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Typhus and Typhuslike Rickettsiae Associated with Opossums and Their Fleas in Los Angeles County, California

SEYMOUR G. WILLIAMS,[1] JOHN B. SACCI, JR.,[1] MARTIN E. SCHRIEFER,[1] ELLEN M. ANDERSEN,[2] KENN K. FUJIOKA,[2] FRANK J. SORVILLO,[2] A. RALPH BARR,[2] AND ABDU F. AZAD[1]*

Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and Los Angeles County Department of Health Services and School of Public Health, University of California at Los Angeles, Los Angeles, California 90024[2]

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The recent discovery of cat fleas (*Ctenocephalides felis*) infected with a typhuslike rickettsia (designated the ELB agent) raises the question of whether similar rickettsial infections exist in wild cat flea populations. We verified the presence of the ELB agent and *Rickettsia typhi* in urban and suburban areas of Los Angeles, Calif. Opossums trapped in close proximity to the residences of human murine typhus cases in Los Angeles county and other areas within the city of Los Angeles were tested for the presence of typhus group rickettsiae by the polymerase chain reaction (PCR). The presence of rickettsiae in the spleen tissues of three opossums ($n = 9$) and in 66 opossum fleas ($n = 205$) was determined by PCR and was verified by dot blot and Southern transfer hybridization. Further analysis of the amplified PCR products generated by a series of primer pairs derived from either the 17-kDa antigen gene or the citrate synthase gene revealed that both *R. typhi* and the ELB agent were present in the tested samples. Dual infection was not noted in the samples; however, the fleas were infected with either *R. typhi* or the ELB agent. The presence of the ELB agent in the cat flea population may have implications for public health. Whether this agent is responsible for the mild cases of human murine typhus in urban and suburban areas of Los Angeles or in other endemic foci remains to be determined.

Murine typhus is a relatively mild febrile illness resulting from infection with *Rickettsia typhi*, a small (0.4 by 1.3 μm), gram-negative, obligate intracellular bacterium. It is usually transmitted to humans by flea bite and contamination of the bite site or skin abrasions with *Rickettsia*-containing flea feces (5). Although murine typhus cases occurred by the thousands annually in the United States during 1931 to 1946 (for reviews, see references 5 and 15), in recent years the disease has been reported infrequently and sporadically. Nonetheless, the 200-plus cases reported in southern California and Texas during the past 10 years have attracted our attention for the following reasons. These cases occurred in the absence of the classical transmission cycle components, i.e., the flea vector (*Xenopsylla cheopis*) and the rodent host (*Rattus norvegicus*). Both the earlier report by Adams et al. (2) and the recent one by Sorvillo et al. (14) emphasized the possible role of peridomestic opossums (*Didelphis marsupialis*) and their fleas (*Ctenocephalides felis*) in the transmission of murine typhus to humans in urban and suburban Los Angeles, Calif., where a cluster of cases has been reported. Experimental infection of cat fleas with *R. typhi* has demonstrated the ability of cat fleas to both maintain rickettsiae and transmit them to laboratory rats (10). Since *C. felis* is abundant on opossums and household pets, this flea may play an important role in maintaining the cycle of infection in suburban and urban areas and may occasionally transmit *R. typhi* to humans.

A *Rickettsia*-like agent (designated the ELB agent) that belongs to the typhus group (which includes *R. typhi*, *R. prowazekii*, and *R. canada*) and that was propagated in a cat flea colony originating from feral cats has been described previously (1, 6). The proximity of this agent and some of the reported human cases prompted us to look for this agent in both *C. felis* and opossums collected as part of investigations of human cases of murine typhus in several locations in Los Angeles County, Calif. The presence of both *R. typhi* and the ELB agent DNA in cat fleas was demonstrated by analysis of products from a polymerase chain reaction (PCR) with conserved primers to the rickettsial 17-kDa common protein antigen and citrate synthase (CS) genes. *Alu*I digestion of the PCR-amplified products was used to differentiate the ELB agent and *R. typhi* as described previously (6). Although fleas collected from both infected and uninfected opossums were infected with either the ELB agent or *R. typhi*, only *R. typhi* was found in opossum tissues.

