APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2009, p. 3230–3237 Vol. 75, No. 10
0099-2240/09/$08.00+0 doi:10.1128/AEM.00220-09

Molecular Investigations of *Rickettsia helvetica* Infection in Dogs, Foxes, Humans, and *Ixodes* Ticks▽

Felicitas S. Boretti,[2]* Andrea Perreten,[2] Marina L. Meli,[1] Valentino Cattori,[1] Barbara Willi,[1,2] Nicole Wengi,[1] Sándor Hornok,[3] Hanspeter Honegger,[4] Daniel Hegglin,[5] Roman Woelfel,[6] Claudia E. Reusch,[2] Hans Lutz,[1] and Regina Hofmann-Lehmann[1]

Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Switzerland[1]; Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Switzerland[2]; Department of Parasitology and Zoology, Faculty of Veterinary Science, Szent István University, Budapest, Hungary[3]; Clinic of Medical Oncology, Triemli Hospital, Zurich, Switzerland[4]; Institute of Parasitology, Vetsuisse Faculty, University of Zurich, Switzerland[5]; and Bundeswehr Institute of Microbiology, Munich, Germany[6]

Received 30 January 2009/Accepted 16 March 2009

***Rickettsia helvetica*, a tick-borne member of the spotted-fever-group rickettsiae, is a suspected pathogen in humans; however, its role in animals is unknown. The aims of this study were to establish a *R. helvetica*-specific real-time TaqMan PCR assay and apply it to the analysis of tick vectors (to determine potential exposure risk) and blood samples from *Canidae* and humans (to determine prevalence of infection). The newly designed 23S rRNA gene assay for *R. helvetica* was more sensitive than a published citrate synthase gene (*gltA*) assay for several rickettsiae. Blood samples from 884 dogs, 58 foxes, and 214 human patients and 2,073 ticks (*Ixodes* spp.) collected from either vegetation or animals were analyzed. Although the maximal likelihood estimate of prevalence was 12% in unfed ticks and 36% in ticks collected from animals, none of the 1,156 blood samples tested PCR positive. Ticks from cats were more frequently PCR positive than ticks from dogs. Sequencing of the 23S rRNA and/or the *gltA* gene of 17 tick pools confirmed the presence of *R. helvetica*. Additionally, *Rickettsia monacensis*, which has not been previously found in Switzerland, was identified. In conclusion, *R. helvetica* was frequently detected in the tick population but not in blood samples. Nevertheless, due to the broad host range of *Ixodes* ticks and the high rate of infestation with this agent (i.e., *R. helvetica* was 13 times more frequent in unfed ticks than the tick-borne encephalitis virus), many mammals may be exposed to *R. helvetica*. The PCR assay described here represents an important tool for studying this topic.**

Tick-borne rickettsioses are caused by intracellular bacteria belonging to the spotted fever group (SFG) of the genus *Rickettsia*. The latter comprises more than 20 different species, of which an increasing number are known to be associated with human and animal diseases. The SFG rickettsiae are distributed worldwide, and their distribution depends upon the occurrence of tick species. The most common tick in Europe is *Ixodes ricinus*, which was found to harbor *Rickettsia helvetica*. *R. helvetica* is transmitted not only transstadially but also transovarially in *I. ricinus*. Therefore, this tick is both a vector and a reservoir for *R. helvetica*. Due to the broad host range of *I. ricinus*, many mammalian species, including humans, can serve as hosts. Therefore, these host species may potentially be exposed to *R. helvetica*. *R. helvetica* is a suspected pathogen in humans, and the symptoms described for infections in humans include fever, headache, arthralgia, and myalgia (1, 3, 7, 21, 34). The agent also has been implicated in two cases of fatal perimyocarditis (20, 22).

Interestingly, despite the wide distribution of *I. ricinus* ticks and the high rate of infection of these ticks with *R. helvetica* that has been reported in several European countries (2, 9, 18, 19, 25, 29, 35, 42), larger studies discussing the prevalence of the infection in humans and animals are scarce. No studies evaluating the importance of *R. helvetica* in pets or farm animals are available as yet. It is unknown whether these animals can serve as a reservoir or develop clinical signs after infection.

Rickettsial infections have been reported to represent the third most common vector-borne disease acquired during international travel and are therefore considered a common cause of fever of unknown origin in returned travelers (24). As the occurrence of tick-borne infectious diseases, and particularly rickettsial infections, is increasing in humans worldwide (26), it may be assumed that the same holds true for companion animals. In dogs, fever of unknown origin that is responsive to antibiotic treatment is frequently observed. In these cases, an infectious agent is suspected but rarely, if ever, confirmed. *R. helvetica* infections may be the underlying cause in some of these cases, even if the patient does not have a travel history, since exposure to *R. helvetica*-infected *I. ricinus* ticks may have occurred locally.

To date, the diagnosis of rickettsial infection has most often been confirmed by serological testing. However, antibodies are not detectable prior to the second week of illness for any rickettsial disease studied thus far. Moreover, except for detection of seroconversion or a fourfold increase in titer, a positive serology result does not necessarily indicate an acute infection. A standardized sensitive and specific molecular method for the confirmation of *R. helvetica* infections would facilitate not only its diagnosis but also prevalence studies. This in turn could increase the awareness of physicians and veterinarians who are confronted with diseased individuals.

Therefore, the aims of the present study were as follows: first, to establish a sensitive real-time PCR assay specific for *R. helvetica*; second, to study tick vectors for *R. helvetica* to assess

* Corresponding author. Mailing address: Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland. Phone: 41 (44) 635 81 12. Fax: 41 (44) 635 89 20. E-mail: fboretti@vetclinics.uzh.ch.

▽ Published ahead of print on 27 March 2009.

