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Characterization of a Spotted Fever Group Rickettsia from *Ixodes ricinus* Ticks in Sweden

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A spotted fever group rickettsia isolated from the common tick, *Ixodes ricinus*, was genetically characterized by PCR and genomic sequencing. This study was performed with nymphal and adult ticks collected in southern and central Sweden. *I. ricinus* is the only North European tick species of medical importance which is regularly collected from humans. No species of the genus *Rickettsia* has previously been found in Scandinavian ticks, nor has any case of domestic rickettsial infection in humans or animals been reported. According to the nucleotide sequencing, the present *Rickettsia* sp. belongs to the spotted fever group of rickettsiae. Ticks are the most common arthropod reservoirs and vectors of the rickettsiae of this group. Among 748 ticks investigated, 13 (1.7%) were positive for a *Rickettsia* sp. *Borrelia burgdorferi* was detected in 52 (7%) of the ticks, a prevalence similar to or somewhat lower than that previously been recorded in other Swedish studies. There was no evidence of ehrlichial or chlamydial DNA in these ticks. The *Rickettsia* sp. was further characterized by 16S ribosomal DNA (rDNA) sequencing and restriction fragment length polymorphism (RFLP). The 16S rDNA sequencing resulted in a sequence identical to that described for *Rickettsia helvetica*, but the pattern obtained with RFLP of the citrate synthetase gene diverged from previously known patterns. The rickettsial agent of one tick which was positive by PCR was confirmed by transmission electron microscopy. The morphology of this rickettsia was similar to that of the spotted fever and typhus group rickettsiae. This represents the first documented isolate of a *Rickettsia* sp. from Swedish ticks.

The genus *Rickettsia* has until recently been considered to consist of three groups of strictly intracellular bacteria, namely, the typhus group (TG), the spotted fever group (SFG), and the scrub typhus group (STG) (30, 34). The classification into species is based on geographical distribution, arthropod hosts, intracellular location, structure of envelopes, conditions for cultivation and multiplication in chicken embryos, and serological patterns (5).

The TG includes the species *Rickettsia prowazekii* and *Rickettsia typhi*. The members of the TG are found mainly in the cytoplasm. Another common trait is serological cross-reactivity in the Weil-Felix agglutination of Proteus OX19 (33). The TG rickettsiae occur in scattered locations worldwide and are transmitted to humans by lice or fleas. The SFG rickettsiae are transmitted mainly by ticks, and 18 different species of SFG rickettsiae have been described. In addition, several other isolates have been identified. They showed differences from the established species and need to be characterized further (6). The definition of SFG species has been based on their serotype as determined by the complement fixation test, the toxin neutralization test in mice, the cross-immunity test in guinea pigs, or the microimmunofluorescence test. However, the serological differentiation of newly isolated strains is difficult because of a significant cross-reactivity among the recognized SFG rickettsiae (2). The third group of rickettsiae, the STG, consists of only one described species, *Orientia tsutsugamushi* (30). This species occurs naturally in southern and southeastern Asia and is transmitted by mite larvae (*Leptotrombidium* spp.). Furthermore, differences in the structure of the cell envelope and the ribosome sequence in comparison with those of the other groups have been recorded (31). European ticks have been shown to harbor SFG rickettsiae such as *Rickettsia helvetica* in Switzerland, *Rickettsia slovaca* in Russia, and *Rickettsia conorii* in France (4, 24). However, no rickettsia has been isolated from ticks in Scandinavia (Sweden, Denmark, Norway, Finland, and Iceland).

The majority of ticks found on humans and other large mammals in Sweden belong to *Ixodes ricinus* (15). Another 10 species are indigenous to Sweden, but more than 95% of all ticks found on humans belong to *I. ricinus*. This tick is an important vector of *Borrelia burgdorferi*, *Francisella tularensis*, and tick-borne encephalitis virus. Closely related to the rickettsiae, and also harbored by ticks, is *Coxiella burnetii*, which causes Q fever (18, 26). The first reported Swedish human case of Q fever occurred only recently (17), and this is the only species of *Rickettsiales* in Sweden that has been investigated epidemiologically (1, 1a). By using modern techniques, including PCR, the present study was carried out to determine the occurrence of *Rickettsia*, *Ehrlichia*, and *Chlamydia* species in Swedish *I. ricinus* ticks. The techniques used facilitate genetic characterization without a need for culturing the bacteria.

