Abstract. Flea-borne spotted fever and flea-borne (murine) typhus are rickettsioses caused by *Rickettsia felis* and *Rickettsia typhi*, respectively, and typically present as undifferentiated febrile illnesses. The relative contribution of these agents to flea-borne rickettsioses in California is unclear. We have developed a duplex reverse transcription real-time polymerase chain reaction (RT-rtPCR) assay targeting *R. felis*– and *R. typhi*–specific 23S ribosomal RNA single nucleotide polymorphisms to better understand the respective roles of these agents in causing flea-borne rickettsioses in California. This assay was compared with an established duplex *R. felis*– and *R. typhi*-ompB rt-PCR assay and was shown to have 1,000-fold and 10-fold greater analytical sensitivity for the detection of *R. felis* and *R. typhi*, respectively. Retrospective testing of clinical specimens with both assays established *R. typhi* as the major etiologic agent of flea-borne rickettsioses in California.

INTRODUCTION

Rickettsioses are acute febrile illnesses caused by small, obligate intracellular, gram-negative bacteria belonging to the genus *Rickettsia*. Infected ticks, fleas, mites, and lice serve as vectors for disease transmission. The flea-borne rickettsioses include flea-borne spotted fever (FBSF) and flea-borne typhus (FBT; also known as murine or endemic typhus) caused by *Rickettsia felis* and *Rickettsia typhi*, respectively. The clinical features of FBSF and FBT are similar and typically include nonspecific symptoms such as fever, headache, and myalgia, although FBSF is associated with milder disease than FBT. A papular to maculopapular rash is associated with most FBSF patients but less frequently observed with FBT patients. Severe clinical manifestations are rare but may include pneumonitis, renal injury, and central nervous system involvement. Therapeutic intervention with a tetracycline class of antibiotic such as doxycycline is highly effective in the management and recovery of patients with flea-borne rickettsioses.

Both *R. felis* and *R. typhi* are distributed worldwide. Flea-borne spotted fever and FBT are not nationally notifiable diseases in the United States; however, reporting of suspected FBT cases is required by some local and state public health departments. The majority of FBT cases in the United States are reported from Texas and California, with 580 and 216 cases, respectively, recorded in 2022. Endemic foci of FBT are maintained through an urban cycle of *R. typhi* transmission, involving rats as the reservoir host and rat fleas as the vector, and a suburban cycle in which opossums serve as the reservoir and cat fleas as the vector. Recent ecological epidemiology investigations have illustrated the relative importance of the suburban cycle for *R. typhi* maintenance and transmission in Texas and California.

Since the initial description of an *R. felis* infection in a patient from Texas, additional cases of FBSF have not been recognized in the United States. In contrast, *R. felis* infections appear to be relatively common in sub-Saharan Africa and Asia; however, reports of *R. felis* detections in both healthy and febrile individuals in Africa have brought into question the true incidence of FBSF in this region and the pathogenic potential of *R. felis*. The invertebrate reservoir host and vector for *R. felis* is the cat flea, *Ctenocephalides felis*. Surveys of cat fleas have shown that *R. felis* infections are far more prevalent than *R. typhi* infections, suggesting that the incidence of FBSF should be much greater than the incidence of FBT in California. These observations suggest that limited awareness of FBSF by clinicians and lack of available diagnostic tests may lead to underestimation of human *R. felis* infections. Alternatively, *R. felis* may have limited infectivity or minimal pathogenic potential for humans.

Serologic detection using indirect immunofluorescence has been the gold standard for laboratory confirmation of rickettsioses, providing group- but not species-level identification. The usefulness of serologic confirmatory testing is also limited by the need to demonstrate seroconversion or a 4-fold rise in titer for paired sera, hindering its timeliness for laboratory diagnosis. Molecular detection of *Rickettsia* nucleic acids offers a rapid genus-, group-, or species-specific alternative to serologic testing. Recently, a Pan-*Rickettsia* reverse transcription real-time polymerase chain reaction (RT-rtPCR) assay was described that targets 23S ribosomal RNA (rRNA), a ribosomal component that is present at high copy numbers within each bacterial cell. With this assay, the relatively abundant 23S rRNA is reverse transcribed, after which the desired target is amplified with specific primers and detected with a specific fluorogenic hybridization probe. The RT-rtPCR assay proved to have superior analytical sensitivity when compared with an earlier Pan-*Rickettsia* 50S ribosomal protein L16 (single-copy DNA target) rPCR assay.

