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Identification of a Novel Rickettsial Infection in a Patient Diagnosed with Murine Typhus

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Identification of ELB agent-infected fleas and rodents within several foci of murine typhus in the United States has prompted a retrospective investigation for this agent among human murine typhus patients. This agent is a recently described rickettsia which is indistinguishable from *Rickettsia typhi* with currently available serologic reagents. Molecular analysis of the 17-kDa antigen gene and the citrate synthase gene has discriminated this bacterium from other typhus group and spotted fever group rickettsiae. Current sequencing of its 16S ribosomal DNA gene indicates a homology of 98.5% with *R. typhi* and 99.5% with *R. rickettsii*. Through a combination of restriction fragment length polymorphism and Southern hybridization analysis of rickettsia-specific PCR products, one of five tested patient blood samples was shown to be infected with ELB while *R. typhi* infections were confirmed in the remaining samples. This is the first reported observation of a human infection by the ELB agent and underscores the utility of PCR-facilitated diagnosis and discrimination of these closely related rickettsial infections.

The genus *Rickettsia* is divided into three antigenically defined groups: the spotted fever group (SFG), the typhus group (TG), and the scrub typhus group. These gram-negative intracellular parasites of eukaryotic cells are found associated with arthropods and often infect vertebrates. Rickettsioses are zoonoses and are geographically limited by the distribution of their infected vectors. The SFG and TG contain at least 12 described species and demonstrate a high degree of relatedness by phenotypic analysis, DNA base ratio determinations, and DNA-DNA hybridization studies (18). These groups contain the well-known agents of epidemic typhus and Rocky Mountain spotted fever (*Rickettsia prowazekii* and *R. rickettsii*).

Often underrecognized and perceived clinically mild, murine typhus occurs worldwide with high frequency in certain geographic regions and may be severe. Roughly 10% of murine typhus patients require admission to an intensive care facility, and case mortality approaches 4% (7). Although the prevalence of typhus is drastically reduced since its peak in the United States during the 1930s and 1940s, several foci of murine typhus persist. These include suburban regions of Los Angeles and Orange County, California, and central and south central Texas (20). Classical disease cycle components involved the rat flea vector *Xenopsylla cheopis* and the rodent hosts *Rattus rattus* and *R. norvegicus* (14). However, more recent epidemiologic studies have prompted a reconsideration of the role of these components in persistent foci of this disease in the United States. Serologic and molecular analysis have implicated the cat flea, *Ctenocephalides felis*, and the opossum, *Didelphis virginiana*, as respective vectors and hosts of *R. typhi* (2, 13, 20). The prevalence of these components in surburban environments and the propensity of cat fleas to feed on humans have raised concern about the potential geographic spread of murine typhus.

Most murine typhus patients initially present with fever of unknown origin, and clinical recognition of systemic manifestations is an early clue toward the proper diagnosis of the infection. The nonspecificity of the clinical presentation and the lack of a diagnostic tool useful during the phase of acute illness makes prospective diagnosis of murine typhus difficult. Despite the previous use of insensitive and nonspecific (e.g., Weil-Felix) or generally unavailable (e.g., indirect hemagglutination) serologic tests, the microimmunofluorescence test (mIF), which utilizes *R. typhi* antigen, is highly sensitive and widely used in endemic areas for confirmation of *R. typhi* infection.

Investigations into the maintenance of the murine typhus disease cycle in several persistent foci in the United States have revealed the presence of a recently identified typhus-like rickettsia (4) among suspected flea vectors and animal hosts of *R. typhi* (3, 12). Infections with this rickettsia, tentatively designated the ELB agent, were determined by molecular analysis to be present in 4% of the cat fleas (n = 399) and 33% of the opossums (n = 9) collected in the vicinity of human murine typhus cases in southern Texas (12). The prevalence of infection in these fleas and opossums has prompted a retrospective investigation of human murine typhus cases from this area for the presence of ELB agent infections and the utility of PCR and restriction fragment length polymorphism (RFLP) and Southern hybridization analysis as diagnostic tools for murine typhus. In addition, to further define the ELB agent, its 16S ribosomal DNA (rDNA) gene was sequenced and compared with those of other members of the SFG and TG.