MATERIALS AND METHODS

Ticks. From a large collection of about 10,000 *I. ricinus* ticks, we used a subsample of 748 ticks (80% adult females, 17% males, and 3% nymphs), which

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were gathered in the central and southern parts of Sweden from 1989 to 1990 and identified by standard taxonomic characteristics (14). Most of them had been found attached to dogs (57%) or cats (23%), and the rest (20%) were from humans, cattle, or wild animals. The ticks were stored in 70% ethanol at room temperature until they were subjected to DNA isolation.

DNA isolation. The ticks were blotted and dried for a few minutes, and all adult ticks were cut in half with a sterile scalpel; one half was stored at −20°C for electron microscopy and/or for later use if required for confirmation. The nymphs were blotted and dried, and the whole organisms were used for DNA preparation. Each tick was then transferred to an Eppendorf tube after trituration and 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 (SDS), and 5 μl of proteinase K (10 mg/ml) (Boehringer, Mannheim, Germany). The mixture was incubated at 55°C for 1 h and heated for 10 min at 95°C. Extraction was performed twice with saturated phenol, once with a mixture of phenol and chloroform, and finally once with chloroform. The DNA was precipitated overnight by addition of 1/10 volume of 4 M sodium acetate and 3 volumes of 99% ethanol and collected by centrifugation at 20,000 × *g* in a microcentrifuge. The pellet was washed with 70% alcohol and then dried under a vacuum and dissolved in 50 μl of distilled water. Volumes of 10 μl from each sample were pooled in groups of 10, evaporated, redissolved in 25 μl of distilled water, and used as templates for PCR.

PCR amplification. The primers used and the thermal cycle conditions for *Chlamydia pneumoniae*, the human granulocytic ehrlichia, and the citrate synthetase gene of the rickettsiae have been described previously (8, 22, 25). For *B. burgdorferi*, primers with the sequences 5′TACCACAGCTCAACTGTGGAA CTA (BBUtp) and 5′TCGGTACTAACTTTTAGTTAACA (BBUrt) were chosen, amplifying a part of the 16S rRNA gene. For 16S ribosomal DNA sequencing of the rickettsia, three primers were chosen. Ric (5′TCTAGAACGAACGC TATCGGTAT) together with Ric U8 (5′TGCGTTAGCTCACCACCTTCA GG) yielded a 1,385-bp fragment encompassing almost the complete gene. A combination of Ric and Ric Rt (5′TTTCATCGTTTAACGGCGTGGACT) yielded a fragment with a size of 757 bp. The thermal cycle began with heating at 95°C for 3 min, followed by denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 60 s for Ric-Ric Rt and 120 s for Ric-Ric U8. This cycle was repeated 30 times. The program ended with 7 min at 72°C and cooling to 4°C until the samples were further used.

The general chemical conditions for the PCR amplification consisted of a mixture of 1 U of *Taq* DNA polymerase (Boehringer) and 2.5 μl of 10× buffer supplied with the enzyme, 2.5 μl of 2 mM deoxynucleoside triphosphates, 5 pmol of each primer, 1 μl of DNA template, and double-distilled water to 25 μl. The reaction was performed in a Perkin-Elmer 9600 thermocycler, and the amplified products were analyzed on a 1.5% agarose (Kodak) gel in 0.5 Tris-borate-EDTA (TBE) buffer (27). Chosen as positive PCR controls were strain IOL 207 for *C. pneumoniae*, clinical isolate 4808 for *Ehrlichia phagocytophila*, AKA1 for *B. burgdorferi*, and *Bartonella henselae* Houston 1 for rickettsiae. As a negative control, PCR buffer treated the same way as the ticks was included.

