Rickettsia felis from Cat Fleas: Isolation and Culture in a Tick-Derived Cell Line

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***Rickettsia felis*, the etiologic agent of spotted fever, is maintained in cat fleas by vertical transmission and resembles other tick-borne spotted fever group rickettsiae. In the present study, we utilized an *Ixodes scapularis*-derived tick cell line, ISE6, to achieve isolation and propagation of *R. felis*. A cytopathic effect of increased vacuolization was commonly observed in *R. felis*-infected cells, while lysis of host cells was not evident despite large numbers of rickettsiae. Electron microscopy identified rickettsia-like organisms in ISE6 cells, and sequence analyses of portions of the citrate synthase (*gltA*), 16S rRNA, *Rickettsia* genus-specific 17-kDa antigen, and spotted fever group-specific outer membrane protein A (*ompA*) genes and, notably, *R. felis* conjugative plasmids indicate that this cultivatable strain (LSU) was *R. felis*. Establishment of *R. felis* (LSU) in a tick-derived cell line provides an alternative and promising system for the expansion of studies investigating the interactions between *R. felis* and arthropod hosts.**

Spotted fever group rickettsiae (SFGR) are obligately intracellular gram-negative bacteria that are typically associated with ticks, with one exception being the etiologic agent of flea-associated spotted fever, *Rickettsia felis*, described predominantly in the cat flea, *Ctenocephalides felis*. There are a growing number of reports implicating *R. felis* as a human pathogen. Serological analysis and PCR amplification of rickettsial DNA in human samples coupled with clinical manifestations, including fever and maculopapular rash, characterize rickettsiosis (reviewed in reference 24). In colonized cat fleas, vertical transmission of *R. felis* is thought to be the primary route of maintenance (3, 43); however, the acquisition mechanism of *R. felis* by vertebrates and uninfected fleas in nature is unknown. Studies on the ecology of *R. felis* identified a role for opossums in the transmission cycle (6, 32, 45). Additionally, a role for companion animals, rodents, and, specifically, their fleas as the potential source of human exposure has been suggested (2, 8, 27).

The relatively recent identification and characterization of *R. felis* yielded novel aspects for the genus *Rickettsia*. A rickettsia-like organism, first observed by electron microscopy in the midgut epithelial cells of colonized adult cat fleas, was designated the “ELB agent” after the source of the fleas, the Elward Laboratory (El Soquel, CA) flea colony (1). Amplification of rickettsial genes encoding citrate synthase (*gltA*) and the genus-specific 17-kDa antigen confirmed the presence of rickettsiae in the Elward Laboratory colony (3), and the name *R. felis* was proposed (10). Subsequent amplification of the genes encoding the 17-kDa antigen and the 190-kDa antigen (*ompA*) from a colony of fleas maintained at Louisiana State

University (LSU) (Baton Rouge, LA) confirmed the molecular characterization of the bacteria and further classified *R. felis* as an SFGR (7). Although attempts were unable to produce a sustained culture of either the ELB or the LSU strains of *R. felis* (7, 28), propagation of *R. felis* isolated from fleas maintained by Flea Data, Inc. (Freeville, NY), proved successful (29). Both Vero (*Cercopithecus aethiops*) and XTC-2 (*Xenopus laevis*) cell lines were reported to be competent host cells for *R. felis* at 28°C (Vero and XTC-2) and 32°C (Vero only) (29). The Flea Data, Inc., isolate was designated strain Marseille-UR-RWXC_{al}2 (also reported as strain California 2) (22) and is the type strain for *R. felis* (16). A second strain of *R. felis* (Pedreira) was isolated in the C6/36 (*Aedes albopictus*) cell line from wild-caught fleas in Brazil (11). The genome of *R. felis* (Marseille-URRWXC_{al}2) has recently been described, and a number of unique characteristics were reported, including the presence of conjugative plasmids pRF and pRFδ (22).

Many of the studies characterizing the relationship between *R. felis* and fleas have utilized the LSU colony of fleas (7, 19, 41–43, 46). By utilizing complementary in vivo and in vitro systems, the biology of this *R. felis* strain could be further characterized; however, the lack of viable cultures limits the scope of this model. Towards the expansion of studies relating to the transmission of *R. felis*, the objective of the current study is to isolate and cultivate *R. felis* (LSU).

The utilization of tick cell lines for the isolation of *Rickettsia* has become an excellent tool for previously uncultivated organisms (36, 37) as well as for protein expression analysis and genetic manipulation of SFGR in an arthropod-derived host cell (4, 5, 14, 26). For example, the infectivity of the SFGR endosymbiont *Rickettsia peacockii* assessed in *Ixodes scapularis* (ISE6), *Boophilus microplus* (BME26), and *Dermacentor variabilis* (DVE1) tick cell lines identify these cells as highly permissive, whereas L929 (*Mus musculus*) and Vero cell lines did not support *R. peacockii* replication (14).

