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

R. Hofmann-Lehmann[1, 2], N. Wagmann[1, 2], M. L. Meli[1, 2], B. Riond[1], M. Novacco[1, 2], D. Joekel[4], F. Gentilini[5], F. Marsilio[6], M. G. Pennisi[7], A. Lloret[8], T. Carrapiço[9], F. S. Boretti[3]

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

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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 Europa. 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. phagocytophilium*. *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

'*Candidatus* Neoehrlichia mikurensis' ist ein durch Zecken übertragener, zoonotischer Erreger von zunehmender Bedeutung, der hauptsächlich immunsupprimierte Patienten befällt. Sowohl Hunde als auch Füchse sind häufig von Zeckenstichen betroffen und leben in engem Kontakt zum Menschen. In dieser Studie untersuchten wir erstmals systematisch das Vorkommen von '*Candidatus* Neoehrlichia mikurensis' in Kaniden in Europa. Die untersuchten Blutproben stammten von 1'739 Hunden aus der Schweiz, Italien, Spanien und Portugal sowie 162 Füchsen (*Vulpes vulpes*) aus der Schweiz. Alle Proben wurden mittels einer bereits publizierten multiplex real-time PCR auf Erreger der Familie *Anaplasmataceae*, des Genus '*Candidatus* Neoehrlichia' und der Spezies '*Candidatus* Neoehrlichia mikurensis' untersucht. Alle *Anaplasmataceae*-positiven Proben wurden danach mittels spezifischer real-time PCR auf *Anaplasma phagocytophilum*, *Anaplasma platys*, *Ehrlichia canis* und *Rickettsia helvetica* getestet. Innerhalb der untersuchten Proben fand sich ein Hund aus Zürich, der mit '*Candidatus* Neoehrlichia mikurensis' infiziert war. Der 12-jährige West Highland White Terrier wurde vorgestellt mit Polyurie/Polydypsie; der Hund war 3 Monate vor der Probenentnahme splenektomiert worden. Aufgrund von

be considered in sick dogs, particularly when immunocompromised. The pathogen seems not to be widespread in Canidae in the investigated countries. Conversely, other *Anaplasmataceae* were more readily detected in dogs and foxes.

Keywords: '*Candidatus* Neoehrlichia mikurensis', *Anaplasmataceae*, *Rickettsia helvetica*, Canidae, zoonosis

einer Glukosurie und Hyperaminoazidurie bei normalen Blutzuckerwerten wurde die Diagnose Fanconi Syndrom gestellt. *A. platys* and *E. canis* wurden in 14/249 Hunden aus Sizilien und Portugal gefunden; zwei Hunde waren mit beiden Erregern infiziert. Vier Schweizer Füchse waren *A. phagocytophilium*- positiv und *R. helvetica* wurde erstmals in einem Fuchs gefunden. Zusammenfassend sollte eine '*Candidatus* Neoehrlichia mikurensis' Infektion bei kranken Hunden als Differentialdiagnose in Betracht gezogen werden, v.a. wenn sie immunsupprimiert sind. Allerdings ist der Erreger in Kaniden in den untersuchten Ländern nicht weit verbreitet, dies im Gegensatz zu anderen *Anaplasmataceae* spp., welche in Hunden und Füchsen öfter gefunden wurden.

Schlüsselwörter: '*Candidatus* Neoehrlichia mikurensis', *Anaplasmataceae*, *Rickettsia helvetica*, Kaniden, Zoonose

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:

889 samples in Zurich, Switzerland, in 2005/2006; 200 samples each in Bologna, Teramo and Messina, Italy, and Barcelona, Spain, and 49 samples from Almacil, Portugal in 2008. One sample from Almacil 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 (*Vulpes vulpes*) 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 DNeasy 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 consequently 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 lotoris' 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 florescent 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 Uracil-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 GCG GCA AGC TTA ACA C-3'), reverse primer Aplat.89r (5'-CGT CTG CCA CTA ATT TTT ATC ATA GC-3') and probe Aplat_34p (5'-FAM-AGC TAC GAC AAA AAT CCG TTC GAC TTG CA-TAMRA-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 a 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).

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). Ultrasound 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 hyperaminoaciduria; 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 normocytic 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.

Species	Origin	No of samples	*Ana_familiy*[a]	'*Candidatus* Neoehrlichia mikurensis'[bg]	*A. phagocytophilum*[cg]	*E. canis*[dg]	*A. platys*[eg]	*R. helvetica*[fg]
Dogs	Switzerland	889	3 (0.3; 0.1-1.0)	1 (0.1; 0[h]-0.6)	0	0	0	0
Foxes	Switzerland	162	44 (27; 21-35)	0	4 (3; 0.7-6)	0	0	1 (1; 0[i] -3)
Dogs	Italy							
	– Bologna	200	1 (0.5; 0j-3)	0	0	0	0	0
	– Teramo	200	0	0	0	0	0	0
	– Messina	200	17 (9; 5-13)	0	0	2[k] (1;0.1-4)	6[k] (3; 1-6)	0
Dogs	Spain	200	0	0	0	0	0	0
Dogs	Portugal	49	7 (14; 6-27)	0	0	1[k] (2; 0.1-11)	7[k] (14;6-27)	0

[a] 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); [b] '*Candidatus* Neoehrlichia mikurensis' real-time PCR, Neo_spec probe (Maurer et al., 2013); [c] *A. phagocytophilum* real-time PCR (Pusterla et al., 1999); [d] *E. canis* real-time PCR (Foley et al., 2007) [e] *A. platys* real-time PCR, this study; [f] *R. helvetica* real-time PCR (Boretti et al., 2009); [g] Only selected samples were tested with these assays, see also M&M; [h] 3×10^{-5}; [i] 2×10^{-4}; [j] 10^{-4}; [k] One dog from Messina and one dog from Portugal were each double-positive for *E. canis* and *A. platys*.