MATERIALS AND METHODS

Human patients. All patients were identified in the Corpus Christi and Nueces County regions of south Texas in the spring of 1991. Murine typhus was suspected in patients who presented with fever and a recent history of flea exposure, flea bite, or exposure to flea-prone animals, especially opossums and cats. Additional clinical features which suggested the diagnosis of murine typhus included headaches, myalgias, rash, stiff neck, a leftward shift of the differential leukocyte count in

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TABLE 1. *Rickettsia*-specific 16S rDNA primers

Primer[a]	Sequence (5′-3′)[b]	*R. rickettsii* 16S rDNA position (nucleotides)
90 f	AATCTAGAACGAACGCTATCGGTAT	28–47
96 f	GCAAGGAAGATAATGACG	426–443
94 f	AATCTAGATATCGGAAGATTCTC	785–804
86 f	AATCTAGATACCAACCCTTGACATGGTGG	939–959
97 r	CGTCATTATCTTCCTTGC	443–426
91 r	AAGGATCCGAGAATCTTCCGATATCT	804–787
87 r	AAGGATCCGCTTCCCTCTGTAAACAC	1221–1204
95 r	AAGGATCCCCAGTCGCTAATTTTA	1446–1431

[a] f, forward direction primer; r, reverse direction primer.
[b] Underlined portions contain restriction sites for cloning (*Bam*HI g/gatcc, *Xba*I t/ctaga). Boldface sequences are derived from *R. rickettsii* 16S rDNA sequence (17).

the absence of leukocytosis, and elevations in serum enzymes (aspartate transaminase, alanine transaminase, lactate dehydrogenase) suggesting hepatic involvement. Serological assays were performed as part of the diagnostic evaluation; Weil-Felix testing was performed in the local hospital laboratories, and specific *R. typhi* mIF testing was performed at the Texas Department of Health Laboratories. Sterile, heparinized or EDTA-anticoagulated blood was obtained from patients after informed consent. Aliquots of the blood samples were either frozen at −70°C or centrifuged at 50 × *g* for 10 min, and the leukocyte-rich plasma was separated and frozen at −70°C. Some patients were evaluated as outpatients, and some hospitalized patients were not available for follow-up serologic studies. Specific mIF tests were not performed for all patients. All patients were treated with doxycycline after blood samples were obtained.

Extraction of DNA from blood samples. Frozen blood samples were thawed at room temperature and heated for 45 min at 55°C. At this point rickettsiae were considered to be completely inactivated, and the blood was diluted with 2 volumes of sterile saline and triturated. Fresh proteinase K and sodium dodecyl sulfate (SDS) were added (final concentrations, 0.2 mg/ml and 0.5%, respectively) to 0.5 ml of the triturate, and the sample was incubated for 4 h at 55°C with mixing. The digested sample was extracted with chloroform-phenol, the aqueous phase was collected, and nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol. One microgram of the nucleic acid extract, as quantified by A_{260}, was utilized as a target for PCR assay.

PCR. PCR conditions for the amplification of a 434-bp portion of the *Rickettsia* 17-kDa antigen gene were as previously described (16). A set of nested primers, delineating a 230-bp portion of the 17-kDa gene, were also utilized. The nested primer sequences are 5′-CATTACTTGGTTCTCAAT TCGGT-3′ for the forward primer and 5′-GTTTTATTAGT GGTTACGTAACC-3′ for the reverse primer. These correspond, respectively, to base positions 181 to 203 and 411 to 389 of the 17-kDa gene of *R. typhi*. For nested PCR, 2 to 5 μl of the first-round PCR products were transferred to tubes containing fresh reagents (internal primers, deoxynucleoside triphosphates, buffer, and polymerase). Thermal cycling conditions for external and nested primer sets were identical.