MATERIALS AND METHODS

Source of samples. Opossums from four areas in Los Angeles County (Fig. 1) were trapped by animal control personnel as part of an investigation of murine typhus cases. The opossums were euthanized, and ectoparasites were collected from each animal by thorough combing and were sorted by species. Necropsy was performed on each animal; and samples of serum, spleen, and liver were collected aseptically and stored at −70°C. Additional cat fleas were obtained from a commercial colony maintained by El Laboratories, Soquel, Calif. Both *R. typhi*-infected and uninfected laboratory colonies of fleas, maintained at the University of Maryland at Baltimore, were used as controls throughout this study. *R. typhi* AZ332 was used to infect fleas as described previously (10).

Direct fluorescent-antibody assay. The presence of rickettsiae in cat fleas was determined by the direct fluorescent-antibody assay by using fluorescein isothiocyanate-labeled anti-*R. typhi* or anti-*R. rickettsii* (convalescent-phase) guinea pig serum (6).

Treatment of samples for amplification of rickettsial DNA by PCR. *Rickettsia*-specific DNA sequences in flea speci-

* Corresponding author.

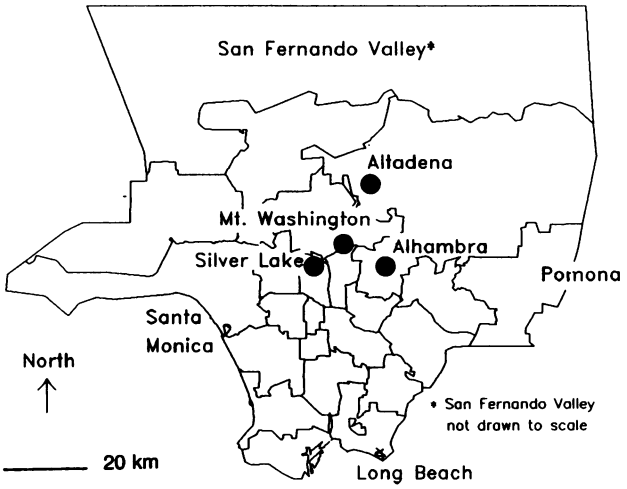

FIG. 1. Geographic locations of the sites in Los Angeles County where opossums were trapped during the study period (1985 to 1987). Opossums were trapped, and ectoparasites were collected and sorted by species and geographic distribution.

mens and opossum tissues were amplified by PCR of the 17-kDa antigen gene as described previously (7, 16). Briefly, individual fleas were triturated in 100 μl of distilled water and boiled for 10 min. PCR was performed by using 10 μl of the boiled suspension as a DNA template (7, 16). DNA was recovered from opossum tissue samples after proteinase K-sodium dodecyl sulfate digestion of the homogenized tissues by repeated phenol and chloroform extraction and ethanol precipitation. The nucleic acids extracted from tissues were quantified by spectroscopy at A_{260}, and 1 μg was used as a DNA template in PCR.

PCR assay and oligonucleotide primers. A pair of oligonucleotide primers (primer 1, 5′-GCTCTTGCAACTTCTAT GTT-3′; primer 2, 5′-CATTGTTCGTCAGGTTGGCG-3′) was synthesized on the basis of the DNA sequence for the 17-kDa protein antigen from *R. rickettsii* (3, 4, 16). Each of the 20-base oligomer primers was complementary to a region of DNA in *R. rickettsii*, *R. conorii*, *R. prowazekii*, *R. typhi*, and the ELB agent where the nucleotide sequences are very similar (1, 3, 4). The length of the rickettsial genome targeted for amplification was predicted to be 434 bp (16). The specificities of these primers in PCR were tested by using purified DNA from various bacteria (7, 16). A single band of the predicted length was obtained only with the members of the spotted fever and typhus groups (16). A second set of primers (primer 3, 5′-GGGGGCCTGCCACGGCGG-3′; primer 4, 5′-ATTGCAAAAAGTACAGTGAACA-3′) (11) was derived from the published sequence of the *R. prowazekii* CS gene (17). This set of primers has been shown to prime the synthesis of a 381-bp product from all species of rickettsiae (except *R. tsutsugamushi*) and *Rochalimaea quantana*, but not *Escherichia coli* or *Mycoplasma* species (11).