We have expanded upon this work by developing a duplex RT-rtPCR assay targeting 23S rRNA single nucleotide polymorphisms (SNPs) for the detection and discrimination of *R. felis* and *R. typhi* in specimens from suspected flea-borne rickettsiosis cases. We describe here the performance characteristics of this new RT-rtPCR assay measured against an established duplex *ompB* rPCR assay. Retrospective testing of 87 clinical specimens revealed the relative contributions of *R. felis* and *R. typhi* as the cause of flea-borne rickettsioses in California.
Nucleic acids. Nucleic acids from Rickettsiales cell culture isolates were obtained from the Rickettsial Zoonoses Branch, U.S. Centers for Disease Control and Prevention, and BEI Resources (Manassas, VA) (Supplemental Table 1). The concentrations of *R. felis* and *R. typhi* nucleic acids were determined by quantitative PCR using the Pan-Rickettsia RCKR assay, absent the reverse transcription step, and a standard curve generated with quantified plasmid DNA; concentrations were expressed as genome copies per microliter. Nucleic acids from non-rickettsial pathogens associated with fever and rash illnesses were obtained from the California Department of Public Health (CDPH) strain and specimen collections (Supplemental Table 1). In addition, nucleic acids extracted from 10 individual *R. felis*-infected cat fleas (*C. felis*) collected within Orange County, CA, were provided by the Orange County Mosquito and Vector Control District (Garden Grove, CA).

**Clinical and contrived specimens.** A total of 117 samples were used to assess assay performance. These included human clinical specimens (81 sera, 4 plasmas, and 2 whole bloods) collected between April 2017 and January 2023 from 87 case-patients that were submitted to the CDPH Viral and Rickettsial Disease Laboratory for confirmatory testing and 30 *R. felis*-contrived specimens. Specimens were collected for public health surveillance and were considered exempt from human subject regulations by the California Health and Human Services Agency Committee for the Protection of Human Subjects (Project #2023-085). Total nucleic acids were extracted from 300 human clinical specimens (81 sera, 4 plasmas, and 2 whole bloods) collected between April 2017 and January 2023 for rtPCR. A nested 23S rRNA RT-PCR sequencing assay was developed and used to resolve discrepant results between the 23S rRNA RT-rtPCR assay and the ompB rPCR assay (Supplemental Materials).

**Assay performance characteristics.** Assay exclusivity was assessed using nucleic acids from 14 members of the order Rickettsiales and 28 pathogens causing fever and rash illnesses (Supplemental Table 1). Analytical sensitivity was determined for the 23S rRNA RT-rtPCR and the ompB rPCR assays using quantified total nucleic acids from *R. felis* and *R. typhi* spiked into pooled nucleic acids from human whole blood or sera at concentrations of descending 10-fold increments from 1,000 to 0.01 genome copies per 5 μL. Each nucleic acid concentration was tested in replicates of five, and the limit of detection (LOD) for each assay was defined as the lowest number of genomic copies at which all five replicates were detected. The agreement between assays for each assay was assessed for a panel of 87 clinical specimens and 30 *R. felis*-contrived specimens. Discrepant results between assays were resolved using the nested 23S rRNA RT-PCR sequencing assay.

**RESULTS**

**SNP identification and RT-rtPCR assay development.** *Rickettsia felis*– and *R. typhi*–specific SNPs were identified through the alignment of 23S rRNA sequences from 35

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**Table 1**

<table>
<thead>
<tr>
<th>Assay Analyte</th>
<th>Oligonucleotide Name</th>
<th>Reference Sequence Coordinates</th>
<th>Oligonucleotide Sequence and Modifications*</th>
<th>Assay Oligonucleotide Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>23S rRNA</strong></td>
<td><em>R. felis</em> R6f23S_S</td>
<td>NR_076359.1: 1322–1343</td>
<td>ABY-AGTTAATCTGCAACAGGTGATAGT-GSY</td>
<td>100 nM</td>
</tr>
<tr>
<td></td>
<td><em>R. typhi</em></td>
<td>NR_076359.1: 1348–1369</td>
<td>GCTTTCAGCTGACGTTTGA</td>
<td>200 nM</td>
</tr>
</tbody>
</table>

