INTRODUCTION

Rickettsia felis is a newly emerging bacterial pathogen that causes flea-borne spotted fever in humans. The bacterium is both competently maintained and biologically-transmitted by several flea species and has been confirmed in cat flea (Ctenocephalides felis) populations from the United States, Ethiopia, Spain, Brazil, Mexico, France, Thailand, the United Kingdom, New-Zealand and Peru.

R. felis clinical infection in humans produces an acute systemic response that is typified by febrile maculopapular exanthema, myalgia, and headache. Involvement of the central nervous system is commonly witnessed in the later stages of the disease. Moreover, this murine typhus-like ailment might well be vague, and patients could be presented with varied nonspecific symptoms. The disease was first reported in the United States in 1994 but has since been detected in humans in Mexico, Brazil, Germany, and South Korea by PCR amplification and in France and Thailand by serologic tests.

In this study, cat fleas collected from cats and dogs in different localities in Israel were tested for R. felis DNA using PCR and sequencing of fragments of 5 different genes. The findings reveal polymorphism among rickettsial isolates in Israel and the presence of two closely related R. felis strains within C. felis fleas.

MATERIALS AND METHODS

Fleas. Seventy-nine pools of 5 to 20 fleas each were collected from individual dogs and cats in private veterinary practices throughout Israel. A total of 480 cat fleas were obtained from 72 cats and 10 dogs from the cities of Ramla, Tel-Aviv, Bat-Yam, Jerusalem, Beer-Sheva, Naharia, and the Sharon region and included in the study. All fleas in a certain pool originated from a single animal except for sample Rf, where fleas from four different cats were merged to a single pool (Table 1). Fleas collected from the city of Ramla were sampled from cats living in an animal shelter maintained by a welfare society, while all other samples were obtained from nonshelter stray and pet animals. Samples were recorded (location, host signalment and medical condition) and kept frozen at −20°C until analyzed. Fleas were classified to the species level (Ctenocephalides felis) by an entomologist, and DNA was detected in 7.6% of the flea pools. Two genotypes similar in their housekeeping gene sequences but markedly different in their surface antigenic genetic milieu were characterized. This is the first detection of this flea-transmitted rickettsia within its vector in Israel and the Middle East. Although no clinical case has been reported in human beings in Israel to date, these findings suggest that this infection is prevalent in Israel.

PCR and sequencing. General rickettsial DNA was initially detected by PCR, using gltA-specific primers (Rp877p and Rp1258a) and sequencing. Positive samples were further tested by RI31 primers (RI31-289-F and -857-R of the gltA gene) and other R. felis–specific primers. These were constructed applying the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/Primer3 www.cgi) to the appropriate RI31 or R. felis GenBank sequences, respectively. Relevant GenBank accession numbers, PCR primers used in the study, and the sizes of the amplicons obtained following amplification are listed in Table 2. Three microliters of each extracted DNA (≥ 100 μg/mL) were added to 22 μL of master mixture for each reaction. The final reagent concentrations were 0.2 μM for each primer, 0.2 mM for each deoxynucleotide triphosphate (Roche Diagnostics, Mannheim, Germany), 2 U of Taq DNA polymerase (Sigma, St. Louis, MO), and its 1× buffer. The following thermal cycler parameters were used for the R. felis–specific reactions: 96°C (90 seconds), followed by 35 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (7 seconds), followed by a final extension period (72°C, 7 minutes). For the amplification of the gltA 569-bp fragment we used 35 cycles and 48°C as an annealing temperature. Positive control material was a British R. felis isolate. Negative controls (DNA-free water, R. typhi DNA, and R. conorii DNA) were included in all R. felis–specific assays. PCR products were purified using the Wizard PCR purification systems (Promega, Madison, WI) and sequenced at least twice (3700 DNA Analyzer, Perkin-Elmer, Foster City, CA). Cloning of the htrA (17-kDa antigen) amplicons (pGEM-T Vector System, Promega) was done according to the manufacturer’s instructions. Sequences obtained were edited using the ClustalW 1.82 program (http://www.ebi.ac.uk/clustalw/index.html), and similarity to published sequences was determined using the BLAST utility (National Center for Biotechnology Information, Bethesda, MD). Translation of the gene sequences into deduced proteins was done using the ExPASy utility (http://www.expasy.org/tools/dna.html). Com-
Detection and sequence identification of *R. felis* from cat fleas by polymerase chain reaction

