Two Pathogens and One Disease: Detection and Identification of Flea-Borne Rickettsiae in Areas Endemic for Murine Typhus in California

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Abstract

Results of an environmental assessment conducted in a newly emergent focus of murine typhus in southern California are described. Opossums, *Didelphis virginiana* Kerr, infested with cat fleas, *Ctenocephalides felis* Buché, in the suburban area were abundant. Animal and flea specimens were tested for the DNA of two flea-borne rickettsiae, *Rickettsia typhi* and *Rickettsia felis. R. felis* was commonly detected in fleas collected throughout this area while *R. typhi* was found at a much lower prevalence in the vicinity of just 7 of 14 case-patient homes identified. DNA of *R. felis*, but not *R. typhi*, was detected in renal, hepatic, and pulmonary tissues of opossums. In contrast, there were no hematologic polymerase chain reaction findings of *R. felis* or *R. typhi* in opossums, rats, and cats within the endemic area studied. Our data suggest a significant probability of human exposure to *R. felis* in the area studied; however, disease caused by this agent is not recognized by the medical community and may be misdiagnosed as murine typhus using nondiscriminatory serologic methods.

Keywords

flea-borne rickettsiosis; *Rickettsia typhi*; *Rickettsia felis*; flea; opossum

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Murine typhus is a febrile disease caused by the gram-negative bacterium *Rickettsia typhi.* It is often referred to as a mild illness with clinical symptoms of fever, headache, rash, meningitis, and retinitis; however, murine typhus has also been described as a master of disguise, causing a variety of atypical symptoms with a fatality rate of up to 4% (Dumler et al. 1991, Azad et al. 1997, Gikas et al. 2002, Khairallah et al. 2009). *R. typhi* is classically maintained in a rat-flea-rat transmission cycle that involves urban rats, *Rattus norvegicus* Berkenhout and *Rattus rattus* L., and their flea, *Xenopsylla cheopis* Rothschild (Azad 1990). However, other rodents and ectoparasites have been reported to be infected with *R. typhi* (Traub et al. 1978, Azad 1990). As earlier reports regarding the ecology and atypical cycles of *R. typhi* were often based on biological or serological characteristics of *R. typhi,* some of these associations may need to be reevaluated with additional testing using modern molecular tools to ensure that other rickettsiae were not actually being detected.

Humans become infected with *R. typhi* through accidental contact with infected fleas and their feces by inhalation or scarification into damaged skin (Azad 1990). While the number of cases reported in the United States has decreased from a peak of several thousand cases per year during the early 1940s to <100 cases per year currently (Civen and Ngo 2008), murine typhus is still an endemic disease and a public health concern in Hawaii, the gulf coast of Texas, and southern California (Fergie et al. 2000; Centers for Disease Control and Prevention 2003, 2009; Purcell et al. 2007; Civen and Ngo 2008; Green et al. 2011).

After the identification of the first case of murine typhus in California in 1919, the number of annual cases increased until the middle of 1940s when 62 cases were reported in both 1945 and 1946 (California Department of Public Health 1950). In total, 473 cases and 21 deaths were attributed to murine typhus in California during 1919–1948, and all cases occurred in five southern counties: Los Angeles, Orange, San Bernardino, San Diego, and Santa Barbara (California Department of Public Health 1950). Since the early 1950s a shift has been seen in the geographic distribution of murine typhus cases in California from urban settings to more suburban areas (Adams et al. 1970). Interestingly, *X. cheopis* was rarely found in association with these suburban murine typhus cases (Adams et al. 1970). Subsequent epidemiological investigations led to the belief that this suburban transmission cycle of murine typhus may differ significantly from the classic urban cycle and involves cats (*Felis catus* L.), opossums (*Didelphis virginiana* Kerr), and the cat flea, *Ctenocephalides felis* Bouché (Beck and Van Allen 1947, Adams et al. 1970, Azad et al. 1997, Sorvillo et al. 1993, Schriefer et al. 1994a). Over the last two decades, it has been established that cat fleas are commonly infected with a different species of *Rickettsia, Rickettsia felis* (reviewed in Reif and Macaluso 2009), which belongs to the spotted fever group (Bouyer et al. 2001, La Scola et al. 2002). However, to the best of our knowledge, no confirmed human infections because of *R. felis* have been reported in California. Only murine typhus is currently diagnosed using serological methods with *R. typhi* antigen and reported in California (Green et al. 2011), despite the significant presence and wide distribution of *R. felis* in both urban and suburban environments (Civen and Ngo 2008, Abramowicz et al. 2011). Sera from humans infected with *R. felis* contain antibody reacting with *R. typhi* antigen detectable by both microimmunofluorescence assay and western blotting assay; similarly, sera from patients with confirmed diagnosis of murine typhus cross-react with *R. felis* antigen (Znasen et al. 2006, Wiggers et al. 2005, Pérez–Arellano et al. 2005, Raoult et al. 2001).

