Molecular Identification of *Rickettsia typhi* and *R. felis* in Co-Infected *Ctenocephalides felis* (Siphonaptera: Pulicidae)

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ABSTRACT *Rickettsia typhi* and *R. felis*, 2 closely related rickettsial species, often have been identified in cat fleas, *Ctenocephalides felis* (Bouché) from the same geographical location. However, no fleas have been found to be naturally infected concurrently with both rickettsial species. To examine whether one rickettsial species can develop simultaneously with another species in the same flea host, cat fleas, naturally infected with *R. felis*, were allowed to feed on blood containing *R. typhi* (Ethiopian strain, 10^7 PFU/ml). Experimental controls consisted of uninfected cat fleas, fleas infected with only *R. typhi*; and fleas naturally infected with only *R. felis*. After 9 d at 28°C, the fleas were examined by PCR amplification and subsequent restriction digest analysis and dot blot hybridization of PCR products. Results from these studies demonstrated that *R. felis* and *R. typhi* are capable of co-existing in the same flea host. Subsequent isolation of *R. typhi* from dually infected fleas by tissue culture indicated that *R. typhi* was viable and capable of being maintained in fleas naturally infected with *R. felis*. As more studies confirm the presence of *R. felis* and *R. typhi* in domestic pets and peridomestic vertebrates in urban areas, the ability of the individual cat fleas, which live on these animals, to support both rickettsial species could be an epidemiologically important consideration.

KEY WORDS *Ctenocephalides felis, Rickettsia typhi, Rickettsia felis*, murine typhus, co-infection

MURINE TYPHUS, a zoonotic disease caused by the obligate intracellular bacterium, *Rickettsia typhi*, has been the focus of several recent studies (Williams et al. 1992, Sorvillo et al. 1993, Schriefer et al. 1994a). Interest in this disease has been due to the reconsideration of the components of the vector–reservoir cycle and their interaction with humans (Adams et al. 1970). The classic cycle of *R. typhi* transmission involves rats, *Rattus rattus* and *R. norvegicus* and, primarily the rat flea, *Xenopsylla cheopis* (Rothschild). The rat flea has been considered to be the main vector, and transmission to humans has occurred either by flea bite or contact with rickettsia–laden flea feces (Traub et al. 1978). Although the rat–flea–rat cycle is still the major source of human *R. typhi* infections throughout the world, murine typhus has been documented in several endemic foci, such as southern Texas (Schriefer et al. 1994a) and the Los Angeles basin of California (Williams et al. 1992, Sorvillo et al. 1993), where rat seropositivity is low and *X. cheopis* is absent. These studies report the maintenance of *R. typhi* in the cat flea, *Ctenocephalides felis* (Bouché) and opossum, *Didelphis virginiana*, cycle. This cycle occurs in peridomestic areas and is, therefore, a public health concern because *C. felis* is a widespread pest that lives on a wide variety of animals and avidly bites humans (Traub et al. 1978, Azad et al. 1990, Azad 1992, Schriefer et al. 1994a).

In addition to these changes in the classic cycle of *R. typhi* transmission, recent studies have identified a typhus-like rickettsia, *Rickettsia felis*, from colonized (Azad et al. 1992; Higgins et al. 1994, 1996) and field-caught cat fleas taken from opossums (Williams et al. 1992, Schriefer et al. 1994a). Furthermore, cats living in the same environments as these rickettsia-infected opossums also have high prevalences of anti-typhus group rickettsial antibodies (Sorvillo et al. 1993, Azad et al. 1997). *R. felis* not only exists in urban environments, but was identified recently in the blood of a patient diagnosed with murine typhus (Schriefer et al. 1994b).

Although cat fleas taken from opossums in southern Texas (Schriefer et al. 1994a) and Los Angeles/Orange counties, California (Williams et al. 1992, Sorvillo et al. 1993) were infected with either *R. typhi* or *R. felis*, no fleas have been found to be infected concurrently with both rickettsial species. This observation is especially interesting because the intermittent feeding behavior of cat fleas associates them with a variety of hosts and thereby increases their likelihood of acquiring pathogenic organism.

To determine whether fleas become infected with both *R. felis* and *R. typhi*, cat fleas, naturally infected with *R. felis*, were given the opportunity to feed on blood containing *R. typhi*. The results from this study indicate that cat fleas are able to acquire and maintain both rickettsial species, a relationship important in the epidemiology of murine typhus in peridomestic and urban environments.

