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# *Rickettsia felis*: a New Species of Pathogenic Rickettsia Isolated from Cat Fleas

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**A flea-borne rickettsia, previously referred to as ELB, has been implicated as a cause of human illness. Using sequence data obtained from a fragment of the citrate synthase gene, we compared ELB, *Rickettsia australis*, *R. rickettsii*, and *R. akari* with the louse-borne *R. prowazekii*. We tallied 24 base pair differences between ELB and *R. prowazekii* and 25 between *R. rickettsii* and *R. prowazekii*; there were 30 base pair differences between *R. australis* and *R. prowazekii* and 29 between *R. akari* and *R. prowazekii*. We observed 32 differences between *Rickettsia typhi* and ELB. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses of ELB, with typing sera against *R. typhi* indicate that ELB surface antigens are more closely related to the flea-borne *R. typhi* than to the mite-borne *R. akari*. On the basis of the results of citrate synthase gene sequence comparisons, as well as previous comparisons with 16S rRNA and 17-kDa-protein gene segments, we found that ELB is sufficiently genetically distinct from other rickettsiae to be designated a new species, *Rickettsia felis*.**

ELB was originally observed in tissue sections of the cat flea, *Ctenocephalides felis*, as detected by electron microscopy (1). Early efforts to cultivate the agent in vitro and in vivo were unsuccessful, and consequently, molecular approaches to the characterization of this bacterium were undertaken. Amplification of a 434-bp segment of the gene for the 17-kDa antigen, an immunologically important surface protein common to rickettsiae, was accomplished by PCR of ELB-infected fleas. Sequencing of the PCR product, as well as restriction fragment length polymorphism data obtained from the digestion of the 17-kDa product, indicated that ELB was distinct from *Rickettsia typhi* Wilmington (2), while serological assays of infected fleas gave results similar to those reported for *R. typhi*. On the basis of these observations, ELB was considered to be allied to the typhus group (TG) rickettsiae, which include *R. typhi* and the agent of epidemic typhus, *R. prowazekii*. Studies of the natural transmission of *R. typhi* in peridomestic habitats in the United States were consequently expanded to investigate the presence of ELB. In California and Texas, ELB and *R. typhi* were detected in fleas and opossums by PCR (11, 13). Interestingly, no fleas were found to be simultaneously infected with ELB and *R. typhi*.

A further indication of the importance of ELB as a component of murine typhus transmission cycles was reported by Schriefer et al. (10), who examined blood samples from Texas patients clinically diagnosed with murine typhus; one patient yielded a PCR amplicon specific for ELB. Sequencing of the 16S rRNA gene of ELB corroborated the results of 17-kDa-protein gene sequence data, indicating that this organism was distinct from *R. typhi* and spotted fever group (SFG) rickettsiae (10). More recently, phylogenetic analyses of the ELB 16S rRNA sequence have placed it either in a clade with the tick-borne *R. australis* and the mite-borne *R. akari* (12) or in a clade of its own, with these species as its nearest neighbors (9).

However, because of the extreme conservation of 16S rRNA among the members of the *Rickettsia* genus (less than 3% sequence divergence), phylogenetic inferences based on the 16S rRNA sequence alone are problematical. In order to further characterize ELB, we have sequenced its citrate synthase (CS) gene and those of *R. rickettsii*, *R. australis*, and *R. akari*. We also determined the protein profile of a purified ELB isolate and the reactivity of *R. typhi* and *R. akari* typing sera to this profile. Here we report the results of those analyses and propose that ELB be henceforth designated the new rickettsial species *Rickettsia felis*.

## MATERIALS AND METHODS

**Rickettsial strains used and PCR of CS gene fragment.** Samples of *C. felis* infected with *R. felis* were obtained from a commercial flea colony maintained by El Labs, Soquel, Calif. Fleas ($n$ = 15 to 20) were triturated in 100 μl of sterile distilled water, and the resulting homogenate was heated at 100°C for 10 min; 10 μl was used as a template for PCR. For *R. rickettsii* Sheila Smith strain VR-149, *R. akari* Kaplan, and *R. australis* JC, 200-μl aliquots of seed were heated at 100°C for 10 min and 3 to 5 μl was used as a template for PCR.

