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Rickettsia helvetica in *Ixodes ricinus* Ticks in Sweden

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In the present study further characterization of the amplified sequence of the citrate synthase gene of the spotted fever group *Rickettsia* isolated from *Ixodes ricinus* ticks in Sweden showed that it has 100% homology with the deposited sequence of the citrate synthase gene of *Rickettsia helvetica*. The restriction fragment length polymorphism (RFLP) pattern of an amplified 382-bp product of the citrate synthase sequence, defined by primers RpCS877 and RpCS1258, yielded fragments for our isolate that could be visualized as a double band that migrated at approximately 44 bp, another double band at 85 bp, and a single band at nearly 120 bp after digestion with the restriction enzyme *Alu*I. When calculating a theoretical PCR-RFLP pattern of the sequence of the citrate synthase gene of *R. helvetica* from the known positions where the *Alu*I enzyme cuts, we arrived at the same pattern that was obtained for our isolate, a pattern distinctly different from the previously published PCR-RFLP pattern for *R. helvetica*. Investigation of 125 living *I. ricinus* ticks showed a higher prevalence of rickettsial DNA in these ticks than we had found in an earlier study. Rickettsial DNA was detected by amplification of the 16S rRNA gene, for which a seminested primer system consisting of two oligonucleotide primer pairs was used. Of the 125 ticks, some were pooled, giving a total of 82 tick samples, of which 20 were found to be positive for the rickettsial DNA gene investigated. When considering the fact that some of the positive samples were pooled, the minimum possible prevalence in these ticks was 20 of 125 (16%) and the maximum possible prevalence was 46 of 125 (36.8%). These prevalence estimates conform to those of other studies of spotted fever group rickettsiae in hard ticks in Europe.

The presence of spotted fever group *Rickettsia* in ticks has been reported from several European countries, such as *Rickettsia slovaca* in Eastern Europe (20) and *Rickettsia conorii* in France, Italy, and Spain (11, 16, 18). In 1979, "the Swiss agent," a rickettsial strain of unknown human pathogenicity, was isolated from the common tick (*Ixodes ricinus*) in Switzerland (7). Identification based on indirect microimmunofluorescence, serologic typing, and microagglutination data indicated that this isolate differed from all prototype strains of spotted fever group rickettsiae that had been studied up to that time. Further characterization by Beati et al. (5) suggested that this *Rickettsia* represented a new member of the spotted fever group of rickettsiae and suggested the name *Rickettsia helvetica*. More than 25% of ticks found on humans and large mammals in Sweden belong to the species *I. ricinus*. In 1996, we discovered a rickettsia of the spotted fever group. This was the first time that a *Rickettsia* species was recorded as being indigenous to Scandinavia (15). A preliminary characterization, including amplification and sequencing of almost the entire 16S rRNA gene, showed 100% homology with the published sequence of *R. helvetica* (GenBank accession no. U59723). On the other hand, digestion of the approximately 380-bp fragment of the citrate synthase gene with *Alu*I revealed a pattern with distinct differences from the published restriction fragment length polymorphism (RFLP) pattern of *R. helvetica* (5, 15). The aim of the present study was to further characterize the genome of the rickettsia that was detected and to obtain more data on its prevalence in live ticks from Sweden.

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MATERIALS AND METHODS

Ticks. A total of 125 *I. ricinus* ticks were collected; these included 79 females, 7 males, 19 nymphs, and 23 larvae. All were collected in the eastern parts of southern and central Sweden during 1996 and 1997 and were identified by standard taxonomic characteristics. The larvae and three adult ticks were collected from vegetation; the others were from dogs, cats, humans, roe deer (*Capreolus capreolus*), and moose (*Alces alces*). The ticks were stored live in moist plastic vials at 4°C until they were subjected to DNA isolation procedures. Most larvae and nymphs and a few of the adults were pooled together for DNA isolation, giving a total of 82 tick samples for use as a template for PCR.

DNA isolation. The ticks were disinfected in 70% alcohol, blotted and dried for a few minutes, transferred to Eppendorf tubes, and homogenized with a mortar prior to DNA preparation. After trituration and the addition of 300 μl of low-salt TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA, 10 mM NaCl), 20 μl of 20% sodium dodecyl sulfate, and 5 μl of 10 mg of proteinase K (Boehringer Mannheim) per ml, the mixture was incubated at 55°C for 1 h and heated at 95°C for 10 min. Extraction was performed twice with saturated phenol, once with a mixture of phenol-chloroform, and finally, once with chloroform. The DNA was precipitated overnight after the addition of a 1/10 volume of 4 M sodium acetate and 3 volumes of 99% ethanol and was collected by centrifugation at 20,000 × *g* in a microcentrifuge. The pellet was washed with 70% alcohol and then dried under vacuum and dissolved in 50 μl of distilled water before being used as a template for PCR.