TABLE 1. Samples, extraction methods, and percentage PCR positive for *R. helvetica*

Sample type	No. of ticks or samples	Source	Origin[a]	No. of ticks pooled per extraction	NA extraction kit[b]	% positive by real-time PCR for:	
						gltA	23S rRNA gene
Ticks from vegetation	1,880	Vegetation	ZH	10	MagNa Pure	9.9[c]	11.7[c]
Ticks from animals	71[d]	39 cats	CH/ZH[e]	1–3	DNeasy	40.9[c]	49.9[c]
	117[f]	66 dogs	CH/ZH[g]	1–4	DNeasy	17.6[c]	28.3[c]
	5	5 horses	ZH		DNeasy	20	20
Blood samples from animals	884[h]	Dogs	CH/ZH[i]		MagNa Pure	0	0
	58	Foxes	ZH		MagNa Pure	0	0
	214	Humans	ZH		MagNa Pure	0	0

[a] ZH, Canton of Zurich, Switzerland; CH, Switzerland.
[b] MagNA Pure, MagNA Pure LC total nucleic acid isolation kit (Roche); DNeasy, DNeasy tissue kit (Qiagen).
[c] Maximal likelihood estimate of prevalence.
[d] 62 single ticks and three tick pools.
[e] The majority (83%) of these ticks were collected from cats in the Canton of Zurich.
[f] 110 out of 117 ticks, as described previously (36); seven additional ticks had been collected from dogs in the Canton of Zurich. In total, 86 single ticks and 12 tick pools were included.
[g] The majority (56%) of these ticks were collected from dogs in the Canton of Zurich.
[h] From the original 889 dogs (38), 884 were included in the study; no sample was available for five dogs.
[i] The majority (70%) of these blood samples were collected from dogs in the Canton of Zurich.

the potential exposure risk for animals and humans; and third, to evaluate blood samples from *Canidae* and humans to assess the occurrence of *R. helvetica* infections.

(These studies were conducted by A. Perreten as partial fulfillment of the requirements for a doctoral thesis at the Vetsuisse Faculty, University of Zurich.)

MATERIALS AND METHODS

Arthropods. To analyze the occurrence of *R. helvetica* in the Swiss tick population, a total of 2,073 ticks were included in the study. The majority of the ticks had been described previously (40) and had been microscopically identified based on their morphology using the key of the University of Neuchatel (6) or assumed to be members of the genus *Ixodes* (40). This tick population included 1,880 unfed ticks that had been collected from vegetation in the area around Zurich (Pfannenstiel, Küsnachter Tobel, and Rumensee area), Switzerland, and 188 ticks collected from 39 cats and 66 dogs. In addition, five *Ixodes* ticks had been collected from five horses in the Canton of Zurich (Table 1). Furthermore, to determine the specificity of the newly designed *R. helvetica* TaqMan PCR assay, 720 *I. ricinus* ticks that were collected from vegetation for an unrelated study and previously differentiated according to sex were also used. They were randomly chosen from a larger pool of ticks to consist of 480 males and 240 females. All arthropods were stored in liquid nitrogen, at −20°C, or in ethanol at 4°C until nucleic acids (NA) were extracted.

Blood samples. A total of 1,156 blood samples were included in the study. They originated from 884 privately owned dogs that were presented for various reasons at the Clinic for Small Animals, Vetsuisse Faculty, University of Zurich, 58 free-ranging wild foxes, and 214 anonymous human patients (Table 1). The canine EDTA-anticoagulated blood samples had been described previously (38); they had been collected throughout a 1-year period. A total of 615 (70%) of the dogs originated from the Canton of Zurich, Switzerland. The foxes originated from three different hunting districts in the Canton of Zurich; samples consisted of coagulated whole blood or serosanguinous fluids. The 214 human EDTA-anticoagulated blood samples had been provided by a hospital in the Canton of Zurich and had been collected for diagnostic purposes from presenting patients.

NA extraction. Some ticks were pooled prior to NA extraction, as previously described (40); the pools consisted of two to ten arthropods of one species collected from one animal (Table 1). All arthropods, as well as coagulated whole-blood samples, were mechanically disrupted with sterile scalpel blades and homogenized in a Mixer Mill MM 300 device (Retsch GmbH, Haan, Germany). NA extraction was performed with a MagNA Pure LC total NA isolation kit (Roche Diagnostics, Rotkreuz, Switzerland) or with a DNeasy tissue kit (Qiagen, Hombrechtikon, Switzerland) (Table 1). From EDTA-anticoagulated blood and serosanguinous samples, NA was extracted from a volume of 100 μl or 200 μl using the MagNA Pure LC total NA isolation kit (38). During each extraction, negative controls consisting of 200 μl phosphate-buffered saline were concurrently prepared with each batch of 11 to 15 samples in order to monitor for cross-contamination.

Eukaryotic NA detection. The presence of amplifiable NA was confirmed for each sample extracted from ticks using a real-time TaqMan PCR assay specific for the 18S rRNA gene (Applied Biosystems, Rotkreuz, Switzerland) on a Rotor-Gene6000 real-time rotary analyzer (Corbett, Mortlake, Australia) using 2× TaqMan fast universal PCR master mix (Applied Biosystems) in a total volume of 25 μl. An initial denaturation step of 20 s at 95°C was followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. In some tick samples, the 18S rRNA assay revealed unexpectedly high threshold cycle (C_T) values (≥30), which could have been attributed to inhibition of the PCR. Inhibition was confirmed by testing a 1:10 dilution of the samples; while a C_T value approximately 3.3 units higher is expected for an uninhibited PCR (sample diluted 1:10), the C_T values obtained from some of our samples were lower after dilution. These samples, which contained substances that inhibited PCRs in their undiluted form, were tested in a 1:10 dilution in subsequent PCR assays. A representative number of randomly selected blood samples from human patients ($n = 50$), dogs ($n = 50$), and foxes ($n = 15$) were also tested for the presence of amplifiable NA. For samples from humans and foxes, the 18S rRNA real-time PCR assay was used; the canine samples were analyzed using the canine GAPDH real-time PCR assay (31). No inhibition of the PCR was found for any of the tested NA samples extracted from blood samples; the C_T values were <22 for all but three samples.