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Materials and Methods

Fleas. The fleas used for this study were provided by 2 laboratories. The Louisiana colony is maintained on cats at Louisiana State University, Baton Rouge, Louisiana, LA. Higgins et al. (1994) reported and more recent studies (unpublished data) confirmed that 86–90% of the fleas from this colony are naturally infected with *R. felis*. The 2nd flea colony is maintained at Heska Corporation, Fort Collins, CO. Heska fleas are checked routinely for the presence of *R. felis* and *R. typhi* by PCR and to date are free from infection.

Infection of *C. felis* with *R. typhi*. Newly emerged, unfed, Louisiana fleas, naturally infected with *R. felis*, and uninfected Heska fleas each were divided into 2 groups ($n = 200$ per group). One group of Heska fleas were fed uninfected, defibrinated human blood (negative controls), whereas the 2nd group was given access to blood containing *R. typhi* (Ethiopian strain, 10^7 PFU/ml of blood) (*R. typhi* controls). One Louisiana group was fed only on uninfected blood (*R. felis* controls), whereas the 2nd group was allowed to feed on blood containing *R. typhi* (10^7 PFU/ml). An "artificial dog" (FleaData, Farmington, NY) with appropriate feeders provided the fleas access to the blood source. Each respective flea group was given access to either uninfected blood or blood containing *R. typhi* at 4-h intervals each day for 3 consecutive days to ensure infection. All fleas then were provided continuous access to uninfected blood for the next 6 d.

Assaying Individual Fleas for Presence of Rickettsial Infections. The procedures for detecting *R. typhi* and *R. felis* in individual fleas followed previously described protocols with slight modification (Williams et al. 1992, Schriefer et al. 1994a). Briefly, individual fleas were triturated with a sterile plastic pestle in 1.5-ml microcentrifuge tubes (Kontes, Vineland, NJ) in 10 μl of sterile water instead of 100 μl as used in the previous studies. Samples were heated at 100°C for 10 min, and 5-μl aliquots, instead of 10 μl used previously, were used as PCR template. A set of primers, delineating a 232 bp portion of the 17-kD gene, were used in the PCR reactions. The primer pair sequences were 5′-CATTACTTGGTTCTCAATTCGGT-3′ for the forward primer and the 5′-GTTTTATTAGGTGTTACGTAACC-3′ for the reverse primer, which corresponded, respectively, to base positions 176–199 and 408–385 of the 17-kD gene of *R. typhi* (Anderson and Tzianabos 1989) and the base positions 43–66 and 224–201 of the 17-kD gene of *R. typhi* (Azad et al. 1992). The PCR products then were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Subsequently, 10 μl of the above PCR product was treated with the restriction endonuclease *Xba*I (GibcoBRL, Gaithersburg, MD) according to the supplier's recommendations, and digested products were separated by electrophoresis on 8% polyacrylamide slab gels (Bio-Rad, Richmond, CA). *Xba*I cuts the PCR product of *R. typhi* once, whereas the *R. felis* product remains uncut. Digested fragments were visualized by ethidium bromide staining.

Dot Blot Detection of Dually Infected Fleas. Oligonucleotide probes specific for *R. typhi* and *R. felis* (Higgins et al. 1994) were labeled at the 3′ end with fluorescein d-UTP via a terminal transferase-catalyzed reaction using the ECL Oligolabelling kit (Amersham, Arlington Heights, IL). For the dot blots, 15 μl of 17 kD PCR product (232 bp) at a concentration of 2 μg/μl were applied to nylon membrane (Hybond-N, Amersham) through a microsample filtration manifold (Schleicher & Schuell, Keene, NH) and linked by a UV Stratalinker 2400 (Stratagene, La Jolla, CA). Hybridization and probe detection followed the established protocols of the ECL kit.

Isolation of *R. typhi* from Fleas. Procedures for cultivating and detecting rickettsiae from infected fleas followed those of Radulovic et al. (1995). Infected and uninfected fleas were surface sterilized with 70% alcohol, triturated in 100 μl of sterile water, and placed onto monolayers of Vero cell cultures (African green monkey kidney cells, ATCC 1008). After 2 passages, indirect immunofluorescent antibody (IFA) (Philip et al. 1978) and Gimenez (Gimenez 1964) staining were used to detect the presence of *R. typhi*. The *R. typhi*-infected Vero cells were harvested, heated at 100°C for 10 min, and then assayed using the PCR described above.