In response to an increased number of reported cases of murine typhus in suburban Los Angeles and Orange counties during 2007, an investigation was conducted to assess the contemporary ecological situation regarding the presence and circulation of flea-borne rickettsiae. We report the prevalence and distribution of *R. typhi* and *R. felis* at sites previously not known for murine typhus endemicity and discuss the ecological and epidemiological implications of these findings.

Materials and Methods

Animal and Flea Collections

Rats (*R. rattus*), opossums (*D. virginiana*), and cats (*F. catus*) were trapped in the vicinity of households with serologically confirmed human murine typhus cases in Orange and Los Angeles counties, CA, by the staff of the Vector-borne Disease Section, California Department of Public Health, the Long Beach Public Health Department, and the Orange County Vector Control District. After notification of each case, Sherman (H.B. Sherman Traps, Tallahassee, FL) and Tomahawk (Tomahawk Live Trap, Tomahawk, WI) live-traps were set up as close to the suspected human exposure site as possible, not to exceed a 1 mile (1,609 m) radius of the case household, and checked daily. Trapped animals were handled according to approved protocols. Fleas were combed from the animals and sent to the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. Fleas from the city of Long Beach were shipped on ice packs while those from Orange County were shipped on dry ice. Blood was drawn from the captured rats and opossums, and shipped to the CDC on cold packs in 2 ml EDTA blood tubes. Sixteen of 79 opossums from Orange County were euthanized and the brain, spleen, lung, liver, adrenal gland, and kidneys removed, frozen, and shipped to the CDC on dry ice. If cats were present in the case household, and owner approval was obtained, flea and blood samples were also collected and shipped to the CDC.

Serological Testing

Blood was centrifuged; plasma was transferred into a clean vial and the buffy coat was collected according to standard laboratory procedures. The plasma was used for serological testing and the buffy coat was used for DNA extraction as described below.

Serology was conducted using indirect immunofluorescent antibody assay (IFA) against *R. typhi* grown in embryonated chicken yolk sacs that were air-dried and acetone fixed onto template slide wells. In each assay, antibodies bound to the antigens were detected using species specific fluorescein-isothiocyanate (FITC) labeled conjugates. FITC-labeled goat anti-rat IgG (H + L) were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD) and used at 1:100 dilution. Rabbit anti-opossum IgG (H + L) was obtained from Bethyl Laboratories (Montgomery, TX) and used at a final dilution of 1:100; the final detection was completed using FITC labeled goat anti-rabbit IgG (H + L) antibody at a final dilution of 1:100. The assay format, buffers, and other reagents were used according to the method described previously (Nicholson et al. 1997). Sera were tested at consecutive two-fold serum dilutions starting at 1:64 in dilution buffer (PBS, pH 7.4 supplemented with 1% BSA and 1% normal goat serum); the last well demonstrating specific fluorescence of the *R. typhi* organisms was recorded as the endpoint titer.

DNA Extraction from Buffy Coat and Tissues

DNA was extracted from the buffy coat preparations and tissue samples using the QiaAmp Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The entire buffy coat was used for the DNA extractions. The opossum organs were thawed and ≈20 mg of tissue used for each extraction; DNA was eluted using AE buffer and stored at 4°C.

Flea Processing

Upon receipt at the CDC, fleas were identified to species using standard taxonomic keys. Fleas from animals were usually placed into 1.8 ml Sarstedt vials in pools of 2–3 to reduce testing costs; however, different species of fleas, or from different animals, were never combined together. The DNA was extracted as previously reported (Eremeeva et al. 2008, Karpathy et al. 2009). Briefly, the fleas were disinfected with 10% bleach and 70% ethanol, pulverized after freezing in liquid nitrogen, and the DNA extracted using the Wizard SV96 Genomic DNA kit (Promega, Madison, WI) on a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA). DNA was eluted with 100 μl of sterile distilled water and stored at 4°C.

Nested Polymerase Chain Reaction

Nested polymerase chain reaction (PCR) was used to test flea samples for the presence of rickettsial DNA as previously published (Massung et al. 2001, Tzianabos et al. 1989). Briefly, 2 μl of sample DNA was used in a 25 μl primary reaction along with primers Rr17.122 and Rr17.500. One microliter of the primary reaction was used as the template for the secondary reaction along with primers RP1D and RP2 for the detection of *R. typhi,* or primers TZ15 and TZ16 for the detection of *R. felis.* All products were resolved on a 1% agarose gel in 1× TAE and sequenced.