Spotted fever and typhus group *Rickettsia gltA* real-time PCR assay. Members of the *Rickettsia* spotted fever and typhus groups were identified using a previously published real-time TaqMan PCR assay specific for a 74-bp fragment of the *gltA* gene (36). The PCR mixtures contained a final concentration of 0.2 μM of primers (CS-F and CS-R) and probe (CS-P) (Table 2), 12.5 μl of 2× TaqMan fast universal PCR master mix (Applied Biosystems), and 5 μl or 2.5 μl of template in a final volume of 25 μl. The *gltA* assay was performed using 60 cycles on a Rotor-Gene6000 real-time rotary analyzer (Corbett), with an initial denaturation step of 20 s at 95°C, which was followed by 60 cycles of 95°C for 3 s and 60°C for 30 s.

Development and specificity of a *R. helvetica*-specific 23S rRNA real-time PCR assay. For the detection of *R. helvetica*, a specific real-time PCR assay based on a 65-bp fragment of the 23S rRNA (GenBank accession number AY125017) was designed: this assay amplified a fragment located in the same region as a previously described real-time PCR (32). The latter assay was not applied in the present study, because the primer and probe sequences and the conditions of the PCR assay were not suitable for a standard TaqMan real-time PCR assay performed in ABI real-time cyclers. The system used the primers Rickhelv.147f and Rickhelv.211r and the probe Rickhelv.170p (Table 2). The real-time PCR mixtures contained 12.5 μl of the 2× quantitative PCR master mix plus low 5-carboxy-X-rhodamine (Eurogentec, Seraing, Belgium), final concentrations of 0.9

TABLE 2. Primers specific to the 23S rRNA gene of *R. helvetica* and the *gltA* gene of several rickettsiae used in this study

Target, purpose, and description	Primer name	Sequence (5′–3′)[a]	Reference	Amplicon length (bp)
23S rRNA gene				
Real-time PCR				
Probe	Rickhelv.170p	6FAM-AAC CGT AGC GTA CAC TTA-MGBNFQ	This study	65
Forward	Rickhelv.147f	TTT GAA GGA GAC ACG GAA CAC A	This study	
Reverse	Rickhelv.211r	TCC GGT ACT CAA ATC CTC ACG TA	This study	
Sequencing				
Forward	rhelv.159f	ACG GAA CAC AGA ACC GTA GC	This study	206
Reverse	rhelv.365r	CTG GAT ACC GTG GAT CAA GC	This study	
Forward	rhelv.26f	AAT GTG TGT AGC TAA CCG ATA CTA AT	This study	330
Reverse	rhelv.356r	GTG GAT CAA GCC ATG ATA TGA C	This study	
gltA				
Real-time PCR				
Probe	CS-P	FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ	36	74
Forward	CS-F	TCG CAA ATG TTC ACG GTA CTT T	36	
Reverse	CS-R	TCG TGC ATT TCT TTC CAT TGT G	36	
Sequencing				
Forward	Rp877p	GGG GAC CTG CTC ACG GCG G	27	381
Reverse	Rp1258n	ATT GCA AAA AGT ACA GTG AAC A	27	
Forward	CS1f	ATG ACT AAT GGC AAT AAT AA	30	496
Reverse	CS477r	TAA GTT CGT AGT CTG CTT CC	30	
Forward	CS477f	GGA AGC AGA CTA CGA ACT TA	30	796
Reverse	CS1273r	GAT AAC CAG TGT AAA GCT GT	30	
Forward	Rp414f	AAA CAG GTT GCT CAT CAT TC	33	163
Reverse	CS477r	TAA GTT CGT AGT CTG CTT CC	30	
Forward	CS1f	ATG ACT AAT GGC AAT AAT AA	30	353
Reverse	CS353r	GAA TGA TGA GCA ACC TGT TT	30	
Forward	Rp877p	GGG GAC CTG CTC ACG GCG G	27	476
Reverse	CS1273r	GAT AAC CAG TGT AAA GCT GT	30	

[a] 6FAM, 6-carboxyfluorescein; MGB, minor groove binder; NFQ, nonfluorescent quencher; BHQ, black-hole quencher.

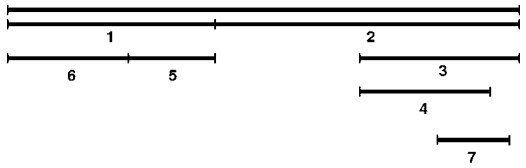
μM of each primer and 0.25 μM of the probe, and 2.5 μl or 5 μl of the template in a total volume of 25 μl. The *R. helvetica*-specific real-time PCR assay was performed using an ABI Prism 7500 fast sequence detection system (Applied Biosystems), with an initial step of 50°C for 2 min and a denaturation step of 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. In the *R. helvetica* 23S rRNA PCR runs, the amplification mixture contained dUTP for use with uracil-*N*-glycosylase in order to prevent the carryover of PCR amplicons. In all the PCR assays, water was used as a negative control. All negative extraction and pipetting controls were PCR negative. NA from the following bacteria was used to determine the specificity of the real-time PCR assays: *R. helvetica*, *Rickettsia africae*, *Rickettsia conorii*, *Anaplasma phagocytophilum*, *Ehrlichia canis*, *Mycoplasma haemofelis*, "*Candidatus* Mycoplasma haemominutum," and "*Candidatus* Mycoplasma turicensis."