<table>
<thead>
<tr>
<th>Flea sample</th>
<th>Origin</th>
<th>No. of fleas/No. of cats in pool</th>
<th>gltA</th>
<th>ompA</th>
<th>ompB</th>
<th>htrA</th>
<th>fusA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R</em>ₙ</td>
<td>Ramla</td>
<td>23/4</td>
<td>+ 100% to <em>R. felis</em> (AF151631)</td>
<td>100% to <em>R. felis</em> (AF191026)</td>
<td>100% to <em>R. felis</em> (AF182279)</td>
<td>2 extracts: 96.95% &amp; 100% to <em>R. felis</em> (AF195118)</td>
<td>99.42% to <em>R. felis</em> (AF502178)</td>
</tr>
<tr>
<td>R4</td>
<td>Ramla</td>
<td>14/1</td>
<td>+ 100% to <em>R. felis</em> (AF151631)</td>
<td>-</td>
<td>-</td>
<td>2 extracts: 96.95% &amp; 100% to <em>R. felis</em> (AF195118)</td>
<td>99.42% to <em>R. felis</em> (AF502178)</td>
</tr>
<tr>
<td>TA22</td>
<td>Tel-Aviv</td>
<td>5/1</td>
<td>+ 100% to <em>R. felis</em> (AF151631)</td>
<td>-</td>
<td>-</td>
<td>96.95% to <em>R. felis</em> (AF195118)</td>
<td>99.42% to <em>R. felis</em> (AF502178)</td>
</tr>
<tr>
<td>TA26</td>
<td>Tel-Aviv</td>
<td>7/1</td>
<td>+ 100% to <em>R. felis</em> (AF151631)</td>
<td>-</td>
<td>-</td>
<td>96.95% to <em>R. felis</em> (AF195118)</td>
<td>99.42% to <em>R. felis</em> (AF502178)</td>
</tr>
<tr>
<td>J8</td>
<td>Jerusalem</td>
<td>5/1</td>
<td>+* 100% to <em>R. felis</em> (AF151631)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>96.95% to <em>R. felis</em> (AF195118)</td>
</tr>
<tr>
<td>Sh10</td>
<td>Bat-Yam</td>
<td>9/1</td>
<td>+* 100% to <em>R. felis</em> (AF151631)</td>
<td>-</td>
<td>-</td>
<td>+*</td>
<td>-</td>
</tr>
</tbody>
</table>

*+, positive PCR; –, negative PCR.

Detection and sequence identification of *R. felis* from cat fleas by polymerase chain reaction

The new sequences obtained from the DNA extracts in this study were submitted to GenBank and assigned the accession numbers AY917126 (*fusA*, 175 bp, common to both “Tel-Aviv” and “Ramla” *R. felis* strains) and AY917127 (17-kDa antigen, 164 bp, “Ramla” *R. felis* strain).

**RESULTS**

*Rickettsia felis* DNA was found in 6 (7.6%) of the 79 flea pools examined. Positive control was found positive, and negative controls were found negative in all *R. felis*-specific PCR assays. One extract (*R*ₙ) was confirmed by PCR and sequencing of fragments of 5 different genes. Three more extracts (R4, TA22, and TA26) were confirmed by PCR and sequencing of fragments of 3 genes. Two other samples, J8 and Sh10, were confirmed by three and two PCR amplifications, respectively (Table 1). Taking into account the number of fleas in each positive pool (total of 63 positive fleas), it was estimated that *R. felis*-infected *C. felis* ranged from 1.25% to 13.13% of the 480 cat fleas collected. All 6 positive flea pools were collected from domestic cats (*Felis catus*), yet no specific signalment or apparent medical condition could be associated with any one of the animals carrying positive fleas.