PCR amplification (100-μl total volumes) was carried out for 35 cycles under conditions optimized for each primer pair, as follows: for primers 1 and 2 (17-kDa gene segment), denaturation for 30 s at 94°C, annealing for 2 min at 57°C, and extension for 2 min at 70°C; for primers 3 and 4 (CS gene segment), denaturation for 20 s at 95°C, annealing for 30 s at 48°C, and extension for 2 min at 60°C. PCR buffer, nucleotides, and *Taq* polymerase conditions were used as suggested by the manufacturer (Perkin-Elmer Cetus, Norwalk, Conn.).

Restriction enzyme analysis of amplified products of the 17-kDa and CS genes. The amplified PCR products obtained from positive opossum tissues and their fleas as well as controls (*Xenopsylla cheopis* fleas infected with *R. typhi* for 9 days and DNA extracted from various rickettsial seeds) were digested with the endonucleases *Aha*II or *Alu*I according to the supplier's recommendations (New England Biolabs, Beverly, Mass.). The digested products were electrophoresed in either 1% agarose gels (SeaKem; FMC Bioproducts, Rockland, Maine) or 1.5-mm-thick 8% polyacrylamide vertical gels (Bio-Rad Laboratories, Richmond, Calif.), and were detected by ethidium bromide staining.

Detection and identification of rickettsial sequences in reaction products. Target DNA sequences amplified by PCR were detected by ethidium bromide staining after agarose gel electrophoresis or by dot blot hybridization. For analysis in gels, 10 μl of each PCR product was resolved in a 1% agarose gel, and a product of the predicted size was identified by comparison with molecular weight standards and positive control samples. The specificities of the PCR products of the predicted sizes in either agarose or polyacrylamide gels were verified by Southern transfer and hybridization as described previously (13) by using a PCR-generated 17-kDa gene probe. For dot blot analysis of PCR products, 40 μl of each sample was transferred to a nylon membrane (Schleicher & Schuell, Keene, N.H.) in a filtration manifold (12). Membranes were probed with a digoxigenin-labeled, primer-delineated, PCR-generated DNA sequence for the 17-kDa *R. typhi* gene. Hybridized probes were detected by using the Genius nucleic acid detection kit (Boehringer Mannheim, Indianapolis, Ind.).

RESULTS

Amplification of rickettsial DNA from opossum spleen specimens. Spleen and liver tissues were obtained from 10 opossums trapped at four study sites during 1987 to 1988 (Fig. 1). The DNA extracted from the spleen samples was amplified with primers 1 and 2 (17-kDa gene). Four DNA extracts from spleens produced the predicted 434-bp PCR product of the 17-kDa gene (Fig. 2, lanes 1 to 4). The rickettsia-positive opossums were from Alhambra (1 of 5; 20%), Altadena (1 of 1; 100%), and Silver Lake (2 of 3; 67%). DNA extracted from renografin-purified *R. typhi* AZ332 seed was used as a positive control for amplification (Fig. 2, lane 5). The identification of PCR products was confirmed by dot blot hybridization by using a labeled probe generated from *R. typhi* 17-kDa antigen gene sequences. Primers 1 and 2 are *Rickettsia* genus specific and produce 434-bp products with members of both the typhus and spotted fever groups (7, 16). However, *Alu*I digestion of the 434-bp PCR products enabled discrimination of *R. typhi*, the ELB agent, and *R. canada* (Fig. 3). The *Alu*I digestion patterns of the PCR products derived from the positive spleens were homologous to those of *R. typhi* but were distinct from those of the ELB agent, *R. rickettsii*, and *R. canada*. Additionally, PCR amplification of the nine opossum spleen tissues with primer pair 3 and 4 (CS gene) resulted in the predicted 381-bp band in the same four samples which tested positive for the 17-kDa gene sequence. Subsequent *Alu*I digestion of the amplified CS gene segment again revealed a digestion pattern homologous to that of *R. typhi* (data not shown), corroborating the results obtained with the first set of primers.