Production of a DNA standard for the 23S rRNA gene of *R. helvetica*. For absolute quantification, a 330-bp sequence of the 23S rRNA gene of *R. helvetica* (AY125017) enclosing the 65-bp-long sequence detected in the TaqMan system was amplified using the *R. helvetica*-specific primers Rhelv.26f and Rhelv.356r (Table 2). The reaction mixture contained 5 μl of 5× Phusion HF buffer (Finnzymes, Espoo, Finland), 0.5 μl of Phusion Hot Start DNA polymerase (Finnzymes), final concentrations of 0.2 mM deoxynucleoside triphosphates (dNTPs) and 0.5 μM of each primer, and 2.5 μl of template NA in a final volume of 25 μl under the following thermal cycling conditions: a denaturation step of 98°C for 3 min followed by 35 cycles of 98°C for 10 s, 65°C for 30 s and 72°C for 1 min, with a final extension of 72°C for 10 min. The amplified fragment was purified using a Gen Elute PCR cleanup kit (Sigma, Fluka GmbH, Buchs, Switzerland) and cloned into the TOPO TA cloning vector (Invitrogen, Basel, Switzerland). Plasmid DNA was purified using a QIAprep spin miniprep kit (Qiagen), and the insert was verified by sequencing (see below). Purified plasmid DNA was linearized by restriction digestion using BamHI (Promega, Wallisellen, Switzerland), purified (QIAquick gel extraction kit; Qiagen), and the copy number was calculated after both spectrophotometric (GeneQuant; Amersham-Pharmacia Biotech, Otelfingen, Switzerland) and agarose gel electrophoresis (Gene Tools; Syngene, Cambridge, United Kingdom) analysis. The *R. helvetica* DNA standard was serially 10-fold diluted in a solution containing 30 μg/ml of salmon sperm DNA (Invitrogen), aliquoted, and frozen at −20°C until use.

Efficiency, linearity, and sensitivity of the 23S rRNA gene real-time PCR assay. For the 23S rRNA assay, the slopes of the dilutions versus C_T curves were assessed for the linear range and as a measure of the amplification efficiency of the assay. Amplification efficiencies were calculated as $(10^{1/-\text{slope}} - 1)$ (14). Moreover, the standard DNA serial dilutions were used to determine the analytic sensitivity of the PCR assay in an endpoint dilution experiment and for spike experiments. For the latter, the dilutions were added to specific-pathogen-free blood samples prior to NA extraction and real-time PCR, or NA extracted from blood samples from specific-pathogen-free cats was supplemented with the standards prior to PCR analysis.

TBEV real-time RT-PCR. For comparison of the prevalence of *R. helvetica* with that of another frequently encountered tick-borne pathogen, the 1,880 ticks collected from the vegetation in the Canton of Zurich were additionally analyzed by real-time RT-PCR for the presence of the tick-borne encephalitis virus (TBEV), as previously described (39).

Sequencing of *gltA*. To confirm the presence of rickettsiae in PCR-positive samples and analyze the *Rickettsia* species, sequencing analyses of the *gltA* gene were performed (Fig. 1 and Table 2). Initially, for this purpose, a 381-bp PCR product was amplified using primers that were described previously (27) (Fig. 1 and Table 2) with the following modifications: the reaction mixture contained 2.5 μl of 10× PCR TaqGold buffer (Applied Biosystems), 0.4 μl of AmpliTaqGold polymerase (Applied Biosystems), 1.5 μl of 25 mM $MgCl_2$, final concentrations of 0.2 mM of dNTPs and 0.5 μM of each primer, and 2.5 μl of template DNA in a total volume of 25 μl. The thermal cycling conditions were as follows: an initial denaturation step of 95°C for 5 min followed by 40 cycles of 95°C for 20 s, 56°C for 30 s, and 72°C for 1 min, and a final elongation step of 72°C for 5 min. The PCR products were purified (GenElute PCR cleanup kit; Sigma) and sequenced using a BigDye Terminator v1.1 cycle sequencing ready reaction kit (Applied Biosystems). The reaction products were purified using a SigmaSpin postreaction



1. CS1f/CS477r: 496 bp
2. CS477f/CS1273r: 796 bp
3. Rp877p/CS1273r: 476 bp
4. Rp877p/Rp1258n: 381 bp
5. Rp414f/CS477r: 163 bp
6. CS1f/CS353r: 353 bp
7. *gltA* TaqMan PCR: CS-F/CS-P/CS-R: 74 bp

FIG. 1. Schematic diagram of the sequencing procedures for the *gltA* gene. The amplification of seven different amplicons was attempted. For sequencing primers, see Table 2.

cleanup kit (Sigma) and run on an ABI Prism 310 genetic analyzer (Applied Biosystems).

In addition, a longer portion of the *gltA* gene was sequenced using five additional published *gltA*-specific PCR assays (27, 30, 33). The primer sequences, lengths of the five PCR products, and corresponding references are provided in Fig. 1 and Table 2. The reaction mixture for the five systems contained 5 μl of 5× Phusion HF buffer (Finnzymes), 0.5 μl of Phusion Hot Start DNA polymerase (Finnzymes), final concentrations of 0.2 mM of dNTPs and 0.5 μM of each primer, and 1.25 μl of template NA in a final volume of 25 μl under the following thermal cycling conditions: initial denaturation step of 95°C for 2 min followed by 40 cycles of 95°C for 30 s, 45°C for 30 s, and 65°C for 55 s and a final elongation step of 72°C for 3 min (27).

Sequencing of the 23S rRNA gene. In order to further confirm the specificity of the newly established *R. helvetica* 23S rRNA TaqMan assays, for the production of the standard, and also to confirm PCR-positive results, two partially overlapping fragments of 206 and 330 bp were amplified. The primers (Table 2) for these assays were designed as follows: for the 206-bp fragment, both primers (Rhelv.159f and Rhelv.365r) were based upon the *R. helvetica* (AY125017) sequence. However, this fragment does not fully enclose the TaqMan sequence. For the fragment of 330 bp, which encloses the complete TaqMan system, a *R. helvetica* specific reverse primer, R.helv.356r, was used (Table 2). The forward primer, Rhelv.26f, was based upon a *Rickettsia* consensus sequence. The reaction mixture and thermal cycling conditions were the same as those described above for production of the standard. The PCR products were sequenced as described above for the *gltA* gene, using the PCR amplification primers.