Sequencing of 16S rDNA gene. Nucleic acid extracts from ELB agent-infected cat fleas (1) were utilized for sequencing of the ELB agent 16S rDNA gene. For comparative purposes, a portion of the *R. rickettsii* 16S rDNA gene was also sequenced. *Rickettsia* genus-specific primers (Table 1) were designed on the basis of published sequences (17). In addition, eubacterial 16S rDNA primers were utilized for initial studies and sequencing of the terminal 5′ and 3′ ends of the gene (21). Thermal cycle conditions for the 16S rDNA were 3 cycles of 94°C for 2 min, 52°C for 1 min, and 68°C for 4 min, followed by 32 cycles of 90°C for 2 min, 55°C for 1 min, and 68°C for 4 min. PCR products were resolved on 1% agarose gels (SeaKem ME; FMC Bioproducts, Rockland, Maine) containing ethidium bromide and visualized with a hand-held UV source. Predicted size bands were cut from gels, and 2 volumes of TE were added. The sample was briefly spun at 14,000 × *g* in order to compress the gel at the bottom of the tube and frozen for several minutes at −80°C. Upon removal to room temperature but prior to complete thawing, the gel was macerated with a plastic pestle. The resulting slurry was vortexed for 1 min. Three volumes of phenol were added, and the sample was vortexed again for 1 min and refrozen at −80°C for 10 min. After being thawed, the aqueous phase was separated by centrifugation and collected. The aqueous phase was extracted once with chloroform-phenol and precipitated with ethanol. Extracted DNA was resuspended at 0.2 μg/μl, and 1.0 μg was utilized directly for *Taq* cycle sequencing with a ABI automated sequencer (model 373A; ABI, Foster City, Calif.), using primers described above.

Quality control. To avoid cross-contamination and amplicon carryover, patient samples were handled individually during nucleic acid extraction and PCR amplification. Additionally, extraction, amplification, and product analysis were performed in physically separated laboratories. All PCR reagents were prealiquoted for individual reactions. Negative PCR controls were run with each of the clinical sample extracts, and positive controls were run separately.

Restriction digestion of PCR products. The amplified PCR products from patient samples as well as controls were endonuclease digested overnight with *Xba*I or *Alu*I according to the supplier's recommendations (New England Biolabs, Beverly, Mass.), electrophoresed in 2% agarose gels (SeaKem ME) with a 123-bp molecular weight ladder (GIBCO BRL, Grand Island, N.Y.), and visualized by ethidium bromide staining.

Southern hybridization. Gel-resolved PCR products (native or restriction endonuclease digested) were alkaline transferred (8) to nylon membranes (Hybond-N; Amersham Corp., Arlington Heights, Ill.). Species-specific oligonucleotide probes for *R. typhi* and ELB agent were synthesized on the basis of sequenced portions of their respective 17-kDa antigen genes (3, 4). The nucleotide sequence of the ELB probe is 5′-GCAA CACCTAGCGGCACTAG-3′, and that of the *R. typhi* probe is 5′-TCTGCTCCTAGCGGTAGTAA-3′. The signature sequence probes were labeled with fluorescein dUTP by terminal transferase according to the manufacturer's instructions (ECL oligolabelling and detection system; Amersham Corp.). Hybridization and detection conditions were also performed as suggested by the manufacturer. Sheared salmon sperm DNA (100 μg/ml) was added to the prehybridization wash, and the stringency washes were performed at 58°C in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS. The specificity of these probes to directed targets and their lack of reactivity to 17-kDa gene sequences of other TG and SFG species have been reported (10).

Nucleotide sequence accession number. The 16S rDNA sequence for the ELB agent has been submitted to GenBank (accession number L28944).

RESULTS

Patients. Patient demographic and epidemiologic data, clinical presentation and outcome, serologic results, therapy, and

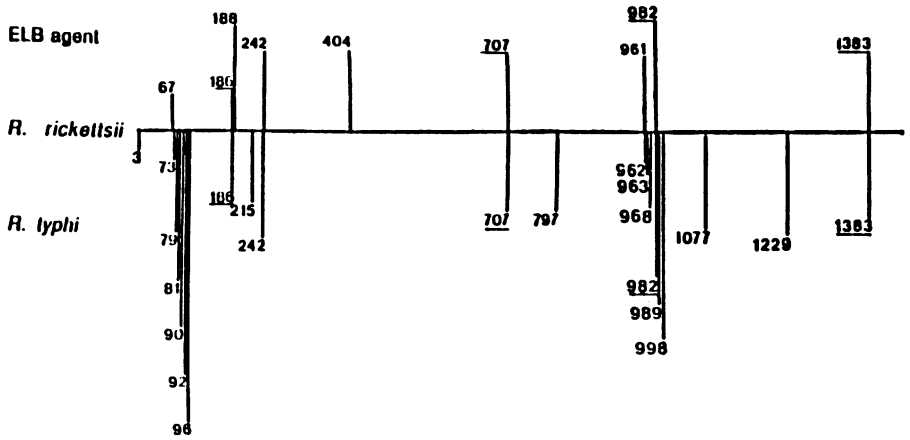

FIG. 1. Comparative sequence alignment of 16S rDNAs from ELB agent, *R. rickettsii*, and *R. typhi*. ELB agent and *R. typhi* 16S rDNAs are aligned with that of *R. rickettsii*. *R. rickettsii* and *R. typhi* sequences are from Weisburg et al. (17). Nucleotide differences from *R. rickettsii* are indicated by their position number. Nucleotide positions which are underlined are base differences from *R. rickettsii* that are shared by ELB and *R. typhi*. The total sequence determined for ELB spans nucleotides 36 to 1443.