Based on previous success with tick cell culture lines for the

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TABLE 1. Primers used for PCR amplification and sequencing of *R. felis* (LSU)

Primer	Gene	Nucleotide sequence (5'-3') ^a	Reference or source
<i>RpCS.877p</i>	Citrate synthase (<i>glcA</i>)	GGGGGCTGCTACGGCGG	30
<i>RpCS.1258n</i>		ATTGCAAAAAGTACAGTGAACA	
<i>fD1</i>	16S rRNA	AGAGTTTGATCCTGGCTCAG	44
<i>rP1-3</i>		ACGGH*TACCTTGTACGACTT	
16S rRNA-walk		AGGGTTGCGY*TCGTTGCK*GR*AC	This study
<i>Rr17.61p</i>	17-kDa genus-specific antigen	GCTCTTGCAACTTCTATGTT	45
<i>Rr17.492n</i>		CATTGTTCGTCAGGTTGGCG	
<i>Rf190.1fw</i>	190-kDa antigen (<i>ompA</i>)	ATGGCGAATATTTCTCTAAAATTA	46
<i>Rf190.1800rev</i>		TTAACTCACCACCACCGTTAGCAAGACCG	
<i>Rf190.2788fw</i>		ACATTACCTGCAAACCTTAACTTAACAG	46
<i>Rf190.3837rev</i>		CCGGCAGCTACGGCA	
<i>Rr190.70p</i>		ATGGCGAATATTTCTCCAAAA	30
<i>Rr190.602n</i>		AGTGCAGCATTCGCTCCCCCT	
<i>Rf190.1790fw</i>		GTCTACAGATGATAGAGTTATCAC	This study
<i>Rf190.2857rev</i>		GTTTAACTTCAGAGCCTGACCG	
<i>Rf190.553fw</i>		CTTGCAGGAAATATAGATGGAG	This study
pRFa	<i>R. felis</i> plasmids	CAAGCTTTTGTAATGCTCTAT	22
pRFc		ACATTCCGTAAAGAATATGAGC	
pRFd		GCTTATGTTTCGCCCTTTAGTATTTA	
pRF.36882p		GCGGATTCTAGGACTGCATCAG	This study

^a *, International Union of Biochemistry codes for mixed bases at a given position (H = A + T + C; Y = C + T; K = G + T; R = A + G).

isolation and propagation of rickettsiae, we utilized colonized *C. felis* (LSU) as a source of *R. felis* for isolation in a susceptible line of tick cells. In this report, we describe the isolation, propagation, and partial characterization of *R. felis* (LSU) in an *I. scapularis* cell line (ISE6).

MATERIALS AND METHODS

Source of fleas. Fleas were maintained on domestic shorthair cats at the Louisiana State University School of Veterinary Medicine (LSU-SVM), as previously described (9). Briefly, fleas were applied directly to the cat and were allowed to feed for up to 1 week. Eggs were collected from trays beneath the cat and transferred into larva-rearing jars containing a mixture of ground dog food, dried beef blood, brewer's yeast, and sand. Fleas were kept at 22°C and 70% relative humidity until adult emergence.

Tick cell culture. ISE6 cells (passage 101) were kindly provided by T. Kurti (University of Minnesota). Cells were cultured in L15B medium (with 80 mM glucose) prepared as described previously by Munderloh and Kurti (20). The medium was supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 10% tryptose phosphate broth (Sigma), and cells were maintained in a humidified 5% CO₂ incubator at 32°C. When confluent, the cells were scraped from the flask and suspended by repeated pipetting. Cells were subcultured (1:5) in vented 25-cm² or 75-cm² tissue culture flasks (Griener). The medium was replaced at 6-day intervals, and cells were typically ready for subculture every 12 to 14 days. Cell viability was assessed by trypan blue exclusion. Cryopreservation of samples utilized L15B medium supplemented with 20% fetal bovine serum and 10% dimethyl sulfoxide.

Rickettsia isolation. Pools of either 10 or 25 newly emerged, unfed adult male and female fleas were sequentially washed three times in 70% ethanol and one time in 10% sodium hypochlorite and rinsed in sterile distilled water. Flea pools were transferred to sterile glass pestle tissue grinders and ground in 25 µl of L15B tick cell culture medium. The flea homogenate was directly transferred to a 25-cm² tissue culture flask containing p105 ISE6 cells (2 days after split). ISE6 cells with flea preparations were immediately placed at 32°C. Cells were visually examined daily and passed every 7 to 14 days as needed. Preparation of cell-free rickettsiae was done by needle lysis and syringe-driven filtration across a 5.0-µm membrane, and they were collected by high-speed centrifugation as previously described (37). Upon each passage of ISE6 cells exposed to flea homogenate, a portion of the cells was prepared for staining using a Cytospin centrifuge (Wescor). Rickettsial infection was assessed by PCR and/or Diff-Quik (Dade Behring) staining according to the manufacturer's protocol.

