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# Real-time TaqMan PCR and molecular investigations of *Rickettsia helvetica* infection in dogs, foxes humans and *Ixodes* spp. ticks

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# 1 Summary

*Rickettsia helvetica*, a tick-borne member of the spotted fever group rickettsiae, was found to be pathogenic in humans. Its importance 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 analyze tick vectors (potential exposure risk) and blood samples from *canidae* and humans (prevalence of infection). The newly designed 23S rRNA gene assay for *R. helvetica* was more sensitive than a published *gltA* gene assay for several rickettsiae. Blood samples from 884 dogs, 58 free-ranging foxes, 214 human patients and 2,073 ticks (*Ixodes* spp.) collected either from the vegetation or from animals were analyzed. Although 12% of the unfed ticks and up to 40% of the ticks collected from animals tested PCR-positive, none of the 1,156 blood samples did. Ticks from cats were more frequently PCR-positive than ticks from dogs. Sequencing of the 23S rRNA and/or *gltA* gene of 17 tick pools confirmed the presence of *R. helvetica*. Additionally, a Rickettsia species not yet described in Switzerland was identified. In conclusion, *R. helvetica* was frequently detected in the tick population but not in blood samples. Nonetheless, due to the broad host range of *Ixodes* ticks and the high infection rate (i.e. *R. helvetica* was found 13-times more frequently in unfed ticks than TBEV) a wide variety of mammals may be exposed to *R. helvetica.* Our newly developed sensitive PCR assay represents an important tool to study this issue.

## 2 Introduction

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 have been associated with human and animal diseases. Depending on the occurrence of tick-species, SFG rickettsiae have a worldwide distribution. The most common tick in Europe is *Ixodes ricinus*, which was found to harbor *Rickettsia helvetica*. *R. helvetica* is transmitted transstadially and transovarially in *I. ricinus*. Therefore, this tick is not only a vector but also a reservoir for *R. helvetica*. Due to the broad host range of *I. ricinus*, many mammalian species including humans can serve as hosts, and, therefore, are potentially exposed to *R. helvetica*. Symptoms described for infections in humans include fever, headache, arthralgia, and myalgia (1, 3, 6, 18, 30). The agent also has been implicated in two cases of fatal perimyocarditis (17, 19).

Interestingly, despite the wide distribution of *I. ricinus* ticks and the high infestation rate of these ticks with *R. helvetica* reported from several European countries (2, 8, 15, 16, 22, 26, 30, 31, 38) larger studies about the prevalence of the infection in humans and animals are scarce. No studies evaluating the importance of *R. helvetica* in companion and farm animals are yet available. It is unknown, whether these animals can serve as 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 therefore are considered a common cause of fever of unknown origin in returned travelers (21). As the occurrence of tick-borne infectious diseases and particularly Rickettsia infections are increasing worldwide in human medicine (23), it can be assumed that the same holds true for companion animals. In dogs, fever of unknown origin but responsive to antibiotic treatment can frequently be observed. In these cases, an infectious agent is suspected but rarely, if ever,

confirmed. *R. helvetica* infections might be the underlying cause in some of these cases even if the patient does not have a travel history, since the exposure to *R. helvetica* infected *I. ricinus* ticks might have occurred locally.

To date, the diagnosis of a rickettsial infection has most often been confirmed by serologic testing. However, antibodies are not detectable prior to the second week of illness for any of the so far studied rickettsial diseases. Moreover, except for detection of seroconversion or a fourfold increase in titer, a positive serology 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 if confronted with diseased individuals.

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

# 3 Materials and Methods

## 3.1 Arthropods

To analyze the occurrence of *R. helvetica* in the Swiss tick population, a total of 2,073 ticks had been included in the study. The majority of the ticks had been described previously and had been microscopically determined or assumed to be of the *Ixodes* sp. (36): this included 1,880 unfed ticks that had been collected from the 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* sp. 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 collected from the vegetation for an unrelated study and differentiated according to sex had been 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 extraction of nucleic acids (NA).

## 3.2 Blood samples

A total of 1,156 blood samples were included in the study. They originated from 884 privately owned domestic dogs 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 (34): they had been collected throughout a one year period and 615 (70%) of the presented dogs originated from the Canton of Zurich, Switzerland. The foxes originated from three different beats in the Canton of Zurich; samples consisted of coagulated whole blood or serosanguineous fluids. The 214 human EDTA-anticoagulated blood samples had been provided by a Hospital

in the Canton of Zurich and had been collected for routine diagnostic purposes.

