Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann¹, 2, N. Wagmann¹, 2, M. L. Meli¹, 2, B. Riond¹, M. Novacco¹, 2, D. Joekel⁴, F. Gentilini⁵, F. Marsilio⁶, M. G. Pennisi⁷, A. Lloret⁸, T. Carrapiço⁹, F. S. Boretti³

¹Clinical Laboratory, ²Center for Clinical Studies, ³Clinic for Small Animal Internal Medicine and ⁴Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland, ⁵Department of Veterinary Science, University of Messina, Polo Universitario dell’Annunziata, Messina, Italy, ⁶Faculty of Veterinary Medicine, University of Teramo, Località Piano d’Accio, Teramo, Italy, ⁷Department of Veterinary Science, University of Messina, Polo Universitario dell’Annunziata, Messina, Italy, ⁸Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra, Spain, ⁹Clinica Veterinaria Canham, Avenida Duarte Pacheco, Almancil, Portugal

Abstract

‘Candidatus Neoehrlichia mikurensis’ is an emerging tick-borne zoonotic agent that primarily affects immunocompromised human patients. Dogs and foxes are frequently exposed to ticks, and both species are in close proximity to humans. This is the first study to systematically investigate the occurrence of ‘Candidatus Neoehrlichia mikurensis’ in Canidae in Europe. We analyzed 1’739 blood samples from dogs in Switzerland, Italy, Spain and Portugal and 162 blood samples from free-ranging red foxes (Vulpes vulpes) in Switzerland. All samples were tested using a previously described multiplex real-time PCR for the Anaplasmataceae family, the ‘Candidatus Neoehrlichia’ genus and the ‘Candidatus Neoehrlichia mikurensis’ species. All Anaplasmataceae positive samples were subsequently tested using specific real-time PCRs for Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia canis and Rickettsia helvetica. Among the tested animals, one dog from Zurich tested positive for ‘Candidatus Neoehrlichia mikurensis’. The 12-year old West Highland white terrier had been splenectomized 3 months prior to the blood collection and presented with polyuria/polydipsia. Fanconi syndrome was diagnosed based on glucosuria with normoglycemia and hyperaminoaciduria. A. platys and E. canis were detected in 14/249 dogs from Sicily and Portugal; two of the dogs were coinfected with both agents. Four Swiss foxes tested positive for A. phagocytophilum, R. helvetica was detected for the first time in a red fox. In conclusion, ‘Candidatus Neoehrlichia mikurensis’ infection should

Nachweis von ‘Candidatus Neoehrlichia mikurensis’ und anderen Anaplasmataceae und Rickettsiaceae in Kaniden in der Schweiz und in Mittelmeerländern

Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann et al.

Introduction

‘Candidatus Neoehrlichia mikurensis’ is a tick-borne agent that has been implicated in human health (Fehr et al., 2010; von Loewenich et al., 2010; Silaghi et al., 2015). The coccoid Gram-negative bacterium belongs within the order Rickettsiales to the family Anaplasmataceae, genus ‘Candidatus Neoehrlichia’ (Dumler et al., 2001). PCR is used to diagnose infections with the uncultivable ‘Candidatus Neoehrlichia mikurensis’. ‘Candidatus Neoehrlichia’ was detected in ticks, such as Ixodes ricinus and other Ixodes species, Rhipicephalus sanguineus and Haemaphysalis concinna and leachi (Silaghi et al., 2015). In Switzerland, the prevalence of ‘Candidatus Neoehrlichia mikurensis’ in I. ricinus was estimated to be 6.4% in the western region and 3.5-8% in the eastern region of the country (Lommano et al., 2012; Maurer et al., 2013). The bacterium has also been detected in ticks in other European countries, including Germany, the Netherlands and Austria and pathogen specific DNA was detected in ticks collected from Danish and German dogs (Schreiber et al., 2014; Stensvold et al., 2015). Rodents may serve as a reservoir for ‘Candidatus Neoehrlichia mikurensis’. In the Neuchâtel region, Switzerland, 8% of mice were infected with ‘Candidatus Neoehrlichia mikurensis’ (Burri et al., 2014).

Neoehrlichiosis is an emerging zoonotic disease (Silaghi et al., 2015). Several patients with Neoehrlichiosis have been described in Europe, including a case in Switzerland (Fehr et al., 2010; von Loewenich et al., 2010; Silaghi et al., 2015). Most of the patients were immunocompromised and displayed signs of systemic inflammation and fever; some had painful muscles or joints and vascular/thromboembolic events. Hematology typically revealed leukocytosis, neutrophilia and anemia, and the C-reactive protein was frequently elevated. The patients fully recovered following doxycycline therapy. Dogs are frequently exposed to ticks. In 2007, ‘Candidatus Neoehrlichia mikurensis’ was detected in an 8-year-old female spayed Irish setter in Germany (Diniz et al., 2011). The dog had undergone bilateral mastectomy and ovariohysterectomy; subsequently, the animal became lethargic and exhibited profuse subcutaneous hemorrhages. The dog was mildly anemic and thrombocytopenic. After therapy with doxycycline the dog had recovered uneventfully (Diniz et al., 2011). ‘Candidatus Neoehrlichia mikurensis’ DNA was also found in paraffin-embedded kidney tissue of a 3.5 month old Croatian dog who died from hemolytic anemia (Beck et al., 2014) and most recently, a Candidatus Neoehrlichia sp. was detected in a red fox from Austria (Hodzic et al., 2015). However, so far no study has systematically tested a large number of dogs for the presence of ‘Candidatus Neoehrlichia mikurensis’.