DNA isolation. Fleas were washed five times in 70% ethanol, followed by three washes with DNase-RNase-free water. Fleas were blotted dry and transferred individually to 1.5-ml microcentrifuge tubes containing 20 µl of DNase-RNase-

free water. Samples were ground with plastic pestles and heated at 95°C for 5 min. After a brief centrifugation to collect the contents, lysates were used as PCR templates. Genomic DNA was purified from control and experimental ISE6 cells using the DNeasy tissue kit (QIAGEN) according to the manufacturer's protocol.

PCR amplification. Flea lysate, genomic DNA from ISE6 cells (uninfected and rickettsia infected), genomic DNA from *Rickettsia montanensis* M5/6 (positive control for the 17-kDa antigen), or water (negative control) was used as a template for PCR. PCR products were amplified using PCR Master Mix (Promega) or Platinum *Taq* DNA polymerase (Invitrogen) together with gene-specific oligonucleotide primers synthesized by Integrated DNA Technologies, Inc. (Table 1). The conditions used were as follows: an initial denaturation step at 94°C for 3 min, which was followed by 35 cycles of denaturation at 94°C for 30 s, primer-specific annealing for 45 s, extension at 72°C for 1 min per kb, and a final extension step at 72°C for 7 min. The annealing temperature for all primer sets was 55°C, with the exception of using 52°C for fD1-rP1-3, 48°C for *Rr190.70p*-*Rr190.602n*, and 50°C and 55°C for pRFa-pRFd primer sets. Amplified products were visualized on ethidium bromide-stained 0.8 to 1.5% agarose gels.

DNA sequencing and analysis. The PCR products were cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer's protocol. At least three clones of each PCR amplicon were sequenced by the dye terminator method on a 3130 genetic analyzer (Applied Biosystems) at LSU-SVM. Sequence analyses were carried out using Vector NTI software (Invitrogen), and nucleotide similarity comparisons were assessed by using the GenBank database.

Electron microscopy. Transmission electron microscopy (EM) of infected ISE6 cells (p110) after five passages of rickettsial infection were prepared as described previously by Ito and Rikihisa (12), with a modified procedure for epon/araldite (EA) embedding. The volume-to-volume ratio of EA to 100% ethyl alcohol was 1:3, 1:1, and 3:1 for 1 h each, followed by 100% EA for 1 h. Samples were then kept in EA-2,4,6-tri(dimethylaminomethyl)phenol-30 for 4 h at room temperature, followed by fresh exchanges of EA-2,4,6-tri(dimethylaminomethyl)phenol-30 and sequential overnight incubations at room temperature and at 70°C. Cells were visualized on a JEM-1011 transmission EM (JEOL) in the Microscopy Center at LSU-SVM.

Nucleotide sequence accession number. The sequence of *ompA* was determined and deposited in the GenBank database under accession number DQ408668.

RESULTS

Rickettsia propagation in ISE6 cells. Unfed adult fleas from the LSU-SVM colony were ground in L15B medium and added to 25-cm² flasks of ISE6 cells. Seven days postinocula-

FIG. 1. Cytospin preparations of tick cells (ISE6) stained with Diff-Quik. *R. felis* (LSU)-infected cells demonstrating increased clearance of cytoplasmic contents and apparent vacuolization of rickettsiae (A) and uninfected ISE6 cells (B) are shown. Both images are at $\times 100$ magnification.

tion (dpi), heavy cell detachment was observed. By 9 dpi, the medium for the cells inoculated with 25 fleas had become contaminated, and the flask was removed from further analysis. At 14 dpi, the sample inoculated with 10 fleas was passed onto ISE6 cells (70% confluent) at a 4:5 and 1:5 ratio in 25-cm² and 75-cm² culture flasks, respectively. A portion of the culture was used for Cytospin preparations, and the presence of rod-shaped bacteria was identified using Diff-Quik staining in both the cells and the extracellular space (Fig. 1). At 14 dpi of new flasks, 90% of cells were detached from the flask, while the remaining cells were scraped free of the flask, and the flasks were combined. Half of the cells were passed directly onto a new 75-cm² flask of ISE6 cells, and the remaining half of the cells were used to confirm cell viability and rickettsial infection; subsequently, the rickettsiae were partially purified and inoculated onto a different 75-cm² flask of ISE6. A frozen stock was

prepared with the second passage and cryopreserved. Passage (every 7 days) of rickettsia-infected ISE6 cells continued, and portions of passages 3 to 10 were utilized for additional cryopreservation. Frozen stock of rickettsiae (passage 2) was reestablished in culture and used in rickettsial characterization studies.

During propagation of rickettsiae in ISE6 cells, a cytopathic effect of increased vacuolization was commonly observed in infected cells compared to uninfected ISE6 cells. Rickettsiae were visualized in cleared areas in the host cells' cytoplasm (Fig. 1). Elongated rickettsiae were observed by staining (data not shown), as reported previously for *Rickettsia bellii* (strain Ac25) (15). Cell lysis was not apparent, but there was an increase in small extracellular debris and rickettsiae in the infected ISE6 cell culture compared to the uninfected cell culture.

PCR amplification and sequence analysis of *R. felis* (LSU).

