenectomy prior to hospitalisation. The patient did not respond to empiric antibiotic therapy with meropenem. Repeated microbiological and immunological analyses as well as various imaging procedures (echocardiography, computed tomography scans of thorax and
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 one year earlier and was treated with 6 cycles of R-CHOP chemotherapy (Rituximab, Cyclophosphamide, Doxorubicin, Vincristin, Prednisolone) 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. However, he frequently went for outdoor walks in nearby forests. Prior to referral, blood cultures were negative, an echocardiogram was normal, serologies for HIV, EBV and CMV were negative or indicated past infection, respectively, and a PET-CT scan was unremarkable beside some diffuse but unspecific glucose uptake of the bone marrow. In particular, PET-CT showed no signs for recurrent lymphoma. On admission, the patient was in a markedly reduced general condition. He was underweight with a body mass index of 19 kg/m², temperature was normal (36.7 °C), blood pressure was low (95/60 mmHg), his pulse was regular (100/min) and there were no cardiac murmurs. Lung, abdomen, joints, neurology and skin were unremarkable. There was oral thrush and an enlarged inguinal lymph node on the left. His blood tests revealed normochromic, normocytic anaemia (haemoglobin 93 g/L), normal platelet and leucocyte counts (6.2 Gpt/L), profound lymphopenia (0.22 Gpt/L), reduced sodium of 127 mmol/L, substantial inflammation...
(erythrocyte sedimentation rate: 94 mm/h, CrP: 92 mg/L), reduced IgM and IgG-levels and a slightly elevated lactate dehydrogenase of 481 U/L. Hepatic and renal parameters were within normal limits and his prothrombin time was on target (INR 2.48). Additional blood cultures remained negative. A bone marrow biopsy was compatible with chronic inflammation or regeneration after R-CHOP chemotherapy but without any signs of recurrent lymphoma.

In both cases, broad-range PCR targeting the bacterial 16S rRNA gene followed by sequence analysis of the amplification product detected *C. Neoehrlichia mikurensis* DNA in peripheral blood and bone marrow samples (Fig. 1; sequence homology analysis showing 0/444 bp and 0/457 bp mismatches with the *C. Neoehrlichia mikurensis* 16S rRNA gene, respectively). Upon diagnosis, both patients received oral antibiotic treatment with doxycycline (100 mg BD) for 6 weeks. Patient 1 became afebrile within two 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 remained negative ever since (Fig. 1). In patient 2, fever subsided within 5 days and after two weeks, when PCR was already negative, the patient had gained 6 kg. Antibiotic treatment was continued and the patient made a full clinical recovery without any signs for recurrence of neoehrlichiosis ever since despite re-initiation 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 (9). In parallel, all samples were routinely tested by broad-range PCR targeting the 16S rRNA gene (3). 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 PCR-grade water (LAL water; Lonza, Belgium) of which 5 µL were tested undiluted and in 1:5 (broad-range PCR) or 1:1000 dilution (species-specific assay) in order to reduce residual inhibitors of DNA polymerase activity.

**Tick populations**

A total of 1916 questing ticks were collected in 2009 at 4 collection sites in the greater Zurich area by flagging low vegetation (Fig. 4). The collection sites were: Winterthur (+47°30'51", +8°43'44", altitude: 480 m), Bassersdorf (+47°26'20", +8°36'40", 470 m), Rümlang (+47°26'20", +8°31'1", 490 m) and Rüti ZH (+47°15'40", +8°52'60", 600 m). Ticks were identified based on morphological characteristics and immediately stored at -80 ºC. 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 5 adult male or female ticks, respectively. DNA extracts from these pools were prepared by adding 600 µL 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 the TissueLyser system (QIAGEN). After a short centrifugation step (5 sec at 3220 x g), the supernatants were collected in separate collection microtubes for further use. Nucleic acid extraction was performed using the QIAsymphony Virus/Bacteria Midi Kit (QIAGEN) and a specially adapted protocol (CP Complex 920 FIX v1, QIAGEN). DNA was eluted in a final volume of 60 µL and either directly used for downstream applications or stored at -80 ºC for further use.