Amplification of rickettsial DNA from fleas. Analysis of

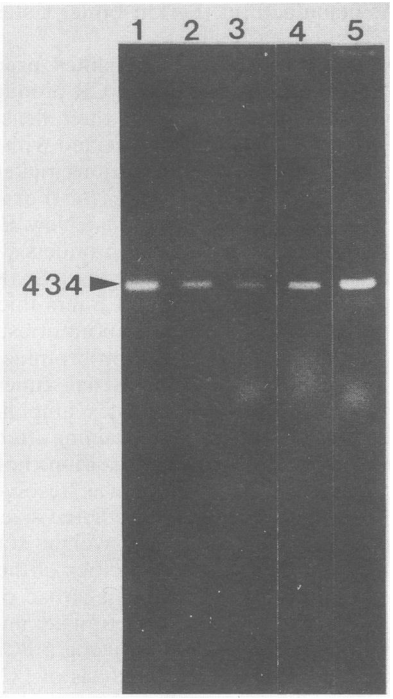

FIG. 2. Detection of *Rickettsia*-specific DNA sequences by PCR amplification of opossum spleen extracts. Opossum spleen tissue was phenol-chloroform extracted, and the resulting DNA was used for PCR with primers specific for a 434-bp sequence of the 17-kDa antigen gene. Lanes 1 to 4, the 434-bp fragment from samples collected in Alhambra (lane 1), Altadena (lane 2), and Silver Lake (lanes 3 and 4); lane 5, positive control, which used DNA extracted from renografin-purified *R. typhi* for the template.

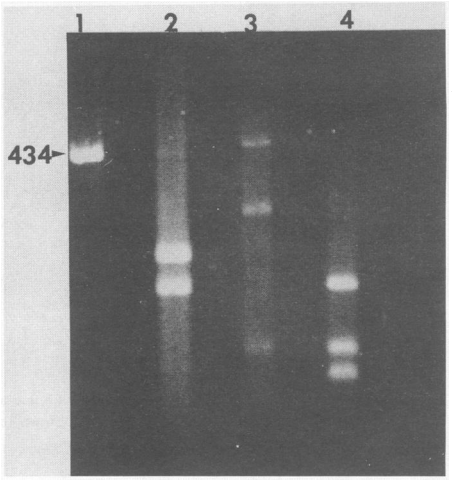

FIG. 3. *Alu*I restriction enzyme digestion of 17-kDa antigen gene (434-bp) PCR products analyzed by agarose gel electrophoresis. Lanes 1, 2, and 4, PCR products amplified from wild fleas caught in Los Angeles county; lane 3, amplified DNA from the McKiel strain of *R. canada*. Lanes: 1 undigested *R. typhi*; 2, *R. typhi* digested with *Alu*I; 3, *R. canada* McKiel digested with *Alu*I (lane 3); 4, the ELB agent digested with *Alu*I.