The presence of *R. felis* in the infected ISE6 cells was confirmed by PCR amplification and sequencing of portions of the citrate synthase (*gltA*), 16S rRNA, *Rickettsia* genus-specific 17-kDa antigen, and spotted fever group-specific outer membrane protein A (*ompA*) genes and, particularly, *R. felis* plasmids. Nucleotide sequences of the *gltA* (382 bp), 16S rRNA (1,461 bp), and 17-kDa antigen (434 bp) genes were identical to those of the sequences reported for *R. felis* in the GenBank database (accession numbers CP000053 and U33922 for the *gltA* gene, CP000053 for the 16S rRNA gene, and CP000053 and AF195118 for the 17-kDa antigen gene). A comparison of the *ompA* sequence (3,792 bp) to the published *R. felis* (LSU) sequences (GenBank accession numbers AF191026 [7] and AY727036 [46]) obtained from the same source of infected fleas (LSU colony) exhibited two nucleotide differences (C-to-T and G-to-A transitions at positions corresponding to bp 1,201 and bp 1,511 of the sequence reported under GenBank accession number AF191026) and one nucleotide difference (G-to-A transition at a position corresponding to bp 1,063 of the sequence reported under GenBank accession number AY727036), respectively, in the coding region, resulting in changes in the amino acid composition (Table 2). We further analyzed the remaining *ompA* sequence and identified 13 additional differences, including a transition, transversions, deletions, and insertions, with respect to the sequence reported previously by Bouyer et al. (7). The *ompA* sequence identified in this report is identical to that reported for *R. felis* (Marseille-URRWXCal₂) (GenBank accession number CP000053) (Table 2).

TABLE 2. Comparison of nucleotide and amino acid compositions of OmpA from *R. felis* LSU and URRWXCal₂ strains

GenBank accession no.	Amino acid position ^a			Reference or source
	242	345	355	
DQ408668 (LSU)	S (TCG)	N (AAT)	I (ATA)	This study
AF191026 (LSU)	P (CCG)	S (AGT)	I (ATA)	7
AY727036 (LSU)	S (TCG)	N (AAT)	V (GTA)	46
CP000053 (URRWXCal ₂)	S (TCG)	N (AAT)	I (ATA)	22

^a The positions indicated are in relation to the first methionine (position 1) in the coding sequence described previously by Bouyer et al. (7). Nucleotides are in parentheses.

FIG. 2. PCR amplification of genes encoding *Rickettsia* genus-specific 17-kDa antigen and plasmids from *R. felis* (LSU)-infected fleas (A) and ISE6 cells (B). For both the *R. felis*-infected flea lysate and ISE6 cells, partial sequences for the genes encoding the 17-kDa antigen and pRF, but not pRF δ , were amplified. *R. montanensis* M5/6 genomic DNA was used as a positive control for 17-kDa primers. Marker (100 bp) sizes are listed to the left of the gel.

Based on a previous report that demonstrated the existence of an *R. felis*-specific conjugative plasmid, both a short form (pRF δ) and a long form (pRF), in *R. felis* (Marseille-URRWX Cal₂) cultivated in the XTC-2 cell line and also in fleas naturally infected with *R. felis* (22), we attempted molecular confirmation of the plasmid content using the above-described primers. Both *R. felis* (LSU)-infected fleas and infected ISE6 cells, validated by a partial sequence of the 17-kDa antigen gene, were subjected to testing by PCR amplification with the primer sets specific to either pRF or pRF δ (Table 1) and verified by sequencing. There was a correlation between *R. felis* infection and the amplification of the pRF plasmid in both fleas and cultured *R. felis* (Fig. 2). However, the pRF δ plasmid

was not amplified in all samples tested, despite attempts to optimize the PCR conditions (data not shown). The PCR-amplified portion (1,342 bp) of the pRF sequence matched (100%) that reported for *R. felis* strain Marseille-URRWX Cal₂ (GenBank accession number CP000053).

Microscopy. In ISE6 cells, *R. felis* (LSU) had a rickettsia-like morphology and was typically identified in large numbers within any given cell (Fig. 3). The rickettsiae were observed in direct contact with the host cell cytoplasm. A distinct “halo” typically associated with SFGR was not observed for individual rickettsiae due to clustering of the bacteria, but an obvious clearance of the cytoplasmic contents was apparent around the clusters. Although a partial vacuolar membrane was occasion-

FIG. 3. Electron micrographs of *R. felis* (LSU) in ISE6 tick cells. Rickettsiae free in the cytoplasm (dark arrows) associated with dilated endoplasmic reticulum (ER) and nuclear (n) membrane (bar, 500 nm) (A), rickettsiae partially enclosed in a vacuole within the host cytoplasm (bar, 500 nm) (B), and high numbers of rickettsiae grouped together in the cytoplasm (bar, 2.0 μ m) (C) are shown.

ally observed in the cells, we were unable to clearly identify vacuolization of rickettsiae by this method; therefore, the vacuolization observed by Diff-Quik staining was not apparent by EM analysis. The rickettsiae were in proximity to the endoplasmic reticulum, and lysis of host cells was not apparent despite large numbers of rickettsiae.