Minimal prevalence of *C. Neoehrlichia mikurensis* was determined for pools of nymphs, female and male ticks based on the assumption that at least one tick in each positive pool was infected.
with *C. Neoehrlichia mikurensis*. The minimal total prevalence per collection site was determined by calculating the weighted means of the respective prevalence rates for males, females and nymphs.

**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, USA, 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'-Cy5-CTA GTA GTA TGG AAT AGC TGT TAG A-BBQ-3'), Ana_family (5'-NED-TAA CAC ATG CAA GTC GAA C-MGB-3'). The forward primer Ana_for was designed by modification of the previously published broad-range *Anaplasmataceae* amplification primer EE1 (2) in order to optimize its melting temperature for the reaction conditions of the *C. Neoehrlichia mikurensis* real-time PCR. For the same purpose, the Neo_genus probe was modified with 4 LNA bases (locked nucleic acid, Exiqon, Vedbaek, Denmark) at the 3' 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.

**Positive control plasmid**
The positive control plasmid pCNM1 containing a 400-bp segment of the 5'-end of the 16S rRNA gene (E. coli 16S rRNA gene positions 14-414), was constructed using in silico design and de novo synthesis and subcloning (Genscript, CA, USA). 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 Nano drop 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 Bioystems 7500 fast instrument with 7500 System software (version V 2.0.4). Each 25.5 µL PCR reaction contained 12.5 µL 2x PCR Mastermix (Roche Diagnostics, Rotkreuz, Switzerland), 2.5 µl 10x Exogenous Internal Positive Control Primer and Probe mix (VIC-labelled), 0.5 µl 50x Exogenous Internal Positive Control target DNA (both Life Technologies), 1.0 µL of each primer (stock concentration 10µM) and probe (stock concentration 2.5µM) and 5.0 µL sample 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 seconds at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds 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 x 10^5 to 5 x 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 extracts of 4 closely related Anaplasmataceae spp, 2 Rickettsia spp., Franciscella 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 C. Neoehrlichia mikurensis probe were sequenced and compared to known sequences using the NCBI BLAST tool.

Results

Development of a real-time PCR for the detection of C. Neoehrlichia mikurensis, Neoehrlichia genus and Anaplasmataceae family members

A TaqMan-based real-time PCR allowing for highly specific and sensitive detection of both C. 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 282-bp fragment was selected at the 5’-end of the 16S rRNA gene, which allowed for the design of a C. Neoehrlichia mikurensis specific probe (Neo_spec), a 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 analysed 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 C. Neoehrlichia mikurensis probe. The Neoehrlichia genus probe showed only cross reactivity with Ehrlichia ruminantium. The Anaplasmataceae family probe detected all Anaplasmataceae spp. 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 *C. Neoehrlichia mikurensis* including the 282-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 *C. 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 $5 \times 10^{-1}$ copies and the negative control, respectively (Table 1 and Fig. 3).

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

To evaluate the suitability of the *C. 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 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 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 days (as calculated from the initiation of antibiotic therapy), were determined based on the obtained Ct values. Bacterial loads were estimated to be $1.48 \times 10^4$ 16S-copies/mL bone marrow (day -30), $3.53 \times 10^7$ 16S-copies/mL bone marrow (day -8), $1.32 \times 10^5$ 16S-copies/mL blood (day 1), $8.67 \times 10^6$ 16S-copies/mL blood (day 6), $4.53 \times 10^5$ 16S-copies/mL blood (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 $1.05 \times 10^6$ 16S-copies/mL bone marrow (day -12) and $7.01 \times 10^6$ 16S-copies/mL blood (day 1). Blood samples obtained on days 15, 29 and 58 after the start of antibiotic therapy were negative using both tests.

**Detection of *C. Neoehrlichia mikurensis* in local *I. ricinus* tick populations**

Since all three patients pursued regular outdoor activities and since ticks are considered to be the main zoonotic vector of *C. Neoehrlichia mikurensis*, four forest sites in close proximity to the patients’ residential areas (< 3 km distance) were analysed for the presence of questing ticks carrying *C. Neoehrlichia mikurensis* (Fig. 4). For this purpose, DNA from 1916 ticks initially collected in 2009 for viral pathogen screening (11), were tested using the *C. Neoehrlichia mikurensis* real-time PCR. To facilitate fast screening of large populations, ticks were separated in nymphs, males and females and pooled (5-10 per pool) before DNA extraction.