The aim of this study was to investigate the occurrence of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries. For this purpose a convenience sample of dog samples from Switzerland, Italy, Spain and Portugal as well as fox samples from the Swiss cantons of Zurich and St Gallen were investigated by pathogen specific PCR.

Animals, Material and Methods

Sample collection from dogs and foxes

A total of 1’739 canine blood samples were available for this study. The samples were collected by veterinarians from dogs that presented at the respective clinics for two previous studies on canine hemoplasma infections (Wengi et al., 2008; Novacco et al., 2010) as follows:
Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann et al.

889 samples in Zurich, Switzerland, in 2005/2006; 200 samples each in Bologna, Teramo and Messina, Italy, and Barcelona, Spain, and 49 samples from Almácil, Portugal in 2008. One sample from Almácil was not obtainable. Data from each dog was recorded as previously described (Wengi et al., 2008; Novacco et al., 2010). Additionally, blood samples from 162 red foxes (>1 of the 691–700) were available; the foxes were shot during routine hunting events in the cantons of Zurich and St Gallen in 2012–2014 under the restrictions of the game laws of Switzerland. Sex and approximate age (young or adult), hunting location and time point were recorded.

Nucleic acid extraction
Total nucleic acids (TNA) were extracted from EDTA anticoagulated blood samples from dogs using the MagnaPure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Rotkreuz, Switzerland) as previously described (Wengi et al., 2008; Novacco et al., 2010). DNA was extracted from 100 μL of the fox samples using the DNaseasy Blood & Tissue Kit 50 (Qiagen, Hombrechtikon, Switzerland). TNA/DNA were eluted in 100 μL and stored at –80°C prior to use. The presence of amplifiable NA was confirmed previously for each canine sample using a real-time PCR either for the canine glyceraldehyde-3-phosphate dehydrogenase or the 18S rRNA gene (Boretti et al., 2009; Novacco et al., 2010). Within the present study, the presence of amplifiable NA was also confirmed for each fox sample using the 18S rRNA real-time PCR (Applied Biosystems, Rotkreuz, Switzerland); samples that showed inhibition were consequent-
ally used at a dilution of 1:10 (n = 35). Negative controls consisting of phosphate-buffered saline were extracted in parallel in all extraction procedures to monitor for cross-contamination.

PCR assays
A previously published real-time PCR was used to detect ‘ Candidatus Neoehrlichia mikurensis’ (Maurer et al., 2013). This PCR used a common primer pair (Ana_for; Neo_rev) and three different probes; one probe (Neo_spec) was designed to amplify all Swiss ‘ Candidatus Neoehrlichia mikurensis’ sequences known at that time; the second probe (Neo_genus) amplified the genus ‘ Candidatus Neoehrlichia’, including ‘ Candidatus Neoehrlichia litoris’ and some closely related agents, such as Ehrlichia ruminantium; and the third probe (Ana_family) amplified the Anaplasmataceae family and some members of the closely related Rickettsiaceae family (Maurer et al., 2013). The three probes were conjugated to different fluorescent reporter dyes so the signals could be differentiated within a run. The reaction contained 500 nM of each primer, 250 nM of each of the three probes, 0.25 μL of Utacil-DNA Glycosylase (UNG, Eurogentec S.A., Ougrée, Belgium) and 12.5 μl of the qPCR Mastermix (Eurogentec).

Ana_family-positive samples were analyzed by real-time PCR as described for Anaplasma phagocytophilum (Pusterla et al., 1999), Ehrlichia canis (Foley et al., 2007) and Rickettsia helvetica (Boretti et al., 2009). The samples were also tested by real-time PCR specific for the A. platys 16S rRNA gene using the following oligos: forward primer Aplat.14f (5′-CTG GGC GCA AGC TTA ACA C-3′), reverse primer Aplat.89r (5′-CTG CTG CCA CTA ATT TTT ATA ATC GC-3′) and probe Aplat_34p (5′-FAM-AGC TAC GAC AAA AAT CCG TTC GAC TTG CAT-AMRA-3′). The mastermix contained 500 nM of each primer, 250 nM probe, 0.25 μl of UNG (Eurogentec) and 12.5 μl of the qPCR Mastermix (Eurogentec). The cycling conditions were as follows: 2 min 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. Finally, if all four specific real-time PCR assays in the Ana_family-positive samples were negative, the samples were tested using a conventional PCR specific for Anaplasma spp. that amplified a 452 bp long target of the 16S rRNA gene as previously described (Goodman et al., 1996).