**Sequencing of the CS gene.** Primers specific for a 381-bp region (nucleotides 877 to 1258) of the *R. prowazekii* CS gene (14) were used to amplify this segment from *R. felis*-infected fleas and from seeds of other rickettsial species. Thermal cycling conditions and product visualization were as described in published protocols (2). The PCR product was purified by using several methods, including the "crush and slush" technique (10), and DNA-binding spin columns (Qiagen Inc., Chatsworth, Calif., and Pharmacia Biotech Inc., Piscataway, N.J.). The purified product was then cycle sequenced with fluorescein-labelled dideoxynucleotide bases and visualized with the Applied Biosystems (Foster City, Calif.) model 373 DNA sequencing system. For *R. rickettsii*, three forward and three reverse primer sequencing runs were performed. For *R. felis*, two forward and two reverse sequencing runs were performed, and for *R. australis*, one forward and one reverse sequencing run were performed. Because of difficulties we encountered in directly sequencing the *R. akari* PCR product, we cloned the product into the PCR II plasmid vector system (Invitrogen, San Diego, Calif.) and sequenced it by using universal forward and reverse primers. A total of three separate forward and reverse runs, on three individual colonies, were performed. In order to obtain data from the 5′ and 3′ ends of the *R. felis* CS gene sequence that were not available from direct sequencing, we also cloned this product into the plasmid vector and performed one universal forward and one universal reverse run.

For interspecies sequence comparisons, in order to avoid artificially inflating the number of nucleotide differences observed, we excluded ambiguous nucleotides at the peripheries of sequences (which are common to templates dye-terminator sequenced directly with specific primers) (5). Consequently, the lengths of the sequences for *R. australis* and *R. rickettsii* were 353 and 351 bp, respectively. Analysis of sequence data was conducted with the Sequence Editor SeqEd 675 software package (Applied Biosystems).

In order to arrive at the most conservative consensus sequence for each species, the following protocol was implemented. First, for each species, all forward and all reverse sequences were compared to arrive at consensus forward and reverse sequences for each species. Where differences in nucleotide bases

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were observed, a predominant base was assigned if the majority of the sequences contained it. In those instances when one base did not predominate, the original chromatographs were consulted in order to resolve ambiguities; peak height became the final criterion of nucleotide assignation. Second, after species-specific consensus forward and reverse sequences were determined, they were compared with the 1,463-bp sequence of *R. prowazekii* (14). If a base change between two species was observed, and was present at the same position in both the forward and reverse consensus sequences used to compare those species, the nucleotide difference was deemed legitimate and was cataloged.

**SDS-PAGE and Western blotting (immunoblotting) of rickettsial proteins.** To characterize the protein profile of Renografin-purified *R. felis* (7), proteins were extracted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.125 M Tris hydrochloride [pH 6.8], 25% glycerol, 0.5% bromphenol blue) with and without 10% 2-mercaptoethanol and heated for 5 min at 100°C; 40 μg of proteins per lane was then electrophoresed on a 15% separating gel (4). Proteins were visualized with silver stain (Bio-Rad Laboratories, Hercules, Calif.).

For immunoblots, *R. typhi*, *R. felis*, and *R. akari* stocks were extracted with SDS-PAGE sample buffer and heated at 100°C for 5 min prior to electrophoresis on 7.5% separating gels (4). A total of 20 μg of total protein was loaded onto each lane. Following electrophoresis, the proteins were electroblotted to reinforced nitrocellulose membranes at 365 mA for 1 h at 4°C. The membranes were blocked with 5% nonfat dry milk in 25 mM Tris-buffered saline (TBS) for 1 h at 21°C and then incubated with rat anti-*R. typhi* (1:100). After three 10-min washing steps in TBS, the membranes were incubated with goat anti-rat immunoglobulin G (heavy plus light chains) (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:1,000 in TBS. After three washes with TBS, the membranes were incubated in 4-chloro-1-naphthol solution and monitored for reactivity.