### 3.3 NA extraction

Some ticks were pooled prior to NA extraction as described (36): 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 the MagNA Pure LC Total Nucleic acid isolation kit (Roche Diagnostics, Rotkreuz, Switzerland) or with the DNeasy tissue kit (QIAGEN, Hombrechtikon, Switzerland) (**Table 1**). From EDTA-anticoagulated blood and serosanguineous samples, NA was extracted from a volume of 100 µl or 200 µl using the MagNA Pure LC Total Nucleic acid isolation kit (34). During each extraction, negative controls consisting of 200 µl phosphate buffered saline were concurrently prepared with each batch of 11 to 15 samples to monitor for cross-contamination.

### 3.4 Real-time PCR assays

The presence of amplifiable nucleic acids was confirmed for each sample 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 2x TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in a total volume of 25µl. An initial denaturation 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 samples the 18S rRNA assay revealed unexpectedly high threshold cycle (Ct) values (≥ 30), which could have been attributable to inhibition of the PCR reaction. Inhibition was confirmed by testing a 1:10

dilution of the samples: while a higher value of approximately 3.3 Ct values higher is expected for an uninhibited PCR (sample diluted 1:10), the Ct values obtained from some of our samples were lower after dilution. These samples, which contained substances that inhibited PCR reactions in their undiluted form, were tested in a 1:10 dilution in subsequent PCR assays.

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 (32). The PCR reactions contained a final concentration of 0.2 µM of the primers (CS-F and CS-R) and the probe (CS-P) (**Table 2**), 12.5 µl of the 2x TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and 5 µl or 2.5 µl 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 of 20 s at 95°C was followed by 60 cycles of 95°C for 3 s and 60°C for 30 s.

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 real-time PCR previously described (28). The latter assay was not applied in the present study, because the primer and probe sequences and the conditions of the PCR run were not suitable for a standard TaqMan real-time PCR assay on 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 reactions contained 12.5 µl of the 2x qPCR MasterMix Plus Low ROX (Eurogentec, Seraing, Belgium), a final concentration of 0.9 µM of each primer and of 0.25 µM of the probe, 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 the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) with an initial step of 50°C for 2 min and a denaturation 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 the use with uracil-N-glycosylase to prevent carryover of PCR amplicons. In all PCR systems, water was used as a negative control. All negative extraction and pipetting controls tested PCR-negative. NA from the following bacteria was used to determine the specificity of the real-time PCR assays: *R. helvetica* and *Rickettsia africae* (kindly provided by Dr. R. Wölfel)*, Rickettsia conorii* (kindly provided by Dr. S. Casati), *Anaplasma phagocytophilum, Ehrlichia canis, Mycoplasma haemofelis, 'Candidatus* Mycoplasma haemominutum*', 'Candidatus* Mycoplasma turicensis'*.* For comparison of the prevalence of *R. helvetica* with that of another frequently encountered tick-born 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 described (35).

### 3.5 Production of DNA standard

For absolute quantification, a 330 bp sequence of the 23S rRNA gene of *R. helvetica* (AY125017) enclosing the 65 bp long TaqMan system was amplified using the *R. helvetica* specific primers Rhelv.26f and Rhelv.356r (**Table 2**). The reaction mixture contained 5 µl of 5x Phusion HF buffer (Finnzymes, Espoo, Finland), 0.5 µl of Phusion Hot Start DNA polymerase (Finnzymes), a final concentration of 0.2 mM of dNTP and of 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 was 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 the Gen Elute PCR Clean-Up Kit (Sigma, Fluka GmbH, Buchs, Switzerland) and cloned into the TOPO TA Cloning vector (Invitrogen, Basel, Switzerland). The plasmid was linearized by restriction digestion using *BamHI* (Promega, Wallisellen, Switzerland), purified (QIAquick Gel Extraction Kit, Qiagen) and the copy

number was calculated after both spectrophotometrical (GeneQuant, Amersham-Pharmacia Biotech, Otelfingen, Switzerland) and agarose gel electrophoresis analysis (Syngene, Gene Tools, Syngene, Cambridge, UK). The *R. helvetica* DNA Standard was serially tenfold diluted in a solution containing 30 µg/ml of salmon sperm DNA (Invitrogen), aliquoted and frozen at -20°C until use.