Restriction fragment length polymorphism (RFLP) analysis. Products from PCR with primers directed to the citrate synthetase gene of *R. prowazekii* (25) were digested with *Alu*I restriction enzymes (Boehringer) according to the supplier's recommendations. Electrophoretic separation was performed in a 2.5% agarose gel and/or homogeneous 20 PHAST polyacrylamide gels (Pharmacia, Uppsala, Sweden). The DNA fragments were visualized with ethidium bromide or silver staining, and fragment sizes were compared with a standard molecular weight marker (Pharmacia).

Direct solid-phase DNA sequencing. Immobilization of the biotinylated PCR products followed by strand separation and template preparation was performed with super paramagnetic beads, M-280 Streptavidin Dynabeads (Dynal, Oslo, Norway). The nucleotide sequences of the 16S rRNA genes were determined in both directions by automated solid-phase DNA sequencing (12, 13, 23, 32) with the ALF (automated laser fluorescence) system (Pharmacia Biotech, Uppsala, Sweden).

Sequencing was also performed manually with ^{35}S-dATP (Amersham) and Sequenase II (U.S. Biochemicals, Cleveland, Ohio) according to the instructions of the suppliers.

Transmission electron microscopy. The frozen halves of one tick yielding a positive PCR were fixed in 1% glutaraldehyde-sodium cacodylate buffer (pH 7.4) overnight. Postfixation was performed in 1% OsO_4 in distilled H_2O. The bacteria and cell material were then dehydrated in an ethanol gradient and embedded in Agar 100 resin (Agar Scientific Ltd., Stanstead, England). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. The cells were inspected in a Philips TEM 420 transmission electron microscope at 60 kV.

RESULTS

Identification by PCR and RFLP. All 748 ticks were examined for intracellular bacteria. Rickettsiae were detected by amplification of the citrate synthetase gene, which has conserved regions shared by all known *Rickettsia* species (25). Twelve pooled tick samples were positive. When all 10 individual samples of each pool were retested, 11 pools were found to contain only one positive tick, while 1 pool contained two positive ticks. The positive samples were not clustered to any distinct geographical area. In order to determine whether the amplified products in the positive samples were related, we used RFLP analysis, which usually discriminates between the species. All 13 individual samples gave similar restriction patterns when digested by *Alu*I. We found a double band with fragments approximately 120 to 130 bp in length, a single band with a 90-bp fragment, and a small fragment (Fig. 1). The small fragment was detected only in silver-stained polyacrylamide gels and had an approximate length of 20 to 30 bp. Comparison of the RFLP patterns of *R. helvetica* and our rickettsial isolate resulted in different-size fragments, reflecting a variability in the structure of the citrate synthetase gene (2). All of the ticks tested were negative for *C. pneumoniae*. We used the primer pair ERB1 and ERB2, developed from the 16S ribosomal DNA sequence of the granulocytic *E. phagocytophila*, to screen for *Ehrlichia* spp. (22). No tick was positive for ehrlichial DNA. *B. burgdorferi* was detected in 52 ticks (7%), a prevalence similar to or somewhat lower than that previously found in Swedish ticks, summarized by Gustafson et al. (9).

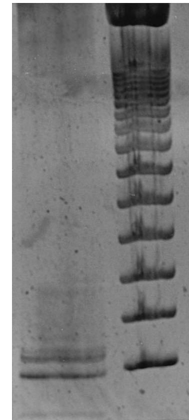

FIG. 1. Silver-stained homogeneous 20 PHAST gel showing the RFLP pattern from the citrate synthetase gene. Left lane, *Alu*I digestion of PCR product from primers RpCS.877p and RpCS.1258n, showing a double band above the 100-bp marker lane and a single band of approximately 90 bp; there are also faint bands of approximately 20 to 40 bp. Right lane, molecular weight marker in the form of a 100-bp ladder.