PCR amplification. For amplification and sequencing of the 16S rRNA gene (rDNA), a seminested primer system consisting of Ric-RicU8 and Ric-RtRic, as described previously (15), was chosen. For amplification and sequencing of the whole citrate synthase gene, we used a seminested system consisting of six oligonucleotide primers (Table 1). A 2-μl portion of the DNA template was amplified in a reaction mixture containing 1 U of *Taq* DNA polymerase (Boehringer Mannheim) and 5 μl of 10× buffer supplied with the enzyme, 5 μl of 2 mM deoxynucleoside triphosphate, 5 pmol of each primer, 2 μl of 0.25 mM $MgCl_2$, and double distilled water to 50 μl. Amplification was carried out under the following conditions: an initial 3-min denaturation step at 95°C was followed by

TABLE 1. Summary of the primers used for PCR amplification of the citrate synthase gene of rickettsia

Primer	Sequence (5′ to 3′)	Citrate synthase gene position
CS1d	ATGACTAATGGCAATAATAA	1–20
RH314	AAACAGGTTGCTCATCATTC	314–333
RH654	AGAGCATTTTTTATTATTGG	654–635
RtRpCS877	CCGCCGTGAGCAGGCCCCC	815–797
RpCS877	GGGGACCTGCTCACGGCGG	797–815
RpCS1258	ATTGCAAAAAGTACAGTGAACA	1178–1157

35 cycles consisting of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 45 s. Amplification was completed by incubation for 5 min at 72°C. The reaction was performed in a Perkin-Elmer 9600 thermocycler, and the amplified products were analyzed on a 1.5% agarose gel (Kodak) in 0.5× Tris-borate-EDTA (TBE) buffer. As positive controls, purified DNA from *Rickettsia prowazekii* Madrid (a kind gift from R. Regnery, Centers from Disease Control and Prevention, Atlanta, Ga.) and/or *Bartonella henselae* Houston 1 was used. As a negative control for each tick sample, pure PCR buffer treated in the same way as the tick samples was included.

DNA sequencing. Direct solid-phase DNA sequencing of fragments of the citrate synthase gene was performed. Immobilization of the biotinylated PCR products, followed by strand separation and template preparation, was carried out with superparamagnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway). The nucleotide sequences of the citrate synthase gene was determined in both directions by automated solid-phase DNA sequencing with the ALF (automated laser fluorescence) system (Amersham Pharmacia Biotech, Uppsala, Sweden) (12, 13, 17, 23). Sequencing was also performed manually with [^{35}P] dATP (Amersham, Pharmacia Biotech) and Sequenase II (United States Biochemicals, Cleveland, Ohio) according to the instructions of the suppliers.

RFLP analysis. Products from the PCR with primers directed to the subterminal part of the citrate synthase gene of *R. prowazekii* (19) were digested with the *Alu*I restriction enzyme (Boehringer Mannheim) by use of the supplier's optimized buffers. Electrophoretic separation was performed in a gel consisting of 2.0% agarose (Kodak) and 3% NuSieve GTG agarose (FMC Bioproducts, Rockland, Maine) in TBE buffer. The DNA fragments were visualized by ethidium bromide staining, and fragment sizes were compared with the sizes from a standard molecular weight marker (Pharmacia, Uppsala, Sweden).

Hemolymph test and isolation of rickettsiae. After a 10-min disinfection in 3% hydrogen peroxidase, the living ticks were washed in 70% alcohol, rinsed in distilled water, and dried on sterile filter paper. One droplet of hemolymph, obtained by cutting a foreleg of the tick, was placed on a slide and was stained by the method of Giménez (9). A further droplet of hemolymph was mixed with 500 μl of brain heart infusion broth and the mixture was inoculated into two shell vials containing monolayers of Vero cells. The vials were centrifuged at 1,500 × *g* for 0.5 h, and the supernatant was discarded and replaced with 1 ml of Eagle's minimum essential medium containing 4% fetal calf serum and 2 mM L-glutamine. After 6 days of incubation in a CO_2 incubator at 32°C, the cells were gently scraped from the bottom of the shell vial and stained for the detection of rickettsiae. The cells in the infected shell vials were detached with trypsin and transferred to 25-cl cell culture flasks, where they were subcultured with Eagle's minimum essential medium (4% fetal calf serum).

RESULTS

Identification by PCR. All 125 ticks were examined for *Rickettsia* spp. For the detection of rickettsial DNA, the 16S rRNA gene was amplified by use of a seminested primer system consisting of the oligonucleotide primers Ric-RicU8 and Ric-RtRic. Twenty of the 82 tick samples were positive for rickettsial DNA. Of these positive samples, 15 consisted of adult females, 3 included an adult male, and 2 included one nymph. In one of these samples, however, one nymph was pooled with 23 larvae (Table 2). A total of 10 of 32 ticks (31%) collected from dogs, 4 of 25 ticks (16%) collected from cats, 3 of 4 ticks (75%) collected from roe deer, 1 of 2 ticks (50%) collected from moose, 1 of 3 ticks (33%) ticks collected from humans, and 2 of 57 ticks (3%) collected from vegetation were positive for rickettsial DNA (Table 3).