Results

In 3 of 4 experiments, individual cat fleas, naturally infected with *R. felis* and allowed to feed on *R. typhi*-infected blood, become infected with both rickettsial species (Table 1). Although the *R. felis* infection rate in the Louisiana flea population remained between 75 and 100%, the prevalence of dually infected Louisiana fleas, exposed to *R. typhi*-infected blood, ranged between 13 and 50%. The *R. typhi* controls were consistently infected (100%), and rickettsial products were not amplified from the negative controls.

A representative PCR–RFLP analysis of individual cat fleas infected with both *R. typhi* and *R. felis* is presented in Fig. 1. Dot blots, using specific probes, were used to confirm individual dually infected fleas identified by PCR–RFLP analysis (Fig. 1). In the representative example, the *R. felis* probe hybridized strongly with the *R. felis* seed control (Fig. 2, panel A,

Table 1. Prevalence of rickettsial infections in cat fleas naturally infected with *R. felis*, allowed to feed on uninfected blood and blood containing *R. typhi*

Experiment no.	*R. felis* controls[a]	Co-infected fleas[b]
1	17/20 (86)	6/23 (26)
2	15/16 (94)	2/15 (13)
3	6/8 (75)	3/6 (50)
4	3/3 (100)	0/0 (0)

Data represent number of positive fleas per number tested (% infection)

[a]Cat fleas, naturally infected with *R. felis*, allowed to feed on uninfected blood

[b]Cat fleas, naturally infected with *R. felis*, allowed to feed on blood containing *R. typhi*.

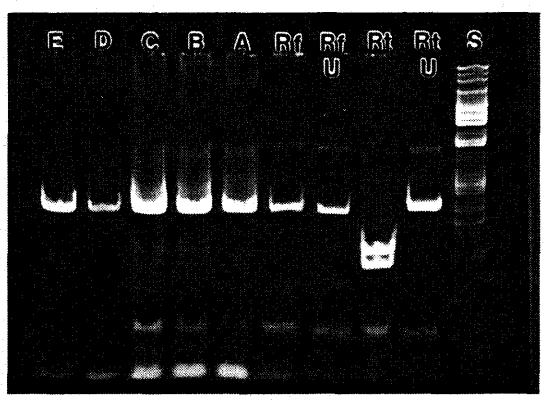

Fig. 1. *Xba*I restriction enzyme analysis of 17kDa PCR products (232 bp) from individual fleas. Rt U, *Rickettsia typhi*, undigested; Rt, *R. typhi*, digested; Rf U, *R. felis*, undigested, Rf, *R. felis*, digested. A, B, C, D, E, fleas infected with both *R. felis* and *R. typhi*. Phi X174 *Hae* III digest standards. Three of the fleas tested (A, D, E) show only the *R. felis* pattern, whereas 2 fleas (B and C) have a pattern consisting of both *R. typhi* and *R. felis*.

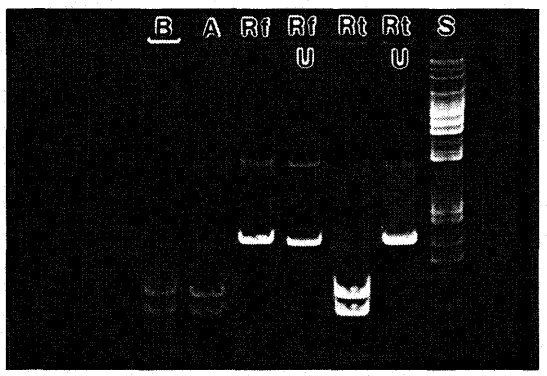

Fig. 3. *Xba*I restriction enzyme analysis of 17-kDa PCR products (232 bp) from Vero cells infected with *R. typhi*-infected fleas. Rt U, *Rickettsia typhi*, undigested; Rt, *R. typhi*, digested; Rf U, *R. felis*, undigested; Rf, *R. felis*, digested. A, Vero cells infected with *R. typhi* from Heska fleas; B, Vero cells infected with *R. typhi* from Louisiana fleas naturally infected with *R. felis*. Phi X174 *Hae* III digest standards.

dot 2) as well as each of the fleas naturally infected with *R. felis* (Fig. 2, panel A, dots 3–7) whereas the *R. typhi* probe recognized the *R. typhi* seed control (Fig. 2, panel B, dot 1) as well as two of the 5 fleas tested (panel B, dots 4–5).