PCR results are presented in Table 1. In brief, all five patients presented with fever, four had headaches, one had a rash, and all patients were exposed to fleas or flea-bearing animals preceding the onset of illness. Because of the retrospective nature of this study, no analysis of fleas or flea-infested animals from the domiciles of the patients was performed. Clinical and laboratory data, including leukocyte counts (mean lowest count, 4,580 cells per μl; range, 2,600 to 5,600 cells per μl), platelet counts (mean lowest count, 107,000 platelets per μl; range, 67,000 to 158,000 platelets per μl), serum sodium concentration (mean lowest level, 130 mEq/liter; range 125 to 133 mEq/liter), serum albumin concentration (mean, 2.73 mg/dl; range, 2.6 to 2.8 mg/dl), and serum aspartate transaminase concentration (129 U/liter; range, 28 to 262 U/liter) are similar to other published data on murine typhus patients (7).

Comparative sequence analysis of 16S rDNA. Initial attempts to amplify portions of the 16S rDNA of the ELB agent were carried out with two pairs of "universal" primers which have demonstrated priming of most eubacteria (21). PCR products of predicted size, derived from individual homogenates of ELB-infected fleas, were gel purified, cloned into PUC 19, and used to transform *Escherichia coli* DH5-α. Sequence analysis of 10 clones indicated the presence of 1 clone with >98% homology with *Rickettsia* genus 16S rDNA. Based on these findings, *Rickettsia*-specific primers were synthesized (Table 1) and used to reamplify ELB agent rDNA. The specificity of these primers was assessed by testing several primer pairs with nucleic acid extracts from uninfected rodent and human blood, laboratory-reared cat fleas, and a panel of bacterial cultures including *Serratia marcescens*, *E. coli*, *Neisseria gonorrhoeae*, *Ehrlichia chaffeensis*, and *Rochalimaea henselae*. When these negative controls were used as target substrates for PCR, no amplification products of the predicted size were generated with the tested *Rickettsia*-specific primers (primer pairs 96f and 97r, 94f and 95r; result not shown).

Seven separate PCR products ranging in size from 274 bp to over 719 bp were generated from ELB agent-infected fleas by using *Rickettsia*-specific 16S rDNA primers (Table 1). These served as templates for cycle sequencing reactions. The fidelity of primer sequences was verified by analysis of overlapping PCR products. A total of 1,407 bp was sequenced three times in the forward and reverse directions. The extreme 5′ and 3′ ends were sequenced by the use of eubacterial primers (21). The ELB agent was found to differ from the spotted fever group rickettsia *R. rickettsii* at 9 of 1,407 bases and from the typhus group rickettsia *R. typhi* at 21 of 1,407 bases (Fig. 1). The ELB agent gene differs from *R. prowazekii* at 25 of 1,407 bases (not shown). A portion of the *R. rickettsii* 16S rDNA gene (bp 42 to 420), which contains five of the nine base differences from the ELB agent, was also sequenced and was found to be identical to the published *R. rickettsii* sequence (17).

