First Detection of Spotted Fever Group Rickettsiae in *Ixodes ricinus* from Italy

Tiziana Beninati,* Nathan Lo,*† Hiroaki Noda,† Fulvio Esposito,‡ Annapaola Rizzoli,§ Guido Favia,‡ and Claudio Genchi*§

*Ixodes ricinus* from Italy were examined for the first time to detect whether rickettsiae were present. Using molecular methods, we detected three different spotted fever group rickettsiae, including *Rickettsia helvetica*. Our results raise the possibility that bacteria other than *R. conorii* are involved in rickettsial diseases in Italy.

The genus *Rickettsia* comprises obligately intracellular, gram-negative bacteria. Before sequence-based classification methods were introduced, the genus was divided into two groups: the typhus group (TG), which included *R. prowazekii*, *R. typhi*, and *R. canadensis*, and the spotted fever group (SFG), which comprised all others. Recent phylogenetic studies of genes such as *gltA*, *ompA*, “gene D,” and that encoding the 17-kDa protein (hereafter referred to as “17kDa”) have shown that these two groupings are not consistent with species relationships; consequently, they have been modified (summarized in [1]). The TG now comprises only *R. prowazekii* and *R. typhi*, while the SFG contains seven divergent lineages: the *Rickettsia* group, *R. japonica*, *R. montana*, the *R. massiliae* group, *R. helvetica*, *R. felis*, and the *R. akari* group. The AB bacterium, *R. bellii*, and *R. canadensis* species have been found in *Ixodes ricinus*; *Rh. sanguineus* in humid, forested habitats (7). While all life stages of *Rh. sanguineus* are mainly associated with dogs, *I. ricinus* can feed on >200 host species, primarily wild rodents and ruminants. In a survey in Liguria of ticks recovered from people, most ticks (89.3%) were *I. ricinus*. A major limitation of MIF is cross-reactivity, which renders it unable to differentiate between various SFG rickettsiae (4). Thus, some cases of MSF in Italy, especially where the disease is not endemic, may in fact be due to other rickettsiae.

*I. ricinus* is found with high prevalence in the Italian Alps and Apennines (reaching 96% of all ticks collected in some areas) and in almost all other Italian regions that contain humid, forested habitats (7). While all life stages of *Rh. sanguineus* are mainly associated with dogs, *I. ricinus* can feed on >200 host species, primarily wild rodents and ruminants. In a survey in Liguria of ticks recovered from people, most ticks (89.3%) were *I. ricinus*; *Rh. sanguineus* was recorded less frequently (9.8%) (8).

To date, no studies have been conducted on potential rickettsiae in Italian ticks, other than *Rhipicephalus* spp. Recently, various *Rickettsia* species have been found in *I. ricinus* from other European countries, including *R. helvetica* in Switzerland, France, Sweden, Slovenia, and Portugal (4) and *Rickettsia* spp. IRS3/4 in Slovakia (9). To check whether such bacteria are also present in Italian *I. ricinus*, we studied specimens from three regions. We used molecular-sequence-based identification techniques, which offer high sensitivity and specificity compared with serologic tests and circumvent the need for bacterial culturing.

### The Study

A total of 109 *I. ricinus* specimens were collected in northern and central Italy (Figure 1), identified by using standard taxonomic keys, and stored at −20°C. Specifically, 89 ticks (70 adults and 19 nymphs) were collected by dragging vegetation in different parts of Trentino Province in April–October 1997 and 1999, and 10 ticks (7 adults and 3 nymphs) by dragging in Feltre (Veneto Region) in March 2000. Ten more ticks (7 adults and 3 nymphs) were collected from a patient at the...
An initial estimate of the overall prevalence in Italian *I. ricinus* is thus 8.25%. To better establish intragenic relationships, the nine positive samples were subjected to further PCR analysis with the primer pairs Rr 17.61p/Rr 17.492n and Rr 190.70p/Rr 190.602n (10), which amplified 394-bp and 488-bp portions of 17kDa and *ompA*, respectively. PCR bands for all three genes were then sequenced directly by using an ABI PRISM sequencer (Perkin-Elmer, Foster City, CA). To compare the sequences obtained during this study with those of other rickettsiae, sequences present in GenBank were selected by means of BLAST as well as on the basis of previous reports (1,12). Sequences were converted to their putative amino acid sequences and aligned by using the program Clustal X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Based on these alignments, nucleotide alignments were performed manually, and phylogenetic relationships were inferred by maximum likelihood (ML). The appropriate model of sequence evolution was determined by Modeltest 3.06 (http://zoology.byu.edu/crandall_lab/modeltest.htm), and trees were produced using the program TreePuzzle 5.0 (www.tree-puzzle.de), which provides branch lengths as well as quartet puzzling support values at each node with >50% support.