Taqman Assay

Primers were designed to amplify a 266 bp region of the citrate synthase gene (*glt*A) of *Rickettsia* (Eremeeva et al. 2008, Karpathy et al. 2009). These primers (gltA-F.287-GAT TTT TTA GAA GTG GCA TAT TTG; gltA-R.552-GGK ATY TTA GCW ATC ATT CTA ATA GC) were used in conjunction with species specific probes to simultaneously detect and differentiate *R. typhi* and *R. felis* DNA. To increase the melting temperature and binding specificity, locked nucleic acid (LNA) nucleotides (in parenthesis) (Koshkin et al. 1998) were incorporated into the probes (*R. typhi glt*A: 5′ CalRed 610-TT(T) A(C)T A(C)A (A)AG (A)T(T) G(C)T (C)A-BHQ2; *R. felis glt*A: 5′ Cy-5-CTA (C)GG A(G)A ATT (G)CC A-BHQ3) (Eremeeva et al. 2008, Karpathy et al. 2009). Reagents from the Brilliant qPCR kit (Stratagene, LA Jolla, CA) were used as previously described (Eremeeva et al. 2008, Karpathy et al. 2009). All samples, including both positive and negative control samples placed on every plate, were tested in duplicate.

DNA Sequencing and Analysis

All nested PCR products and selected qPCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to the manufacturer's instructions. Purified amplicons were sequenced using the BigDye Terminator v3.1 cycle

sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions on an ABI 3130xl genetic analyzer (Applied Biosystems). The sequencing reads were assembled and analyzed using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI) and compared with the nr database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/).

Data Analysis

Minimum infection rates (MIR) were calculated for both *R. typhi* and *R. felis* by dividing the total number of flea pools positive for that agent (pools positive for that agent alone plus those pools positive for both agents) by the total number of fleas tested. To assess the distribution of flea infection rates in proximity to the human case households, trap locations were classified as being within areas of one acre or closer to a case household (≈36 m radius), a half mile radius from a household (>36–800 m), or between 0.5 and 1 mile radius (>800–1,600 m) from a case household. Opossum home-range was estimated between 10 and 50 acres (113–252 m radius or 4–20 ha area) (Jackson 2011). Flea infection rates obtained from animals trapped further than a mile from any case household were grouped together and were used to determine the background level of *R. typhi* and *R. felis* in the area. Statistical assessment was done using Student's *t*-test and χ^2 test.

Results

Characteristics of Trapped Animals and Collections of Fleas

After reporting of diseases, an environmental investigation was conducted for each of 14 serologically diagnosed human cases of murine typhus in the City of Long Beach area of Los Angeles County and the neighboring part of Orange County. In total, 177 opossums (*D. virginiana*), 20 rats (*R. rattus*), and 16 cats (*F. catus*) were captured during 14 mo between January of 2007 and February of 2008 (Table 1; Figs. 1 and 2). Most of the opossums were caught in the City of Long Beach during March–April and August–October months of 2007 (30 and 44 animals, respectively, 38 and 56% of the total number trapped), while in Orange County, most animals were trapped in January–February of 2007 (11 opossums, 29%) and 2008 (14 opossums, 37%) (Fig. 2).

In total, 4,586 fleas were collected including *C. felis* (*n*=4443, 97%), 114 *Pulex irritans* L., 19 *Echidnophaga gallinacea* Westwood, 5 *Xenopsylla cheopis*, 4 *Diamanus montanus* Baker, and 1 *Leptosylla segnis* Schoenherr (Table 1). Many animals had severe flea infestations particularly opossums. Male adult opossums typically had a higher flea index than females (*P*=0.04 and *P* = 0.006 [χ^2 test], respectively, in Los Angeles and Orange counties). In total, 56 *C. felis* fleas were collected from 6 of 16 cats examined. Only two rats from Orange County of the 16 rats captured in both locations had fleas, of which one was infested by *C. felis* (*n* = 11) and *P. irritans* (*n* = 1), the other rat had only one *L. segnis,* and neither harbored *X. cheopis.*

Laboratory Testing of Animal Specimens

DNA of buffy coats from 138 animals was tested for the presence of DNA from *R. typhi* and *R. felis* using the multiplex TaqMan assay. No rickettsial DNA was detected in any of the

blood samples tested (Table 1). Serological testing did not detect the presence of antirickettsial antibodies reacting with *R. typhi* antigen in the plasma of these animals (Table 1). In contrast, DNA of *R. felis* was detected in tissue samples from several opossums captured in Orange County. Of the 81 tissue samples from 16 animals examined, five DNA samples from four animals tested positive for *R. felis* DNA by TaqMan assay: lung ($n = 1$), kidney ($n = 1$), liver ($n = 1$), and adrenal gland ($n = 2$). DNA of *R. typhi* was not detected in any of the 81 animal tissue samples tested.