Amplification and identification of rickettsia 17-kDa antigen gene sequences from blood extracts of murine typhus patients. Nucleic acid extracts of blood samples from murine typhus patients were utilized as targets for the amplification of a portion of the 17-kDa antigen gene by using rickettsia-specific primers. Amplification of a 434-bp portion of the gene was variable under standard PCR conditions; of the five samples tested, only two samples were successful sources of target DNA as assessed by visualization in ethidium bromide-stained gels (results not shown). In light of the suspected low numbers of circulating rickettsiae in the original blood samples, nested PCR was performed on the same extracts. Under these conditions, nested products (231 bp) were consistently obtained from all five of the murine typhus patient extracts (Fig. 2). Amplification products of the predicted size were not generated from extracts of uninfected control blood samples (although only one example of these controls is shown in Fig. 2, this type of control sample was initially run in parallel with each murine typhus patient sample). Discrimination between the ELB agent and *R. typhi* was accomplished by detection of restriction endonuclease digest polymorphisms and Southern hybridization using signature-specific oligonucleotide probes. Restriction digestion with *Xba*I cleaved the nested *R. typhi* product at one site producing a 125- and a 106-bp product. The nested product from the ELB agent is not cleaved by *Xba*I (Fig. 3, upper portion). Two murine typhus patient samples (no. 2 and 5), representative of a *R. typhi* and an ELB agent infection, are shown. The restriction pattern of nested PCR products from patient no. 2 (Fig. 3B) is identical with that of the *R. typhi* control, and that of patient no. 5 (Fig. 3A) is the same as that of the ELB control. A similar strategy using *Alu*I, which cuts the *R. typhi* product once and the ELB product twice, was also used to corroborate the restriction-based diagnosis (not shown). The nested products were indepen-

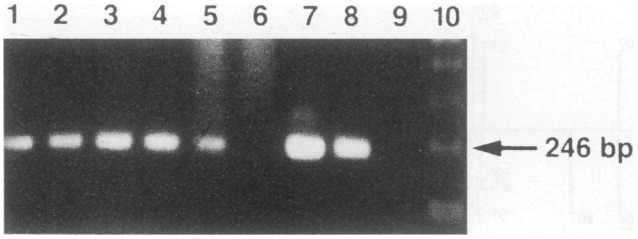

FIG. 2. Agarose gel electrophoresis of nested PCR-amplified 17-kDa gene fragments from nucleic acid extracts of blood samples. Nucleic acid extracts from acute-phase blood samples of murine typhus patients were utilized for nested PCR amplification of a portion (231 bp) of the rickettsia-specific 17-kDa antigen gene. PCR products were resolved on a 2% agarose gel. Lanes 1 through 5 represent products from patients 1 through 5, respectively. Lane 6, product from noninfected control blood extract. Lane 7, product from ELB-infected cat flea extract. Lane 8, product from *R. typhi* seed. Lane 9, negative control (no added DNA extract). Lane 10, 123-bp molecular weight ladder (the position of the 246-bp marker is indicated by the arrow).

dently identified by Southern hybridization analysis (Fig. 3, lower portion). Fluorescein-labeled oligonucleotide probes complementary to a signature sequence of the nested product were utilized to identify both endonuclease-cleaved and noncleaved products. Under the stringency conditions described, the ELB and *R. typhi* probes hybridize only with authentic and predicted-size PCR products or endonuclease-cleaved fragments. Results of the Southern analysis confirmed those of the restriction digest and indicated that one of the five patients (patient no. 5) was infected with the ELB agent and the remaining four patients were infected with *R. typhi* (Table 2).

DISCUSSION

The patients described herein presented with a clinical illness with laboratory and epidemiologic features consistent with other published series of cases of murine typhus (7). All had a fever, 80% had a headache, 20% had a rash, and all had been recently exposed to fleas or animals known to harbor

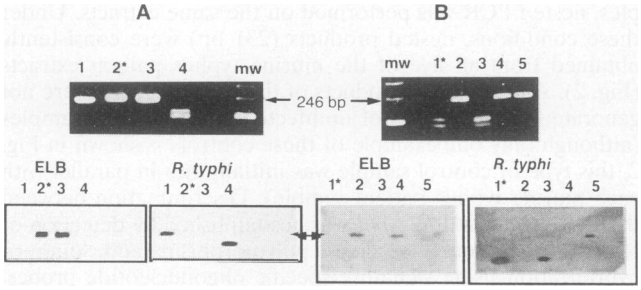

FIG. 3. Identification of ELB and *R. typhi* PCR products by restriction endonuclease digestion and southern hybridization. Nested PCR products of the 17-kDa antigen gene were digested with *Xba*I and resolved on 2% agarose gels (upper portion of figure). The ELB product is not cleaved by *Xba*I, whereas the *R. typhi* product is cleaved at one site. Replicate gels of A and B were alkaline transferred to nylon membranes (lower portion of figure) and hybridized with *R. typhi*- or ELB-specific oligo-probes as indicated. (A) Probes (lanes): 1, *R. typhi* undigested; 2*, patient number 5 digested; 3, ELB digested; 4, *R. typhi* digested. (B) Probes (lanes): 1*, patient number 2 digested; 2, ELB digested; 3, *R. typhi* digested; 4, ELB undigested; 5, *R. typhi* undigested. mw, 123-bp molecular weight ladder (the position of the 246-bp marker is indicated by arrowheads).