**Nucleotide sequence accession numbers.** Sequences were submitted to GenBank and assigned accession numbers as follows: *R. felis*, U33922; *R. australis*, U33923; and *R. akari*, U41752.

## RESULTS

We cataloged 24 base pair differences between the sequence of *R. felis* and *R. prowazekii*; this was of a magnitude similar to that of the base pair differences observed between *R. rickettsii* and *R. prowazekii* (25 differences), *R. australis* and *R. prowazekii* (30 differences), and *R. akari* and *R. prowazekii* (29 differences) (Fig. 1).

We compared the sequence of *R. felis* and a published *R. typhi* sequence (3) and tallied 32 nucleotide differences (Fig. 1).

The SDS-PAGE profile of *R. felis* proteins indicated that this species shared several prominent polypeptides with other species of rickettsiae, particularly the 17-kDa proteins (Fig. 2). No reproducible differences in protein sizes due to treatment with 2-mercaptoethanol were observed. We were not able to detect a protein band with a molecular mass above 150 kDa, confirming immunoblotting and PCR data that this species lacks the 190-kDa surface antigen common to SFG rickettsia (2, 8).

We probed *R. felis*, fellow clade member *R. akari*, and *R. typhi* with anti-*R. typhi* polyclonal sera (Fig. 3). The sera recognized a prominent 120-kDa band in all species; however, more reactive peptides were shared by *R. typhi* and *R. felis* than by *R. felis* and *R. akari*.

In Table 1, we compare and contrast aspects of the biology of *R. felis* and the other flea-borne rickettsia, *R. typhi*, as well as the tick-borne *R. rickettsii*, the mite-borne phylogenetic relative *R. akari*, and louse-borne *R. prowazekii*. Three of our comparison parameters are immunologically important antigens: the 190-kDa surface antigen prevalent in SFG rickettsiae, the 120- or 135-kDa outer membrane protein, and the 17-kDa protein, analogous to the lipopolysaccharide (LPS) present in other genera of gram-negative bacteria. With the exception of the vector insect, *R. felis* shares four characteristics (absence of a 190-kDa antigen, presence of 120-kDa and 17-kDa antigens, and cytopathology) with *R. typhi* and *R. prowazekii*, rather than with *R. akari*.

## DISCUSSION

Until the mid-1980s, identification of new species of rickettsia was a tedious and time-consuming process which required successful propagation of the organism in cell culture systems in order to characterize such parameters as growth and cytopathology. It was also necessary to obtain large quantities of the rickettsia to perform SDS-PAGE and immunological assays. The advent of PCR technology allowed investigators to amplify conserved portions of rickettsial genes, particularly 16S rRNA, from unpurified isolates without resorting to cell culture. Sequence data from these genes could then be used to determine if the organism in question was a new species.

16S rRNA sequences have conventionally been used in phylogenetic analyses of bacteria (6). Stothard and Fuerst (12), in their analysis of 14 rickettsial taxa, found that *R. felis* forms a unique clade with *R. akari* and *R. australis*; interestingly, all of these species have different vectors. The other flea-borne rickettsia, *R. typhi*, forms a clade with the louse-borne *R. prowazekii*. More recently, phylogenetic analyses of rickettsial taxa with sequence data from the 17-kDa-antigen gene placed *R. felis* in a clade of its own, with the *R. australis*-*R. akari* clade constituting its nearest neighbors (3a); these characterizations are also supported by another 16S rRNA phylogenetic analysis (9). We compared CS gene sequences of *R. felis* and *R. typhi* and tallied 32 nucleotide differences; additionally, *R. felis* exhibits a 24-nucleotide difference from *R. prowazekii*, a magnitude shared by *R. rickettsii*, *R. australis*, and *R. akari* (Fig. 1). As recently reported by Balayeva et al. (3), 11 base pair differences exist between *R. typhi* and *R. prowazekii*; we interpret those results to indicate a higher degree of relatedness of *R. typhi* to *R. prowazekii* than to *R. felis*. In conjunction with sequence data mentioned above, this information is convincing evidence that *R. felis* is sufficiently genetically distant from *R. typhi* to be named as a species.