Testing of Fleas

In total, 4,586 fleas were removed from opossums (n=112 of 117), cats (n=6 of 16), and rats ($n = 2$ of 20) captured at peridomestic sites in the vicinity of households of patients with suspected or serologically confirmed cases of murine typhus in Los Angeles and Orange Counties, CA; 3,690 fleas were tested in 1,950 pools for the presence of DNA from *R. typhi* and *R. felis* (Table 2). The fleas were tested in samples of 1, 2, or 3 fleas (average =1.9 fleas per pool) with each pool consisting of a single species of flea, all from the same animal. Of the 1,950 pooled samples tested, 29 (1.5%) were positive for the presence of only *R. typhi* DNA and 900 (46.2%) were positive only for *R. felis* DNA. Furthermore, 33 (1.7%) samples tested positive for both *R. typhi* and *R. felis* DNA.

When analyzed based on the host animal species, significant differences in flea infestation and prevalence of infected fleas were detected (Tables 1 and 2). Only 2 of the 20 rats captured were infested with fleas including 1 *L. segnis,* 1 *P. irritans,* and 11 *C. felis.* No rickettsial DNA was detected from the *P. irritans* or *L. segnis* collected from rodents, while 1 of the 6 *C. felis* pools was positive for *R. felis* DNA. In total, 56 *C. felis* were collected from 6 cats and screened in 29 pools. Of these, 22 pools (75.9%) tested positive for the presence of *R. felis* DNA alone, while one pool was positive for the DNA of both *R. felis* and *R. typhi.* The remaining 3,621 fleas, including 3,472 *C. felis,* 111 *P. irritans,* 19 *E. gallinaceae,* 5 *X. cheopis,* and 2 *D. montanus,* were collected from 112 opossums. These fleas were tested in 1,914 pools and 877 (45.8%) tested positive only for *R. felis* DNA, 29 (1.5%) tested positive only for *R. typhi* DNA, and 32 pools (1.7%) tested positive for the presence of both DNAs.

For *C. felis,* 47.1% ($n = 882$) of the pools tested positive for *R. felis* DNA only, 1.3% ($n = 25$) were positive for *R. typhi* DNA only, and 1.7% (n=32) were positive for DNA from both species. Of the 58 *P. irritans* pools, 17.2% ($n = 10$) were positive for *R. felis* DNA only and 6.9% ($n = 4$) were positive for *R. typhi* DNA only. The 19 *E. gallinacea* fleas were tested in 11 pools; 4 (36.4%) were positive for *R. felis* DNA only, and 1 (9.1%) tested positive for both *R. typhi* and *R. felis* DNA. Two (50%) of the four *X. cheopis* flea pools tested positive for *R. felis* DNA only, as did two (66.7%) of the three *D. montanus* pools. No *R. typhi* DNA was detected in any of the *X. cheopis* or *D. montanus* fleas tested. No rickettsial DNA was detected in the lone *L. segnis* flea tested.

Spatial Distribution of *R. felis* and *R. typhi* in Fleas Relative to Case Households

Of the 749 fleas removed from 26 animals captured within a one acre area around the case household, 63.7% of flea pools were positive for *R. felis* DNA and 4.1% were positive for *R.*

typhi DNA. Nine animals were captured within 0.002 mile to 0.5 mile radius from a case household from which 354 fleas were collected. Of these, 35.9% of the pools tested positive for *R. felis* DNA and 4.9% tested positive for *R. typhi* DNA. Thirteen animals were captured within a 0.5–1 mile radius zone from a case household, from which 294 fleas were removed. *Rickettsia felis* DNA was detected in 37.7% of such flea pools, while *R. typhi* DNA was not detected in any fleas. In total, 2,293 fleas were removed from 69 animals trapped further than 1 mile from any reported human cases; 46.9% of flea pools were positive for the presence of *R. felis* DNA while 3.9% were positive for *R. typhi* DNA. Respectively, there was no significant difference in the MIRs for *R. typhi* of fleas collected within a 1 mile radius zone from cases compared with the MIR in fleas collected more than a mile from any case ($P = 0.33$; χ^2 test). Similarly, the MIR of *R. felis* in the same fleas was 26.7% and did not exhibit significant differences compared with 26.2% MIR detected in the fleas collected from remote locations ($P = 0.73$; χ^2) (Table 3).