PCR successfully amplified both gltA fragments (381 and 569 bp) in all extracts (Table 1) and sequence analysis of the combined 853 bp gltA fragment amplified from sample *R*ₙ (Ramla) identified a 100% homology with the Thai-Burmese RF31 isolate. Nevertheless, numerous attempts to amplify the gltA region upstream to the 5' end, using various primer sets constructed for the RF31 deposited sequence and different PCR conditions proved futile (data not shown). PCR amplifications of *R. felis*-specific ompA and ompB gene fragments (237 bp and 236 bp, respectively) proved negative in all extracts, except for *R*ₙ (Ramla) and the British positive control, despite repeated trials using variable PCR primers and conditions. Sequence analyses of *R*ₙ’s ompA and ompB amplicons were identical to the previously reported sequence for *R. felis* (GenBank AF191026) between positions 194 and 430 and to *R. felis* (GenBank AF182279) between positions 2774 and 2966, respectively. PCR successfully amplified fusA (175 bp) and htrA (17-kDa antigen; 164 bp) gene fragments in all extracts, except for fusA in Sh10. Sequence analysis of the fusA amplicon demonstrated 99.42% similarity (C instead of T in position 252; C/T) with that of *R. felis* (GenBank AF502178) between positions 156 and 330. Sequencing of the htrA amplicons manifested genetic polymorphism, delineating one polymorph found in all Tel-Aviv (TA22 and TA26) and Ramla (*R*ₙ and R4) samples with 96.95% similarity (G/A, C/T). A second htrA polymorphism was found only in the Ramla extracts (*R*ₙ and R4), having 100% similarity to *R. felis* (AF195118) between positions 218 and 381 (Figure 1). Thus, both Ramla samples (*R*ₙ and R4) harbored the two htrA DNA polymorphs (Table 1).

Analysis of the deduced protein sequences derived from the polymorphic gene sequences described above showed that the single nucleotide difference in both isolates’ fusA gene (C/T) when compared with *R. felis* did not enforce an amino acid (proline) change in the protein product. On the other hand, the 5 point differences between the 17-kDa antigen gene of the “Tel-Aviv” and “Ramla” strains potentially inflict 3 amino acid substitutions in the rickettsial surface protein: V₆₅ instead of A₆₅ (V65A), K73A, and A94T. Further, computational analysis of the 17-kDa protein topology showed that 2 of these 3 substituted amino acids (K73A and A94T) are predicted to lie in the extracellular domain of the protein (data not shown).

**DISCUSSION**

This is the first molecular detection of *Rickettsia felis* in cat fleas collected in Israel and to the best of our knowledge also the first report of this flea-transmitted rickettsia in the Middle East. Our results are supported by the use of primers targeting different genes, including genes for metabolic enzymes and outer membrane protein-coding genes that potentially hold antigenic significance. This polygenic analytic approach, which included evaluation of both types of genes, is in accordance with the Ad Hoc Committee for the Re-Evaluation of the Species Definition in Bacteriology. 21
The implied prevalence of *R. felis*-infected flea pools found in this study (7.6%) is in scale with findings from southern Texas (4%),16 France (8.1%),10 and the United Kingdom (6–12%).12 However, it is noteworthy that the flea samples were largely collected from stray cats (data not shown) and not from catteries (except for the Ramla series), where higher infection rates of *R. felis* are often recorded.10,22 Hence, it may well be that infection rates in closed animal populations in Israel are higher than those presented. Furthermore, although this study detected *R. felis* in Israeli cat fleas (*C. felis*), systematic sampling was not performed in all regions of Israel, and hence the precise prevalence or distribution of *R. felis* in Israel cannot be provided.