Sequencing of the 16S rRNA gene. Solid-phase sequencing resulted in an unambiguous determination of 1,380 nucleotides of the 16S rRNA gene. Of the sequences, 1,146 bp were reconfirmed from two to five fragments. The sequence obtained was identical to the deposited sequence of *R. helvetica* (accession no. L36212) and was therefore not deposited in the data bank.

Electron microscopy. One of the PCR-positive ticks was examined by transmission electron microscopy. As seen in Fig. 2, most of the rickettsiae contained a vacuole-like structure, which previously has been shown to be characteristic of *R. prowazekii* in the stationary phase of growth (34). A cell envelope similar to that previously described for both the SFG and the TG of rickettsiae (31) is shown in Fig. 3. The STG rickettsia *O. tsutsugamushi* has a cell envelope with a much thicker outer leaflet, thus differing fundamentally from our rickettsia.

DISCUSSION

We show for the first time the existence of a *Rickettsia* species in Scandinavian *I. ricinus* ticks. Ticks are known to be

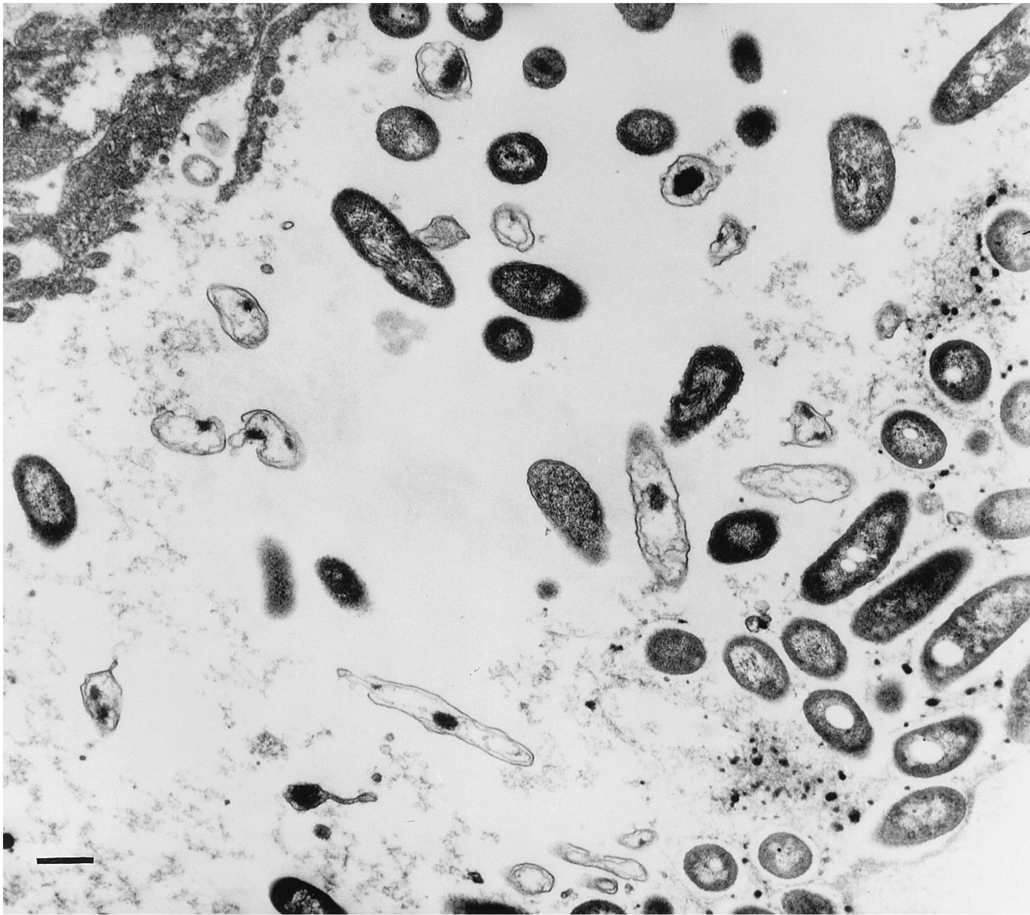

FIG. 2. Transmission electron micrograph of a triturated whole tick, showing a number of rickettsiae, some with vacuoles typical for the bacteria in the stationary phase of growth. Bar, 0.2 μm.

the most common arthropod reservoirs and vectors of SFG rickettsiae. The prevalence of SFG rickettsiae varies from a few up to 50% in different collections of ticks from other parts of the world (19, 20). Rickettsioses are not known to be endemic or epidemic among humans or animals in Scandinavia.