PCR products was performed on a total of 205 fleas that comprised 167 *Ctenocephalides felis*, 15 *Pulex simulans*, and 23 *Echidnophaga gallinacea*. These fleas were collected from 10 opossums at four study sites (Fig. 1). Fleas were triturated individually in 100 μl of distilled water and boiled for 10 min. PCR was performed with 10 μl of the boiled triturate as a template for primers 1 and 2. Initial analysis of PCR products was accomplished by ethidium bromide staining of agarose gel-resolved samples. By this method of analysis, the predicted 434-bp products were detected only in *C. felis*; 12 of 167 (5.8%) *C. felis* fleas were positive. No other fleas produced a visible 434-bp PCR product. Additionally, 434-bp products were not observed in the negative control (no rickettsial DNA) samples. However, dot blot analysis of samples detected rickettsial DNA in 31% of *C. felis*, 27% of *P. simulans*, and 43% of *E. gallinacea*. All samples that were positive in ethidium bromide-stained agarose gels were also positive by dot blot analysis. Uninfected laboratory control fleas ($n = 50$) were all negative by gel and dot blot analyses. Table 1 summarizes the combined results of PCR-dot blot for 205 fleas, in which there was an overall infection rate of 32.2% (range, 19.6 to 39.4%). Restriction enzyme analysis of the PCR products from positive fleas digested with *Aha*II or *Alu*I confirmed the presence of *R. typhi* (9 of 12 fleas) or the ELB agent (3 of 12 fleas) (data not shown). The specificity of the 434-bp PCR products amplified from fleas was confirmed by hybridization to a *R. typhi* probe in both Southern and dot blot analyses (Fig. 4). The 17-kDa gene probe hybridized only to the 434-bp band of *R. typhi*, the ELB agent, and *R. canada* (Fig. 4A). Similarly, the PCR products from positive fleas amplified with primers 3 and 4 were subjected to *Alu*I digestion to further confirm the presence of the ELB agent in these fleas (Fig. 5, lanes 1 to 3). All the flea samples that produced the 434-bp products with the primer 1 and 2 produced the expected products (381 bp) with the second set of the primers and the appropriate restriction enzyme pattern. To confirm that positive fleas were not missed by analysis of PCR products, a sample of negative fleas was tested by direct fluorescent-antibody assay by using anti-*R. typhi* and anti-*R. rickettsii* serum. None of the 20 fleas tested was positive for either typhus or spotted fever group rickettsiae. Positive fleas harbored either the ELB agent or *R. typhi*. Both the ELB agent and *R. typhi* were present in *C. felis* collected on opossums from Altadena (seven *R. typhi*, one ELB agent), Silver Lake (one *R. typhi*, one ELB agent), Mt. Washington (one ELB agent), and Alhambra (one *R. typhi*) areas.

DISCUSSION

Maintenance of urban murine typhus has classically been attributed to the infection of rodents belonging to the genus

TABLE 1. Oligonucleotide probing of fleas collected from opossums from a suburban focus of murine typhus in Los Angeles County by using probe complementary to the rickettsial 17-kDa protein antigen gene[a]

Collection sites	No. of positive fleas/total no. tested (% positive)			
	C. felis	*P. simulans*	*E. gallinacea*	Total
Alhambra	21/55 (38.0)	3/8 (37.5)	2/3 (66.7)	26/66 (39.4)
Altadena	10/54 (18.5)	0/0	1/2 (50.0)	11/56 (19.6)
Silver Lake	10/29 (34.5)	1/7 (37.5)	7/18 (38.9)	18/54 (33.3)
Mt. Washington	11/29 (37.9)	0/0	0/0	11/29 (37.9)
Total	52/167 (31.1)	4/15 (26.7)	10/23 (43.5)	66/205 (32.2)

[a] Further details of the probes and study sites are given in the text.