DISCUSSION

In the present report, we describe the cultivation of *R. felis* in a tick-derived cell line. Although this is the third report of the successful cultivation of *R. felis*, this is the first cultivation of this strain (LSU) and the first use of a tick cell line (ISE6) for this flea-associated SFGR. Additionally, differences between *R. felis* (LSU) and previous isolates include culture characteristics and genetic composition. Taken together, the data indicate that there is a unique strain of *R. felis* that is cultivatable in the ISE6 cell line.

Tick cell lines created for several genera of ixodid (hard) and argasid (soft) ticks have unique characteristics (14, 21) and are useful for the isolation of SFGR in natural host cells. Both *R. peacockii* and *Rickettsia monacensis* have been cultivated in cells derived from naturally infected *Dermacentor andersoni* and *Ixodes ricinus* ticks, respectively (36, 37). The infectivity of the endosymbiont *R. peacockii* was assessed and was reported to multiply in several tick cell lines and in an insect cell line but not in mammalian L929 or Vero cell lines and is enhanced by high seeding densities and centrifugation (14). Likewise, isolation of *R. felis* (Marseille-URRWXCal₂ and Pedreira) in amphibian (XTC-2) and insect (C6/36) cell lines utilizes centrifugation via the shell vial technique (29). Additionally, both *R. felis* isolates display optimal growth at temperatures below 32°C, inferring a low-temperature requirement for *R. felis* propagation (11, 16, 29). For the present study, we chose what is considered to be a permissive cell line from *I. scapularis* (14) for the isolation of *R. felis* from colonized fleas. Both infected host cells and cell-free rickettsiae were infective for new cells during passage at 32°C, without a temperature shift or the use of centrifugation. It is likely that a combination of factors, including a high prevalence of *R. felis* infection in the flea colony (W. Pornwiroon and K. R. Macaluso, unpublished results) and a permissive cell line, facilitated the isolation of this previously uncultivated strain of *R. felis*. These findings suggest either the presence of undefined conserved arthropod-derived factors (e.g., receptors) for rickettsia on the host cell or that the rickettsiae express the required molecules needed for attachment to and entry into tick cells.

Assessment of cultured and flea-associated *R. felis* (LSU) genetic composition demonstrates differences in comparison to previous reports of *R. felis* (LSU). The sequence for *ompA* in *R. felis* (LSU) contains several stop codons that potentially result in a truncated protein (7, 46). In the present study, a coding sequence mixed between previously reported sequences of *R. felis* (LSU) (7, 46) was identified using primers described previously by Zavala-Castro et al. (46) and novel primers. However, analysis of our entire sequence (3,792 bp) revealed three coding sequences identical to that reported for *R. felis* (Marseille-URRWXCal₂) (GenBank accession number CP000053, under locus tags RF_1303, RF_1304, and RF_1305), and several stop codons were not found. Although

R. felis (LSU) *ompA* transcription analysis has been addressed previously (46), the assessment of functional protein expression of OmpA is lacking for *R. felis*. The significance of the modified OmpA, in comparison to other SFGR, must be assessed, as SFGR OmpA has been demonstrated to be involved in rickettsial adherence to, and invasion of, host cells (17). Although transcribed, the lack of functional OmpA in the endosymbiont *R. peacockii* has been associated with decreased pathogenicity and the absence of horizontal transmission (5, 14, 37). The partial sequences for genes encoding citrate synthase, the 17-kDa antigen, 16S rRNA, and plasmid pRF match previously identified sequences for *R. felis*. One difference between *R. felis* (LSU) and the type strain Marseille-URRWXCal₂ is the lack of a detectable short-form plasmid, pRF δ , in *R. felis* (LSU). The reason for the plasmid count discrepancies between the two strains is not clear. The fleas from which the type strain was isolated were maintained on an artificial feeding system (40), which typically utilizes defibrinated sheep or cow blood as a source of blood meal. Conversely, the LSU fleas are maintained only on cat hosts. The possibility of host-associated differences (e.g., blood meal composition) influencing rickettsial genetics is intriguing; however, a portion of *R. felis* pRF δ was amplified in 100% of unidentified wild-caught *R. felis*-infected fleas from unidentified hosts in Algeria, France, and New Zealand (22). Wild-caught fleas from naturally infested dogs that were the source of *R. felis* (Pedreira) were not assessed for plasmid composition (11).