To further evaluate these observations, we analyzed the same parameters on a case by case basis (Fig. 3). *R. typhi* was detected in fleas near 7 of 14 case households but was not detected in any flea collected within a mile of cases 3, 4, 5, 6, and 9, and only infrequently (MIR = 0.8%) in fleas collected within an acre area of case 8. However, *R. felis* was detected in these same fleas in varying rates (MIR = 34.3–48.1%), often exceeding or even nearly doubling the background rate (MIR = 26.2%). Fleas removed from animals captured 0.5 miles (37–800 m) from cases one and two had MIRs for *R. typhi* (3.2 and 6.9%, respectively) much higher than the background rate (1.6%). Cases 10, 11, 12, and 14 represent a cluster of neighboring cases and the same fleas were associated with all four cases. Both *R. typhi* (MIR = 15.0%) and *R. felis* (MIR = 46.6%) were detected at rates much higher than the background rate and either agent could have caused disease. No animals were trapped within a mile from either case 7 or 13.

Discussion

The state of California is one of two mainland U.S. states that continues surveillance and reporting of murine typhus because of its recognized potential public health impact. By the end of the 20th century the number of reported cases fell off dramatically because of efforts aimed at controlling the populations of rats and their associated ectoparasites. Current endemic foci are mostly confined to a few southern counties, with Los Angeles County accounting for 42 to 91% of all cases in California (Civen and Ngo 2008). Observations made during the 1970–1990s led to the conclusion that the distribution of human cases has shifted from urban to suburban foci and, respectively, associated with two different ecological cycles, the classic urban cycle involving *R. typhi* circulating in rat and rat fleas and the suburban cycle involving opossums, cats, and cat fleas that were later found to be primarily infected with *R. felis* (Adams et al. 1970, Reif and Macaluso 2009). The classic endemic focus continues to be associated with its historic area in North Central Los Angeles County and central Los Angeles. Starting in 2006, murine typhus cases were observed first in the western part of Los Angeles County and then south and eastward in areas where murine typhus had not typically been reported (Adams et al. 1970, Civen and Ngo 2008). Here we provide the details of our ecological assessment of areas in the City of Long Beach

area of Los Angeles County and an adjacent region of Orange County where emerging cases of murine typhus have been reported.

To ensure sensitive high throughput testing we developed a multiplex TaqMan assay that allows simultaneous detection of two flea-borne rickettsiae, *R. typhi* and *R. felis* (Eremeeva et al. 2008, Karpathy et al. 2009). First, 216 *C. felis* DNA samples were used to compare the performance of our TaqMan assay which targets a GltA gene fragment (Eremeeva et al. 2008, Karpathy et al. 2009) with a nested PCR assay targeting the 17 kDa protein gene of *Rickettsia* (Tzianabos et al. 1989, Massung et al. 2001). We established that the TaqMan assay is more sensitive than the nested PCR for detection of *R. felis* in DNA samples of wild-caught cat fleas as it detected 3 times as many positive samples as did the nested PCR assay. The sensitivity for detection of *R. typhi* DNA was demonstrated using a homologous recombinant plasmid, because *R. typhi* DNA was not detected in this set of samples and is typically much less prevalent in cat fleas as shown here and reported by others (Boostrom et al. 2002, Henry et al. 2007). The sensitivity of this *R. typhi* TaqMan assay was similar as that established for *R. felis* DNA with limits of detection 1–2.5 DNA copies per reaction (Karpathy et al. 2009).

In total, 3,690 fleas that had been removed from 120 animals captured in the suburban environment associated with suspected or serologically confirmed cases of human murine typhus were tested for *R. typhi* and *R. felis* DNA. Overall the MIR in these samples was 1.7% for *R. typhi* and 25.3% for *R. felis* (Table 2). When the samples from two counties were compared, the MIR of fleas with *R. typhi* was estimated to be 1.3% in Los Angeles County and 2.2% in Orange County and did not exhibit significant differences ($P = 0.72$; χ^2), while the MIR of fleas with *R. felis* was much lower in Los Angeles County (21.8%) than that found in Orange County (30.3%). When the presence and prevalence of *R. felis* and *R. typhi* in fleas captured within a 1 mile distance from each case household was estimated, we found *R. typhi* only in the vicinity of seven patient households (Fig. 3). No *R. typhi* (five cases) or even animals (two cases) were found for the other seven cases also diagnosed as murine typhus using serologic methods (Fig. 3). The reported home range of Norway rats is within a 25–50 m radius, but may vary depending on habitat conditions and density of rodent population (Russell et al. 2010). In Southern California, the roof rat home range varies from 0.5 to 1.25 acres (a 2.5–12 m radius) and shape depending on the microstructure of the habitat (Recht 1988). While we expected opossums to range much more widely (Jackson 2011), our trapping results suggested that their distribution around case households is patchy so some exposures may have actually occurred at sites other than their households.