All real-time PCR assays in this study were run on an ABI 7500 FAST Real-Time PCR System (Applied Biosystems) with 5 μl of DNA/TNA in a 25 μl reaction. The conventional PCR assays were run on a Biometra TPersonal Thermocycler (BioLabo Scientific Instruments, Châtel-Saint-Denis, Switzerland). Negative, positive and extraction controls were included in each run.

Sequencing
The PCR products of the conventional Anaplasma spp. PCR (Goodman et al., 1996) were separated on a 2% agarose gel, and appropriately sized products (452 bp) were excised and purified using the GenElute Gel Extraction Kit (Sigma-Aldrich, Buchs, Switzerland). Direct sequencing of the purified amplicons or sequencing after cloning of the purified amplicons in the pCR4 vector using a TOPO TA Cloning Kit for sequencing (Life Technologies, Zug, Switzerland) was performed at a commercial laboratory (Microsynth, Balgach, Switzerland) under standard conditions. Plasmid DNA was purified using a QIAprep Spin Miniprep kit (Qiagen) and the M13 forward and reverse primers (Microsynth) if the amplicons were cloned. Moreover, the near-complete 16S rRNA gene of A. phagocytophilum, E. canis and A. platys positive samples were sequenced using nested PCR protocol as previously described (Barlough et al., 1996). The sequences were submitted to GenBank (KX180944 - KX180948).

Statistical analysis
For the observed prevalence, 95% confidence intervals were calculated and frequencies were compared using the Chi-square test (Graph-Pad Prism Software V6.04, San Diego, CA, USA).
Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries
R. Hofmann-Lehmann et al.

Results

‘Candidatus Neoehrlichia mikurensis’ in a splenectomized dog in Switzerland

Out of the 1'739 tested dog samples, one sample from a Swiss dog was positive for ‘Candidatus Neoehrlichia mikurensis’ (Tab. 1). The blood load was too low for sequencing (Ct-value of 37.7, which corresponds to 1.1 × 10^4 copies/mL of blood). The positive sample originated from a 12-year-old male West Highland white terrier that lived in Zurich. The dog was splenectomized due to a splenic mass in May, 2006. He recovered well after surgery but was referred due to polydipsia and polyuria at the blood collection time point for this study in September 2006. Results of the clinical examination were unremarkable except for a slightly tense abdomen. Hematology revealed mild neutrophilia, lymphopenia, monocytosis and thrombocytosis. The clinical blood chemistry analysis detected a mild increase in alkaline phosphatase, very mild hypokalemia and hypocalcemia. Moreover, the C reactive protein was slightly increased but the serum amyloid A was within the reference range. Urinalysis revealed profound glucosuria, mild proteinuria (urine protein to creatinine ratio 1.3) and pyuria (4-8 leukocytes per average high-power field in the sediment). Ultrasonic examination of the abdomen demonstrated the prostate to be moderately enlarged with multiple small cysts and small hyperechoic densities in the medulla of both kidneys. As a first line treatment, the dog received amoxicillin/clavulanate acid (12.5 mg/kg BID PO) because a urinary tract infection could not be excluded at that time. Further chromatographic urine analysis revealed severe hyperaminocuria; when combined with the glucosuria (normoglycemia and fructosamine at the lower end of the reference interval), the dog was diagnosed with Fanconi syndrome. Due to the good general condition and the only mildly decreased potassium, treatment was withheld at that time but the owner was encouraged to present the dog at regular intervals for re-checks. In April 2007, the dog was presented due to acute vomiting, diarrhea and hyporexia. The dog was in good clinical condition but had lost 1 kg of body weight since its first presentation. The blood analysis demonstrated a moderate increase in the blood urea nitrogen (BUN), creatinine and phosphate concentrations, moderate thrombocytosis (704,000/μL; 95% quantile of reference values: 394,000/μL) and moderate non-regenerative, normochromic anemia (packed cell volume 29%; 5% quantile of reference values: 42%). After fluid therapy, the renal parameters improved but remained above their respective reference intervals. Chronic kidney disease (CKD) was diagnosed (IRIS stage II), and an ACE inhibitor and a phosphate-binding agent were prescribed. A therapeutic renal diet was also recommended.

Blood values (renal parameters, electrolytes, and packed cell volume) were monitored at regular intervals thereafter (once a month) and remained stable until October 2007 when they started to deteriorate. The dog had lost another kg of body weight since the diagnosis of CKD. In December 2007, the dog had to be euthanized for humane reasons due to severe kidney failure.