In addition to demonstrating that cat fleas can be infected concurrently with both *R. felis* and *R. typhi*, *R. typhi* was isolated from dually infected fleas and *R. typhi* controls using Vero cell culture (Fig. 3).

Discussion

This study demonstrated that *R. typhi* and *R. felis* can co-exist in the same flea host and indicated that fleas, naturally infected with *R. felis*, may not acquire *R. typhi* as readily as uninfected fleas. The possibility that *R. felis* interferes in some capacity with infection by *R. typhi* may be associated with the presence of *R. felis* in the tracheal matrix, muscle, hypodermis, ovaries, and midgut epithelial lining in naturally infected cat fleas (Adams et al. 1990).

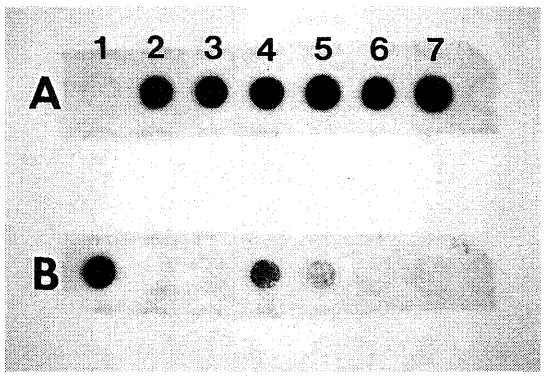

Fig. 2. Autoradiograph depicting hybridization of *Rickettsia typhi* and *R. felis*-specific oligonucleotide probes of dot blots of 231 bp PCR product of the 17-kDa gene. 15 μl of products (containing 2 μg of DNA) from *R. typhi* seed (1), *R. felis* seed (2), and individual fleas (3–7) were dot blotted onto nylon membranes, then probed with oligonucleotides specific for *R. felis* (panel A) and *R. typhi* (panel B).

The best studied example of interference in dually infected arthropods is the *R. peacockii* (formerly the East Side agent)–*R. rickettsii* relationship in *Dermacentor andersoni* Stiles ticks in the Bitterroot Valley of Montana. Transmitted transtadially and transovarially, *R. peacockii* is a nonpathogenic rickettsial species found primarily in the ovaries of *Dermacentor andersoni* Stiles. When given the opportunity to feed on *R. rickettsii*-infected blood, these *R. peacockii*-infected *D. andersoni* are able to acquire *R. rickettsii* which colonizes most of the tissues in nymphal and adult ticks. However, although transmissible to guinea pigs after colonizing the host tick, *R. rickettsii* are not able to invade the ovaries of *R. peacockii*-infected ticks and, therefore, are not passed on to the progeny. Through some unknown mechanism, *R. peacockii* interferes with the ability of *R. rickettsii* to invade the ovaries and thereby blocks the transovarial transmission of *R. rickettsii*. This interference phenomenon may explain why there are so few Rocky Mountain spotted fever cases on the eastside of the Bitterroot Valley where 70% of the *D. andersoni* are naturally infected with *R. peacockii* (Burgdorfer et al. 1981, Niebylski et al. 1997).

Although no other relationships involving dually infected arthropods have been studied as thoroughly, the results from other studies consistently show that the prevalence of dual infections in tick vectors are commonly low. For example, the agent of human granulocytic ehrlichiosis was identified recently in 2.2% (Pancholi et al. 1995) and 4.0% (Telford et al. 1996) of *Ixodes scapularis* Say ticks co-infected with *Borrelia burgdorferi*. Additionally, *B. burgdorferi* and *Babesia microti* were identified in 19–24% of individual *I. scapularis* collected from Naushon and Nantucket Island (Piesman et al. 1986). Finally, 24% of the *I. ricinus*

L. ticks collected in the Rambouillet Forest, France, were found to be concurrently infected with various combinations of 3 species of *B. burgdorferi sensu lato* (Pichon et al. 1995).