At least two polymorphic strains of *R. felis* were detected in this study by the revealing of polymorphism in the 17-kDa antigen (*htrA*) gene. These two strains, *R. felis* “Tel-Aviv” (found in Tel-Aviv, TA22 and TA26; and in Ramla, Rp and R4) and *R. felis* “Ramla” (found only in the Ramla samples), exhibited complete match in their housekeeping gene sequences (*gltA* and *fusA*), but deviated in their out-projecting “antigenic” gene sequences (*htrA* and possibly *ompA* and *ompB*). Failure in PCR amplification of *ompA*, but not *ompB*, has already been documented in various rickettsial isolates—including those of *R. felis*.23,24 The fact that the designed primers consistently failed to amplify both the *ompA* and the *ompB* gene fragments in the “Tel-Aviv” strain despite copious trials under different PCR conditions, could probably be explained by the primers’ failure to attach mismatching *ompA* and *ompB* sequences at the target rickettsial genes. Alternatively, this dual failure could also be attributed to the probable existence of flea-derived PCR inhibitors affecting these assays.25

The “Tel-Aviv” strain has also shown 96.95% similarity to the *R. felis* *htrA* gene fragment (AF195118), resulting in a distinct antigenic typing. The “Ramla” strain genetic typing was more complex, due to the containment of both 17-kDa gene polymorphs in the Rp and R4 samples (Table 1). The possibility that two or more different genotypes existed in the

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### Table 2

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Nucleotide sequence (5’–3’)</th>
<th>Amplicon size (bp)</th>
<th>Accession number [positions]</th>
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<tbody>
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<td><em>gltA</em></td>
<td>Rp877p*</td>
<td>GGGGGCGCTGCTCACGGCGG</td>
<td>382</td>
<td>AF516331</td>
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<td><em>gltA</em></td>
<td>Rpi1258n*</td>
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<td>569</td>
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<tr>
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<td>Rs1-298-F</td>
<td>GAATTCAGCGAAGCGAGCA</td>
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<td>AF516331</td>
</tr>
<tr>
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<td>Rs1-857-R</td>
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<td>AF516331</td>
</tr>
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<td>AY394853</td>
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<tr>
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<tr>
<td><em>ompB</em></td>
<td>RtopmB2986-R</td>
<td>CGTCAAGCTATGCTATCA</td>
<td>237</td>
<td>AY394853</td>
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<tr>
<td><em>htrA</em> (17-kDa Ag)</td>
<td>RthtrA228-F</td>
<td>GGAGACGCTTTGCGGAGGGGG</td>
<td>164</td>
<td>AF195118</td>
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<tr>
<td><em>htrA</em> (17-kDa Ag)</td>
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<td>TTCTTGTTACGGGCTACCTGC</td>
<td>164</td>
<td>AF195118</td>
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<tr>
<td><em>fusA</em></td>
<td>RffusA156-F</td>
<td>GTAGGGCAAGCGAAGCAGAAG</td>
<td>175</td>
<td>AF502178</td>
</tr>
<tr>
<td><em>fusA</em></td>
<td>RffusA330-R</td>
<td>TGCTACGCGCCTAATCTG</td>
<td>175</td>
<td>AF502178</td>
</tr>
</tbody>
</table>

* These *gltA* primers are widely used general rickettsial primers.20 All other primers were specifically constructed for the Thai isolate Rf31 and other *R. felis* sequences.

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**Figure 1.** *htrA* (17-kDa rickettsial antigen) sequence alignment of *R. felis* “Tel-Aviv” and “Ramla” strains. (A) Gene level (the 164-bp amplicon) and (B) deduced amino acid sequence. Arrows indicate point changes at the gene level. Framed amino acids indicate the possible antigenic variation at the deduced protein level. The *htrA* fragment of the “Ramla” strain manifested complete homology, both at the nucleotide and the protein levels, to the deposited *R. felis* sequences (AF195118).
Ramla samples cannot be excluded. Sample $R_p$ was the only sample to amplify both ompA and ompB gene fragments while both $R_p$ and $R_q$ samples exclusively contained R. felis DNA with 100% similarity to the R. felis htrA gene fragment, along with the 96.95% similarity morph of this gene. The “Ramla” strain of R. felis might carry both the ompA and ompB genes (that were not detected in R. felis “Tel-Aviv”), and the 100% similarity morph of the 17-kDa gene, which differs from the 96.95% similarity morph, in R. felis “Tel-Aviv.” However, further work necessitating this genotype’s pure DNA has to be done to validate this notion.

