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Genetic characterization and transovarial transmission of a typhus-like rickettsia found in cat fleas

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ABSTRACT **The identification of apparently fastidious microorganisms is often problematic. DNA from a rickettsia-like agent (called the ELB agent) present in cat fleas could be amplified by PCR with conserved primers derived from rickettsial 17-kDa common protein antigen and citrate synthase genes but not spotted fever group 190-kDa antigen gene. *Alu* I sites in both the 17-kDa and citrate synthase PCR products obtained with the rickettsia-like agent and *Rickettsia typhi* were different even though both agents reacted with monoclonal antibodies previously thought specific for *R. typhi*. The DNA sequence of a portion of the 17-kDa PCR product of the rickettsia-like agent differed significantly from all known rickettsial sequences and resembled the 17-kDa sequences of typhus more than spotted fever group rickettsiae. The rare stable transovarial maintenance of this rickettsia in cat fleas has important implications for the disease potential of cat fleas.**

Invertebrate species, particularly arthropods and protozoa, are often observed to contain regularly associated bacteria that are variously called rickettsia-like, chlamydia-like, or mycoplasma-like depending on their size, pleomorphism, and cell wall structures (1–3). Less often plants (4) and vertebrate hosts may also contain such bacteria (5). The morphologic descriptions of these bacteria cannot be assumed to have any taxonomic validity because they are rarely supplemented by established antigenic or biochemical relationships or even biological or pathogenic properties until methods for their cultivation have been developed. Until recently, the primary obstacles in the characterization of such bacteria were their apparent "fastidious" growth requirements, the minute amounts of material for characterization that could be obtained directly from their hosts, and contamination of cultures by other less-fastidious adventitious flora of the hosts. Now the availability of information on conserved gene sequences combined with the PCR method for amplification of small amounts of DNA has permitted another approach to the identification of fastidious or, heretofore, uncultured microorganisms (6–9). We describe here the molecular identification of a typhus-like rickettsia found in tissues of the cat flea *Ctenocephalides felis*¶ (10). We also examined the maintenance and transovarial transmission of this agent and speculate on the implications of these findings for public health. The animal experiments reported herein were conducted strictly in accordance with ref. 11.

MATERIALS AND METHODS

Source of Infected Fleas. The samples of cat fleas obtained in 1985 and 1989 were from a commercial colony maintained by El Labs, Soquel, CA (ELB), from a laboratory colony maintained at the University of Maryland at Baltimore (UMAB), and from the American Biological Supply, Baltimore (ABS). Control fleas were *C. felis* and *Xenopsylla cheopis* fleas that were infected with the Ethiopian strain of *Rickettsia typhi* (AZ332) as described (12).

Transmission and Seroconversion Assays. Rat seroconversion assay was used for selected samples of ELB cat fleas (newly emerged unfed and fed). Individual laboratory rats were inoculated i.p. with 0.2 ml of flea homogenates (pool = 10 fleas per 1 ml of brain heart infusion broth). Inoculated animals (three animals per flea pool) were observed for 28 days, and seroconversion was measured by the indirect immunofluorescent antibody test to measure specific antibodies to *R. typhi* in serum as described (12).

Direct Fluorescent Antibody Test (DFA) and ELISA Procedures. The presence of *R. typhi* in ELB fleas was determined by DFA and ELISA tests. In DFA the smears of 30 newly emerged unfed fleas were stained with fluorescein isothiocyanate-labeled anti-*R. typhi* (convalescent) guinea pig serum (12). A double sandwich antigen-capture ELISA test using two monoclonal antibodies (T65-1G9.3 and T66-1E8.1) with specificity for *R. typhi* was also used as described (13).

PCR. Detection of *Rickettsia*-specific DNA sequences in flea specimens by PCR amplification of 17-kDa antigen gene was done as described (14, 15). In brief, individual fleas were triturated in 100 μl of brain heart infusion broth and boiled for 10 min, and PCR was done by using 10 μl of the boiled suspension (14, 15). Each 100 μl of sample was amplified for 35 repeated cycles of denaturation at 94°C for 30 sec; annealing was at 57°C for 2 min, and the sequence extension step was at 70°C for 2 min in the presence of *Taq* polymerase (Perkin–Elmer/Cetus) and each of the four deoxynucleotide triphosphates in the reaction mixture (100 μl total). The target DNA sequence amplified by PCR was detected by agarose gel after electrophoresis and ethidium bromide staining. PCR amplification with conserved primers derived from *Rickettsia rickettsii* 190-kDa antigen and *Rickettsia prowazekii* citrate synthase genes was done as described (16).