Many tick-borne SFGR are maintained primarily via transovarial transmission within the tick, and the same is thought to be true for the flea-associated SFGR *R. felis*. Therefore, the utilization of tick cells as host cells provides an excellent model system for the expansion of studies examining the interactions between *R. felis* and arthropod hosts. Microscopic analysis of *R. felis* (LSU) infection of ISE6 cells identified a cytopathic effect that was not previously reported for this organism. When stained, the increased vacuolization or clearance of cytoplasmic contents within the host cell (spongy appearance) was associated with *R. felis* infection and was not obvious in uninfected ISE6 cells. Additionally, host cell lysis of rickettsia-filled cells is not apparent, despite the cell detachment from the flask. EM analysis confirmed the heavy rickettsial load in the cells, thereby supporting the possibility of a persistently infected cell line, as seen for other SFGR in tick and vertebrate cell lines (26, 37, 39). Based on the stained cell preparations, we suspected vacuolization, as observed for *R. felis* (LSU) infection in fleas (7); however, our EM analysis did not confirm vacuolization, as a definitive vacuolar membrane was not identified. *R. felis* infection in tick cells resulted in the dilation of the nuclear membrane and the endoplasmic reticulum, which is associated with cell lysis during *Rickettsia rickettsii* infection (34, 35). However, similar to *R. rickettsii* cultures in tick cell lines, tick cells are able to tolerate a high rickettsial burden with reduced cell lysis (26).

In addition to rickettsial comparative genomics and metabolism studies in an arthropod host cell, *Rickettsia*-arthropod interactions that are critical to our understanding of the ecology of this emerging vector-borne disease will be enhanced by the propagation of *R. felis* (LSU) in vitro. The relatively recent description of *R. felis* has been followed by the molecular identification of *R. felis* in cat fleas collected from rodents,

opossums, and domestic and feral cats. In fact, the distribution of *R. felis* appears to be cosmopolitan, as the molecular detection of *R. felis* in fleas from Brazil (11, 23), Cyprus (27), Ethiopia (29), France (31), Spain (18), the United Kingdom (33), Thailand (25), and New Zealand (13) has been described previously, in addition to the original description from the United States (1). While the primary identification of *R. felis* infection in arthropods has been in cat fleas (2, 6, 18, 23, 25, 31), molecular identification of *R. felis* has been reported for three other species of flea, including *Ctenocephalides canis* (dog flea) (25), *Pulex irritans* (human flea) (2), and, most recently, *Anomopsyllus nudata* (38). The identification of infected *A. nudata* fleas from *Neotoma albigula* wood rats collected in the western United States suggests that transmission cycles exist independent of domestic and peridomestic animals and their ectoparasites. The role of small rodents and their fleas must be explored further, and the cultivation of *R. felis* will also allow for vector competence studies using species of fleas other than *C. felis*.

Horizontal transmission of *R. felis* by cat fleas has not been demonstrated. The pathogenic nature of *R. felis* should be defined, and the isolation of several strains of *R. felis* will facilitate epidemiological studies. Cats seroconvert due to exposure to infected fleas; however, overt disease was not observed, and viable rickettsiae have not been recovered from cats (42). Although the specificity of the reactions has been limited due to the high cross-reactivity of antibodies to different rickettsial species, serological assays are the primary techniques for diagnosing *R. felis* infection in vertebrate hosts. Defining the mechanisms of *R. felis* transmission, specifically, the respective roles of invertebrate and vertebrate hosts, is essential to fully appreciate the epidemiology of flea-associated spotted fever and will greatly impact the application of appropriate control measures.