*Anaplasma phagocytophilum* and *Rickettsia helvetica* in red foxes in Switzerland

Out of the 162 Swiss fox samples, 44 tested positive in the *Ana_family* PCR. When these samples were tested using specific real-time PCR, 4 were confirmed to be positive for *A. phagocytophilum* (Tab. 1). All 4 foxes originated from the canton of Zurich and 3 of them were young animals (Tab. 2). Sequencing in one sample (Oct12_29) was successful: the 902 bp long nested PCR product showed 100% identity with *A. phagocytophilum*

Table 1: Summary of PCR results in dogs and red foxes from Switzerland and dogs from Mediterranean countries. For the different PCR assays, the number of PCR-positive samples is provided; the percentage of PCR-positive samples and the 95% confidence interval are provided in parentheses.

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
<th>No of samples</th>
<th><em>Ana_family</em></th>
<th>‘Candidatus Neoehrlichia mikurensis’</th>
<th><em>A. phagocytophilum</em></th>
<th><em>E. canis</em></th>
<th><em>A. platys</em></th>
<th><em>R. helvetica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs</td>
<td>Switzerland</td>
<td>889</td>
<td>3 (0.3; 0.1-1.0)</td>
<td>1 (0.1; 0^-0.6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>162</td>
<td>44 (27; 21-35)</td>
<td>0</td>
<td>4 (3; 0.7-6)</td>
<td>0</td>
<td>0</td>
<td>1 (1; 0^-3)</td>
</tr>
<tr>
<td></td>
<td>Italy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bologna</td>
<td>200</td>
<td>1 (0.5; 0^-3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Teramo</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Messina</td>
<td>200</td>
<td>17 (9; 5-13)</td>
<td>0</td>
<td>0</td>
<td>2^- (1;0-1.4)</td>
<td>0^- (3; 1^-6)</td>
<td>0</td>
</tr>
<tr>
<td>Dogs</td>
<td>Spain</td>
<td>200</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Portugal</td>
<td>49</td>
<td>7 (14; 6-27)</td>
<td>0</td>
<td>0</td>
<td>1^- (2; 0-1.1)</td>
<td>7^- (14;6-27)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Real-time PCR, Ana_family probe (Maurer et al., 2013); the primers and probe of this PCR also fit Pseudomonas sequences (observation from this study; for details see text); ‘Candidatus Neoehrlichia mikurensis’ real-time PCR, Neo_spec probe (Maurer et al., 2013); ‘A. phagocytophilum’ real-time PCR (Pusterla et al., 1999); ‘E. canis’ real-time PCR (Foley et al., 2007); ‘A. platys’ real-time PCR, this study; ‘R. helvetica’ real-time PCR (Boretti et al., 2009); Only selected samples were tested with these assays, see also M&M; ‘3 x 10^-6; ‘2 x 10^-4; ‘10^-4; ‘1 one dog from Messina and one dog from Portugal were each double-positive for *E. canis* and *A. platys*. 694 SAT ASMV 10|2016 Band 158, Heft 10, Oktober 2016, 691-700, © GST | SVS
Six additional Ana_family-positive foxes were positive in the conventional Anaplasma spp. assay and were sequenced (452 bp of the 16s RNA gene). Sequence comparisons demonstrated closest identity with *Pseudomonas* spp. (97%; e.g., NR_113578) but not with *Anaplasmataceae*. Subsequent NCBI BLASTN analysis of the primers and probe of the Ana_family PCR (Ana-family, Ana_for, Neo_rev (Maurer et al., 2013)) revealed that these oligonucleotides also fit 95-100% of the *Pseudomonas* sequence (e.g., HQ706105). A similar finding was made after BLASTN analysis of the primers of the conventional *Anaplasm* spp. PCR (Goodman et al., 1996): 95-100% identity to *Pseudomonas* sequences for 19-20 bp was found; however, the PCR products of of *Anaplasma* and *Pseudomonas* have a slightly different length (~450 bp versus ~480 bp).

### Discussion

Tick-borne diseases represent major public and animal health issues. ‘*Candidatus Neoehrlichia mikurensis*’ is the causative agent of the emerging zoonosis Neoehrlichiosis and has been recognized in many tick species in European and Asian countries (Silaghi et al., 2015). To date, only a limited number of human and canine cases have been described, but it may be assumed that the infection has been underdiagnosed because diagnostic assays are not yet widely available. This is the first study to investigate the occurrence of ‘*Candidatus Neoehrlichia mikurensis*’ in a large Canidae sample in Europe.

Overall, one out of 1’739 dogs tested positive for ‘*Candidatus Neoehrlichia mikurensis*’. The dog had been splenectomized. This finding parallels human cases, where most infections with ‘*Candidatus Neoehrlichia mikurensis*’ have been asymptomatic. However, in a recent case report, a 6-year-old boy presented with fever, pain in the left knee, splenomegaly, and hemophagocytic syndrome (Melissari et al., 2019). This report highlights the importance of recognizing and diagnosing *Candidatus Neoehrlichia mikurensis* infections in humans, especially in at-risk populations such as children and immunocompromised individuals.