While initial immunofluorescence assays of *R. felis* indicated that it had a close affinity to TG rickettsiae (2), analysis of sequence data for a 434-bp 17-kDa-protein gene segment demonstrated that *R. felis* was more akin to members of the SFG rickettsiae. More recently, the isolation of *R. felis* from infected fleas by mammalian-cell culture (7) permitted initial studies of the antigenic and biologic properties of this rickettsia. The growth pattern of *R. felis* in Vero cells, and the delayed formation of plaques, resemble those reported for TG rather than SFG rickettsiae. In addition, immunoblotting studies suggest that *R. felis* contains an LPS which is recognized by TG-reactive sera but not by antisera to SFG LPS. In light of the phylogenetic data identifying *R. akari* and *R. australis* as relatives of *R. felis* (9, 12), we were interested in assaying *R. felis* by immunoblotting with anti-*R. typhi* typing sera. Results of this assay (Fig. 3) correlate with earlier immunofluorescence data, namely, that *R. felis* displays antigenic similarity to *R. typhi*. We do not believe that these results necessarily contradict our conclusions about *R. felis* based on sequence comparisons, in light of the fact that numerous variables, such as properties of the immunizing agent, the immunization regimen, preparation of monoclonal antibodies, and variations in the immunoblotting techniques from one laboratory to another, make definitive interpretations of such data difficult. Because such immunologic assays are unable to differentiate between *R. felis* and *R. typhi*, clinical investigators should supplement their assays with molecular analyses if exact identification of the etiologic agent is of importance. We are currently testing the use of *R. felis*-specific PCR with the 120-kDa-antigen gene in addressing this issue.

Given that *R. felis* and *R. typhi* are both flea-borne rickettsiae, is it possible that *R. felis* represents a strain of *R. typhi* which has undergone enough divergence to arise as a new species? And would this explain the sharing of characteristics such as the presence of a 120-kDa antigen, the lack of a