The weighted means for the prevalence of *C. 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 *C. 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 3 tick pools, which were tested positive in the *C. Neoehrlichia mikurensis* TaqMan assay, were sequenced. Furthermore, DNA from the same pools was separately analysed 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 *C. Neoehrlichia mikurensis* sequences obtained from the clinical specimens. In addition, several *C. Neoehrlichia mikurensis* negative, but *Anaplasmataceae* probe positive pools
showed 16S rRNA sequences of *Rickettsia helvetica*, *Wolbachia pipientis* and *Hamiltonella defensa*.

**Discussion**

*C. Neoehrlichia mikurensis* is an emerging human pathogen causing septicaemia and clinical symptoms such as relapsing fever, malaise and weight loss (9). So far, six human cases were 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 *C. Neoehrlichia mikurensis* is widespread over Europe (9, 22, 31, 32). This is supported by several reports of the detection of *C. Neoehrlichia mikurensis* in questing tick populations in different European countries with an average prevalence between 5-8% (24). In this study, we describe two new human cases of neoehrlichiosis in immunocompromised patients. Both were identified in close geographic proximity to each other as well as to the first patient we diagnosed in 2009 (Fig. 4) (9). This indicates that the greater Zurich region is a high-risk area for *C. 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 were tested negative for *C. 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 were tested positive for *C. Neoehrlichia mikurensis* DNA (Fig. 4) and (iii) only 50-70% of patients with Lyme disease remember a tick bite (12). In addition, none of the patients reported having come into contact with rodents, being another potential source of infection.
Until now, in all patients infected with C. Neoehrlichia mikurensis, the pathogen has been detected by rather laborious broad-range 16S rRNA gene analysis followed by sequencing. However, human infections with C. 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 C. Neoehrlichia mikurensis reverse line blot has been developed and used to test ticks, but has not been applied to 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 C. Neoehrlichia mikurensis (Fig.2). The amplification product of this assay is 282 bp in size, which allows for sequencing and specificity control. Results show that the limit of detection is approximately 5 copies of the C. Neoehrlichia mikurensis 16S rRNA gene per reaction (Fig.3). Since chromosomes of Anaplasmataceae species contain only a single 16S rRNA gene (8, 17), we made the same assumption for C. Neoehrlichia mikurensis and equalled the 16S rRNA gene count to the number of bacterial cells.

Homology analysis showed a certain degree of variability in the 16S rRNA gene of different C. 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 C. 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 hybridise with all currently published sequence variants of C. Neoehrlichia spp., including...
Candidatus Neoehrlichia lotoris (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 allow to discover new sequevars. 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 as an additional control and can also detect other infectious agents of the Anaplasmataceae and Rickettsiaceae families. Using this probe, we found that almost all tick pools analysed were positive for DNA of Rickettsiales, 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 *C. 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 four weeks after initiation of antibiotic treatment with doxycycline. In patient 2, both broad-range and real-time PCR were already negative after two weeks of antibiotic treatment. In addition to the rapid improvement of clinical symptoms, these data prove the excellent therapeutic efficacy of doxycycline against *C. Neoehrlichia mikurensis*.  

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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 1000-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 2000 ticks using 96-well microtiter plates. The minimal prevalence rates for *C. 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 *C. 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 *C. Neoehrlichia mikurensis* without a clear preference for nymphs, 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 *C. 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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Ethics statement

All patients gave their written informed consent for the study.