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

Sample ID	Sex	Age	Hunting place	Time point of collection	Positive PCR[a]
Oct12_29	Female	Young	Winterthur	October 2012	*A. phagocytophilum*
Jan13_09	Male	Young	Wädenswil	January 2013	*A. phagocytophilum*
Jan13_58	Male	Adult	Zurich Seebach	January 2013	*A. phagocytophilum*
Jan14_29	Male	Young	Zurich Oerlikon	January 2014	*A. phagocytophilum*
Jan14_12	Female	Young	Winterthur	January 2013	*R. helvetica*

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

(e.g., NR_044762). Moreover, one Ana_familiy positive young fox from Winterthur tested positive for *R. helvetica* by specific real-time PCR (Tab. 2). None of the foxes tested positive for *E. canis* and *A. platys*. Overall, the free-ranging foxes in Switzerland had a higher chance of being infected with *Anaplasmataceae* and *Rickettsiaceae* (5/162) compared to the Swiss domestic dog population (0/889; $p_{chi2} < 0.0001$; odds ratio 62; 95% confidence interval 3-1130).

Six additional Ana_familiy-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 *Anaplasma* 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).

Ehrlichia canis and *Anaplasma platys* in dogs from Sicily and Portugal

In the Mediterranean countries, 25 out of 849 samples were positive in the Ana_family PCR (Tab. 1). To confirm and characterize these samples, they were tested with real-time PCRs specific for *A. phagocytophilum*, *E. canis*, *A. platys* and *R. helvetica*; if all of the tests were negative, the samples were applied to a conventional *Anaplasma* spp. PCR. Only dog samples from Messina, Italy, and Portugal tested positive; the 3 samples from Switzerland and Bologna, Italy, were negative. Out of the 17 Ana_family-positive samples from Messina, 6 were positive for *A. platys* and 2 for *E. canis*, including one dog coinfected with *A. platys* and *E. canis*. Out of the 7 Ana_family-positive samples originating from Portugal, 6 tested positive only for *A. platys* and one was coinfected with *A. platys* and *E. canis* (Tab. 1). Three of the positive dog samples were further analyzed by sequencing of the near-complete 16S rRNA gene: in the Italian dog M48A the 1239 bp sequence showed 100% to *A. platys* (e.g., JQ396431); in the Italian dog M66 the sequence was 100% identical to *E. canis* (e.g., KJ513197) over 1292 bp; and in the Portuguese dog P30 a 1358 bp long sequence was 100% identical to *A. platys* (e.g., EF139459). None of the tested dogs was positive for *A. phagocytophilum* and *R. helvetica*.

Overall, 4 of the *A. platys*-positive dogs were clinically healthy. The other *A. platys* and/or *E. canis*-infected dogs demonstrated tick and/or flea infestations, some had low body condition score and some had enlarged lymph nodes. The data available for the dogs are summarized in Tab. 3. Eight out of the 14 infected dogs were stray or rescue dogs, including the *A. platys-E. canis*-double positive dog from Portugal; this dog was a stray animal without a vaccination record and had been rescued two month prior to presentation. The dog had developed acute unilateral epistaxis and displayed a poor body condition and rough coat. The animal had a severe infestation of dandruff, ticks and fleas and had pale mucous membranes and pyrexia. Infection with hemoparasites was suspected. The animal recovered under subsequent doxycycline and imidocarb therapy.

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

Table 3: Description of the *E. canis* and *A. platys*-positive dogs

Origin	Sample ID	Sex	Age (years)	Breed	Clinics	Positive PCR[a]
Messina	M2	Female	0.9	Mixed	Low body condition score, lameness, leukocytosis, neutrophilia	*A. platys*
	M90	Male	9	Beagle	Healthy, vaccinated, dewormed	*A. platys*
	M109	Female	0.7	Unknown	Low body condition score, slightly enlarged lymph nodes, fleas, received antiparasitics	*A. platys*
	M130	Male	3	Mixed	Healthy, received antiparasitics	*A. platys*
	M48A	Male	1.5	Mixed	Low body condition score, *Leishmania* positive, received antiparasitics	*A. platys*
	M66	Male	13	German Shepherd	Vaccinated, thin, myiasis, slightly enlarged lymph nodes, pale mucous membranes, ticks, fleas	*E. canis*
	M1A[b]	Female	Unknown	Unknown	Low body condition score, slightly enlarged lymph nodes	*E. canis & A. platys*
Portugal	P4[c]	Male	1	Mixed	Low body condition score, ticks, fleas	*A. platys*
	P6[c]	Female	1	Mixed	Dermatitis, fleas	*A. platys*
	P13[c]	Female	1	Mixed	Ticks, fleas	*A. platys*
	P19[c]	Female	0.7	Mixed	Fleas	*A. platys*
	P30[c]	Female	0.7	Mixed	Healthy	*A. platys*
	P39[c]	Female	0.7	Mixed	Healthy	*A. platys*
	P44[b]	Male	Adult	Siberian Husky	Poor body condition, epistaxis, pale mucous membranes, pyrexia, ticks, fleas	*E. canis & A. platys*

[a] 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 coinfected 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_familiy-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 *Anaplasma* spp. PCR (Goodman et al., 1996). However, in the latter case, the PCR products from *Anaplasma* 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 Neoehrlichosis has been recognized as a zoonosis. Thus, the improvement of routine diagnostic assays and raising the awareness of clinicians for this infection are important.

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

'*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 Zürich 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. platys* 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. phagocytophilium* 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.

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 immunodepressi. 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 sistematico 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. platys* 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 risultate 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 immunodepressi.

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Corresponding author

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