Restriction Enzyme Analysis of 17-kDa and Citrate Synthase-Amplified Products. The PCR products from the rickettsia-like agent (or ELB agent) and 9-day *R. typhi*-infected *X. cheopis* fleas were digested with the following endonucleases: *Aha* II and *Alu* I, according to the supplier's recommendations (New England Biolabs). Digested products were then electrophoresed in 1% agarose gel, followed by ethidium bromide staining and UV transillumination. Control DNA was prepared from *R. typhi* AZ332-infected fleas by proteinase K/SDS digestion followed by repeated phenol and

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Abbreviations: UMAB, University of Maryland at Baltimore; ELB, El Labs; ABS, American Biological Supply; DFA, direct fluorescent antibody test; SFG, spotted fever group.

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¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M82878 and M82879).

chloroform extractions and ethanol precipitation. *Alu* I was used to digest the PCR products obtained with citrate synthase primers with ELB and *R. typhi* templates. The digestion products were separated on 1.5-mm-thick, 8% polyacrylamide vertical gels and detected by ethidium bromide staining and UV transillumination.

Preparation and Sequencing of PCR Products. DNA sequencing of both strands of a portion of the 17-kDa gene was accomplished by using the dideoxynucleotide chain-termination method (17). The sequencing template was generated in a PCR reaction in which the PCR template was DNA derived from rickettsiae and one of the PCR primers was biotinylated. Selective elution of the nonbiotinylated single-strand template from Dynabeads M-280 streptavidin (Dynal, Oslo, Norway) was accomplished with alkali as described (18). Sequencing reactions were done with Sequenase (United States Biochemical) and reagents and conditions described by the manufacturer; electrophoretic separation was performed and analyzed by using a Genesis 2000 automated DNA sequencer (DuPont).

RESULTS AND DISCUSSION

Because the cat flea agent had an ultrastructure and tissue distribution that was similar to that of *R. typhi* in the oriental rat flea *X. cheopis* and because it reacted with monoclonal antibodies thought specific for *R. typhi*, we first tested whether a specific PCR assay for typhus and spotted fever rickettsiae (14, 15) could be used to detect the presence of this agent in single or pooled cat fleas (Fig. 1). The expected amplified 434-base-pair (bp) fragment derived from the conserved 17-kDa protein gene was readily detected in all samples of cat fleas obtained in 1985 and 1989 from a commercial colony maintained by ELB. No specific PCR product was obtained with uninfected *X. cheopis* or *C. felis* from colonies maintained at UMAB and ABS (Fig. 1). This result also demonstrated that the cat flea agent belonged to the genus *Rickettsia* because no other genera are known to have the 17-kDa protein gene (18–20). To determine whether the 434-bp gene product was derived from *R. typhi* rather than from another typhus or spotted fever group rickettsia, the PCR products obtained with DNA from the cat flea rickettsia and from the AZ332 strain of *R. typhi* were analyzed by digestion with the restriction enzymes *Aha* II and *Alu* I (Fig. 2). With each restriction enzyme the cleavage products obtained with *X. cheopis* infected with *R. typhi* AZ332 or purified AZ332 DNA were identical to those predicted from the *R. typhi* Wilmington 17-kDa protein gene sequence (14). Surprisingly, however, *Aha* II failed to cleave the cat flea PCR product, and *Alu* I digestion yielded three

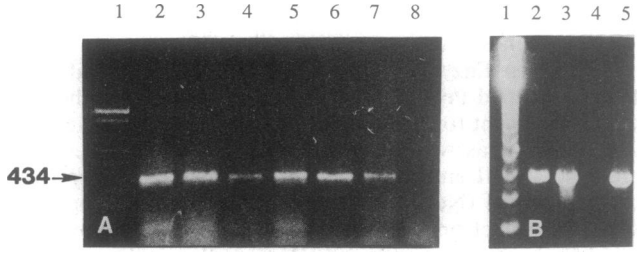

FIG. 1. Detection of *Rickettsia*-specific DNA sequences in ELB *C. felis* colony by PCR amplification of DNA. (*A*) Lanes: 1, DNA size standards; 2–5, four individual fed *C. felis* sampled in 1985; 6, 10^6 *R. typhi* AZ332-seeded brain heart infusion broth; 7, laboratory *R. typhi* AZ332-infected *X. cheopis* fleas; and 8, uninfected *X. cheopis* flea. (*B*) Lanes: 1, 123 bp-DNA ladder; 2 and 3, newly emerged unfed ELB flea pools (n = four fleas per pool) sampled in 1989; 4, unfed *C. felis* pool (UMAB, n = four fleas per pool); and 5, laboratory *R. typhi* AZ332-infected *X. cheopis* fleas.