Phylogenetic analyses. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (16). The sequences obtained were compared to known sequences deposited in the GenBank database, and the percent identity was calculated by pairwise comparison (Kimura two-parameter model) (13). The sequences were aligned using CLUSTAL W (37) and manually adjusted. Only positions where the nucleotide composition was known in all of the sequences being compared were used in the phylogenetic analysis. The phylogenetic tree was constructed after trimming of the primer sequences by the neighbor-joining method (28) from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model. The data set was resampled 1,000 times to generate bootstrap values.

Statistics. The maximal likelihood estimate of prevalence and the 95% confidence intervals (CI) were calculated as described previously (41) using the open-source program R (http://www.r-project.org/). This program allows the calculation of prevalence data from samples consisting of single ticks only or tick pools only but also from samples containing single ticks and tick pools combined. The rickettsial loads of the female and male tick pools were compared using the Mann-Whitney U test (4); the NA content of each pool was controlled using an 18S rRNA gene assay. The frequencies of PCR-positive results for ticks collected from cats and those for ticks collected from dogs were compared using the χ^2 test (4). Ticks from horses were not included in this analysis because of the small number of ticks ($n = 5$).

Nucleotide sequence accession numbers. The partial *gltA*/23S rRNA gene nucleotide sequences generated from *R. helvetica* isolates have been submitted to GenBank and given the accession numbers EU359279 to EU359283, EU359285 to EU359297, and EU359301 for *gltA* and EU359273 to EU359278 for the 23S rRNA gene. In addition, sequences for tick pool 73 have been submitted to GenBank and given the accession numbers EU359284, EU359298 to EU359300, and EU359302.

RESULTS

Specificity of the 23S rRNA gene real-time PCR assay. A new real-time PCR assay for the detection and quantification of the 23S rRNA gene of *R. helvetica* was designed in this study. To test the specificity of the PCR assay, NA of various bacteria (see Materials and Methods) were analyzed. They all tested negative by real-time PCR, with the exception of *R. helvetica*. To confirm that the PCR system did not amplify endosymbionts, which are present only in female ticks, 24 tick pools consisting of 20 male ticks and 24 pools containing 10 female ticks were assayed. All pools tested positive using the 23S rRNA real-time PCR assay. No significant differences were found in the rickettsial NA load between the female and male tick pools using the 23S rRNA real-time PCR assay for quantification (data not shown). To further confirm the specificity of the assay and PCR-positive results, five 23S rRNA gene TaqMan PCR-positive *I. ricinus* tick pools collected from vegetation were sequenced (four PCR products of 206 bp and two PCR products of 330 bp) (Table 2). All resulting sequences were 100% similar to that of *R. helvetica* (AY125017).

Linear range of amplification and sensitivity of the real-time PCR assay. The 23S rRNA gene *R. helvetica* DNA standard was serially 10-fold diluted to assess the linear range and sensitivity of the assay. The amplification of the serial dilutions showed linearity over 8 orders of magnitude and a mean efficiency of the assay of 98.8%. The highest dilution still yielding a positive signal contained an average of one copy of the standard per reaction; in an endpoint dilution experiment, six out of eight replicates (75%) of this dilution were positive. In the spike experiments, between one and ten copies of the DNA standard were detectable when the DNA standard was added to blood or to NA samples prior to processing. This calculated a lower detection limit of 100 to 2,000 copies/ml of blood, depending on the extraction procedures.

Rickettsiae in arthropods collected from vegetation. The NA from 188 tick pools collected from vegetation in the area around Zurich, Switzerland, were analyzed by the *gltA* real-time PCR for the presence of rickettsiae of the SFG and typhus group: 122 of the 188 pools tested positive. This resulted in a maximal likelihood estimate of the prevalence of spotted fever and typhus group rickettsiae of 9.9% (95% CI, 8.3 to 11.8%) (Table 1).

In addition, arthropods were analyzed by the 23S rRNA gene real-time PCR for the presence of *R. helvetica* NA. When unfed ticks from Swiss vegetation were analyzed, the majority of the pools (134 out of 188) were found to be positive for the 23S rRNA gene. This resulted in a maximal likelihood estimate of prevalence of *R. helvetica* in the Swiss *I. ricinus* tick population under investigation of 11.7% (95% CI, 9.9 to 13.8%) (Table 1).

The readout of the real-time PCR assays, e.g., the 23S rRNA gene and *gltA* gene assays, is the so-called C_T value. The C_T value is an inverse measure of the template load; the higher the template load, the lower the C_T value. Analyzing the 188 tick pools from the vegetation, all of the C_T values from the *gltA* gene real-time PCR assay were higher than the C_T values from the 23S rRNA gene real-time PCR assay, except for one tick

pool, no. 73. Twelve pools tested positive in the 23S rRNA assay but negative in the *gltA* assay. Tick pool 73 and some additional pools were analyzed further by sequencing.

For comparison, the 188 tick pools were also analyzed for TBEV: 16 of 188 pools tested positive. This resulted in a maximal likelihood estimate of prevalence of 0.9% (95% CI, 0.5 to 1.4%).

Rickettsiae in arthropods collected from animals. Overall, 43 out of 153 ticks and 6 out of 15 tick pools tested real-time PCR-positive with the *gltA*-specific TaqMan PCR system. This resulted in an overall maximal likelihood estimate of prevalence of 26.2% (95% CI, 20.2 to 32.8%). When the samples were classified according to the animal species they had been collected from, 26 out of 62 ticks and two out of three tick pools from cats were found to be PCR positive. This resulted in a sample prevalence of rickettsiae of the spotted fever and typhus groups in ticks from cats of 40.9% (95% CI, 29.8 to 52.9%) (Table 1). Ticks from 18 out of 39 cats were found to be *gltA* PCR positive. For ticks collected from dogs, 16 out of 86 ticks and 4 out of 12 pools were positive. The maximal likelihood estimate of prevalence in ticks from dogs was 17.6% (95% CI, 11.4 to 25.3%) (Table 1). Ticks collected from 18 out of 66 dogs tested PCR positive. One out of five ticks collected from horses was positive. Ticks collected from cats were significantly more frequently *gltA* PCR positive than ticks collected from dogs (P [χ^2] = 0.0019). In addition, ticks from a higher percentage of cats than of dogs were PCR positive (P [χ^2] = 0.0489).