The prevalence of *Candidatus Neoehrlichia mikurensis* in domestic dogs in Switzerland was 0.2%, which is similar to the prevalence reported in other European countries. This finding underscores the need for ongoing surveillance and early detection of *Candidatus Neoehrlichia mikurensis* infections in domestic dogs to prevent zoonotic transmission.

### Table 2: Description of the *A. phagocytophilum* and *R. helvetica*-positive red foxes in the Canton Zurich, Switzerland

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Sex</th>
<th>Age</th>
<th>Hunting place</th>
<th>Time point of collection</th>
<th>Positive PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct12_29</td>
<td>Female</td>
<td>Young</td>
<td>Winterthur</td>
<td>October 2012</td>
<td><em>A. phagocytophilum</em></td>
</tr>
<tr>
<td>Jan13_09</td>
<td>Male</td>
<td>Young</td>
<td>Wädenswil</td>
<td>January 2013</td>
<td><em>A. phagocytophilum</em></td>
</tr>
<tr>
<td>Jan13_59</td>
<td>Male</td>
<td>Adult</td>
<td>Zurich Seebach</td>
<td>January 2013</td>
<td><em>A. phagocytophilum</em></td>
</tr>
<tr>
<td>Jan14_29</td>
<td>Male</td>
<td>Young</td>
<td>Zurich Oerlikon</td>
<td>January 2014</td>
<td><em>A. phagocytophilum</em></td>
</tr>
<tr>
<td>Jan14_12</td>
<td>Female</td>
<td>Young</td>
<td>Winterthur</td>
<td>January 2013</td>
<td><em>R. helvetica</em></td>
</tr>
</tbody>
</table>

* For PCR assays refer to Table 1 and M&M. a Dog in rescue center/kennel. b Stray animal.
Table 3: Description of the *E. canis* and *A. platys*-positive dogs

<table>
<thead>
<tr>
<th>Origin</th>
<th>Sample ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Breed</th>
<th>Clinics</th>
<th>Positive PCR a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Messina</td>
<td>M90</td>
<td>Male</td>
<td>9</td>
<td>Beagle</td>
<td>Healthy, vaccinated, dewormed</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>M109</td>
<td>Female</td>
<td>0.7</td>
<td>Unknown</td>
<td>Low body condition score, slightly enlarged lymph nodes, fleas, received antiparasitics</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>M130</td>
<td>Male</td>
<td>3</td>
<td>Mixed</td>
<td>Healthy, received antiparasitics</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>M48A</td>
<td>Male</td>
<td>1.5</td>
<td>Mixed</td>
<td>Low body condition score, <em>Leishmania</em> positive, received antiparasitics</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>M66</td>
<td>Male</td>
<td>13</td>
<td>German Shepherd</td>
<td>Vaccinated, thin, myiasis, slightly enlarged lymph nodes, pale mucous membranes, ticks, fleas</td>
<td><em>E. canis</em></td>
</tr>
<tr>
<td></td>
<td>M1A b</td>
<td>Female</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Low body condition score, slightly enlarged lymph nodes</td>
<td><em>E. canis &amp; A. platys</em></td>
</tr>
<tr>
<td>Portugal</td>
<td>P4 c</td>
<td>Male</td>
<td>1</td>
<td>Mixed</td>
<td>Low body condition score, ticks, fleas</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>P6 c</td>
<td>Female</td>
<td>1</td>
<td>Mixed</td>
<td>Dermatitis, fleas</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>P13 c</td>
<td>Female</td>
<td>0.7</td>
<td>Mixed</td>
<td>Ticks, fleas</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>P19 c</td>
<td>Female</td>
<td>0.7</td>
<td>Mixed</td>
<td>Fleas</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>P30 c</td>
<td>Female</td>
<td>0.7</td>
<td>Mixed</td>
<td>Healthy</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>P39 c</td>
<td>Female</td>
<td>0.7</td>
<td>Mixed</td>
<td>Healthy</td>
<td><em>A. platys</em></td>
</tr>
<tr>
<td></td>
<td>P44 c</td>
<td>Male</td>
<td>Adult</td>
<td>Siberian Husky</td>
<td>Poor body condition, epistaxis, pale mucous membranes, pyrexia, ticks, fleas</td>
<td><em>E. canis &amp; A. platys</em></td>
</tr>
</tbody>
</table>

For PCR assays refer to Table 1 and M&M. b Dog in rescue center/kennel. c Stray animal.