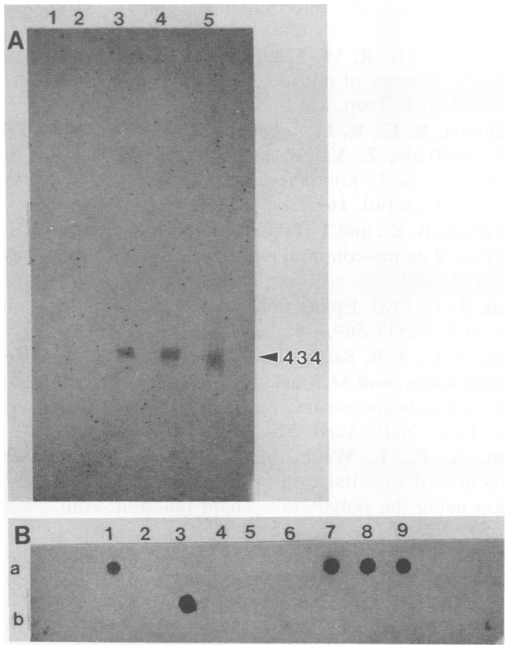

FIG. 4. (A) Southern and dot blot analysis of 434-bp PCR products. Amplified extracts negative by agarose gel electrophoresis (lanes 1 and 2) and PCR products from *R. canada*, *R. typhi*, and ELB agent (lanes 3 to 5, respectively) from fleas caught in the wild which gave positive agarose gel bands and were positive when they were Southern blotted (lanes 3 to 5) were tested. (B) Dot blot analysis of PCR products from gel-negative (a-1 to a-6) and gel-positive (a-7 to a-9) fleas caught in the wild. An uninfected and an *R. typhi*-infected laboratory flea served as negative and positive controls, respectively (b-2 and b-3, respectively). Samples were hybridized with a PCR-generated probe and developed by a nonradioactive alkaline phosphatase procedure.

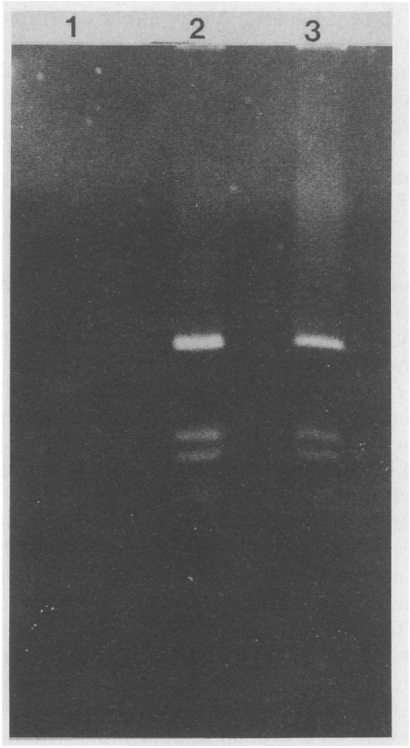

FIG. 5. *Alu*I digestion of PCR products by using CS gene primers amplified from fleas either caught in the wild or reared in the laboratory. Lane 1, restriction pattern of DNA from wild caught fleas, which was consistent with that of the rickettsialike organism (ELB agent); lane 2, restriction pattern of DNA from a flea caught in the wild containing *R. typhi*; lane 3, restriction pattern of DNA from a laboratory-reared flea infected with *R. typhi*.

Rattus and the transmission of rickettsiae by their fleas, in particular, *X. cheopis* (5, 15). However, during the past two decades, the persistence of murine typhus foci in suburban areas of Orange and Los Angeles counties in California and the association of these foci with human cases (on the basis of serological and clinical diagnoses) has not been attributed to the classical rat-flea cycle because of the absence of rats or *X. cheopis* fleas in the affected areas (3, 4). In light of these findings, Adams et al. (2) have suggested the involvement of peridomestic opossums and their fleas, in particular, *C. felis*, in some of the sporadic human cases in Orange County. In their study, 443 serum samples from animals (14 species) were examined for the presence of complement-fixing serum antibody (2). Serum samples from eight of 75 (11%) opossums and one of seven (14%) skunks examined were positive for antibodies to *R. typhi*. *R. typhi* was successfully isolated from the spleen of one of those opossums (2). Using the immunofluorescent antibody method, Sorvillo et al. (14) reported that 90% (9 of 10) of resident cats and 22% (21 of 97) of the opossums trapped in close association with human cases were seropositive for *R. typhi*. In contrast, none of the 21 resident cats and 130 opossums collected from control areas (with no reported human cases) were seropositive for *R. typhi*. Results of these two studies strongly suggest the involvement of an opossum-flea cycle, rather than the classical rat-flea cycle, in murine typhus cases in the study areas.