For each serial dilution of the 23S rRNA assay, the slope of the dilutions versus threshold cycle curve was assessed as a measure for the amplification efficiency. Amplification efficiencies were calculated as $(10^{1/(-\text{slope})}-1)$ (12).

### 3.6 Sequencing of *gltA* gene

To confirm the presence of rickettsiae in PCR-positive samples and analyze the prevailing 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 previously described (24) (**Fig. 1** and **Table 2**) with the following modifications: the reaction mixture contained 2.5 µl of 10x PCR TaqGold Buffer (Applied Biosystems), 0.4 µl of AmpliTaqGold Polymerase (Applied Biosystems), 1.5 µl of 25 mM $MgCl_2$, a final concentration of 0.2 mM of dNTP and of 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 of 72°C for 5 min. The PCR products were purified (Gen Elute PCR Clean-Up Kit, Sigma) and sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The reactions were purified using the SigmaSpin Post-reaction cleanup Kit (Sigma) and run on an ABI310 sequencer (Applied Biosystems).

In addition, a longer portion of the *gltA* gene was analyzed using five additional published *gltA*-gene specific PCR assays (24, 27, 29). Primer sequences,

length of the five PCR products and according references are given in **Fig. 1** and **Table 2**. The reaction mixture for the five systems contained 5 µl of 5x Phusion HF buffer (Finnzymes), 0.5 µl of Phusion Hot Start DNA polymerase (Finnzymes), a final concentration of 0.2 mM of dNTP and of 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 of 72°C for 3 min (24).

### 3.7 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 standard production and to confirm PCR-positive results, two partially overlapping fragments of 206 and 330 bp, respectively, were amplified. The primers (**Table 2**) for these assays were designed as follows: for the 206 bp fragment both primers (Rhelv.159f/Rhelv.365r) were based on the sequence of *R. helvetica* (AY125017). However, the 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 on a rickettsia consensus sequence. The reaction mixture and thermal cycling condition were the same as described above for the standard production. PCR products were sequenced as described above for the *gltA* gene, using the PCR amplification primers.

### 3.8 Phylogenetic analyses

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (13). The sequences obtained were compared to known

sequences deposited in the GenBank database and percentage identity was calculated by pairwise comparison (Kimura 2-Parameter model) (11). The sequences were aligned using CLUSTAL W (33) and manually adjusted. Only positions where the nucleotide composition was known in all sequences being compared were used in the phylogenetic analysis. The phylogenetic tree was constructed after trimming of the primer sequences with the neighbor-joining method (25) from a distance matrix corrected for nucleotide substitutions by the Kimura 2-parameter model. The data set was resampled 1,000 times to generate bootstrap values.

### 3.9 Statistics

Prevalence and confidence intervals (CI) were calculated as described (37) using the open-source program R (http://www.r-project.org/). Rickettsial loads of female and male tick pools were compared using the Mann-Whitney *U*-test (4); the NA content of each pool was controlled for using the 18S rRNA gene assay. Frequencies of PCR-positive results from ticks collected from cats versus those collected from dogs were compared using the $Chi^2$ test (4). Ticks from horses were not included in this analysis because of small numbers of ticks (n = 5).

### 3.10 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-EU359283, EU359285-EU359297 and EU359301 for the *gltA*-gene and EU359273-EU359278 for the 23S rRNA gene. In addition sequences found for the tick pool #73 have been submitted to GenBank and given the accession numbers: EU359284, EU359298-EU359300 and EU359302.

# 4 Results

## 4.1 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 were analyzed. They all tested real-time PCR-negative with the exception of *R. helvetica*. To confirm that the PCR system did not amplify endosymbionts, which are only present in female ticks, 24 tick pools consisting of 20 male ticks and 24 pools containing ten female ticks were assayed. All pools tested positive using the 23S rRNA real-time PCR assay. No significant difference was found in the rickettsial NA load between the female and male tick pools using the 23S rRNA real-time PCR assay quantitatively (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 the 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 *R. helvetica* (AY125017).

## 4.2 Linear range of amplification and sensitivity of the real-time PCR assay

The 23S rRNA gene R. helvetica DNA standard was serially tenfold diluted to assess the linear range and sensitivity of the assay. The amplification of the serial dilutions showed linearity over eight 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.