*Oligonucleotide modifications: ABY and 6-FAM (6-carboxyfluorescein) are fluorescent dyes; QSY, ZEN, and IABKFO (Iowa Black Fluorescent Quencher) are nonfluorescent acceptor dyes.
Rickettsia species and subspecies. An *R. felis* 23S rRNA G1333A SNP and an *R. typhi* 23S rRNA T1976C SNP were selected for the design of dual-labeled allelic discrimination probes and the development of the duplex RT-rtPCR assay. The regions amplified by the RT-rtPCR assay included nucleotides 1302–1369 of the *R. felis* 23S rRNA sequence NR_076359.1 and nucleotides 1929–2020 of the *R. typhi* 23S rRNA sequence NR_076209.1 and correspond to ampiclon sizes of 67 and 91 bp, respectively (Table 1). A search of NCBI databases indicated that the respective primer and probe sequences were conserved among the available 23S rRNA sequences for *R. felis* strains (URRWXCal2, Pedreira, LSU, LSU-Lb, and BBayA_MAG) and *R. typhi* strains (Wilmington, TM2540, TH1527, and B991CWP). Alignments of the LSU-Lb, and BBayA_MAG) and species at this time, the 23S rRNA sequence from *Rickettsia senegalensis*, a genetic near neighbor of *R. felis* (Figure 1A). The alignments illustrate that the pesviruses 1 coxsackieviruses A6 and A16, enterovirus A71, human herpesvirus 1–6, measles virus, rubella virus, human immunodeficiency virus, West Nile virus, dengue virus types 1–4, Zika virus, Coxielia burnetii, Bartonella quintana, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Treponema pallidum*, *Streptococcus pyogenes*, *Salmonella typhi*, and *Staphylococcus aureus*. Cross reactivity was not observed with any of the nucleic acids tested.

**Analytical sensitivity and assay agreement.** The analytical sensitivity of the 23S rRNA RT-rtPCR and *ompB* rtPCR assays was determined for the detection of total nucleic acids from the *R. felis* strain Baton Rouge and *R. typhi* strain Wilmington. Among a background of nucleic acids derived from blood or serum specimens, the LOD of the 23S rRNA RT-rtPCR assay for *R. felis* and *R. typhi* was 0.1 and 1 genomic copies per reaction, respectively, whereas the LOD of the *ompB* rtPCR assay for *R. felis* and *R. typhi* was 100 and 10 genomic copies per reaction, respectively (Tables 2–5).

The agreement between the 23S rRNA RT-rtPCR and the *ompB* rtPCR assays was assessed by testing the panel of clinical specimens from 87 individuals with suspected Rickettsia infections. Cases from 15 counties were represented in this study, with the majority (75.9%) collected from patients residing in Los Angeles and Orange Counties. Most case-patients (82.8%) were seropositive for *Rickettsia* as determined by a commercial or clinical laboratory prior to submission to the CDPH. Clinical information was available for 81 case-patients, of whom 49.4% reported a rash and 63% met the clinical criteria for defining suspected rickettsioses cases for surveillance purposes.15 The number of days elapsed between the onset of symptoms and the date of specimen collection ranged from 1 to 17 days, with a median of 6 days. The panel was supplemented with 30 contrived *R. felis* specimens, bringing the total number of specimens tested with both assays to 117. The agreement between assays for *R. felis* and *R. typhi* detection was 93.2% and 95.7%, respectively (Tables 6 and 7).

*Rickettsia felis* was detected in 23 specimens, all contrived, by both the 23S rRNA RT-rtPCR and *ompB* rtPCR assays. Discrepant *R. felis* results were obtained for eight specimens.