Overall, 57 out of 153 ticks and 9 out of 15 tick pools collected from animals tested real-time PCR positive for the 23S rRNA gene of *R. helvetica.* This resulted in an overall maximal likelihood estimate of prevalence of 36.1% (95% CI, 29.4 to 43.3%). When the samples were classified according to the animal species from which they were collected, 32 out of 62 ticks and two out of three pools containing three ticks per pool collected from cats were positive; the overall sample prevalence for ticks from cats was 49.9% (95% CI, 38.1 to 61.7%) (Table 1). Ticks from 21 out of 39 cats tested PCR positive. In ticks from dogs, 24 out of 86 ticks and 7 out of 12 tick pools tested PCR positive. The maximal likelihood estimate of prevalence in ticks from dogs was 28.3% (95% CI, 20.4 to 37.2%) (Table 1). Ticks collected from 27 out of 66 dogs tested PCR positive. One out of five ticks collected from horses was PCR positive. Ticks collected from cats were significantly more frequently 23S rRNA gene PCR positive than ticks collected from dogs (P [χ^2] = 0.0033).

Again, the C_T values for the 23S rRNA assay were, with one exception (tick 17), lower than those for the *gltA* assay; 17 samples tested positive using the *R. helvetica*-specific 23S rRNA assay but negative in the *gltA* assay. Tick 17 could not be analyzed further by sequencing due to a low bacterial load. It originated from a 9-year-old Japanese Chin dog from the Canton of Zurich.

Rickettsiae in blood samples from dogs, foxes, and human patients. All 1,156 NA samples extracted from blood from dogs, foxes, and human patients in Switzerland tested negative in the *gltA* and the 23S rRNA gene real-time PCR assays.

Sequencing results from rickettsiae detected in ticks. To confirm the *gltA* PCR-positive results and to determine the *Rickettsia* species, different fragments of the *gltA* gene were sequenced. A 381-bp-long PCR product (Table 2 and Fig. 1) was amplified from 10 PCR-positive tick pools collected from the vegetation. Nine out of the ten pools yielded a sequence with 100% identity to that of *R. helvetica* (AM418450). In contrast, sequencing of the 10th pool, tick pool 73, resulted in a sequence with 16 mismatches to *R. helvetica* (AM418450). This sequence was most closely related (98 to 99% identity) to the following *Rickettsia* species: *Rickettsia* sp. strain IRS4 (AF141906), found in *I. ricinus* in Slovakia; *Rickettsia monacensis* (DQ100163), reported in Germany; and *Rickettsia* sp. strain PoTiR6dt (EF501756), from *I. ricinus* in Portugal.

For tick pool 73 and four additional tick pools (pools 15, 20, 21, and 41), several other fragments could be amplified and sequenced. For all five pools, the 163-bp PCR product was amplified, and for all but pool 15, the 796-bp fragment was amplified (Fig. 1 and Table 2). Moreover, for three ticks, the 476-bp fragment was amplified, and for tick pool 15, the 353-bp PCR product was successfully amplified (Fig. 1 and Table 2). The sequences from the additional four tick pools (pools 15, 20, 21, and 41) were most similar (98 to 99%) to *R. helvetica* (U59723). However, tick pool 73 had 34 mismatches to the *R. helvetica* (U59723) sequence and was most closely related (99%) to *Rickettsia* sp. strain IRS3 (AF140706) and *Rickettsia* sp. strain IRS4 (AF141906), found in *I. ricinus* ticks collected in Slovakia, and *R. monacensis* (DQ100163), which has been reported in Germany.

Phylogenetic analyses. Phylogenetic analyses were performed based on the 348-bp-long sequence of the *gltA* gene of 14 sequenced tick pools (Fig. 2). These sequences were obtained from 10 tick pools after amplification of the 381-bp fragment, from three tick pools after amplification of the 796-bp PCR product, and from one tick pool after amplification of the 476-bp fragment. Thirteen out of 14 sequences clustered with *R. helvetica* (U59723). Only tick pool 73 branched away from the remaining sequenced tick pools, and sequences from this pool were most closely related to *R. monacensis* (DQ100163), *Rickettsia* sp. strain IRS4 (AF141906), and *Rickettsia* sp. strain PoTiR6dt (EF501756) (Fig. 2).

DISCUSSION

This study expands our knowledge of the distribution of *R. helvetica* infection in ticks, foxes, dogs, and human patients within a geographical region by employing a newly developed sensitive real-time TaqMan PCR assay. We concentrated on the investigation of humans and *Canidae*, because both are known to develop disease when infected with SFG rickettsiae. Despite the fact that *R. helvetica* was frequently detectable in *Ixodes* ticks, none of the 1,156 blood samples tested positive. Nonetheless, ticks collected from animals, and particularly from cats, were more frequently positive than those collected from vegetation. With one exception, all identified rickettsiae were assumed to be *R. helvetica*. In addition, *R. monacensis*, which to our knowledge had not been previously detected in Switzerland, was identified according to the sequencing results.

The real-time TaqMan PCR assay developed in this study amplifies a fragment of the 23S rRNA gene of *R. helvetica.* A recently described assay for the *R. helvetica* 23S gene (32) could not be used in the present study because the oligonucleotide sequences and the run conditions were not compatible with a standard TaqMan assay on our ABI real-time cyclers.