### 4.3 Rickettsia in arthropods collected from the vegetation

The 188 NA tick pools collected from the vegetation in the area around Zurich, Switzerland, were analyzed by the *gltA* gene real-time PCR for the presence of rickettsiae of the spotted fever group and typhus group: 122 out of the 188 pools tested positive. This calculated into an estimated prevalence of rickettsiae of the spotted fever and typhus group 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. Analyzing unfed ticks from the vegetation of Switzerland, the majority of the pools (134 out of 188) were found to be positive for the 23S rRNA gene. This calculated into an estimated 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 threshold cycle (Ct) value. The Ct value is an inverse measure of the template load: the higher the template load, the lower the Ct value. Analyzing the 188 tick pools from the vegetation, all of the Ct values from the *gltA* gene real-time PCR assay were higher than the Ct values from the 23S rRNA gene real-time PCR assay, except for one tick pool, #73. Twelve pools tested positive in the 23S rRNA assay but negative in the *gltA* assay. Tick pool #73 and some additional pools were further analyzed by sequencing.

For comparison, the 188 tick pools have also been analyzed for TBEV: 16 out of 188 pools tested positive. This calculated into an estimated prevalence of 0.9% (95% CI, 0.5 to 1.4%).

### 4.4 Rickettsia in arthropods collected from animals

Overall, 49 out of 168 arthropod samples (29%), 43 out of 153 ticks (28%) and 6 out of 15 tick pools, tested real-time PCR-positive with the *gltA* gene specific TaqMan PCR system. When the samples were classified according to the animal species they had been collected from, 26 out of 62 (42%) ticks and two out of three tick pools from cats were found positive. This calculated in an overall sample prevalence of rickettsiae of the spotted fever and typhus group in ticks from cats of 40.9% (95% CI, 29.8 to 52.9%; **Table 1**). Ticks from 18 out of 39 cats (46%) were found *gltA* PCR-positive. For ticks collected from dogs, 16 out of 86 ticks (19%) and four out of 12 pools containing two to four ticks per pool were positive. The sample prevalence in ticks from dogs was 17.6% (95% CI, 11.4 to 25.3%; **Table 1**). Ticks collected from 18 out of 66 (27%) dogs tested PCR positive. One out of five ticks (20%) collected from horses was positive. Ticks collected from cats were significantly more frequently *gltA* PCR-positive than ticks collected from dogs ($pChi^2 = 0.0019$). In addition, ticks from a higher percentage of cats then of dogs were PCR-positive ($pChi^2 = 0.0489$).

Overall, 66 of 168 arthropod samples (40%) - 57 out of 153 ticks (37%) and nine out of 15 tick pools - collected from animals tested real-time PCR-positive for the 23S rRNA gene of *R. helvetica.* When the samples were classified according to the animal species from which they were collected, 32 out of 62 ticks (51%) and two out of three pools contained three ticks per pool collected from cats were positive; the overall sample prevalence for ticks from cats calculated into 49.9% (CI, 38.1 to 61.7%; **Table 1**). Ticks from 21 out of 39 cats (54%) tested positive. In ticks from dogs, 24 out of 86 (28%) ticks and seven out of twelve tick pools tested positive. The sample prevalence in ticks from dogs was 28.3% (CI, 20.4 to 37.2%; **Table 1**). Ticks collected from 27 out of 66 dogs (40%) tested positive. One out of 5 ticks (20%) collected from horses was positive. Ticks collected from cats were significantly more

frequently 23S rRNA gene PCR-positive than ticks collected from dogs ($pChi^2$ = 0.0033).

Again, Ct values for the 23S rRNA assay were, with one exception (tick #17), lower than those of 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 further analyzed by sequencing because of a low bacterial load. It originated from a Japanese Chin dog nine years of age from the Canton of Zurich.

### 4.5 Rickettsia in blood samples from dogs, foxes and humans

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

### 4.6 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 tenth pool, tick pool #73, resulted in a sequence with 16 mismatches to *R. helvetica* (AM418450). This sequence was most closely related (98-99% identity) to the following Rickettsia species: *Rickettsia* sp. *IRS4* (AF141906) found in *I. ricinus* in Slovakia, *Rickettsia monacensis* (DQ100163) reported in Germany and *Rickettsia* sp. *PoTiR6dt* (EF501756) from *I. ricinus* in Portugal.