![Figure 1. Alignment of Rickettsia felis (A) and Rickettsia typhi (B) 23S rRNA primer and probe sequences with sequences from 34 Rickettsia species and subspecies. The R. felis- and R. typhi-specific single nucleotide polymorphisms are located near the center of the probe sequence. Dots indicate identical nucleotide at that position. A dash indicates a nucleotide insertion/deletion. RC = reverse complement.](image-url)
resided in either Los Angeles or Orange Counties.

design of analytically sensitive RT-rtPCR assays for bacterial
cellular in a bacterial cell offers multicopy targets for the

The overall case positivity rate for R. typhi detection in clinical specimens was 39.1%, with the detections ranging from 1 to

The authors demonstrated that a 23S rRNA RT-rtPCR testing results: detections in four specimens by only the 23S rRNA

Assay limit of detection comparison in blood matrix

<table>
<thead>
<tr>
<th>Genomic Copies/Reaction</th>
<th>R. felis 23S rRNA RT-rtPCR</th>
<th>R. felis ompB rtPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>22.32 (0.27)</td>
<td>30.97 (0.11)</td>
</tr>
<tr>
<td>10</td>
<td>25.56 (0.29)</td>
<td>34.07 (0.10)</td>
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<td>1</td>
<td>28.94 (0.24)</td>
<td>36.85 (0.54)</td>
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<tr>
<td>0.1</td>
<td>32.31 (0.23)</td>
<td>38.09 (0.99)</td>
</tr>
<tr>
<td>0.01</td>
<td>35.87 (0.25)</td>
<td>38.14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genomic Copies/Reaction</th>
<th>R. typhi 23S rRNA RT-rtPCR</th>
<th>R. typhi ompB rtPCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>23.70 (0.17)</td>
<td>29.08 (0.14)</td>
</tr>
<tr>
<td>10</td>
<td>26.89 (0.37)</td>
<td>32.76 (0.28)</td>
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<td>1</td>
<td>30.73 (0.27)</td>
<td>35.97 (0.72)</td>
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<tr>
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<td>33.87 (1.07)</td>
<td>38.04</td>
</tr>
<tr>
<td>0.01</td>
<td>36.50 (0.71)</td>
<td>Not Detected</td>
</tr>
<tr>
<td>1</td>
<td>35.83</td>
<td>Not Detected</td>
</tr>
</tbody>
</table>

The lack of significant 16S rRNA sequence divergence between species. To circumvent this shortcoming, we targeted
distlespecific 23S rRNA SNPs for the detection of R. felis and R. typhi by duplex RT-rtPCR. Compared with an estab-
lished duplex ompB rtPCR assay, the 23S rRNA RT-rtPCR assay was 1,000-fold and 10-fold more analytically sensitive
for the detection of R. felis and R. typhi, respectively. The superior analytical sensitivity of the 23S rRNA RT-rtPCR assay
carried over to the testing of clinical specimens and R. felis–

tected by either assay for the remaining two specimens. Testing of five specimens with the nested RT-PCR sequencing assay agreed with the original 23S rRNA RT-rtPCR testing results: R. typhi detected in four specimens and not detected in one specimen. The overall case positivity rate for R. typhi detection in clinical specimens was 39.1%, with the detections ranging from 1 to 14 days after symptom onset. All positive R. typhi cases resided in either Los Angeles or Orange Counties.

**DISCUSSION**

The presence of hundreds to thousands of rRNA molecules in a bacterial cell offers multicopy targets for the design of analytically sensitive RT-rtPCR assays for bacterial detection.  Recently, Chung et al. applied ribosomal RT-rtPCR for the genus-level detection of Rickettsia in clinical specimens. The authors demonstrated that a 23S rRNA RT-rtPCR assay had a 100-fold higher analytical sensitivity for Rickettsia detection than a single-copy 50S ribosomal protein L16 gene rtPCR assay. The design of species-specific ribosomal RT-rtPCR assays can be challenging because of the lack of significant rRNA sequence divergence between species. To circumvent this shortcoming, we targeted species-specific 23S rRNA SNPs for the detection of R. felis and R. typhi by duplex RT-rtPCR. Compared with an established duplex ompB rtPCR assay, the 23S rRNA RT-rtPCR assay was 1,000-fold and 10-fold more analytically sensitive for the detection of R. felis and R. typhi, respectively. The superior analytical sensitivity of the 23S rRNA RT-rtPCR assay carried over to the testing of clinical specimens and R. felis–contrived specimens. Initial testing of samples with both assays revealed eight additional R. felis and four additional R. typhi detections with the 23S rRNA RT-rtPCR assay. The six contrived R. felis and all four of the clinical R. typhi detections were confirmed by a nested RT-PCR sequencing assay. However, two R. felis clinical specimen detections with the 23S rRNA RT-rtPCR assay could not be confirmed because either the results were falsely positive or the analyte concentrations were at or beyond the lower LOD for the resolver test.