Sequencing and RFLP analysis of the citrate synthase gene. By combining a system of six oligonucleotide primers, the complete citrate synthase gene could be amplified and sequenced. The sequence of our isolated rickettsia showed 100% similarity with the deposited sequence of *R. helvetica* (accession no. U59723). The theoretical digestion of the amplified 382-bp product, of the citrate synthase sequence of *R. helvetica*, defined by primers RpCS877 and RpCS1258, with the *Alu*I restriction enzyme yields fragments of 6, 43, 44, 81, 86, and 122 bp. Digestion of the amplified sequence from our isolate with *Alu*I gave an RFLP pattern similar to that calculated theoretically, as shown in Fig. 1.

TABLE 2. Distribution of ticks, tick samples, and pooled samples and distribution of various developing stages in different samples[a]

Parameter	Adult males	Adult females	Nymphs	Larvae	Total
No. of ticks	7	78	17	23	125
No. of samples	4	70	14	1	82[a]
No. of positive samples	3	15	2	1	20
No. of ticks in positive samples	3	15	5	23	46
No. of positive pooled samples	0	0	2	1	3
No. of individual ticks in positive pools[b]			4 n, 23 l, and 1 n	23 l and 1 n	
% Positive ticks	42.9	19	11.7–29.4	4.3–100	16–36.8

[a] Some sample pools contained individuals from different groups, and so the total number is smaller than the number of individual groups.
[b] n, nymph; l, larvae.

Isolation of rickettsia. In the cell culture, growth which could be detected both by staining by the method of Giménez (9) and by its ability to yield positive PCR products was found. This procedure had the advantage of giving suitable and generous DNA preparations for use in further molecular analyses. However, bacterial and fungal contamination of the shell vial, probably due to a lack of antibiotic and antifungal additives, made it necessary to discard most of these cultures from the cell culture incubator.

DISCUSSION

The prevalence of rickettsial DNA in *I. ricinus* in the present study is considerably higher than that in our previous study (15), in which alcohol-preserved ticks were used. From a total of 125 living ticks, 82 tick samples were prepared, and of these, 20 were positive for the 16S rRNA gene when a seminested primer system combining the oligonucleotide primers Ric-RicU8 and Ric-RtRic was used for the detection of rickettsial DNA. Of the positive samples, a few contained more than one tick specimen. With the assumption that only one tick in each of the pooled samples was positive for rickettsial DNA, the minimum prevalence would be 16% (20 of 125). If, on the other hand, all the ticks in the pooled sample were positive, the

TABLE 3. Distribution of complete tick material by host animal

Host animal positive for ticks	No. of ticks	Minimal no. of positive ticks	Minimal % positive ticks
Dog	32	10	31
Cat	25	4	16
Human	3	1	33
Roe deer	4	3	75
Moose	2	1	50
In vegetation	59	2	3
Total	125	21	16.8

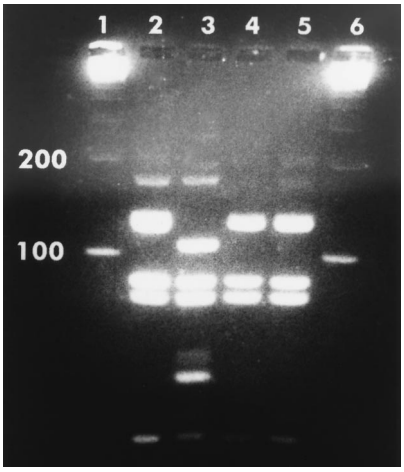

FIG. 1. Ethidium bromide-stained agarose gel (2.0% agarose plus 3% NuSieve agarose). The RFLP pattern of the PCR product from the terminal citrate synthase gene digested with *Alu*I is shown. Lanes 1 and 6, molecular weight markers; lane 2, *R. helvetica* (our isolate); lane 3, *R. prowazekii*; lane 4, *R. canada*; lane 5, PCR product from positive *I. ricinus* tick tested directly, without DNA isolation.