For the latter (tick pool #73) and four additional tick pools (#15, #20, #21, #41), several other fragments could be amplified and sequenced. For all five pools

the 163 bp PCR product and for all but #15 796 bp were amplified (**Fig. 1** and **Table 2**). Moreover, for three ticks the fragment of 476 bp and for tick pool #15 the PCR-product with a length of 353 bp was successfully amplified (**Fig. 1** and **Table 2**). The sequences that resulted for the additional four tick pools (#15, #20, #21, #41) were most similar (98-99%) to *R. helvetica (*U59723). Tick pool #73, however, showed overall 34 mismatches to *R. helvetica* (U59723) and was most closely related (99%) to *Rickettsia* sp. *IRS3* (AF140706) and *R.* sp. *IRS4* (AF141906) found in *I. ricinus* ticks collected in Slovakia, and *R. monacensis* (DQ100163) reported in Germany.

### 4.7 Phylogenetic analyses

Phylogenetic analyses were based on the 348 bp long sequence of the *gltA* gene of 14 sequenced tick pools (**Fig. 2**). These sequences were obtained from ten 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 fourteen sequences clustered with *R. helvetica* (U59723). Only the tick pool #73 branched away from the remaining sequenced tick pools and was most closely related to *R. monacensis* (DQ100163), *R.* sp. *IRS4* (AF141906) and *R.* sp. *PoTiR6dt* (EF501756) (**Fig. 2**).

## 5 Discussion

This study expands the knowledge on the distribution of *R. helvetica* infection by employing a newly developed sensitive real-time TaqMan PCR assay in ticks, foxes, dogs and humans within a geographic region (Canton of Zurich, Switzerland). We concentrated on the investigation of humans and *canidae* because both are known to develop disease when infected with SFG-rickettsiae. While *R. helvetica* was frequently detectable in *Ixodes* spp. 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 the vegetation. With one exception, all identified rickettsiae were assumed to be *R. helvetica*. In addition, a Rickettsia never described previously in Switzerland was identified according to the sequencing results.

The herein described real-time TaqMan PCR assay amplifies a fragment of the 23S rRNA gene of *R. helvetica.* A recently described assay for the *R. helvetica* 23S (28) 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. Our newly designed PCR system did not amplify endosymbionts; the latter is of major concern when developing diagnostic PCR assay for rickettsiae 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 group (32). This can be explained by the higher specificity of the primers and probe for *R. helvetica* of the newly designed assay compared to the published more generic assay (32). Thus, for most sensitive detection of *R. helvetica* the 23S rRNA gene assay should be used because with the *gltA* assay *R. helvetica* prevalence could be underestimated.

The Ct difference between the two assays (23S and *gltA*) could be used as a marker for the presence of *R. helvetica* compared to other rickettsiae. Samples with lower Ct values for the 23S rRNA gene compared to the *gltA* gene were identified by sequencing to be *R. helvetica* (n=16). Remarkably, the one sample with a higher Ct value in the 23S rRNA assay was a Rickettsia species not yet reported in Switzerland. Therefore, although the 23S rRNA gene PCR assay was designed to specifically amplify only *R. helvetica*, other rickettsiae particularly so far uncharacterized strains may be amplified although probably with a low efficiency. We suggest using the above mentioned method of comparison of Ct values resulting from the two TaqMan PCR assays (23S and *gltA*) for screening purposes and further confirmation by sequencing of

discordant samples. The estimated prevalence of *R. helvetica* in approximately 2,000 analyzed ticks was between 10 and 40%. Confirmed by sequence analyses, *R. helvetica* was the only detected rickettsial agent with the exception of one positive tick pool sample, which revealed a different Rickettsia species not yet reported in Switzerland. Its sequence was most closely related to those reported from ticks from Portugal (EF501756.1: *Rickettsia* sp. *PoTiR6dt*), Slovakia (AF141906.1: *Rickettsia* sp. *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 *Ixodes ricinus* ticks collected in Upper Palatine in South-Eastern Germany. The Rickettsia species was identified by sequencing of *gltA*, *rOmpA* and *rOmpB* genes. Based on the obtained data an identity of > 99% between *R. monacensis* and *R.* spp. *IRS3* and *IRS4* could be demonstrated (*Wölfel et al.*, personal communication). The results show that *R. monancensis* has a much larger area of distribution than known so far. Furthermore, the molecular characterization indicates that *R. monacensis* and the *R.* spp. *IRS3* and *IRS4* belong to only one single species.