The results mentioned above were taken from field surveys, whereas our study was done in the laboratory where certain parameters, not observable under natural conditions, may influence the results. First, the Louisiana fleas come from a flea colony maintained on cats in St. Gabriel, LA. In our study, these fleas were fed on a membrane feeder and not on a live animal. Although the feeding success of the Louisiana fleas over a 3-d period was 90–100%, large numbers of fleas died 4 d after the experiment began. Moreover, the surviving fleas did not lay any eggs, so that transovarial studies could not be carried out to evaluate whether *R. typhi* was passed to the progeny along with the *R. felis*. A 2nd confounding variable may involve the obligate intracellular nature of rickettsial species. The Louisiana colony fleas usually are maintained on cats and do not respond quickly to blood offered via the membrane feeder. In contrast, the Heska fleas, which are maintained on a membrane system, feed quickly on blood containing *R. typhi* (100% fed 10 min after being offered blood) and produce consistent infection rates of 100%. Therefore, because it took longer for Louisiana fleas to feed on the membrane feeders, the possibility exists that a portion of the rickettsiae may have died in the membrane feeders, thereby decreasing the chances of infection. However, because virulent *R. typhi* successfully was isolated from Louisiana fleas, we conclude that some rickettsiae remained viable. Finally, as reported in the *R. peacockii–R. rickettsii* relationship in *D. andersoni*, a number of other unknown and unmeasurable biological factors, such as differences in growth patterns and physiological requirements of the 2 rickettsial species in the fleas, may have affected the results of our study.

Although such issues are inherent in laboratory models, the current study was done in response to the inability to detect dually infected fleas in 2 field surveys done in southern California and southern Texas (Williams et al. 1992, Schriefer et al. 1994a). The absence of dual infections in these surveys is highlighted by the fact that the fleas infected with either *R. felis* or *R. typhi* were taken from opossums infected with the converse rickettsia. Even though field and laboratory studies often are difficult to compare, the current laboratory study may provide some insights into why dually infected fleas were not detected in the field-derived flea samples. First, a simple improvement in technique, specifically in the trituration of individual fleas in smaller volumes of water, allowed for a more concentrated template to be acquired from dually infected fleas, thereby enhancing PCR detection of 2 rickettsial species. Second, the current study indicates that, even under laboratory conditions, the prevalence of dual infections may be low. Therefore, large sample sizes may be required to detect dual infections in the field samples.

Finally, we have demonstrated that *R. typhi* colonized the midguts of fleas transovarially infected with *R. felis* by the isolating *R. typhi* from the midguts of dually infected fleas in Vero cell culture. Because the midgut is the primary organ through which *R. typhi* infects its flea host during the first 9 d after infection (Azad et al. 1984), these results indicate that the rickettsiae present in the flea midgut were viable and, therefore, capable of being maintained in dually infected fleas for a period of time.

These results could have important epidemiological ramifications. In suburban areas, vector fleas are most often associated with human habitation through their natural hosts. Opossums, free-ranging cats, and rats in both urban and suburban habitats where food and hospitable environments are plentiful, may live their entire lifetime in the same backyards. The cat fleas of these peridomestic animals could be picked up easily by household pets and brought into homes. These fleas, apart from being a nuisance, may carry both *R. felis* and *R. typhi* which are of concern to human health (Traub et al. 1978; Azad 1990; Schriefer et al. 1994a, b).

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References Cited

Adams, J. R., E. T. Schmidtmann, and A. F. Azad. 1990. Infection of colonized cat fleas, *Ctenocephalides felis* (Bouché), with a rickettsia-like organism. Am. J. Trop. Med. Hyg. 43: 400–409.

Adams, W. H., R. W. Emmons, and J. E. Brooks. 1970. The changing ecology of murine (endemic) typhus in southern California. Am. J. Trop. Med. Hyg. 19: 311–318.

Anderson, B. E., and T. Tzianabos. 1989. Comparative sequence analysis of a genus-common rickettsial antigen gene. J. Bacteriol. 171: 5199–5201.

Azad, A. F. 1990. Epidemiology of murine typhus. Annu. Rev. Entomol. 35: 553–569.

Azad, A. F., R. Traub, M. Sofi, and C. L. Wisseman, Jr. 1984. Experimental murine typhus infection in the cat flea, *Ctenocephalides felis* (Siphonaptera: Pulicidae). J. Med. Entomol. 21: 675–680.

Azad, A. F., J. B. Sacci, Jr., W. M. Nelson, G. A. Dasch, E. T. Schmidtman, and M. Carl. 1992. Genetic characterization and transovarial transmission of a novel typhus-like *Rickettsia* found in cat fleas. Proc. Natl. Acad. Sci. 89: 43–46.