Comparisons of the sequences identified with those from closely related SFG *Rickettsia* spp. are shown in the Table; Figure 2 shows the results of phylogenetic analysis. *gltA*-based results (Figure 2a) show that all strains detected are SFG rickettsiae. For 17kDa (Figure 2b), no identical sequences for IrITA2 and IrITA3 were present in GenBank, and they clustered with *R. cooleyi* (isolated from *I. scapularis* in Texas [13]). *ompA* was the most variable of the three genes analyzed (Figure 2c) and could only be amplified from IrITA2 and IrITA3. Consistent with the results from *gltA*, *ompA* from IrITA2 was 100% identical to IrR/Munich; however, two substitutions were found between these two sequences and that of IRS4. Notably, for *ompA*, the cluster to which IrITA2 and IrITA3 belong also contains a strain detected in Spain (14). This finding suggests that these bacteria may be widespread in Europe. On the basis of *ompA* (and 17kDa) sequences, the clade containing IrITA2 and IrITA3 was closest to a clade containing *R. cooleyi* and an endosymbiont (10), both hosted by *I. scapularis*. All previous attempts to amplify *ompA* from *R. helvetica* by using various primers have failed, which suggests that the gene is either absent or too variable to work with primers designed from other SFG bacteria (12). This would explain...
why we were unable to amplify $ompA$ from IrITA1. Taken together, the results from the three genes indicate that the clade containing IrITA2 and IrITA3 represents a lineage divergent from the seven described previously (1).

Conclusions

Our results represent the first demonstration of rickettsiae in Italian *I. ricinus* and the first use of molecular-sequence–based methods to identify rickettsiae in Italy. One bacterium, *R. helvetica*, occurs in several parts of Europe and has been implicated as a human pathogen. The other two strains have only recently been discovered in *I. ricinus* from Slovakia and Germany. Whether they are pathogenic is not known, but since other rickettsiae of previously unknown pathogenicity have subsequently been shown to be associated with disease (*R. helvetica* and *R. slovaca* [15]), these new strains warrant attention.

Several studies on rickettsioses in Italy have been published in the last two decades, and they all report *R. conorii* as the causative agent. As MSF is the only known rickettsiosis in Italy, diagnostic tests use *R. conorii* as the only antigen for serologic assays (16,17). However, since SFG rickettsiae cause cross-reactions, confusion about the source of the illness may occur. Although antibiotic therapy is generally effective for all SFG-related diseases, a better understanding of how different rickettsiae cause different symptoms will only come with their correct identification. During 1996–1999 in the regions we sampled, 23 rickettsioses (assumed to be MSF) were reported from Veneto, 42 from Toscana, and 3 from Trentino Province (Italian Ministry of Health, unpub. data). While many were likely to be MSF cases, the possibility exists that some were caused by other SFG (perhaps *R. helvetica*).

Unlike most studies, one serosurvey in northeastern Italy (18) used the complement-fixation test, which is less prone to cross-reactions (19); none of the sera tested was found positive for antibodies to rickettsiae. This finding may be explained by the use of *R. conorii*, *R. rickettsii*, *R. typhi*, and *R. akari* as the only antigens. Serosurveys such as these could therefore benefit from the use of antigens from the bacteria identified in our study.

*I. ricinus* is one of the most abundant tick species in Italy, having a very low host specificity and a record of attacking large numbers of humans (8). The results reported here add SFG rickettsiae to the list of potentially dangerous pathogens that Italian *I. ricinus* carry.

Acknowledgments

We thank C. Bandi for advice; V. Tagliapietra, L. Agostini, S. De Felici, R. Luise, and A. Iori for help with tick collection; and A. Bartolini for ticks and clinical information about the patient in Firenze.

This work was supported by the Centro di Ecologia Alpina, Trento. N. L is supported by the Science and Technology Agency of Japan.

Ms. Beninati is a doctoral candidate of the veterinary faculty of the University of Milan, Italy. Her research interests include tick-borne diseases and population genetics of *I. ricinus*.
References


Address for correspondence: Nathan Lo, Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università di Milano, Via Celoria 10, 20133 Milano, Italy; fax: 39 02 5031 8095; e-mail: nathanlo@affrc.go.jp