Investigation of tick populations can 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 the vegetation was found PCR-positive for *R. helvetica*. Interestingly, in ticks collected from animals the estimated prevalence was higher than in ticks collected from the vegetation. Our results are in agreement with a study by Nielsen and coworkers (15) in which the presence of *R. helvetica* was highest in adult ticks collected from dogs and roe deer. These findings might point to larger mammals as reservoir hosts for *R. helvetica*, an assumption which is further supported by a study from Inokuma and coworkers (9). The authors analyzed blood samples collected from Sika deer in Japan and found 8 out of 102 animals PCR-positive for *R. helvetica*. Since deer are often infested with high numbers of

ticks, they can be important reservoir animals for tick-borne pathogens. Remarkably, in the present study more ticks from cats than from dogs were PCR-positive for *R. helvetica*. For all ticks collected from cats, the Ct values were lower in the 23S rRNA than in the *gltA* gene PCR assay. From an ongoing study we know that *R. felis* - that could potentially occur in feline samples but has not yet been detected in Switzerland - led to a higher Ct value in the 23S rRNA than in the *gltA* assay (S. Hornok, personal communications). From this we assume that the high prevalence observed in ticks from cats was not due to *R. felis* and, therefore, cats actually may be a reservoir for *R. helvetica*. In a study from Africa, up to 34% of the included cats were found seropositive for *Rickettsia conorii*, another member of the spotted fever group rickettsiae that is transmitted by *Rhipicephalus sanguineus*. The authors concluded that cats can be used as indicators for the presence of these organisms (14).

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 blood samples from different mammals, including wild animals (foxes) and humans, 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 it has been shown, e.g., for *R. rickettsii* in rickettsiemic patients (5) (10). However, this seems unlikely because the lower detection limit of the applied PCR assay was one copy per reaction, which calculates into 200 copies/ml blood. A second explanation could be a very focal distribution of *R. helvetica* leading to high geographic variations of the prevalence. This seems also 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 rickettsiemia 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 an entire year. The lack of detection of *R. helvetica* in blood samples could also have resulted from an only short-lived bacteriemia. This has been demonstrated for other rickettsiae, such as *R. rickettsii*, which were detectable only up to 10 days after experimental infection of dogs (20). Thus infection might indeed have taken place, but the time point of the blood collection might not have been optimal. Yet, if bacteriemia is only short-lived in larger mammals, the likelihood of them being a major reservoir for *R. helvetica* is low.

Nonetheless, despite all blood samples tested PCR-negative, a pathogenic potential of *R. helvetica* in animals should not be excluded. For comparison the 1,880 ticks collected from the vegetation in the Canton of Zurich were not only analyzed for *R. helvetica* but also for TBEV, the infectious agent leading to encephalitis, e.g., in humans and *canidae*. The exposure risk to *R. helvetica* was found to be 13-times higher (prevalence 11.7%) than that to TBEV (prevalence 0.9%). Therefore, we anticipate that *R. helvetica* infections may occur also in Swiss individuals as has been demonstrated in other countries with a high prevalence of *R. helvetica* in *I. ricinus* ticks (7, 15). Further studies are necessary to clarify this issue. The ability of our newly developed real-time PCR to detect even very low copy numbers and the specific nature of our assay make it a valuable tool to further evaluate the importance of *R. helvetica* infections

## 6 Tables and Figures

TABLE 1. Species, number, source and extraction methods of ticks, which were collected throughout Switzerland and number and percentage that tested PCR-positive for *R. helvetica.*