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REFERENCES

- Adams, J. R., E. T. Schmidtman, and A. F. Azad. 1990. Infection of colonized cat fleas, *Ctenocephalides felis* (Bouche), with a rickettsia-like microorganism. *Am. J. Trop. Med. Hyg.* **43**:400–409.
- Azad, A. F., S. Radulovic, J. A. Higgins, B. H. Noden, and J. M. Troyer. 1997. Flea-borne rickettsioses: ecologic considerations. *Emerg. Infect. Dis.* **3**:319–327.
- 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 typhus-like rickettsia found in cat fleas. *Proc. Natl. Acad. Sci. USA* **89**:43–46.
- Baldrige, G. D., N. Burkhardt, M. J. Herron, T. J. Kurtti, and U. G. Munderloh. 2005. Analysis of fluorescent protein expression in transformants of *Rickettsia monacensis*, an obligate intracellular tick symbiont. *Appl. Environ. Microbiol.* **71**:2095–2105.
- Baldrige, G. D., N. Y. Burkhardt, J. A. Simser, T. J. Kurtti, and U. G. Munderloh. 2004. Sequence and expression analysis of the *ompA* gene of *Rickettsia peacockii*, an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni*. *Appl. Environ. Microbiol.* **70**:6628–6636.
- Boostrom, A., M. S. Beier, J. A. Macaluso, K. R. Macaluso, D. Sprenger, J. Hayes, S. Radulovic, and A. F. Azad. 2002. Geographic association of Rickettsia felis-infected opossums with human murine typhus, Texas. *Emerg. Infect. Dis.* **8**:549–554.
- Bouyer, D. H., J. Stenos, P. Crocquet-Valdes, C. G. Moron, V. L. Popov, J. E. Zavala-Velazquez, L. D. Foil, D. R. Stothard, A. F. Azad, and D. H. Walker. 2001. *Rickettsia felis*: molecular characterization of a new member of the spotted fever group. *Int. J. Syst. Evol. Microbiol.* **51**:339–347.
- Case, J. B., B. Chomel, W. Nicholson, and J. E. Foley. 2006. Serological survey of vector-borne zoonotic pathogens in pet cats and cats from animal shelters and feral colonies. *J. Feline Med. Surg.* **8**:111–117.
- Henderson, G., and L. D. Foil. 1993. Efficacy of diflubenzuron in simulated household and yard conditions against the cat flea *Ctenocephalides felis* (Bouche) (Siphonoptera: Pulicidae). *J. Med. Entomol.* **30**:619–621.
- Higgins, J. A., S. Radulovic, M. E. Schrieffer, and A. F. Azad. 1996. *Rickettsia felis*: a new species of pathogenic rickettsia isolated from cat fleas. *J. Clin. Microbiol.* **34**:671–674.
- Horta, M. C., M. B. Labruna, E. L. Durigon, and T. T. Schumaker. 2006. Isolation of *Rickettsia felis* in the mosquito cell line C6/36. *Appl. Environ. Microbiol.* **72**:1705–1707.
- Ito, S., and Y. Rikihisa. 1981. Techniques for electron microscopy of rickettsiae, p. 213–240. In W. Burgdorfer and R. L. Anacker (ed.), *Rickettsiae and rickettsial diseases*. Academic Press, Inc., New York, N.Y.
- Kelly, P. J., N. Meads, A. Theobald, P. E. Fournier, and D. Raoult. 2004. *Rickettsia felis*, *Bartonella henselae*, and *B. clarridgeiae*, New Zealand. *Emerg. Infect. Dis.* **10**:967–968.
- Kurtti, T. J., J. A. Simser, G. D. Baldrige, A. T. Palmer, and U. G. Munderloh. 2005. Factors influencing in vitro infectivity and growth of *Rickettsia peacockii* (Rickettsiales: Rickettsiaceae), an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni* (Acari: Ixodidae). *J. Invertebr. Pathol.* **90**:177–186.
- Labruna, M. B., T. Whitworth, D. H. Bouyer, J. McBride, L. M. Camargo, E. P. Camargo, V. Popov, and D. H. Walker. 2004. *Rickettsia bellii* and *Rickettsia amblyommii* in *Amblyomma* ticks from the State of Rondonia, Western Amazon, Brazil. *J. Med. Entomol.* **41**:1073–1081.
- La Scola, B., S. Meconi, F. Fenollar, J. M. Rolain, V. Roux, and D. Raoult. 2002. Emended description of *Rickettsia felis* (Bouyer et al. 2001), a temperature-dependent cultured bacterium. *Int. J. Syst. Evol. Microbiol.* **52**:2035–2041.
- Li, H., and D. H. Walker. 1998. rOmpA is a critical protein for the adhesion of *Rickettsia rickettsii* to host cells. *Microb. Pathog.* **24**:289–298.
- Marquez, F. J., M. A. Muniain, J. M. Perez, and J. Pachon. 2002. Presence of *Rickettsia felis* in the cat flea from southwestern Europe. *Emerg. Infect. Dis.* **8**:89–91.
- Moron, C. G., D. H. Bouyer, X. J. Yu, L. D. Foil, P. Crocquet-Valdes, and D. H. Walker. 2000. Phylogenetic analysis of the *ompB* genes of *Rickettsia felis* and *Rickettsia prowazekii* European-human and North American flying-squirrel strains. *Am. J. Trop. Med. Hyg.* **62**:598–603.
- Munderloh, U. G., and T. J. Kurtti. 1989. Formulation of medium for tick cell culture. *Exp. Appl. Acarol.* **7**:219–229.
- Munderloh, U. G., Y. Liu, M. Wang, C. Chen, and T. J. Kurtti. 1994. Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. *J. Parasitol.* **80**:533–543.
- Ogata, H., P. Renesto, S. Audic, C. Robert, G. Blanc, P. E. Fournier, H. Parinello, J. M. Claverie, and D. Raoult. 2005. The genome sequence of *Rickettsia felis* identifies the first putative conjugative plasmid in an obligate intracellular parasite. *PLoS Biol.* **3**:e248.
- Oliveira, R. P., M. A. Galvao, C. L. Mafra, C. B. Chamone, S. B. Calic, S. U. Silva, and D. H. Walker. 2002. *Rickettsia felis* in *Ctenocephalides* spp. fleas, Brazil. *Emerg. Infect. Dis.* **8**:317–319.
- Parola, P., B. Davoust, and D. Raoult. 2005. Tick- and flea-borne rickettsial emerging zoonoses. *Vet. Res.* **36**:469–492.
- Parola, P., R. S. Miller, P. McDaniel, S. R. Telford III, J. M. Rolain, C. Wongsrichanalai, and D. Raoult. 2003. Emerging rickettsioses of the Thai-Myanmar border. *Emerg. Infect. Dis.* **9**:592–595.
- Policastro, P. F., U. G. Munderloh, E. R. Fischer, and T. Hackstadt. 1997. *Rickettsia rickettsii* growth and temperature-inducible protein expression in embryonic tick cell lines. *J. Med. Microbiol.* **46**:839–845.
- Psaroulaki, A., M. Antoniou, A. Papaconstathiou, P. Toumazos, F. Loukaidis, and Y. Tselentis. 2006. First detection of *Rickettsia felis* in *Ctenocephalides felis* fleas parasitizing rats in Cyprus. *Am. J. Trop. Med. Hyg.* **74**:120–122.
- 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.
- Raoult, D., B. La Scola, M. Enea, P. E. Fournier, V. Roux, F. Fenollar, M. A. Galvao, and X. de Lamballerie. 2001. A flea-associated *Rickettsia* pathogenic for humans. *Emerg. Infect. Dis.* **7**:73–81.
- Regnery, R. L., C. L. Spruill, and B. D. Plikaytis. 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J. Bacteriol.* **173**:1576–1589.
- Rolain, J. M., M. Franc, B. Davoust, and D. Raoult. 2003. Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipiensis* in cat fleas, France. *Emerg. Infect. Dis.* **9**:338–342.
- Schrieffer, M. E., J. B. Sacci, Jr., J. P. Taylor, J. A. Higgins, and A. F. Azad. 1994. Murine typhus: updated roles of multiple urban components and a second typhus-like rickettsia. *J. Med. Entomol.* **31**:681–685.
- Shaw, S. E., M. J. Kenny, S. Tasker, and R. J. Birtles. 2004. Pathogen