The opossum tissue and flea samples examined in the present study came from the same areas that were associated with human cases that were sampled by Sorvillo et al. (14). Analysis of PCR products revealed the presence of *R. typhi* DNA in both spleen and flea samples and ELB agent DNA in the flea samples alone. In contrast to PCR analysis that detected only *R. typhi* DNA in some opossum spleen samples, serum samples from three opossums were positive by the immunofluorescent antibody assay against the ELB agent and negative for other typhus group rickettsiae. This is the first report of a widespread distribution of the ELB agent in *C. felis* populations caught in the wild. The relationship between the ELB agent and human cases of murine typhus cannot be established at this time because of the lack of serum samples from both the reported cases and the rickettsial isolates. Intraperitoneal inoculation of laboratory rats with homogenates of ELB agent-infected *C. felis* has resulted in the production of an anti-ELB agent antibody which cross-reacts with *R. typhi*-infected Vero cells (1, 6). Similarly, anti-*R. typhi* and, to a lesser extent, anti-*R. rickettsii* antibodies react with the ELB agent in infected fleas.

In contrast to a colonized *C. felis* population that has maintained a stable ELB agent infection rate of 100%, the fleas collected from opossums in Los Angeles County had a much lower infection rate (3%). It is unclear whether *C. felis* acquires the infection from opossums or whether a low level of transovarial and transstadial transmission is responsible for this low infection level. In experimentally infected *X. cheopis*, the transovarial transmission of *R. typhi* ranged

only from 3 to 11% (9). Very effective transovarial and transstadial transmission of rickettsiae is required to allow the maintenance of the high infection rates observed in the colonized cat fleas which have been maintained in El Laboratories over the past two decades. The ELB agent DNA was found by PCR in the newly emerged, unfed, and fed fleas as well as in their surface-sterilized eggs (6). This colonized flea population repeatedly tested negative for *R. typhi* DNA during the sampling period of 1985 to 1991 (6).

The increased sensitivity of dot blot analysis enabled the detection of rickettsial DNA in both *E. gallinacea* and *P. simulans* as well as in increased numbers of *C. felis*. However, because of the low frequency of occurrence on opossums and household pets and their feeding habits, *E. gallinacea* and *P. simulans* are less important than *C. felis* in the transmission of rickettsiae to humans. Nonetheless, the infection of these fleas, i.e., *E. gallinacea*, which remains affixed to the host for days, can be used to document the host infection with the rickettsial agent.

The absence of dual infection with *R. typhi* and the ELB agent in fleas collected from an *R. typhi*-infected opossum is of interest and may support the results of earlier reports (8) that infection with one rickettsial agent precludes the maintenance of the other species that share antigenic relatedness. Burgdorfer et al. (8) reported that *Dermacentor andersoni* ticks infected with the nonpathogenic East Side agent were refractory to superimposed infection with a virulent *R. rickettsii* isolate. It is crucial to determine whether the stable maintenance of infection of cat fleas subsequently favors the selection of an ELB agent-infected population of cat fleas that are refractory to *R. typhi* infection. Further research in our laboratory is aimed at answering the questions of whether infection with the ELB agent in a colonized population of cat fleas prohibits the growth and establishment of *R. typhi* infections in those fleas and whether animal infection with the ELB agent provides cross protection against *R. typhi* infection. In addition, it should be established whether the ELB agent is pathogenic to humans and laboratory animals, and its role in the endemic foci of murine typhus should also be established. Until the agent is cultivated in tissue culture, our studies will be limited to the use of nucleic acid probes to determine the extent of ELB agent distribution in otherwise known foci of murine typhus infection.

ACKNOWLEDGMENTS

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