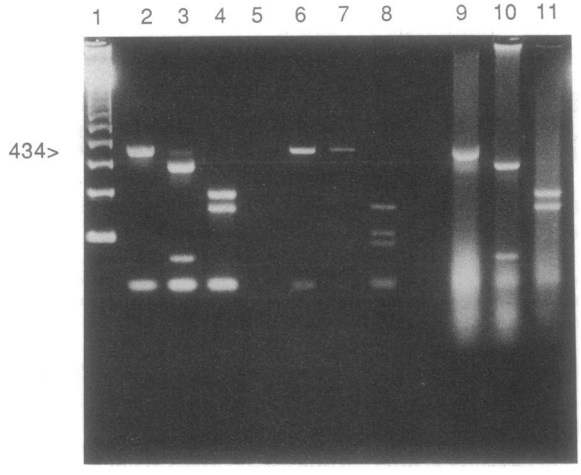

FIG. 2. Restriction enzyme analysis of PCR products by agarose gel electrophoresis. PCR products from purified *R. typhi* DNA (lanes 2–4), uninfected *C. felis* (lane 5), ELB fleas (lanes 6–8), and 9-day *R. typhi* AZ332-infected *X. cheopis* fleas (lanes 9–11). Lanes: 1, 123-bp DNA ladder; 2, 6, and 9, uncut PCR product; 3, 7, and 10, *Aha* II digested; 4, 8, and 11, *Alu* I digested.

fragments, each differing in size from the two fragments obtained for *R. typhi*. This fragmentation pattern also suggested that the cat flea rickettsia was different from the other typhus and spotted fever rickettsiae (15, 21). However, because *R. typhi* might exhibit interstrain variability in its 17-kDa protein genes and *Rickettsia canada*, the third species found in the typhus group, has also been isolated in California (22), we determined the DNA sequences of a portion of the 17-kDa PCR products obtained from the cat flea agent, *R. canada*, and three different isolates of *R. typhi* with different passage histories (Fig. 3). The sequences obtained from the three different *R. typhi* strains were identical to that reported for Wilmington strain (19). Further, as expected from its biologic, genetic, and antigenic properties (23), the 17-kDa protein gene of *R. canada* exhibited features in common with both typhus and spotted fever rickettsiae as well as specific sequence differences. In this region nucleotides 209, 290, 311, 323, 347, 357, and 374 of the 17-kDa protein genes of *R. typhi*, *R. prowazekii*, and *R. canada* each exhibited identical differences from those found in *Rickettsia conorii* and *R. rickettsii*. The ELB agent sequence is typhus-like for only 4 of these 7 positions. For the 13 nucleotides where *R. typhi* and *R. prowazekii* but not *R. canada* differ from spotted fever group (SFG) sequences (218, 224, 279, post-307 insertion, 317, 336, 338, 387, 389, 419, 422, 425, 428), ELB agent also exhibits four identical changes and two different base changes. For the 3 nucleotides where all three typhus species differ from spotted fever but the change is not identical (239, 251, 313), the ELB sequence is identical to that of *R. rickettsii* and *R. conorii* in two cases. Although the variability of these signature nucleotide positions among other SFG rickettsiae has not yet been fully determined, the ELB agent clearly differs from all three typhus rickettsiae at least as much as *R. canada* does from *R. typhi* and *R. prowazekii*. Only *Rickettsia australis* (7 nucleotides) exhibits more than 1 base identical to the 21 typhus group signature sequences among the seven SFG species for which sequence data is available. However, its 17-kDa sequence is not identical to that of the cat flea agent (unpublished data).

At the protein level, the ELB agent and *R. canada* 17-kDa sequences differ from *R. rickettsii* at 6 and 11, respectively, of the 80 amino acids for which sequence was determined but only share three changes. *R. prowazekii* and *R. typhi* also differ from *R. rickettsii* at 6 and 8, respectively, of these 80 positions, but each shares only one amino acid change