*mikurensis* were documented in immunocompromised patients (von Loewenich et al., 2010). The dog exhibited mild signs of inflammation and thrombocytosis, which could be signs of pathological changes in the vascular/thromboembolic system. The above-mentioned human patients suffered from thrombotic or hemorrhagic events. In another dog infected with *Candidatus Neoehrlichia mikurensis* diagnosed in Germany (Diniz et al., 2011), persistent neutropenia and a platelet count at the lower end of the reference range were observed. Moreover, a profuse subcutaneous hemorrhage occurred after surgery with a normal coagulation panel. Although we could not document any clinical signs of thrombosis or hemorrhages in our case, this result points out an interesting aspect. In another species (infected rats), *Candidatus Neoehrlichia mikurensis* was detected in endothelial cells lining the splenic venous sinuses but not within blood cells. This endothelial tropism might be an explanation for the observed disturbances in the vascular/thromboembolic system. The present case of *Candidatus Neoehrlichia mikurensis* infection was noted in a dog with renal Fanconi syndrome. Interestingly, *Candidatus Neoehrlichia mikurensis* was detected in the kidneys of a Croatian dog; however, this dog presented with a severe hemolytic anemia and the authors did not mention any signs of kidney disease (Beck et al., 2014). We speculated that local colonization of the kidneys with the infectious agent in our dog could have resulted in damage of the renal tubular system and consequently the observed glucosuria and hyperaminoaciduria, which were described as renal Fanconi syndrome.

In laboratory rats infected with *Candidatus Neoehrlichia mikurensis*, the agent was only detectable in the blood, spleen, and liver but not in the kidney (Kawahara et al., 2004; Naitou et al., 2006). Therefore, this finding remains speculative because a necropsy of the dog to evaluate the renal tissue was unavailable. Of note, the *Candidatus Neoehrlichia mikurensis* blood load in our dog was approximately 10- to 1000-fold lower compared to the two human patients from Zurich (Maurer et al., 2013), which may explain why all attempts to sequence the 16S rRNA or groEL gene of *Candidatus Neoehrlichia mikurensis* from the dog were unsuccessful.

Red foxes are among the most abundant wild canid species in Europe and have invaded many urban areas, such as the City of Zurich, where they live in close proximity to domestic animals and humans. We speculated that free-ranging foxes might be more frequently exposed to ticks than domestic dogs. Moreover, a *Neoehrlichia* sp. closely related to *Candidatus Neoehrlichia lotoris* was reported recently in a red fox (Hodzic et al., 2015). However, none of the 162 Swiss red fox samples in the present study tested positive for Neoehrlichia. To date, *Candidatus Neoehrlichia mikurensis* infections were described primarily in immunocompromised individuals, including the herein described dog. Free-ranging foxes with immune suppression would probably not survive long in the natural environment; thus, if immunosuppressed foxes were primarily infected by *Neoehrlichia* sp., the window of opportunity to detect an infected fox would be small.
Four of the foxes tested positive for *A. phagocytophilum* – the infectious agent of granulocytic anaplasmosis in domestic animals and humans in Switzerland. *A. phagocytophilum* is primarily transmitted by *Ixodes* ticks, and PCR-positive ticks are abundantly present in the sampled area (Pusterla et al., 1999). *A. phagocytophilum* has been detected by PCR in red foxes from several European countries, including Italy and Germany (Ebani et al., 2011; Hartwig et al., 2014). To the best of our knowledge, our study provides the first molecular evidence of *A. phagocytophilum* infection in red foxes in Switzerland; in comparison with other countries where up to 17% of the foxes were infected (Ebani et al., 2011), the prevalence in the sampled area was rather low. Our results complements early serological data where only 1% of the foxes from central Switzerland had antibodies to *A. phagocytophilum* (Pusterla et al., 1999).

*A. platys* and *E. canis* are the infectious agents of canine cyclic thrombocytopenia and monocytic ehrlichiosis, respectively. The vector for these two agents (*R. sanguineus*) is autochthonous in Mediterranean countries but not north of the Alps where the Swiss foxes were sampled. Consistent with this fact, none of the Swiss foxes but several dogs from Sicily and Portugal were positive for *A. platys* and/or *E. canis*. The highest sample prevalence was identified for *A. platys* in dogs in Portugal. This result parallels observations of the hemoplasma prevalence in the same samples: 40% of the samples from Portugal were positive for canine hemoplasmas, which was significantly higher than Italy (9.5%) and Spain (2.5%) (Novacco et al., 2010). A potential explanation for the high prevalence was the origin of the samples: the majority of the Portuguese samples were obtained from stray animals and rescue dogs kept in kennels (Novacco et al., 2010). Dogs infected with *A. platys* experience cyclic thrombocytopenia. The clinical disease is often mild, although some animals may have clinical evidence of bleeding (epistaxis or petechiation), particularly during the initial bacteremic phase when the platelet counts may be reduced to < 20,000 cells/µl. Coinfection with other tick-borne infectious agents, such as *E. canis*, can result in more severe clinical manifestations of fever, lethargy, pale mucous membranes, petechial hemorrhages, epistaxis, and lymphadenopathy. This was the case in one of the dogs co-infected with *A. platys* and *E. canis*. None of the 200 dogs from Spain tested positive for *Anaplasmataceae*. Again, this may be due to the tested samples; the dogs typically presented at the sampling clinic were well cared for, had good nutritional status and were regularly treated with antihelmintics and ectoparasiticides. All of the dogs were kept in private homes, and 75% were purebred dogs (Novacco et al., 2010).