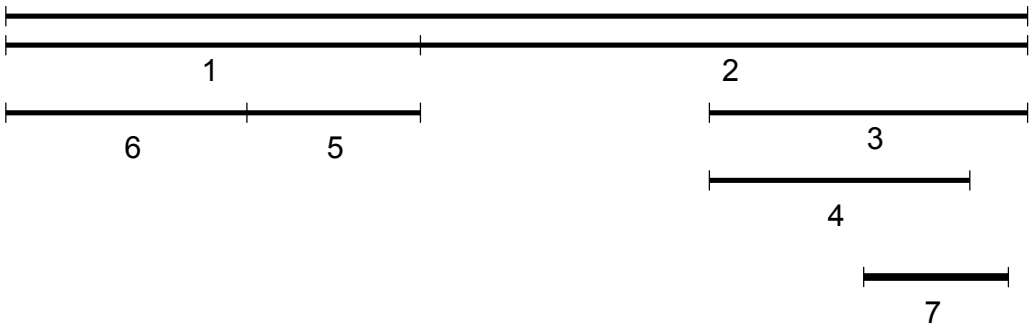
| Sample group | Number of ticks / blood samples | Source | Origin | Number of ticks pooled/ extraction | NA extraction | *gltA* gene real-time PCR positive (percentage) | 23S rRNA gene real-time PCR positive (percentage) |
|---|---|---|---|---|---|---|---|
| **Ticks from vegetation** | 1880 | vegetation | ZH | 10 | MagNa Pure[1] | 9.9% | 11.7% |
| **Ticks from animals** | | | | | | | |
| ***Ixodes* sp.** | 71[2] | 39 cats | CH/ZH[3] | 1-3 | DNeasy tissue kit[4] | 40.9% | 49.9% |
| | 117[5] | 66 dogs | CH/ZH[6] | 1-4 | DNeasy tissue kit | 17.6% | 28.3% |
| | 5 | 5 horses | ZH | - | DNeasy tissue kit | 120% | 20% |
| **Blood samples** | 884[7] | dogs | CH/ZH[8] | - | MagNa Pure | 0 | 0 |
| | 58 | foxes | ZH | - | MagNa Pure | 0 | 0 |
| | 214 | humans | ZH | - | MagNa Pure | 0 | 0 |

[1]MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche). [2](36): the sample consisted of 62 single ticks and 3 tick pools. [3]The majority (83%) of these ticks had been collected from cats in the Canton of Zurich. [4]Qiagen. [5] 110 out of 117 samples 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. [6]The majority (56%) of these ticks had been collected from dogs in the Canton of Zurich. [7]From the originally 889 dogs (34), 884 were included in the study; no sample was available anymore from 5 dogs. [8]The majority (70%) of these blood samples had been collected from dogs in the Canton of Zurich. ZH: Canton of Zurich; TI: Canton of Ticino; CH: Switzerland.

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

| Target | Oligo nucleotide | Sequence | Reference | Apmlicon lengths |
|---|---|---|---|---|
| **23S-Gen** | | | | |
| **Real-time PCR** | | | | |
| probe | Rickhelv.170p | 6FAM-AAC CGT AGC GTA CAC TTA-MGBNFQ | This study | |
| 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 | 65bp |
| **Sequencing primer** | | | | |
| forward | rhelv.159f | ACG GAA CAC AGA ACC GTA GC | This study | |
| reverse | rhelv.365r | CTG GAT ACC GTG GAT CAA GC | This study | 206bp |
| forward | rhelv.26f | AAT GTG TGT AGC TAA CCG ATA CTA AT | This study | |
| reverse | rhelv.356r | GTG GAT CAA GCC ATG ATA TGA C | This study | 330bp |
| ***gltA*-Gen** | | | | |
| **Real-time PCR** | | | | |
| probe | CS-P | FAM-TGC AAT AGC AAG AAC CGT AGG CTG GAT G-BHQ | [1] | |
| forward | CS-F | TCG CAA ATG TTC ACG GTA CTT T | [1] | |
| reverse | CS-R | TCG TGC ATT TCT TTC CAT TGT G | [1] | 74bp |
| **Sequencing primer** | | | | |
| forward | Rp877p | GGG GAC CTG CTC ACG GCG G | [2] | |
| reverse | Rp1258n | ATT GCA AAA AGT ACA GTG AAC A | [2] | 381bp |
| forward | CS1f | ATG ACT AAT GGC AAT AAT AA | [3] | |
| reverse | CS477r | TAA GTT CGT AGT CTG CTT CC | [3] | 496bp |
| forward | CS477f | GGA AGC AGA CTA CGA ACT TA | [3] | |
| reverse | CS1273r | GAT AAC CAG TGT AAA GCT GT | [3] | 796bp |
| forward | Rp414f | AAA CAG GTT GCT CAT CAT TC | [4] | |
| reverse | CS477r | TAA GTT CGT AGT CTG CTT CC | [3] | 163bp |
| forward | CS1f | ATG ACT AAT GGC AAT AAT AA | [3] | |
| reverse | CS353r | GAA TGA TGA GCA ACC TGT TT | [3] | 353bp |
| forward | Rp877p | GGG GAC CTG CTC ACG GCG G | [2] | |
| reverse | CS1273r | GAT AAC CAG TGT AAA GCT GT | [3] | 476bp |

[1] (32); [2] (24); [3] (27); [4] (29). 6FAM: 6-carboxyfluorescein; MGB: minor groove binder; NFQ: non-fluorescent quencher; BHQ: black-hole quencher.