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  189.          .         .         .         .         .         .         .    268
Rr GGTTCTCAATTCGGT-AAGGGCAAAGGACAGCTTGTTGGAGTAGGTGTAGGTGCATTACTTGGAGCAGTTCTTGGTGGACA
Rco               -
ELB              C-                    C
Rca     C  G      -     T            A            CT  A        A  T  TA C      AAT
Rp                C    -T        A     C              C           G
RtW               -C C  T        A     C              C           G
Rt2               -C C  T        A     C              C           G
Rt3               -C C  T        A     C              C           G
Rt4               -C C  T        A     C              C           G
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  269.         .         .         .          .         .         .         .    348
Rr AATCGGTGCAGGTATGGATGAACAGGATAGAAGACTTGC-AGAGCTTACCTCACAGAGAGCTTTAGAAACAGCTCCTAGTG
Rco                                       -
ELB   A                 G                 T-  A  C  T     A            G  A A     C
Rca   T        C     C  G                 -   A  C     G  A     G         A A     C
Rp       C   A          G               - T   A  A  A     A            T T  A     C
RtW          A  C       G          A    - T   A  A  A     A            T T        C
Rt2          A          G          A    - T   A  A  A     A            T T        C
Rt3          A          G          A    - T   A  A  A     A            T T        C
Rt4          A          G    N     A    - T   A  A  A     A            T T        C
-----------------------------------------------------------------------------------
  349.         .         .         .         .         .         .         .     429
Rr GTAGTAACGTAGAATGGCGTAATCCGGATAACGGCAATTACGGTTACGTAACACCTAATAAAACTTATAGAAATAGCACTG
Rco
ELB C C  G                           T   C T                                       *
Rca   C  G A                A        T           T          GC          A
Rp         A          G     A            C T        C                    G  C  TG A
RtW        A          C     A     T      C T                             G  C  T  A
Rt2        A          C     A     T      C T                             G  C  T  A
Rt3        A          C     A     T      C T                             G  C  T  A
Rt4        A          C     A     T      C T                             G*
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FIG. 3. Comparison of aligned DNA sequences of the 17-kDa protein antigen genes of species of *Rickettsia* with ELB agent. *R. rickettsii* Sheila Smith (Rr), *R. conorii* Morrocan (Rco), *R. prowazekii* Breinl (Rp), and *R. typhi* Wilmington (RtW) sequences from ref. 12. ELB, *R. canada* McKiel (Rca), and *R. typhi* AZ331, AZ357, and Bb (Rt2–4, respectively) were obtained by sequencing of PCR-amplified 17-kDa protein gene. Bases are numbered from ref. 17, deletions are indicated (–), and only bases different from those of *R. rickettsii* are indicated. *, End of sequence.

(different ones) with *R. canada* and the ELB agent. *R. typhi* and ELB agent differ at 11 of these amino acids.

To further define the genetic relationship of the ELB agent to typhus and SFG rickettsiae and to exclude the possibility that an unusual event had occurred in the 17-kDa antigen gene during passage of a strain of *R. typhi* in cat fleas, we used conserved primers derived from the citrate synthase gene of *R. prowazekii* and the 190-kDa antigen of *R. rickettsii* to amplify DNA (16) from ELB agent and *R. typhi* by PCR. Neither set of 190-kDa primers gave detectable product with either DNA, although PCR amplification with these primers could be confirmed with a number of SFG species (data not shown). Because these primers do not yield PCR products with DNA from typhus rickettsiae, ELB agent appears more closely related to these agents than to SFG. PCR products of the expected size were obtained with the more conserved citrate synthase primers for both *R. typhi* and ELB agent DNA (Fig. 4). The *Alu* I digestion pattern of the citrate synthase PCR product (122, 89, 83, and 44 bp) obtained with ELB agent closely resembled those of *R. canada* and most SFG rickettsiae but clearly differed from those of *R. typhi* (160, 82, 67, 43, and 21 bp) and *R. prowazekii* (108, 88, 82, 59, and 43 bp) (16). This result is not consistent with the hypothetical origin of the ELB agent from *R. typhi* by transovarial maintenance in cat fleas. Together all the PCR and DNA sequence data suggest that ELB agent is a previously undescribed agent belonging to the typhus group of rickettsiae.

Pathogenic rickettsiae are largely transmitted to vertebrate hosts by blood-feeding arthropod vectors, such as ticks, mites, lice, and fleas (23). Ticks, in particular, and some mites as well not only vector rickettsiae, but owing to vertical and horizontal transmission of rickettsiae within populations, serve also as reservoirs of rickettsiae in nature (24). *R. typhi* is transmitted vertically, by transovarial transmission, from experimentally infected female fleas, *X. cheopis*, to a small percentage of offspring (25), but all other rickettsiae known to be transmitted by transovarial transmission are associated with ticks and mites. We tested for the presence of ELB agent in unfed *C. felis* by DFA, ELISA, and animal inoculation (Table 1). Although we could detect the ELB agent in ELB fleas by DFA (30/30), PCR (29/30), ELISA (5/8 flea pools), or animal inoculation (3/3 flea pools), we were unable to detect presence of ELB agent in 79 *C. felis* or 50 *X. cheopis* from ABS or UMAB by any of these methods (Table 1). The DFA uses a guinea pig antiserum against *R. typhi* for detection, and the ELISA assay uses two *R. typhi*-specific monoclonal antibodies against the major surface protein antigen. Despite the obvious differences in the 17-kDa protein genes of these agents, both assays were highly efficient in detecting the ELB agent. These results also suggested that both agents shared important epitopes on the major immunoreactive surface protein antigen. Similarly, rats produced antibodies reactive with *R. typhi* after inoculation with homogenates of