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Transparency declarations

None do declare.
References


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<tr>
<td></td>
<td>$10^4$ copies</td>
<td>27.95 ± 0.37</td>
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<td>$10^2$ copies</td>
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<td>$10^1$ copies</td>
<td>34.15 ± 0.49</td>
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<td></td>
<td>$10^{-1}$ copies</td>
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<tr>
<td>Water</td>
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**Table 1:** Analytical sensitivity of the three TaqMan hydrolysis probes used in the *C. Neoehrlichia mikurensis* multiplex real-time PCR. Shown are the average Ct values and standard deviations for serial dilutions of the positive control plasmid pCNM1, which contains a partial 16S rRNA gene of *C. Neoehrlichia mikurensis*. Each data point has been measured in 10 separate PCR reactions.
Figure 1: Detection of C. 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 C. Neoehrlichia mikurensis TaqMan species probe (Neo_spec) obtained from the same samples. Day 0 represents the start of antibiotic therapy. Panel A: Patient 1, blood and bone marrow samples taken on days -30 (1.48 x 10^4 16S-copies/mL bone marrow), -8 (3.53 x 10^7 16S-copies/mL bone marrow), 1 (1.32 x 10^5 16S-copies/mL blood), 6 (8.67 x 10^5 16S-copies/mL blood), 14 (4.53 x 10^5 16S-copies/mL blood), 28 and 49. Panel B: Patient 2, blood and bone marrow samples obtained on days -12, 1, 15, 29 and 58. The Ct values for samples taken on days -12 and 1 correspond to 1.05 x 10^6 16S-copies/mL bone marrow and 7.01 x 10^6 16S-copies/mL blood, respectively. Samples were tested undiluted and in 1:5 (16S broad-range PCR) or 1:1000 (multiplex real-time PCR) dilutions to reduce PCR inhibition. MW: molecular weight standard; Rn: Normalized reporter dye fluorescence; E.coli chromosomal DNA was used as positive control for broad-range 16S rRNA PCR assay. Dilutions were tested to detect possible PCR inhibitions.
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<table>
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<td>baumannii</td>
<td>JCM01955-12</td>
<td>R1</td>
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Note: Additional information or context is not provided in the image.
Figure 2: Homology analysis of 2 regions within a 282-bp fragment of the 16S rRNA gene including hypervariable regions V1 and V2 in members of the Anaplasmataceae family. Sequences were aligned using the ClustalW algorithm. The reference sequence (GenBank accession no. GQ501090) was derived from a human case of neoehrlichiosis diagnosed by broad-range PCR targeting the 16S rRNA gene (9). The lines immediately above the reference sequence depict the position of the forward (Ana_for) and reverse (Neo_rev) primers as well as of the TaqMan probes designed for C. Neoehrlichia mikurensis species, Neoehrlichia genus and Anaplasmataceae family detection (Neo_spec, Neo_genus and Ana_family, respectively).
Figure 3: Analytical sensitivity of the multiplex real-time PCR test for detection of *C. Neoehrlichia mikurensis*, *Neoehrlichia genus* and *Anaplasmataceae* species. Panel A: *C. Neoehrlichia mikurensis* specific probe (Neo_spec). Panel B: *Neoehrlichia genus* probe (Neo_genus). Panel C: *Anaplasmataceae* family probe (Ana_family). A dilution series ranging from $10^5$ copies to $10^1$ copies of the plasmid pCNM1 containing a partial 16S rRNA gene of *C. Neoehrlichia mikurensis* (one copy per plasmid) was used for determining the
analytic sensitivity of the probes. The experiment was performed 10 times of which one representative run is shown. All runs showed comparable results. Rn: Normalized reporter dye fluorescence. H2O: negative water control
Figure 4: Geographic association between three patients diagnosed with neoehrlichiosis in 2009 (Kloten area), 2011 (Rütli area) and 2012 (Winterthur area) and infection rates of *C. Neoehrlichia mikurensis* in nymphal and adult *Ixodes ricinus* ticks (total n=1916) collected in four forests in the greater Zurich area in September 2009 (black arrows). The grey circles depict an area (radius 3km), which includes both tick collection sites and the patients’ residential areas. Of note: Only Ct values <35.0 were counted as positive for the *Anaplasmataceae* probe (12 pools showed Ct values >35.0). The map in the figure is used with permission from swisstopo.

*a* based on the fact that at least 1 tick per pool must have carried *C. Neoehrlichia mikurensis* DNA.