Azad, A. F., S. Radulovic, J. A. Higgins, and B. H. Noden. 1997. Flea-borne rickettsiosis: some ecological considerations. Emerg. Inf. Dis. 3: 319–327.

Burgdorfer, W., S. F. Hayes, and A. J. Mavros. 1981. Nonpathogenic rickettsiae in *Dermacentor andersoni*: a limiting factor for the distribution of *Rickettsia rickettsii*, pp. 585–594. *In* W. Burgdorfer and R. L. Anacker [eds.], Rickettsiae and rickettsial diseases. Academic, New York.

Gimenez, D. F. 1964. Staining rickettsiae in yolk-sac cultures. Stain Technol. 39: 135–140.

Higgins, J. A., J. B. Sacci, Jr., M. E. Schriefer, R. G. Endris, and A. F. Azad. 1994. Molecular identification of rickettsia-

like microorganisms associated with colonized cat fleas (*Ctenocephalides felis*). Insect Mol. Biol. 3: 27–33.

Higgins, J. A., S. Radulovic, M. E. Schriefer, and A. F. Azad. 1996. *Rickettsia felis:* a new species of pathogenic rickettsia isolated from cat fleas. J. Clin. Microbiol. 34: 671–674.

Niebylski, M. L., M. E. Schrumpf, W. Burgdorfer, F. R. Fischer, K. L. Gage, and T. G. Schwan. 1997. *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western Montana. Int. J. Syst. Bacteriol. 47: 446–452.

Pancholi, P., C. P. Kolbert, P. D. Mitchell, K. D. Reed, J. S. Dumler, J. S. Bakken, S. R. Telford III, and D. H. Persing. 1995. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J. Infect. Dis. 172: 1007–1012.

Philip, R. N., E. A. Casper, W. Burgdorfer, R. K. Gerloff, L. E. Hughes, and E. J. Bell. 1978. Serologic typing of rickettsiae, of the spotted fever group by microimmunofluorescence. J. Immunol. 121: 1961–1968.

Pichon, B., E. Godfroid, B. Hoyois, A. Bollen, F. Rodhain, and C. Perez-Eid. 1995. Simultaneous infection of *Ixodes ricinus* nymphs by two *Borrelia burgdorferi* sensu lato species: possible implications for clinical manifestations. Emerg. Infect. Dis. 1: 89–90.

Piesman, J., T. N. Mather, S. R. Telford III, and A. Spielman. 1986. Concurrent *Borrelia burgdorferi* and *Babesia microti* infection in nymphal *Ixodes dammini*. J. Clin. Microbiol. 24: 446–447.

Radulovic, S., J. A. Higgins, D. C. Jaworski, G. A. Dasch, and A. F. Azad. 1995. Isolation, cultivation and partial characterization of the ELB agent associated with cat fleas. Infect. Immun. 63: 4826–4829.

Schriefer, M. E., J. B. Sacci, Jr., J. P. Taylor, J. A. Higgins, and A. F. Azad. 1994a. Murine typhus: updated roles of multiple urban components and a second typhus-like rickettsiae. J. Med. Entomol. 31: 681–685.

Schriefer, M. E., J. B. Sacci, Jr., J. S. Dumler, M. G. Bullen, and A. F. Azad. 1994b. Identification of a novel rickettisial infection in a patient diagnosed with murine typhus. J. Clin. Microbiol. 32: 949–954.

Sorvillo, F. J., B. Gondo, R. Emmons, P. Ryan, S. H. Waterman, A. Tilzer, E. M. Anderson, R. A. Murray, and A. R. Barr. 1993. A suburban focus of endemic typhus in Los Angeles county: association with seropositive domestic cats and opossums. Am. J. Trop. Med. Hyg. 48: 269–273.

Telford, S. R. III, J. E. Dawson, P. Katavolos, C. K. Warner, C. P. Kolbert, and D. H. Persing. 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc. Natl. Acad. Sci. U.S.A. 93: 6209–6214.

Traub, R., C. L. Wisseman, and A. F. Azad. 1978. The ecology of murine typhus: a critical review. Trop. Dis. Bull. 75: 237–317.

Williams, S. G., J. B. Sacci, Jr., M. E. Schriefer, E. M. Anderson, K. K. Fujioka, F. J. Sorvillo, A. R. Barr, and A. F. Azad. 1992. Typhus and typhus-like rickettsia associated with opossums and their fleas in Los Angeles county, California. J. Clin. Microbiol. 30: 1758–1762.

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