maximum prevalence would be 36.8% (46 of 125). On the basis of the fact that the frequency of positive ticks in the pooled samples can be expected to be the same as that in the entire selection of tick material, it is probable that the most accurate prevalence rate is 22% (27 of 125). In our previous study PCR analyses of 748 adult and a few nymphal *I. ricinus* ticks revealed that 13 (1.7%) were positive for rickettsial DNA. The higher prevalence rate observed in the present study might be explained by differences in the tick materials as well as methodological differences. In the earlier study the ticks had been stored for 5 to 6 years in 70% alcohol at room temperature until they were subjected to DNA isolation. In this study the ticks were kept alive. The time that elapsed from the time of collection of the ticks to the time of DNA extraction varied from less then 1 h to usually 1 to 2 days and, more rarely, up to 3 weeks. These different procedures for the storage of the collected ticks may have affected the amounts of rickettsial DNA available. It has been suggested that the number of viable bacteria might be increased and might thereby improve the in vitro cultivation result if the engorged tick is kept at 37°C for 2 days before primary isolation. In analogy, the detection limit for the PCR assay might not be reached if the majority of the rickettsiae are in the stationary phase of growth or dead. Furthermore, since we are the first to use a seminested primer system for studies of the prevalence of rickettsiae, our prevalence rates may be expected to be somewhat higher than those found in previous investigations. In a study of *I. ricinus* ticks collected in Switzerland (7), up to 11.7% were found to be positive for spotted fever group rickettsiae ("Swiss agent") in tissues and hemolymph by staining by the method of Giménez (9). A subsequent study of ticks from Switzerland by the hemolymph test showed that 10.3% of the ticks (*Dermatocenter marginatus* and *I. ricinus*) were positive for spotted fever group rickettsiae (2). The rickettsia detected in Switzerland was *R. helvetica* sp. nov. (5). Regarding other *Ixodes* ticks, a study with *Ixodes cookei* ticks in which the rates of prevalence of spotted fever group rickettsiae and *Borrelia* spp. were compared showed a high rate of spotted fever group rickettsiae; in some cases up to 50% of the female ticks were positive for this rickettsia, with an overall rate of 15%, whereas none of the female ticks were positive for *Borrelia* spp. (14). The prevalence of related spotted fever group *Rickettsia* spp. in ticks has been shown to have large geographic variations in different studies and depends on the tick species, the tick host, and the detection method used. For example, dogs infected with *R. conorii* harbored brown dog ticks (*Rhipicephalus sanguineus*) positive for SFG rickettsiae at the highest frequency, namely, 84.6%, as demonstrated by immunofluorescence with polyclonal antibodies raised against the Moroccan strain of *R. conorii*, which is pathogenic for humans (18). The presence of dogs harboring infected ticks correlates with the prevalence of Mediterranean spotted fever in France (1, 3, 4, 10, 11, 16, 22). In another study, 1 of 70 ticks of the species *Rhipicephalus sanguineus* were found to be positive for *Rickettsia* spp. when the ticks were analyzed by the hemolymph test (6). Our study differs from previous ones by the fact that we tested all the ticks by a nested PCR. By using a seminested primer system for the citrate synthase gene, an approximately 1,230-bp product was amplified from the isolated strain. Sequencing of the whole fragment showed that it had 100% homology with the deposited sequence for *R. helvetica*. The usefulness of RFLP analysis of PCR-amplified fragments of the citrate synthase gene for the differentiation of rickettsial isolates has been demonstrated previously (8, 19, 21). The technique is sensitive and practical, and species-specific RFLP profiles can be stored in a database and used for subsequent identifications. When the sequence of the gene is known, a theoretical digestion with the enzyme could serve as a means of checking the RFLP pattern result. The reason for carrying out the study was that the theoretically calculated fragment sizes did not correspond to those from the published RFLP pattern. A theoretical digestion with *Alu*I of the citrate synthase sequence of *R. helvetica*, defined by primers RpCS877 and RpCS1258, yields fragment sizes of 6, 43, 44, 81, 86, and 122 bp. This corresponds to the fragment sizes that we obtained by digestion with the restriction enzyme *Alu*I (Fig. 1). However, this differs from the RFLP pattern published earlier for that rickettsia, in which fragment sizes of 45, 95, 105, and 135 bp were found (5). Our RFLP pattern is also slightly different from the pattern which we observed in a previous investigation (15). The difference might be due to digestion of weakly amplified products that in the previous study needed separation on silver-stained polyacrylamide gels to make the fragments detectable. As shown in Fig. 1, the close relationship of the spotted fever group rickettsiae is exemplified by *Rickettsia canada* and *R. helvetica*, for which only small differences in two fragments are found: fragments of 81 and 86 bp in *R. helvetica* compared to fragments of 83 and 89 bp in *R. canada*. It is not possible to demonstrate such small differences by agarose gel electrophoresis, but they can be calculated theoretically. As expected, there is an obvious difference between *R. helvetica* and *R. prowazekii*, which was used as a control and which has fragments of 43, 59, 82, 88, and 108 bp. So far no *Rickettsia*-caused disease is known to be endemic in Sweden. The present study shows that the prevalence of *Rickettsia* organisms in *I. ricinus* ticks is greater than was previously thought. The implication of this finding and its possible association with human disease will require further investigations.

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