vector fleas of murine typhus. Early diagnosis of murine typhus is largely based on clinical suspicion. To avoid severe and potentially fatal infections, prompt and specific antirickettsial therapy is indicated and should not be withheld pending laboratory confirmation. Current laboratory confirmation is serologic and largely retrospective since antibodies are infrequently detectable during the acute illness. Although the mIF test is generally regarded as highly sensitive and specific, its exact diagnostic utility remains undetermined. For example, of 47 patients with suspected murine typhus whose serum samples was tested for *R. typhi* antibody at the Texas Department of Health in 1991, murine typhus was confirmed in only 16 (33%) (unpublished results). Similarly, low rates of immunofluorescence assay confirmation have been reported by the Centers for Disease Control and Prevention for suspected rickettsial illnesses (11).

In contrast to seroconfirmation, PCR is clearly capable of identifying an acute rickettsial infection even in a patient without subsequent seroconversion despite appropriate specimen collection and therapy (e.g., patient no. 2). In fact, this methodology has been successfully adapted for the diagnosis of both murine typhus and Rocky Mountain spotted fever during the acute phase of the illness (6, 15). As an extension to these approaches we have chosen to utilize nested PCR amplification of a portion of the 17-kDa antigen gene. Under these conditions, gene products of the predicted size were amplified from all five of the patient samples. To avoid amplicon carryover, sample extraction, PCR amplification, and product analysis were each conducted in separate facilities.

The ability to definitively identify the organisms causing murine typhus with PCR-RFLP and Southern hybridization enables an important retrospective review of the clinical observations of murine typhus patients. Among the samples investigated, seroconversion, as assessed by a fourfold rise in *R. typhi* mIF titers or a single titer $\geq$1:128, was observed in three of four samples in which a *R. typhi* infection was confirmed by PCR. In the fourth PCR-confirmed *R. typhi* infection, a convalescent-phase titer of $<$1:64 in serum was observed. Interestingly, this patient had a preexisting mixed connective tissue disorder and was treated with corticosteroids. We speculate that this treatment regimen may have diminished the development of *R. typhi* specific antibody and resulted in aberrant serologic results. Analysis of the fifth patient's acute-phase blood sample further highlights the utility of PCR-RFLP and Southern hybridization analysis and provides the first evidence of a human infection with ELB. Unfortunately, *R. typhi* mIF was not done for this patient. Nonetheless, rickettsia-specific gene products were readily generated from nucleic acid extracts of an acute-phase blood sample, and identification of an ELB infection was corroborated independently by RFLP and Southern hybridization analysis. This same approach (i.e., RFLP and Southern hybridization) has been previously utilized to diagnose *R. typhi* and ELB agent infections in fleas and to discriminate these bacteria from other SFG and TG *Rickettsia* spp. (4, 20). Although no accurate comparison of the clinical consequences of ELB and *R. typhi* infection is possible, it is clear that the patient with ELB infection suffered a moderately severe illness with close clinical similarity to murine typhus. This result suggests that the clinical-epidemiological syndrome currently recognized as murine typhus represents an infection by more than one etiologic agent.

Establishment of an ELB agent culture isolate and further taxonomic characterization of this rickettsia are in progress. Sequence analysis of the 16S rDNA gene, which is highly conserved and stable among the *Rickettsia* spp. (17), reveals

TABLE 2. Patient presentation, serology, and PCR-based diagnosis

Patient no.	Age/race/sex[a]	Presenting diagnosis	Rash/hosp[b]	Serology[c]		Complications	Onset (mo)	Final clinical diagnosis	PCR result[d]
				IFA[e]	Weil-Felix[f]				
1	80/W/F	Bronchitis	No/yes	1:128A	OX19, 1:40A	None	March	MT[g]	Rt
2	31/W/M	Cerebral vasculitis	No/yes	<1:64AB	OX19, 1:40B	Confusion, lethargy	March	Mixed connective tissue disorder	Rt
3	30/H/F	MT	No/no	1:1,024B 1:4,096B	OX19, 1:1,280B OX19, 1:10,240B	None	March	MT	Rt
4	19/W/M	Viral syndrome	Yes/yes	1:512A 1:4,096B	OX19, 1:160A	None	March	MT	Rt
5	44/H/M	MT	No/yes	ND	OX19, negAB OX2, 1:20A OXK, negAB	Cholecystitis	April	MT	ELB