Our data indicate a very significant prevalence of *R. felis* in multiple species of fleas collected from two suburban areas of southern California identified as newly emergent foci of murine typhus. Furthermore, the significant presence of opossums with a high rate infestation by *C. felis* was also established, while attempts to detect the classic rat–flea cycle of *R. typhi* were not successful in this study. Indeed, only a few roof rats were captured and they had very limited flea infestation, a result in good agreement with previous findings in 1985 reporting lack of ectoparasites on these animals (Schwan et al. 1985). However, *C. felis* that were recovered from case homes with pet cats exhibited a high MIR infection rate

by *R. felis* only (38 and 41% from three infested cats each in Orange and Los Angeles Counties, respectively), suggesting the possible importance of domestic cats as transport hosts of fleas to humans. Cat fleas are very promiscuous feeders and can establish transient parasitism on many different animals; furthermore, once infected they maintain persistent rickettsial infection through very effective transovarial transmission (Azad 1990). To the contrary, the efficiency of horizontal transmission is low and it appears that only a small portion of animals infested with infected fleas may develop a transient rickettsiaemia (Wedincamp and Foil 2000, Boostrom et al. 2002, Adjemian et al. 2010, Horta et al. 2010). Seroconversion in animals to *R. felis* has also been reported; however, it appears to require a prolonged exposure before reaching detectable levels in cats and opossums, and infection does not always correlate with the degree of flea infestation (Reif et al. 2008, Horta et al. 2010) and occurs at a low prevalence in cats (Case et al. 2006). Therefore, it is plausible to conclude that the absence of detectable antirickettsial antibodies in the blood of animals tested in this study may be because of limited transmission from cat fleas that were mostly infected with *R. felis.* The source and mechanism of acquisition and maintenance of *R. typhi* by cat fleas in this area is not known and it may be because of spillover from wildlife that was not sampled. Norway rats and mice are particularly susceptible to *R. typhi* infection and may establish a long-term persistent intracerebral infection after a short-term transient rickettsiemia (Traub et al. 1978). Although roof rats are often described as a natural reservoir of *R. typhi* (Traub et al. 1978), their significance in the maintenance of *R. typhi* in California appears to be less important and may require further evaluation as noted previously (Schwann et al. 1985) and found in this study. Another source of *R. typhi* might be *P. irritans,* a very promiscuous feeder. In this study we obtained two large collections of 62 and 42 *P. irritans* both from two opossums from Orange County that were also infested with *C. felis.* For one of these animals, *P. irritans* tested positive either for *R. felis* or *R. typhi* but not both while *C. felis* were positive for *R. felis* alone or tested positive for both organisms.

R. felis has been detected on several occasions in the same animal and flea populations as *R. typhi,* and its presence often correlates with identification of human cases of murine typhus (Boostrom et al. 2002, Abramowicz et al. 2011). Experimentally infected fleas were shown to be able to maintain co-infection with both rickettsiae (Noden et al. 1998), and wild caught *X. cheopis* can be also coinfected with *R. felis* and *R. typhi* (Eremeeva et al. 2008). Those observations raise questions about the role of both bacteria in the pathogenesis and epidemiology of human infections diagnosed as murine typhus. Although, *R. felis* is a recognized human pathogen with well-confirmed cases reported from many parts of the world (Pérez–Osorio et al. 2008, Richards et al. 2010), in the United States only one single confirmed case due to *R. felis* was identified in 1994 in Texas (Schriefer et al. 1994b). The relatively low incidence of *R. felis* cases reported in other countries either suggests that only a small portion of the human population may be susceptible to this pathogen or other factors may affect susceptibility to infection including genetic variations in the pathogen itself (Bauer et al. 2006, Rolain et al. 2009). It is unknown whether there is a human genetic factor associated with susceptibility to this infection. Molecular diagnostic practices and procedures should be implemented to ensure accurate clinical treatment and etiological identification of cases suggestive of flea-borne rickettsioses because these infections may be

because of either *R. typhi* or *R. felis* based on the presence of both agents in fleas in many locations. PCR based diagnostic assays are recommended to detect the presence of rickettsial DNA in EDTA whole-blood samples collected during the acute stage of illness before administration of antibiotics. If only serological testing can be performed, simultaneous testing of acute and convalescent serum specimens against both *R. typhi* and *R. felis* antigens is needed, ideally using cross-absorption of the sera to ensure the accurate differential detection of the correct etiological agent (Pérez–Arellano et al. 2005).