In addition to superior analytical sensitivity, in silico analysis of Rickettsia 23S rRNA sequences and exclusivity testing indicated that the RT-rtPCR assay is highly specific for R. felis and R. typhi. Several R. felis–like organisms recently have been described and the genomes sequenced, including R. asembonensis, Rickettsia hooegastraali, and Candidatus R. senegalensis. All three of these Rickettsia have an alternate SNP allele at nucleotide 1333 of the R. felis 23S rRNA target sequence and are predicted to be nonreactive in the 23S rRNA RT-rtPCR assay. Indeed, R. asembonensis was included in the assay exclusivity panel and was found to be nonreactive. In contrast, the R. felis genetic near neighbors R. asembonensis and Candidatus R. senegalensis shared sequence identity with the R. felis ompB rtPCR target, reducing the specificity of this assay for R. felis detection.

The number of FBT cases recorded annually by Texas and California has increased in the last 10 years. Texas has also noted a geographic expansion in case distribution, whereas reported cases in California remain mostly restricted
to Los Angeles and Orange Counties, with 90% of the cases acquired in a suburban setting.\textsuperscript{24,25} In this environment, the transmission of flea-borne rickettsioses to humans likely is enabled by opossums serving as the reservoir host and cat fleas functioning as the vector. Field studies conducted in regions of southern California with endemic foci of flea-borne rickettsioses have found that cat flea infections with \textit{R. felis} are much more prevalent than infections with \textit{R. typhi} and have led to the proposal that \textit{R. felis} may be the principal cause of flea-borne rickettsioses.\textsuperscript{7,9} Our study counters this hypothesis by demonstrating that 39.1\% of the suspected flea-borne rickettsiosis cases were detected as \textit{R. typhi} infections, whereas only 0–2.3\% of the cases were detected as \textit{R. felis} infections; our data support the counter proposal that most California cases are caused by \textit{R. typhi}.\textsuperscript{26}

There are two limitations to our study. First, the study was geographically restricted to California and does not account for strain variation that may occur in other parts of the world. In addition, very few \textit{R. felis} and \textit{R. typhi} 23S rRNA sequences are available in public databases to assess sequence variation for strain variation that may occur in other parts of the world. Further evaluation of the assay with geographically diverse \textit{R. felis} and \textit{R. typhi} samples and expansion of \textit{Rickettsia} 23S rRNA sequences in public databases are warranted. Second, most specimens in this study were serum samples. Although frequently unavailable because of lags in case reporting, the case positivity rate may have benefitted from the use of more productive specimen types such as whole blood and, optimally, skin biopsies of rash lesions.\textsuperscript{27–30} However, even for these specimen types, the detection of \textit{Rickettsia} can be challenging owing to transient bacteremia and diurnal fluctuations in bacterial loads for blood specimens and the variable presentation of a rash for the collection of skin biopsies.\textsuperscript{1,31} Nonetheless, the RT-rtPCR assay targeting multiple copies of 23S rRNA offered a significant advantage over rtPCR assays targeting single-copy DNA sequences and promises to provide a powerful new surveillance tool for detecting \textit{R. felis} and \textit{R. typhi} cases.

We have described the development of an improved duplex molecular diagnostic test for the detection of flea-borne rickettsioses. The assay demonstrated enhanced analytical sensitivity and specificity for \textit{R. felis} and \textit{R. typhi} detection relative to an established duplex rtPCR assay. Testing of surveillance specimens collected over the last 6 years with these two assays demonstrated that \textit{R. typhi} is the predominant cause of flea-borne rickettsioses in California and confirmed that FBT is largely restricted to Los Angeles and Orange Counties. The implementation of this rapid, analytically sensitive, and accurate test will facilitate public health surveillance efforts to monitor flea-borne rickettsiosis trends, identify outbreaks and epicenters of disease transmission, and help guide targeted intervention to reduce infection rates.
REFERENCES