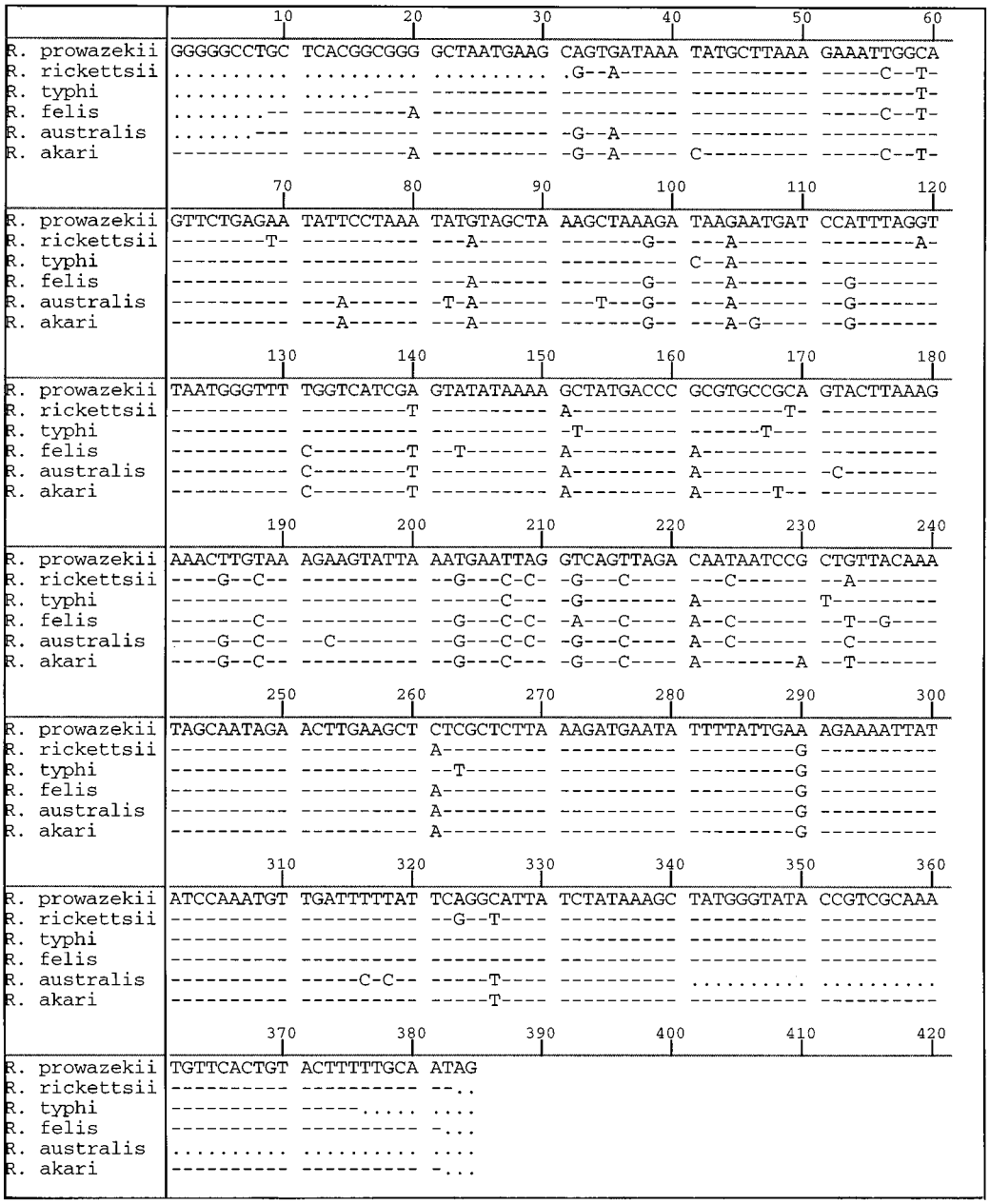


FIG. 1. Comparison of nucleotide sequences for a segment of the CS gene amplified from various species of rickettsiae. Uppercase letters indicate substitution of a base different from the *R. prowazekii* reference sequence, dots indicate missing sequence, and dashes indicate homology.

190-kDa antigen, and cell lysis and plaque formation in cell culture?

While this is a logical assumption, the large sequence differences observed in three gene segments, the 16S rRNA gene, the 17-kDa-protein gene, and the CS gene, seem to indicate that such a divergence must have occurred quite early in the evolutionary history of the rickettsiae, particularly in light of the fact that the 16S rRNA and CS genes are "housekeeping" genes and therefore are less likely to be subjected to pressure from host immunological responses than are surface antigen genes such as the 120-kDa-protein gene. It may be that *R. felis* is a rickettsial species which independently evolved into a flea symbiont. This question is further complicated by our observation that wild fleas have not been found to be simultaneously infected with *R. typhi* and *R. felis* (2, 11), although this may reflect sampling variation and not a genuine ecological phenomenon. In any event, the evolutionary history of *R. typhi* and *R. felis* will remain speculative until further data become available.

In conclusion, we propose that the pathogenic, flea-borne rickettsia formerly known as ELB be designated *R. felis* on the basis of the fact that sequence analyses of the 16S rRNA, 17-kDa-protein, and CS genes indicate that this bacterium is sufficiently different from *R. typhi* and other members of the genus to warrant assignation as a new rickettsial species. The use of the appellation *felis* recognizes the origin of this microbe in cat fleas, which were maintained on infected cats, *Felis domesticus*, at Elward laboratories in California; we have re-