- carriage by the cat flea *Ctenocephalides felis* (Bouche) in the United Kingdom. *Vet. Microbiol.* **102**:183–188.
34. **Silverman, D. J.** 1984. *Rickettsia rickettsii*-induced cellular injury of human vascular endothelium in vitro. *Infect. Immun.* **44**:545–553.
 35. **Silverman, D. J. and C. L. Wisseman, Jr.** 1979. In vitro studies of rickettsia-host cell interactions: ultrastructural changes induced by *Rickettsia rickettsii* infection of chicken embryo fibroblasts. *Infect. Immun.* **26**:714–727.
 36. **Simser, J. A., A. T. Palmer, V. Fingerle, B. Wilske, T. J. Kurtti, and U. G. Munderloh.** 2002. *Rickettsia monacensis* sp. nov., a spotted fever group rickettsia, from ticks (*Ixodes ricinus*) collected in a European city park. *Appl. Environ. Microbiol.* **68**:4559–4566.
 37. **Simser, J. A., A. T. Palmer, U. G. Munderloh, and T. J. Kurtti.** 2001. Isolation of a spotted fever group rickettsia, *Rickettsia peacockii*, in a Rocky Mountain wood tick, *Dermacentor andersoni*, cell line. *Appl. Environ. Microbiol.* **67**:546–552.
 38. **Stevenson, H. L., M. B. Labruna, J. A. Monteneri, M. Y. Kosoy, K. L. Gage, and D. H. Walker.** 2005. Detection of *Rickettsia felis* in a New World flea species, *Anomopsyllus nudata* (Siphonaptera: Ctenophthalmidae). *J. Med. Entomol.* **42**:163–167.
 39. **Todd, W. J., W. Burgdorfer, and A. J. Mavros.** 1982. Establishment of cell cultures persistently infected with spotted fever group rickettsiae. *Can. J. Microbiol.* **28**:1412–1416.
 40. **Wade, S. E., and J. R. Georgi.** 1988. Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouche (Siphonaptera: Pulicidae). *J. Med. Entomol.* **25**:186–190.
 41. **Wedincamp, J., Jr., and L. D. Foil.** 2003. *Rickettsia felis* infection in the cat flea (Siphonaptera: Pulicidae). *J. Entomol. Sci.* **38**:234–239.
 42. **Wedincamp, J., Jr., and L. D. Foil.** 2000. Infection and seroconversion of cats exposed to cat fleas (*Ctenocephalides felis* Bouche) infected with *Rickettsia felis*. *J. Vector Ecol.* **25**:123–126.
 43. **Wedincamp, J., Jr., and L. D. Foil.** 2002. Vertical transmission of *Rickettsia felis* in the cat flea (*Ctenocephalides felis* Bouche). *J. Vector Ecol.* **27**:96–101.
 44. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.
 45. **Williams, S. G., J. B. Sacci, Jr., M. E. Schriefer, E. M. Andersen, K. K. Fujioka, F. J. Sorvillo, A. R. Barr, and A. F. Azad.** 1992. Typhus and typhus-like rickettsiae associated with opossums and their fleas in Los Angeles County, California. *J. Clin. Microbiol.* **30**:1758–1762.
 46. **Zavala-Castro, J. E., M. Small, C. Keng, D. H. Bouyer, J. Zavala-Velazquez, and D. H. Walker.** 2005. Transcription of the *Rickettsia felis* ompA gene in naturally infected fleas. *Am. J. Trop. Med. Hyg.* **73**:662–666.