PCR analyses of 748 adult and a few nymphal *I. ricinus* ticks revealed that 13 (1.7%) were positive for rickettsial DNA. These ticks represented a minor part of a large collection of ticks gathered in 1989 and 1990 from the central and southern parts of Sweden. The samples analyzed were chosen to represent various regions of the country, but the outcome was randomized with respect to geographical regions.

The amplified product was sequenced, and a 1,380-bp sequence of the 16S rRNA gene from the isolated strain was compared with those of previously described rickettsial species. We found 100% homology with *R. helvetica*. Species of the genus *Rickettsia* are closely related, and the genetic differences between their 16S rRNA genes are only 2%. For example, 11 nucleotides differentiate this isolate from *R. conorii*, a species endemic in southern Europe and phylogenetically the most closely related characterized SFG rickettsia and, furthermore, the only rickettsia that has been found in *I. ricinus* so far. In comparison with *R. prowazekii*, which is not as closely related and belongs to the TG, our strain differs in 25 nucleotide positions. As no rickettsial agent has been found or amplified at our laboratory, and as the positive control used was *B. henselae*, we can be quite sure that no amplicon contamination could have taken place.

The RFLP pattern of the citrate synthetase gene improves the discrimination between members of the *Rickettsiales* as demonstrated by Regnery et al. and Eremeeva et al. for both *Rickettsia* and *Bartonella* spp. (7, 25). The RFLP pattern obtained for our strain, compared to those of known type strains, is most similar to that of *Rickettsia bellii* and distinctly different from the published pattern of *R. helvetica* (2). According to the nucleotide sequencing results, the detected *Rickettsia* sp. should be classified as an SFG rickettsia (26). Furthermore, the RFLP pattern suggests that the strain should be regarded as a subtype of *R. helvetica*. However, this has to be regarded as tentative until isolation and cultivation of this strain have been performed, so that DNA-DNA reassociation experiments and serological studies will be possible in order to determine whether the strain is a new species, a new subspecies, or a new serotype.

Transmission electron micrographs of one PCR-positive tick showed that rickettsia-like structures were clearly present in large numbers (Fig. 2). A correspondingly high frequency was found in a study comparing the frequency of rickettsia species with *B. burgdorferi* in *Ixodes cookei* ticks in Connecticut as judged from immunofluorescence microscopy of stained hemolymph preparations (20). *B. burgdorferi* was detected in 52 ticks (7%) of the present collection, which is a frequency sim-