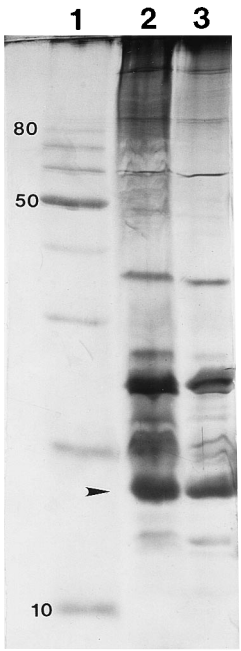


FIG. 2. SDS-PAGE profile of *R. felis* proteins (40 μg per lane). Lane 1, 10-kDa molecular mass marker; lane 2, *R. felis* protein extract; lane 3, *R. felis* protein extract, treated with 2-mercaptoethanol. The numerals next to bands are sizes in kilodaltons. The arrowhead indicates the location of the 17-kDa antigen.

cently assayed sera collected from febrile cats referred to veterinarians in the northeastern United States and observed a seropositivity rate of 8% (unpublished data), indicating a possible role for household pets in *R. felis* transmission. We are continuing our epidemiologic investigations, as well as molecular genetic analyses, to improve our knowledge of this emerging pathogen.

**Description of *R. felis*.** *R. felis* (from *F. domesticus*, the domestic cat) is an obligate intracellular, gram-negative bacterium in the order *Rickettsiales*. It was isolated from cat fleas, *C. felis*, and can be grown in Vero, L929, and Huvec cells, as well as chicken embryos (7). *R. felis* induces cytopathic foci and plaque formation in Vero cells at approximately 11 days postinoculation; the bacterium is present in the cytoplasms of infected cells and not the nuclei. *R. felis* can be detected in infected cells by immunofluorescence with rat polyclonal anti-*R. felis* antibody (7). *R. felis* DNA can be amplified with rickettsial-specific primers for the 17-kDa-protein, CS and 16S rRNA genes and can be identified to the species level by restriction fragment length polymorphism assays of the PCR products of these genes (2, 7, 10). *R. felis* has been amplified from the blood of a Texas patient suspected of having *R. typhi* infection (10).

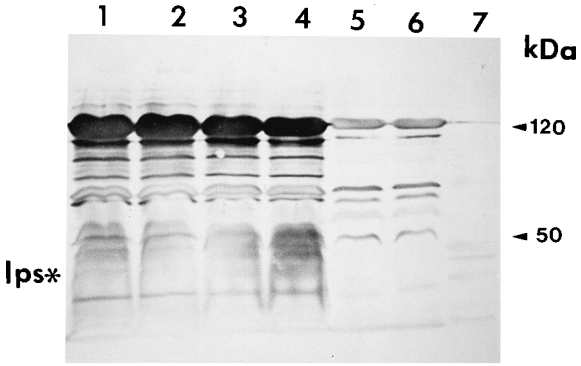


FIG. 3. Immunoblots of rickettsial proteins (20 μg per lane) probed with rat anti-*R. typhi* serum. Lanes 1 and 2, *R. typhi* Wilmington; lanes 3 and 4, *R. felis*; lanes 5 and 6, *R. akari* Kaplan; lane 7, 10-kDa molecular mass marker, with 120- and 50-kDa bands indicated. lps*, LPS residue.

TABLE 1. Comparison of *R. felis* with other vector-borne rickettsiae

| Species | Vector | Presence of antigen and cytopathologic characteristic[a] | | | |
|---|---|---|---|---|---|
| | | 190 kDa[b] | 120 or 135 kDa[c] | 17 kDa[d] | Cell lysis/ plaque formation |
| *R. felis* | Flea | − | + | + | +/+ |
| *R. akari* | Mite | + | + | + | −/+ |
| *R. rickettsii* | Tick | + | + | + | −/+ |
| *R. typhi* | Flea | − | + | + | +/+ |
| *R. prowazekii* | Louse | − | + | + | +/+ |

[a] The "+" sign expression of the gene or protein and presence of cell culture characteristic; the "−" sign indicates their absence.
[b] Also referred to as rickettsial outer membrane protein A.
[c] Also referred to as rickettsial outer membrane protein B.
[d] Also referred to as LPS or rickettsial inner membrane protein A.

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