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**

FIGURE 1. Schematic diagram of the sequencing procedures for the *gltA* gene. The amplification of six different amplicons was attempted. For sequencing primers see TABLE 2.

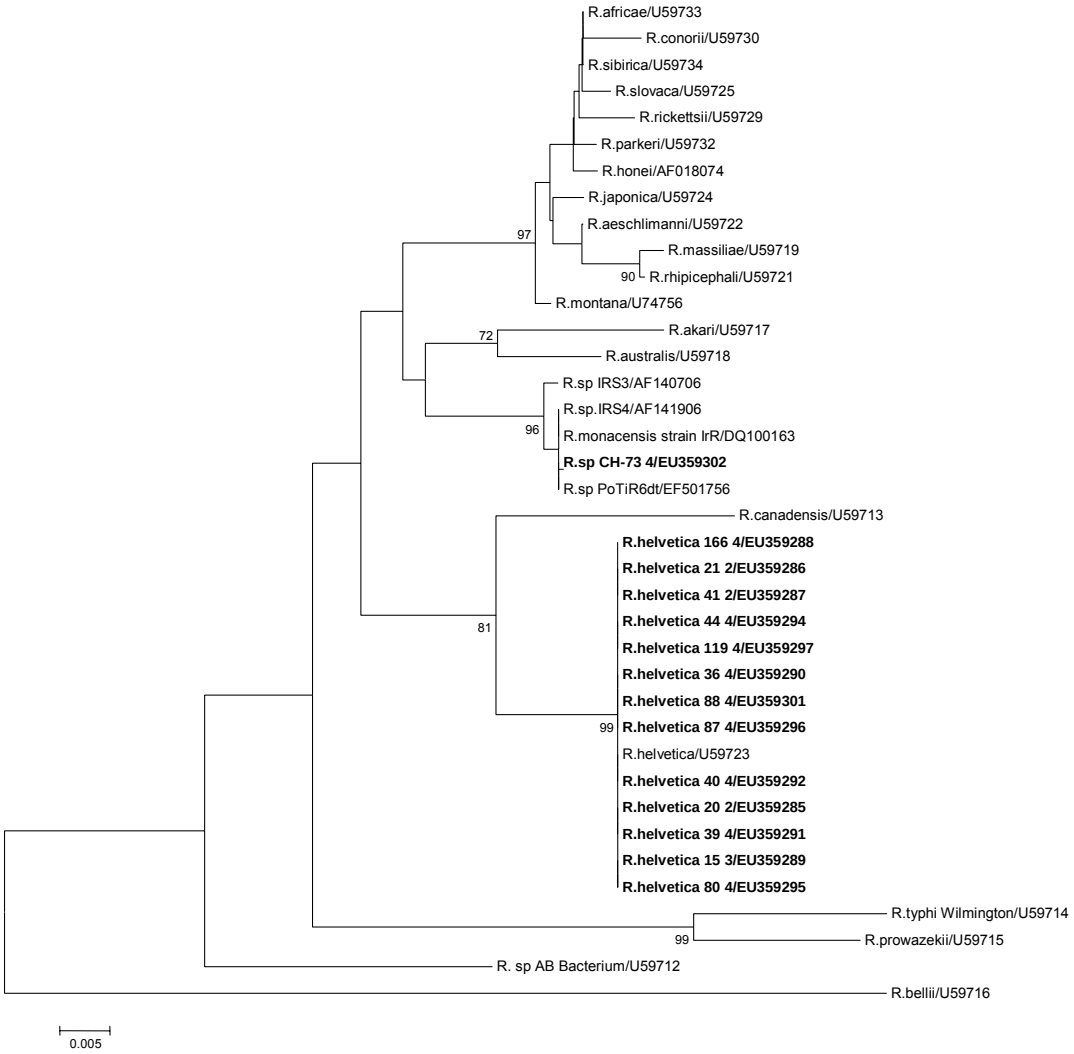



FIGURE 2. Phylogenetic tree.

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

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