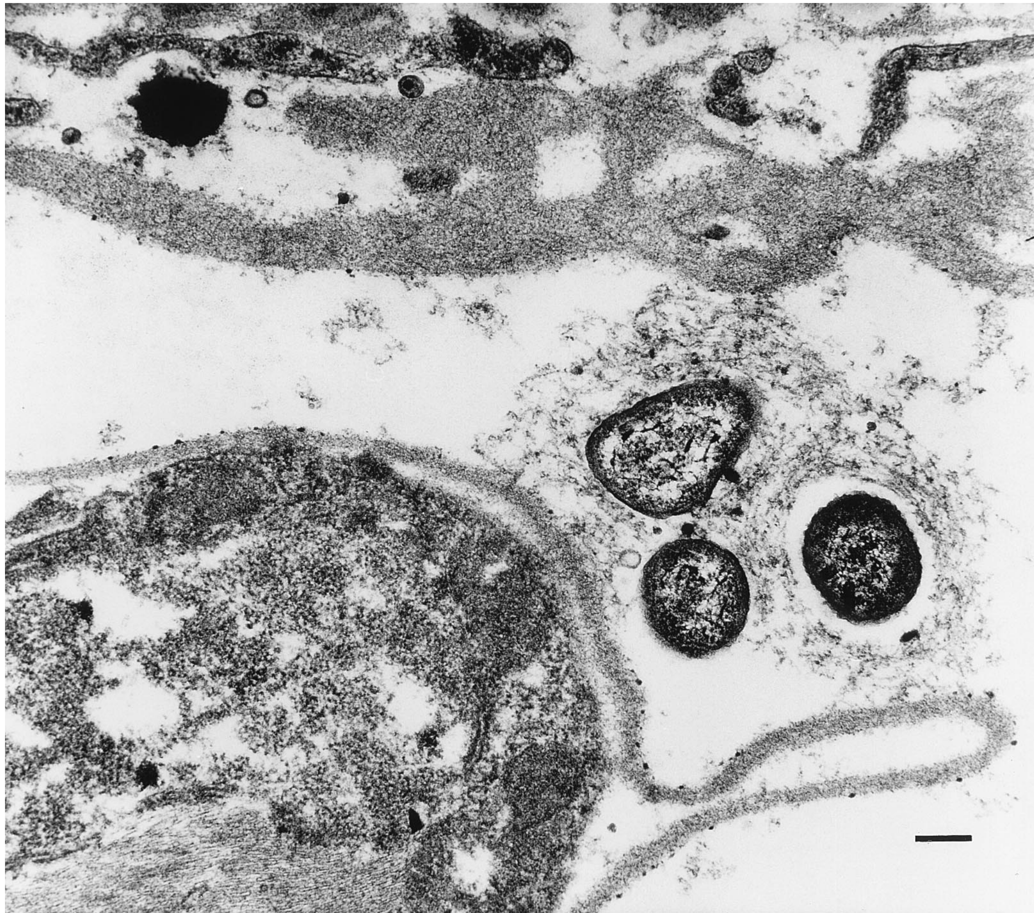

FIG. 3. Transmission electron micrograph of a *Rickettsia* sp. showing three bacteria in close contact with a tick cell. Note the bacterial cell envelope, including the microcapsular layer. Bar, 0.1 μm.

ilar to or somewhat lower than those reported from previous epidemiological studies of that organism in ticks collected in Sweden (3, 9). This possibly lower frequency in our study might be explained by methodological factors, such as storage of the ticks for a long time before DNA extraction. If there is an analogous influence on the observed frequency of rickettsia DNA compared with that of *B. burgdorferi*, it may be assumed that the observed frequency of 1.7% is the lowest level that is to be expected.

The failure to detect ehrlichial DNA was a somewhat surprising finding, since granulocytic ehrlichiae have been found in Swedish dogs, horses, and cattle (16, 22). One explanation could be that there is no known rodent cycle for *Ehrlichia* spp. in Sweden and that the majority of our tested ticks were collected in areas not used for breeding of sheep or cattle, domestic animals that are the main reservoir for *Ehrlichia* spp.

The fact that the clinical significance of rickettsial infections cannot be overestimated has recently been emphasized (1b, 6, 11). Many studies have shown that the signs and symptoms of rickettsial diseases may differ widely. This is valid for, e.g., Rocky Mountain "spotless" fever, for which the clinical findings are very similar to those for Lyme disease but for which the serological evaluation suggests an infection with *R. rickettsii* (11). Another example is a new rickettsial agent, ELB, for which the definitive clinical diagnosis was murine typhus (29). Extended investigations have now shown that the ELB agent is a distinct rickettsial species with the proposed name *Rickettsia felis* (10). Therefore, further studies are needed to establish the geographical distribution, the vector and host relations, and the prevalence of rickettsiae in the Scandinavian tick population, as well as their role as potential pathogens for humans and animals.

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