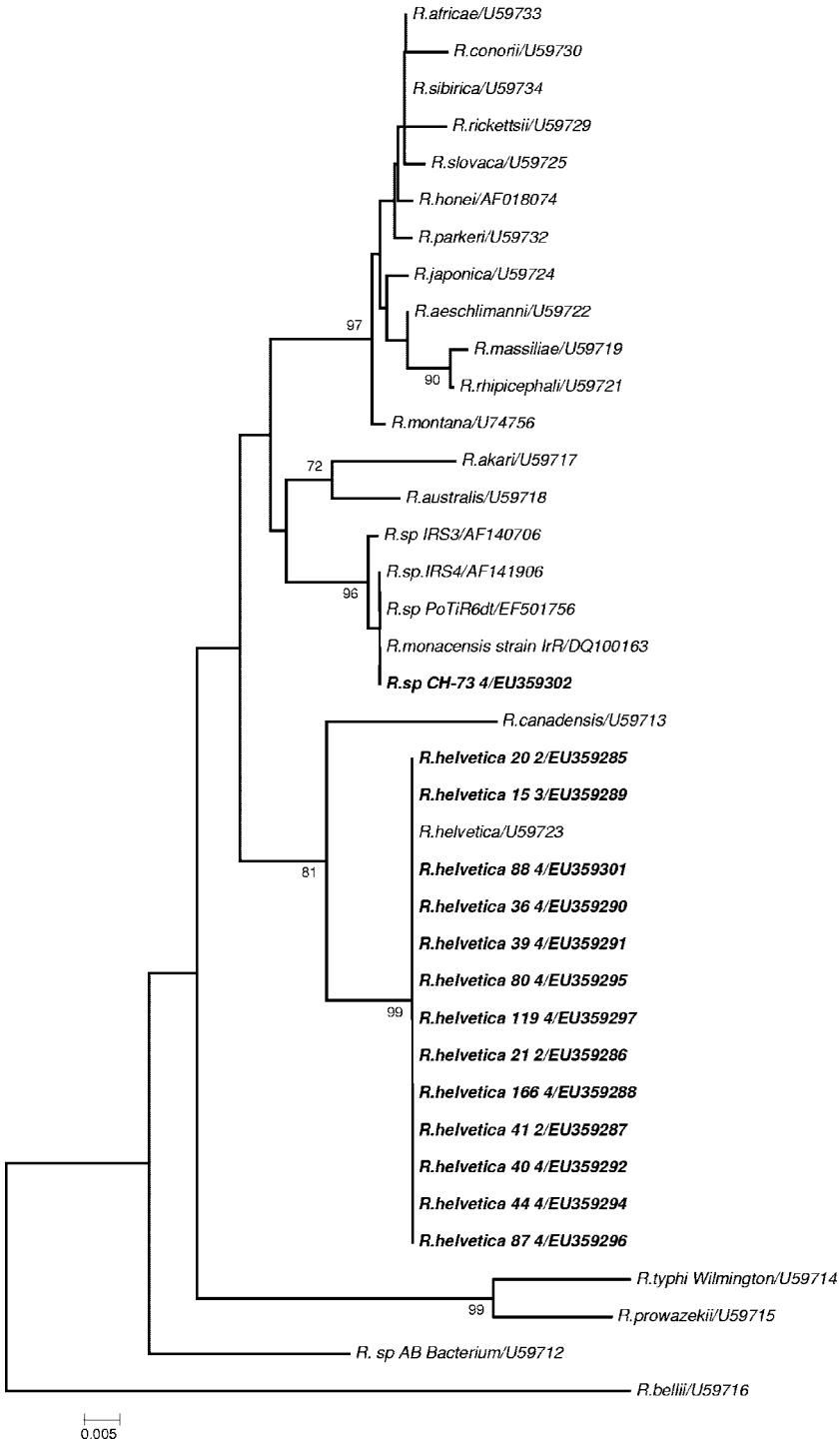


FIG. 2. Bootstrap phylogenetic tree of 14 partial *gltA* sequences from tick pools (1,000 bootstrap resamplings). Only bootstrap values of >70% are shown. The bar represents the mean number of differences per 100 sites. Sequences of 10 tick pools (EU359288, EU359290 to EU359292, EU359294 to EU359297, EU359301, and EU359302) were derived from a 381-bp PCR fragment, three other pools (EU359285 to EU359287) from a 796-bp PCR fragment, and one pool (EU359289) from a 476-bp PCR fragment.

Our newly designed PCR system did not amplify endosymbionts; the latter is of major concern in the development of diagnostic PCR assays for rickettsial and related agents. The assay was found to be more sensitive for the detection of *R. helvetica* than a previously described real-time TaqMan assay that detects the *gltA* gene of rickettsiae of the spotted fever and typhus groups (36). This observation may be explained by the higher specificity of the primers and probe for *R. helvetica* in the newly designed assay compared to the more generic published assay (36). The latter assay was demonstrated to have

three mismatches in the target sequence (36), which may lead to a reduced efficiency (15) and in turn to a lower sensitivity of the assay. Thus, for the most sensitive detection of *R. helvetica* the 23S rRNA gene assay should be used, because with the *gltA* assay, the prevalence of *R. helvetica* could be underestimated.

The C_T difference between the two assays (23S RNA gene and *gltA*) could be used as a marker for the presence of *R. helvetica* compared to other *Rickettsia* species. Samples with lower C_T values for the 23S rRNA gene than for the *gltA* gene were identified as *R. helvetica* ($n = 16$) by sequencing. Remarkably, the one sample with a higher C_T value in the 23S rRNA assay was a *Rickettsia* species that had not been previously reported in Switzerland. Therefore, although the 23S rRNA gene PCR assay was designed to specifically amplify only *R. helvetica*, other rickettsiae, in particular uncharacterized strains, may also be amplified; however, the efficiency at which this would be expected to occur would probably be low. We suggest using the above-mentioned method of comparison of C_T values resulting from the two TaqMan PCR assays (23S and *gltA*) for screening purposes and further confirmation of discordant samples by sequencing. The estimated prevalence of *R. helvetica* in approximately 2,000 analyzed ticks was between 10% and 40%. This was confirmed by sequence analyses, and *R. helvetica* was the only detected rickettsial agent, with the exception of one positive tick pool sample that revealed a different *Rickettsia* species. Its sequence was most closely related to those reported from ticks from Portugal (EF501756.1; *Rickettsia* sp. strain PoTiR6dt), Slovakia (AF141906.1; *Rickettsia* sp. strain IRS4), and Germany (DQ100163.1; *R. monacensis*). This observation is in accordance with other results recently obtained in Germany: *R. monacensis* was isolated and characterized from *I. ricinus* ticks collected in Upper Palatine in southeastern Germany. The *Rickettsia* species was identified by sequencing the genes *gltA*, *rompA*, and *rompB*. Based on the data obtained, an identity of >99% between *R. monacensis* and *Rickettsia* strains IRS3 and IRS4 was observed (R. Wölfel, personal communication). The results show that *R. monacensis* has a much larger area of distribution than previously believed. Furthermore, molecular characterization indicates that *R. monacensis* and the *Rickettsia* strains IRS3 and IRS4 belong to a single species.