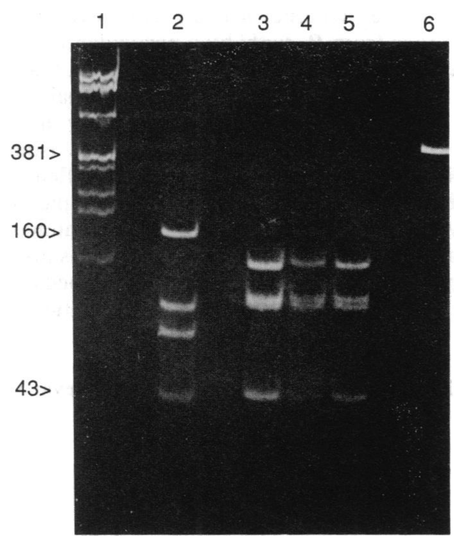

FIG. 4. *Alu* I restriction endonuclease-digested PCR products derived from the citrate synthase gene of *R. typhi* (lane 2), *R. canada* (lane 3), *R. rickettsii* (lane 4), ELB flea (lane 5). ϕX174RF DNA/*Hae* III fragments were used as size standards (lane 1). The uncut PCR product of an ELB flea appears in lane 6.

Table 1. Evaluation of direct fluorescent antibody test, PCR, antigen-capture ELISA, and rat seroconversion assay for detecting ELB rickettsial infection of fleas

Flea sample	Flea species	Method			
		DFA*	PCR*	ELISA*	RSA†
ELB	*C. felis*	30/30	29/30	5/8	3/3
ABS	*C. felis*	0/20	0/8	0/10	0/3
UMAB	*C. felis*	0/20	0/4	0/10	ND
UMAB	*X. cheopis*	0/20	0/10	0/10	0/3

RSA, rat seroconversion assay; ND, not done.
*Number of positive individual fleas per total number examined.
†Number of flea pool positive per total number examined.

ELB *C. felis* but not homogenates of *C. felis* from UMAB or ABS. Like the rat sera, the sera of three cats used to maintain the ELB flea colony were also highly reactive with *R. typhi* but not *R. rickettsii* by the indirect fluorescent antibody test. Because only adult fleas take a blood meal, the consistent detection of 17-kDa antigen sequences as well as rickettsial antigen by three different methods in unfed ELB *C. felis* (Table 1, Fig. 1) clearly demonstrates that the ELB rickettsial agent is transmitted both transtadially and transovarially. The presence of the agent in freshly deposited ELB flea eggs has also been demonstrated directly with the PCR assay (data not shown). The ELB agent was previously demonstrated by electron microscopy to be present in both male and female reproductive tissues (10).

The demonstration of a typhus-like rickettsial agent that can be efficiently maintained in cat fleas by transovarial and transtadial transmission has several important implications for public health. The finding demonstrates that an arthropod other than ticks and mites can serve as a reservoir for a rickettsia. The infection of ELB fleas might conceivably date from the original collection of adult fleas from cats at a rodent-infested granary in the Central Valley area of California in 1969, although the continuous presence of the rickettsia in ELB fleas can only be demonstrated with certainty between 1985 and 1989 (Fig. 1). The maintenance of this rickettsia in the fleas has occurred despite the presence of significant antibody titers to the agent in the cat used to maintain the fleas. We do not know yet whether this rickettsial agent is pathogenic for man, whether the agent is widespread in nature, whether it may be transmitted by other vectors, or indeed whether it is a rapidly evolving different form that arose from *R. typhi* by a particular set of selective circumstances. Moreover, the demonstration that a typhus-like agent can occur in cat fleas raises additional concern that *R. typhi* can also be maintained vertically in this host. Although *R. typhi* is known principally as isolates from rodents (*Rattus* sp.) and their vector fleas, cat fleas, and other arthropods have often been suspected in the epidemiology of murine typhus (25). The presence and maintenance of *R. typhi* in cat fleas, a ubiquitous, hematophagous insect, would be of particular concern to public health because it may present a high probability of human acquisition of murine typhus.

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