[a] Age (in years)/race (W, white; H, Hispanic)/sex (F, female; M, male).
[b] Presence of rash/hospitalization of patient.
[c] A, acute-phase serum (<10 days after presentation) used; B, convalescent-phase serum (≥10 days after presentation) used.
[d] Rt, *R. typhi*.
[e] IFA, immunofluorescence assay. ND, not done.
[f] OX designations indicate O antigens of *Proteus* strains OX19, OX2, and OXK. neg, negative.
[g] MT, murine typhus.

features that are both conserved and unique with respect to the SFG and TG. Of the 1,407 bases sequenced, the ELB agent is 99.5% homologous with *R. rickettsii* and 98.5% homologous to *R. typhi*. A similar degree of homology (98.5%) exists between *R. rickettsii* and *R. typhi* (17). To date, serologic (1) and genetic (4, 20) analysis have suggested that the ELB agent lies between the SFG and TG rickettsiae, demonstrating antigenic cross-reactivity with TG rickettsiae, RFLP patterns similar to those of the citrate synthase gene with SFG rickettsiae, and both conserved and unique sequence features to these groups in the 17-kDa antigen gene.

Recent investigations of a febrile illness in a cluster of individuals from New York and Virginia have suggested a typhus-like etiologic agent (9, 19). Attempts to isolate the causative agents from acute blood samples, postmortem tissues, or fleas and ticks collected in the vicinity of the patients' residences were unsuccessful. However, through a combination of immunofluorescence assay and Western immunoblot analyses, utilizing antigens from a panel of SFG, TG, and other suspected etiologic agents, both studies indicate a typhus-like, non-spotted fever group rickettsia or an unknown agent, sharing common antigens with *R. typhi*, as a likely cause of illness. Further identification of these pathogens and other unconfirmed rickettsial disease agents may be afforded through a PCR-based approach as described herein.

Currently, reported cases of murine typhus in the United States are largely focused in central and south central Texas and the Los Angeles-Orange County area of California. Although maintenance of the murine typhus disease cycle by infected rats (*R. rattus* and *R. norvegicus*) and rat fleas (*X. cheopis*) was well documented during the first half of the 20th century (14), it has become increasingly difficult to identify the presence of these infected components within the persistent foci of this disease in California and Texas (2, 12, 13). These findings suggested the presence and involvement of alternative mammalian hosts and flea vectors. Indeed, evidence implicating infected cat fleas (*C. felis*), opossums, and possibly cats has been the subject of several recent studies. A California-based investigation (13) reported the association of 33 cases of locally acquired murine typhus in Los Angeles County with seropositive domestic cats and opossums. Over 40% (16 of 38) of the opossums and 90% (9 of 10) of the domestic cats collected from the case areas were seropositive for *R. typhi* antibodies. In contrast, no seropositive cats ($n = 21$) or opossums ($n = 36$) were found in the control areas. Although no investigation of flea infection was attempted in that study, opossums were found to be most heavily infected with cat fleas ($\bar{X} = 104.7$/animal). The cat flea was also the most prevalent flea species (97%) collected from opossums, cats, and dogs sampled in the areas surrounding the human murine typhus of the current study (12). The ELB agent, originally described in a population of colonized cat fleas (1), was detected in fleas collected from Los Angeles and Orange Counties of California (20) and recently identified in fleas and opossums collected in the vicinity of human murine typhus cases in southern Texas (12).

In summary, the maintenance of murine typhus in a cat flea-opossum cycle is of considerable public health concern since the cat flea, *C. felis*, is a prevalent and widespread pest that avidly bites humans (5, 7, 14). The potential for increased involvement of opossums in the murine typhus cycle is also of importance since these marsupials are found in at least 42 states in the United States and readily adapt to urban and suburban habitats where food and hospitable environments are plentiful. PCR-facilitated identification of ELB agent infections among murine typhus patients indicates the presence of more than one causative agent and underscores the utility of this methodology in the diagnosis and discrimination of rickettsial disease etiologies.

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