Acknowledgments

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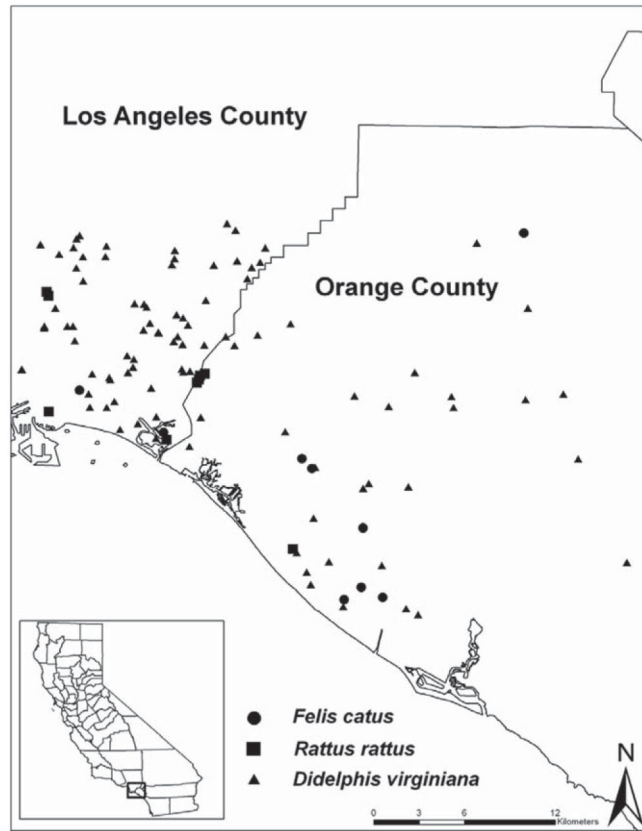
Fig. 1.
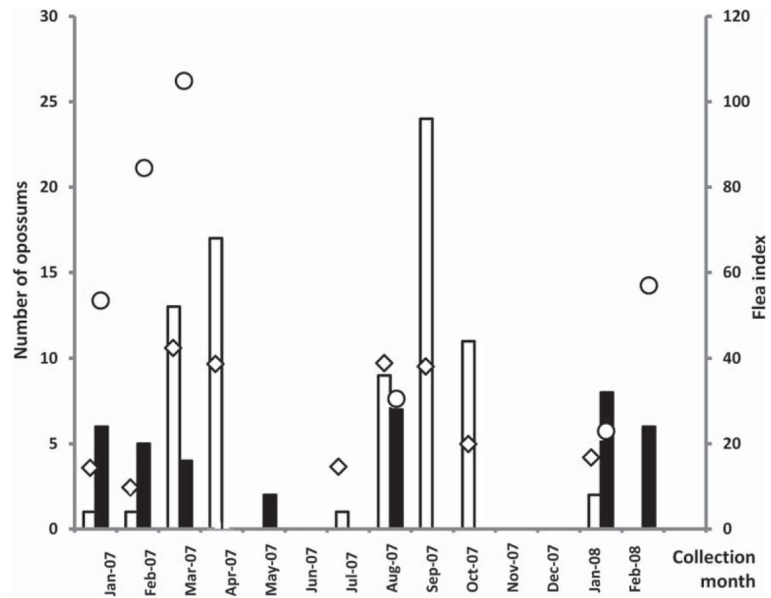Map of animal trap sites in Los Angeles and Orange Counties.

Fig. 2.
Seasonal distribution of opossum trapping in Los Angeles and Orange Counties, CA. White bars, number of opossums captured in Los Angeles County; black bars, numbers of opossums captured in Orange County; diamond (◇) indicates flea index in Los Angeles County; circle (○) indicates flea index in Orange County.
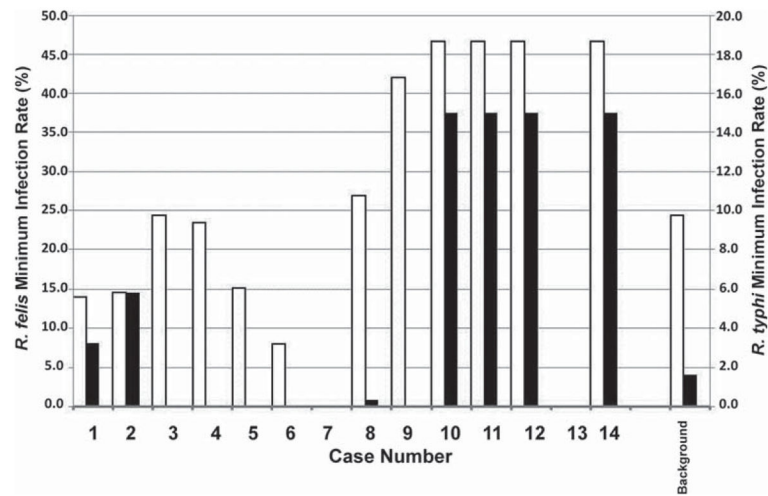
Fig. 3.
Minimum infection rates for *R. typhi* and *R. felis* in fleas collected within one mile of individual case households. Minimum infection rates were calculated as the ratio of the numbers of PCR positive flea DNA samples divided by total number of flea DNA samples tested. The background prevalence was calculated from all fleas collected more than 1 mile from any known case household. No animals were collected within 1 mile of cases 7 and 13.