Identification of at least 2 distinct strains of R. felis divergent in their expected antigenic properties (e.g., the htrA gene K73A substitution), in a small-scale area (Ramla series cat- tery) might point to the presence of antigenic variation in these isolates. Moreover, the extent of this variation—5 point differences of 164 (3.05%) and 3 of 54 (5.55%) at the partial 17-kDa antigen nucleotide and deduced amino acid levels, respectively—suggests it is an “active” rather than a haphazard phenomenon (Figure 1). The qualitative and quantitative descriptions of this variation in the “antigenic” but not in the “metabolic” genomic loci lend support to this finding. Furthermore, the well-established presence of antigenic variation in two other members of the order Rickettsiales, Anaplasma marginale26 and Ehrlichia chaffeensis,27 and the recognition of Neisseria spp. RS3-like repeat motifs, which participate in the exchange of bacterial genetic matter in the R. felis genome,28 further support this possibility. Four general mecha- nisms for antigenic variation have been described in vector- born pathogens: modification of transcript levels by gene silencing/activation, gene conversion, DNA rearrangement, and multiple point mutations.29 Although the latter mecha- nism might seem apt in the case of the current study, further comparative genomic and antigenic analyses among various R. felis isolates are yet needed to point to the mechanisms that commanded the potential antigenic differences found here.

Both of the R. felis extracts from Israel manifested complete similarity (100%) with the Thai isolate-RF31’s gltA gene in their 853-bp fragment. This sequence identity might suggest their unity at first glance, however our repetitive failure to amplify gltA 5’ region in the new extracts using an array of primers derived from the RF31 deposited sequence (AF516331) opposes this concept. These abortive PCR trials could be explained by differences in nucleotide sequences in the rickettsial gltA 5’ regions that possibly discriminate the RF31 from the Israeli isolates. The gltA gene (1–1258 bp) is the only RF31 deposited sequence of this field isolate.3 Thus, further comparative inquiries on homologies in other genes are required to establish or deter this unity concept. Finally, the reported identification of RF31 on its unique gltA se- quence in Thailand only3 and the detection of its closely re- lated R. felis isolates in Israel might bear epidemiologic importance in terms of Asian endemicity. Sampling of more rickettsial DNA, both from arthropod carriers and human rickettsiosis cases, from scattered Asian and other settings is needed to validate this isolate’s distribution and zoonotic sig- nificance.

Although human cases of flea-borne spotted fever are yet to be reported in Israel, it would seem likely that this infection is more common than is currently recognized. Because extensive antigenic cross-reactivity exists among SFG rickettsiae, available serological or immunohistochemical assays are only SFG-specific and cannot be used to ascribe etiology to a spe- cific pathogen.30 Forty-seven cases of human murine typhus were reported in Israel in the past decade,31 with 19 of these (45.2%) originating from the Ramla and Tel-Aviv districts, where we traced the presence of R. felis. Given our findings, it is likely that R. felis could be implicated in murine typhus-compatible and Mediterranean spotted fever cases in Israel and that some of these reported cases might have well been murine typhus-like disease caused by R. felis. Consequently, we believe Israeli physicians should consider this bacterium a potential causative agent of fever and/or rash in patients with a history of possible flea contact.

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Authors’ addresses: Omri Bauer, Gad Baneth, and Shimon Harrus, School of Veterinary Medicine, The Hebrew University of Jerusalem, POB 12, Rehovot 76100, Israel. Tamar Eshkol, “Let the Animals Live” Association, Moshav Talmei-Menashe, Israel. Susan E. Shaw, Department of Clinical Veterinary Sciences, University of Bristol, Langford House, Langford, North Somerset BS40 5DU, UK.

Reprint requests: Dr. Shimon Harrus, School of Veterinary Medicine, The Hebrew University of Jerusalem, POB 12, Rehovot 76100, Israel, Telephone: 972-8-9489633, Fax: 972-8-9489956, E-mail: harrus@agri.huji.ac.il.

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