The investigation of tick populations may be used as an epidemiological tool to determine the importance of an infectious agent in a specific area. A high percentage of *I. ricinus* ticks collected from vegetation were found to be PCR positive for *R. helvetica*. Interestingly, the estimated prevalence was higher in ticks collected from animals than in ticks collected from vegetation. Our results are in agreement with a study from Denmark by Nielsen and coworkers (18), in which the presence of *R. helvetica* was highest in adult ticks collected from dogs and roe deer. These findings may indicate that large mammals act as reservoir hosts for *R. helvetica*, an assumption which is further supported by a study from Inokuma and coworkers (10). Those authors analyzed blood samples collected from sika deer in Japan and found that 8 out of 102 animals were PCR positive for *R. helvetica*. Since deer are often infested with high numbers of ticks, they can be an important reservoir for tick-borne pathogens. Remarkably, in the present study, more of the ticks from cats than from dogs were PCR positive for *R. helvetica*. For all the ticks collected from cats, the C_T values were lower in the 23S rRNA PCR assay than the *gltA* assay. From an ongoing study, we know that *Rickettsia felis*, which could potentially be found in feline samples but has not been detected in Switzerland as yet, had a higher C_T value in the 23S rRNA assay than in the *gltA* assay (S. Hornok, personal communication). Based on this information, we assume that the high prevalence of *R. helvetica* observed in ticks from cats was not due to *R. felis*, and therefore, cats may actually be a reservoir for *R. helvetica*. In a study from Africa (17), up to 34% of the included cats were found to be seropositive for *R. conorii*, another member of the SFG rickettsiae that is transmitted by *Rhipicephalus sanguineus*. Those authors concluded that cats can be used as indicators for the presence of these organisms (17).

Due to the high abundance and the broad host range of *I. ricinus* and the high prevalence of *R. helvetica* in this tick species, the likelihood of transmission of the agent to tick-exposed individuals should be high. Nonetheless, all the blood samples from different mammals, including wild animals (foxes) and human patients, tested PCR negative. Several reasons have to be considered to explain this unexpected result. First, the sensitivity of our PCR assay may not have been sufficient. Rickettsia loads may be very low, as has been shown for *Rickettsia rickettsii* in rickettsemic patients (5, 11, 12). However, this seems very unlikely, given that the lower detection limit of the applied PCR assay was one copy per reaction and as little as 100 copies/ml blood could still be detected in a spike experiment. In addition, the NA extracted from blood samples did not inhibit the PCRs, in contrast to the NA from some tick samples. A second explanation could be a very focal distribution of *R. helvetica*, leading to high geographical variations in prevalence. This also seems unlikely, because in the present study, the majority of ticks and blood samples originated from the same region (Canton of Zurich). A third explanation could be a seasonal occurrence of rickettsemia associated with the seasonal fluctuation of the tick vector *I. ricinus*; we aimed to avoid this potential source of bias by collecting canine blood samples throughout the year. The lack of detection of *R. helvetica* in blood samples could also have resulted from a short-lived bacteremia. This has been demonstrated for other rickettsiae, such as *R. rickettsii*, which were detectable for up to only 10 days after experimental infection of dogs (12, 23). Thus, infection may indeed have taken place, but the time point of the blood collection may not have been optimal for detection of infection. However, if bacteremia is short-lived in large mammals, the likelihood that they are a major reservoir for *R. helvetica* is low.

Nonetheless, despite the fact that all the blood samples tested PCR negative, the pathogenic potential of *R. helvetica* in animals should not be overlooked. For comparison, the 1,880 ticks collected from vegetation in the Canton of Zurich were analyzed not only for *R. helvetica* but also for TBEV, the infectious agent that causes encephalitis in humans and *Canidae*. The exposure risk of *R. helvetica* was found to be 13 times higher (prevalence, 11.7%) than that of TBEV (prevalence, 0.9%). Therefore, we anticipate that *R. helvetica* infections may also occur in Swiss individuals, as has been demonstrated in other countries with a high prevalence of *R. helvetica* in *I. ricinus* ticks (8, 18). Further studies are necessary to clarify this issue. The ability of our newly developed real-time PCR assay to detect even very low copy numbers and the specific nature of

our assay make it a valuable tool for further evaluation of the importance of *R. helvetica* infections.

ACKNOWLEDGMENTS

We thank Simona Casati, Istituto Cantonale di Microbiologia, Bellinzona, Switzerland, for providing *R. conorii*. Special thanks go to B. Riond, G. Dasen, R. Tandon, K. Hungerbühler, Y. Kessler, K. Museux, G. Wolf, and C. Braun for their helpful contributions and excellent support and to B. Weibel, T. Meili Prodan, B. Pineroli, B. Sigrist, A. Ivos, and E. Gönczi for their excellent laboratory assistance. Laboratory work was performed using the Center for Clinical Studies at the Vetsuisse Faculty of the University of Zurich.

This work was supported by a research grant (Forschungskredit 2006) of the University of Zurich and by the Roche Research Foundation, Basel, Switzerland. R.H.-L. is the recipient of a professorship sponsored by the Swiss National Science Foundation (PP00B-102866 and PP00P3-119136).

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