Table 1

Summary of trapping success and laboratory findings in animal blood

Location and animal	Characteristic (no.)	Flea species collected	Flea index (range)[a]	Laboratory findings in animals	
				IFA	PCR[b]
Los Angeles County					
Didelphis virginiana (79)	Adult, female (40)	*Ctenocephalides felis* (1,189)	40 (2–144)	0/24	0/38
		Xenopsylla cheopis (5)			
		Pulex irritans (1)			
		Echidnophaga gallinaceae (2)			
	Adult, male (25)	*Ctenocephalides felis* (1,151)	66 (4–136)	0/18	0/24
		Pulex irritans (1)			
		Echidnophaga gallinaceae (2)			
	Young (10)	*Ctenocephalides felis* (174)	17 (1–50)	0/10	0/10
		Pulex irritans (1)			
		Echidnophaga gallinaceae (13)			
	Unclassified (4)	*Ctenocephalides felis* (99)	25 (14–42)	0/1	0/4
Rattus rattus (6)	Adult, female (3)	None		0/3	0/3
	Adult, male (3)	None		0/3	0/3
Felis catus (3)	Adult (3)	*Ctenocephalides felis* (27)	9 (3–11)	ND[c]	0/3
Orange County					
Didelphis virginiana (38)	Adult, female (11)	*Ctenocephalides felis* (504)	46 (0–197)	0/6	0/10
		Diamanus montanus (2)			
	Adult, male (7)	*Ctenocephalides felis* (606)	87 (0–216)	0/6	0/7
		Echidnophaga gallinaceae (2)			
		Diamanus montanus (2)			
		Pulex irritans (104)			
	Young (5)	*Ctenocephalides felis* (103)	21 (0–51)	0/3	0/2
	Unclassified (15)	*Ctenocephalides felis* (550)	37 (2–221)	0/3	0/4
		Pulex irritans (6)			
Rattus rattus (14)	Adult, female (6)	None		0/6	0/6
	Adult, male (4)	*Ctenocephalides felis* (11)		0/2	0/4

Location and animal	Characteristic (no.)	Flea species collected	Flea index (range)[a]	Laboratory findings in animals	
				IFA	PCR[b]
		Pulex irritans (1)			
	Young (3)	None		0/3	0/3
	Unclassified (1)	*Leptosylla segnis* (1)		0/1	0/1
Felis catus (13)	Adult (13)	*Ctenocephalides felis* (29)	2 (0–29)	ND	0/13

[a] Flea index and range were calculated for *C. felis* only because of the low collection sizes of other flea species collections.

[b] PCR only blood results are shown. *R. felis* PCR positive issue samples were found in 4 of 16 opossums trapped in Orange County (details are provided in the text).

[c] ND, testing not performed.

Table 2

Detection of *R. typhi* and *R. felis* DNA in flea DNA samples

Location, flea species	No. of flea pools tested (total fleas)	No. of positive pools (%)			Minimum infection rate (%)[a]	
		R. typhi	R. felis	Dual positive	R. typhi	R. felis
Orange County						
Ctenocephalides felis	727 (1405)	7 (0.9)	428 (58.9)	22 (3.0)	2.1	32
Pulex irritans	55 (109)	4 (7.3)	9 (16.4)	0	3.7	8.3
Echidnophaga gallinacea	2 (2)	0	0	0	0	0
Diamanus montanus	3 (4)	0	2 (66.7)	0	0	50
Leptosylla segnis	1 (1)	0	0	0	0	0
Los Angeles County						
Ctenocephalides felis	1,146 (2144)	18 (1.6)	454 (39.6)	10 (0.9)	1.3	21.6
Pulex irritans	3 (3)	0	1 (33.3)	0	0	33.3
Xenopsylla cheopis	4 (5)	0	2 (50.0)	0	0	40
Echidnophaga gallinacea	9 (17)	0	4 (44.4)	1 (11.1)	5.9	29.4

[a] Minimum infection rate: no. of PCR positive flea pools/total no. of fleas tested. Assumes that each PCR positive pool contains DNA of at least one flea infected with either *R. typhi* or *R. felis*, respectively.

Table 3

Flea minimum infection rates vs distance to case household

Distance to nearest case	Fleas	Animals	MIR *R. felis*	MIR *R. typhi*
36 m (0.02 mi)	749	26	32.9	2.1
37–800 m (<0.5 mi)	354	9	18.6	2.5
801–1,600 m (0.5–1 mi)	294	13	20.7	0
>1,600 m (>1 mi)	2,293	69	26.2	2.2