The Ana_family PCR also detects *R. helvetica* and closely related members of the *Rickettsiaceae* family (Maurer et al., 2013). Therefore, we also tested the Ana_family-positive samples for *R. helvetica* by specific real-time PCR. *R. helvetica* is a suspected pathogen in humans, and cardiac and neurologic problems have been reported. Remarkably, in the present investigation one red fox was *R. helvetica* positive. In a previous study, we had found up to 36% of *Ixodes* spp. ticks in Switzerland positive for *Rickettsia* but none of the blood samples from 884 dogs and 58 foxes primarily from the canton of Zurich (Boretti et al., 2009). We had suggested that the latter negative results may at least partially be related to the presumably short-lived bacteremia of *R. helvetica* in mammalian species. While in recent years, wild cervids, rodents, other small mammals and birds have been shown to be potential reservoirs for *R. helvetica* (Hornok et al., 2014; Elfving et al., 2015) to the best of our knowledge this is the first report of *R. helvetica* in a red fox.

When further investigating the Ana_family-positive samples in the present study by sequencing analyses, we realized that the previously published real-time PCR (Maurer et al., 2013) also amplifies *Pseudomonas*. This cross-reactivity had not been reported so far. Moreover, we identified cross-reactivity with *Pseudomonas* for the primers of the conventional *Anaplasmataceae* spp. PCR (Goodman et al., 1996). However, in the latter case, the PCR products from *Anaplasmataceae* and *Pseudomonas* can be differentiated by their length by agarose gel electrophoresis.

In conclusion, *Candidatus Neoehrlichia mikurensis* was identified in a splenectomized Swiss dog; whether the clinical signs were related to the infection remains unclear. The occurrence of *Candidatus Neoehrlichia mikurensis* in ticks may be on an upward trend (Coipan et al., 2013), and Neoehrlichiosis has been recognized as a zoonosis. Thus, the improvement of routine diagnostic assays and raising the awareness of clinicians for this infection are important.

Acknowledgments

The authors thank E. Gönczi, T. Meili, B. Weibel, D. Seda and S. Childers for excellent laboratory assistance. The laboratory work was performed with logistical support from the Center for Clinical Studies, Vet suisse Faculty, University of Zurich. This study was part of the master thesis of NW.
Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann et al.

‘Candidatus Neoehrlichia mikurensis’ est un agent de zoonose transmis par les tiques qui gagne en importance et concerne principalement les patients immunosupprimés. Les chiens comme les renards sont souvent concernés par des morsures de tiques et vivent en contact étroit avec les êtres humains. Dans le présent travail, nous étudions pour la première fois systématiquement la présence de ‘Candidatus Neoehrlichia mikurensis’ chez les canidés en Europe. Les échantillons sanguins analysés provenaient de 1'739 chiens de Suisse, d’Italie, d’Espagne et du Portugal ainsi que de 162 renards (Vulpes vulpes) de Suisse. Tous les échantillons ont été examinés avec un test de PCR multiplex en temps réel déjà publié quant à la présence d’agents de la famille des Anaplasmataceae, du genre ‘Candidatus Neoehrlichia’ et de l’espèce ‘Candidatus Neoehrlichia mikurensis’. Les échantillons positifs aux Anaplasmataceae ont ensuite été testés avec un test PCR en temps réel spécifique quant à Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia canis und Rickettsia helvetica. Parmi les échantillons examinés se trouvait celui d’un chien de Zurich qui était infecté par ‘Candidatus Neoehrlichia mikurensis’. Ce West Highland White Terrier de 12 ans avait été présenté pour polyurie/polydipsie; il avait été splénectomisé trois mois avant la prise de l’échantillon. Au vu d’une glycosurie et d’une hyperaminoacidurie accompagnées d’une glycémie normale, on a posé le diagnostic de syndrome de Fanconi. A. platsy et E. canis ont été mis en évidence chez 14/249 chiens provenant de Sicile et du Portugal; deux chiens étaient infectés par les deux agents pathogènes. Quatre renards suisses étaient positifs à A. phagocytophilum et R. helvetica a été trouvé pour la première fois chez un renard. En résumé, on peut dire qu’une infection à ‘Candidatus Neoehrlichia mikurensis’ chez un chien malade doit être prise en considération comme diagnostic différentiel, particulièrement chez les anomaux immunosupprimés. Toutefois cet agent n'est pas très répandu chez les canidés des pays examinés, contrairement aux autres Anaplasmataceae spp. qui ont été trouvées plus souvent chez les chiens et les renards.

Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann et al.

‘Candidatus Neoehrlichia mikurensis’ è un agente patogeno zoonotico emergente trasmesso dalle zecche che colpisce soprattutto i pazienti immunodepresi. Cani e volpi vivono a stretto contatto con l’uomo e spesso sono esposti a infestazioni da zecche. In questo studio, si è analizzata per la prima volta in modo sistematica la presenza di ‘Candidatus Neoehrlichia mikurensis’ nei canidi in Europa. I campioni di sangue esaminati sono stati ottenuti da 1.739 cani provenienti da Svizzera, Italia, Spagna e Portogallo e da 162 volpi (Vulpes vulpes) provenienti dalla Svizzera. Tutti i campioni sono stati analizzati mediante un saggio multiplex real-time PCR già pubblicato che individua patogeni della famiglia delle Anaplasmataceae, del genere ‘Candidatus Neoehrlichia’ e della specie ‘Candidatus Neoehrlichia mikurensis’. Tutti i campioni Anaplasmataceae-positivi sono stati poi testati con saggi real-time PCR specifici per Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia canis e Rickettsia helvetica. Tra i campioni studiati è stato identificato un cane, proveniente da Zurigo, positivo per ‘Candidatus Neoehrlichia mikurensis’. Il cane, di razza West Highland White Terrier, età 12 anni, soffriva di poliuria/polidipsia ed era stato splenectomizzato 3 mesi prima del campionamento. Al cane è stata diagnosticata la sindrome di Fanconi sulla base di glicosuria normoglicemica e iperaminoaciduria. A. platsy ed E. canis sono stati riscontrati in 14/249 cani provenienti dalla Sicilia e dal Portogallo; due cani erano infetti da entrambi i patogeni. Quattro volpi svizzere sono state positive ad A. phagocytophilum e, per la prima volta, è stata riscontrata in una volpe R. helvetica. In sintesi, sebbene ‘Candidatus Neoehrlichia mikurensis’ non sia molto diffuso nei canidi dei Paesi esaminati a differenza di altre specie di Anaplasmataceae più comuni, l’infekzione sostenuta da questo patogeno dovrebbe essere presa in considerazione in cani ammalati e in particolare in quelli immunodepressi.

Mise en évidence de ‘Candidatus Neoehrlichia mikurensis’ et d’autres Anaplasmataceae et Rickettsiaceae chez les canidés en Suisse et dans les Pays méditerranéens

Presenza di ‘Candidatus Neoehrlichia mikurensis’ e altre Anaplasmataceae e Rickettsiaceae nei canidi in Svizzera e in Paesi mediterranei

‘Candidatus Neoehrlichia mikurensis’ è un agente patogeno zoonotico emergente trasmesso dalle zecche che colpisce soprattutto i pazienti immunodepresi. Cani e volpi vivono a stretto contatto con l’uomo e spesso sono esposti a infestazioni da zecche. In questo studio, si è analizzata per la prima volta in modo sistematica la presenza di ‘Candidatus Neoehrlichia mikurensis’ nei canidi in Europa. I campioni di sangue esaminati sono stati ottenuti da 1.739 cani provenienti dalla Svizzera, Italia, Spagna e Portogallo e da 162 volpi (Vulpes vulpes) provenienti dalla Svizzera. Tutti i campioni sono stati analizzati mediante un saggio multiplex real-time PCR già pubblicato che individua patogeni della famiglia delle Anaplasmataceae, del genere ‘Candidatus Neoehrlichia’ e della specie ‘Candidatus Neoehrlichia mikurensis’. Tutti i campioni Anaplasmataceae-positivi sono stati poi testati con saggi real-time PCR specifici per Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia canis e Rickettsia helvetica. Tra i campioni studiati è stato identificato un cane, proveniente da Zurigo, positivo per ‘Candidatus Neoehrlichia mikurensis’. Il cane, di razza West Highland White Terrier, età 12 anni, soffriva di poliuria/polidipsia ed era stato splenectomizzato 3 mesi prima del campionamento. Al cane è stata diagnosticata la sindrome di Fanconi sulla base di glicosuria normoglicemica e iperaminoaciduria. A. platsy ed E. canis sono stati riscontrati in 14/249 cani provenienti dalla Sicilia e dal Portogallo; due cani erano infetti da entrambi i patogeni. Quattro volpi svizzere sono state positive ad A. phagocytophilum e, per la prima volta, è stata riscontrata in una volpe R. helvetica. In sintesi, sebbene ‘Candidatus Neoehrlichia mikurensis’ non sia molto diffuso nei canidi dei Paesi esaminati a differenza di altre specie di Anaplasmataceae più comuni, l’infezione sostenuta da questo patogeno dovrebbe essere presa in considerazione in cani ammalati e in particolare in quelli immunodepresi.
References


2011, 47: 276–284.

Detection of Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann et al.
Detection of ‘Candidatus Neoehrlichia mikurensis’ and other Anaplasmataceae and Rickettsiaceae in Canidae in Switzerland and Mediterranean countries

R. Hofmann-Lehmann et al.

Corresponding author
Regina Hofmann-Lehmann
Clinical Laboratory
Winterthurerstr. 260
CH-8057 Zurich
E-Mail: rhofmann@vetclinics.uzh.ch