



University
of Glasgow

Jiang, J., Maina, A.N., Knobel, D.L., Cleaveland, S., Laudisoit, A., Wamburu, K., Ogola, E., Parola, P., Breiman, R.F., Njenga, M.K., and Richards, A.L. (2013) Molecular detection of *Rickettsia felis* and *Candidatus Rickettsia Asemboensis* in fleas from human habitats, Asembo, Kenya. *Vector-Borne and Zoonotic Diseases*, 13 (8). pp. 550-558. ISSN 1530-3667

Copyright © 2013 Mary Ann Liebert, Inc.

<http://eprints.gla.ac.uk/85391>

Deposited on: 12 September 2014

Enlighten – Research publications by members of the University of Glasgow
<http://eprints.gla.ac.uk>

Molecular detection of *Rickettsia felis* and *Candidatus Rickettsia Asemboensis* in Fleas from Human Habitats, Asembo, Kenya

Ju Jiang,¹ Alice N. Maina,^{1,2,3} Darryn L. Knobel,^{3,4} Sarah Cleaveland,⁵ Anne Laudisoit,^{6,10} Kabura Wamburu,⁷ Eric Ogola,³ Philippe Parola,⁸ Robert F. Breiman,⁷ M. Kariuki Njenga,⁷ and Allen L. Richards^{1,9}

Abstract

The flea-borne rickettsioses murine typhus (*Rickettsia typhi*) and flea-borne spotted fever (FBSF) (*Rickettsia felis*) are febrile diseases distributed among humans worldwide. Murine typhus has been known to be endemic to Kenya since the 1950s, but FBSF was only recently documented in northeastern (2010) and western (2012) Kenya. To characterize the potential exposure of humans in Kenya to flea-borne rickettsioses, a total of 330 fleas (134 pools) including 5 species (*Xenopsylla cheopis*, *Ctenocephalides felis*, *Ctenocephalides canis*, *Pulex irritans*, and *Echidnophaga gallinacea*) were collected from domestic and peridomestic animals and from human dwellings within Asembo, western Kenya. DNA was extracted from the 134 pooled flea samples and 89 (66.4%) pools tested positively for rickettsial DNA by 2 genus-specific quantitative real-time PCR (qPCR) assays based upon the citrate synthase (*gltA*) and 17-kD antigen genes and the Rfelis qPCR assay. Sequences from the 17-kD antigen gene, the outer membrane protein (*ompB*), and 2 *R. felis* plasmid genes (pRF and pRFd) of 12 selected rickettsia-positive samples revealed a unique *Rickettsia* sp. ($n=11$) and *R. felis* ($n=1$). Depiction of the new rickettsia by multilocus sequence typing (MLST) targeting the 16S rRNA (*rrs*), 17-kD antigen gene, *gltA*, *ompA*, *ompB*, and surface cell antigen 4 (*sca4*), shows that it is most closely related to *R. felis* but genetically dissimilar enough to be considered a separate species provisionally named *Candidatus Rickettsia asemboensis*. Subsequently, 81 of the 134 (60.4%) flea pools tested positively for *Candidatus Rickettsia asemboensis* by a newly developed agent-specific qPCR assay, Rasemb. *R. felis* was identified in 9 of the 134 (6.7%) flea pools, and *R. typhi* the causative agent of murine typhus was not detected in any of 78 rickettsia-positive pools assessed using a species-specific qPCR assay, Rtyph. Two pools were found to contain both *R. felis* and *Candidatus Rickettsia asemboensis* DNA and 1 pool contained an agent, which is potentially new.

Key Words: *Rickettsia*—Fleas—PCR—Multilocus sequence typing—Surveillance.

Introduction

RICKETTSIAE ARE OBLIGATE INTRACELLULAR gram-negative bacteria that are associated with various arthropod vectors, including ticks, mites, lice, and fleas, and can cause mild

to life-threatening human disease (Kelly et al. 2002). The first rickettsia associated with fleas was *Rickettsia typhi*, the causative agent of murine typhus. *R. typhi*, a typhus group (TG) rickettsia, has been most commonly associated with *Xenopsylla cheopis* (oriental rat flea), although at least 10 other

¹Viral and Rickettsial Diseases Department, Naval Medical Research Center, Silver Spring, Maryland.

²Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

³Kenya Medical Research Institute, Kisumu, Kenya.

⁴Department of Veterinary Tropical Diseases, University of Pretoria, Onderstepoort, South Africa.

⁵College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, Glasgow, United Kingdom.

⁶Institute of Integrative Biology, School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom.

⁷US Centers for Disease Control and Prevention, Nairobi, Kenya.

⁸WHO Collaborative Center for Rickettsial and Other Arthropod-borne Bacterial Infections, UMR 7278CNRS IRD, IFR 48, Aix Marseille University, Marseille, France.

⁹Uniformed Services University of the Health Sciences, Bethesda, Maryland.

¹⁰Evolutionary Ecology Group, University of Antwerp, Antwerpen, Belgium.

species of fleas have been found to be naturally or experimentally infected with *R. typhi*. In addition, other arthropods (lice and mites) have been found to be infected with *R. typhi* (Azad 1990). Murine typhus is endemic to Kenya, but at a low level, especially in rural areas (Heisch and Harvey 1959, Maina et al. 2012). During the early 1960s it was reported that of batches of the fleas *Ctenocephalimus cabirus*, *Dinopsyllus lyppus*, and *X. cheopis* collected from wild rodents only *X. cheopis* were infected with *R. typhi* (Heisch et al. 1962), although in experimental infections both *X. cheopis* and *Xenopsylla braziliensis* could be infected equally well with *R. typhi* in the laboratory (Heisch 1969). More recently a serosurvey conducted in western Kenya showed that of a randomly selected subset of stored sera ($n=357$) collected from January, 2007, through October, 2008, from patients who presented to a rural health clinic with respiratory illness, jaundice, and acute febrile illness with or without a clinically apparent cause, 5 (1.4%) of the serum specimens contained immunoglobulin G (IgG) antibodies that reacted against *R. typhi*, as determined by an indirect fluorescence antibody assay (IFA) (Maina et al. 2012). This low prevalence of antibodies reactive to typhus group rickettsiae corroborates the earlier assertion by Heisch and Harvey (1959) that murine typhus is rare in Kenya, especially in rural areas.

A second rickettsia associated with fleas, *Rickettsia felis*, was discovered in *Ctenocephalides felis* during the 1990s (Adams et al. 1990, Azad et al. 1992). *R. felis*, initially characterized as a TG rickettsia because of its reactivity to guinea pig antiserum against *R. typhi*, was subsequently placed in the spotted fever group (SFG) of rickettsiae due to presence of *ompA* and phylogenetic studies. More recently, some have further separated *R. felis* from both the TG and SFG rickettsiae into a transition group of rickettsiae (Gillespie et al. 2007). It was successfully cultivated in the XTC-2 cell line derived from *Xenopus laevis* and fully described in 2001 (Raoult et al. 2001). Early on, *R. felis* was determined to be a human pathogen (Shriefer et al. 1994) causing flea-borne spotted fever (FBSF) that now is frequently diagnosed around the world, especially in sub-Saharan Africa (Parola 2011). Two recent reports from our group have shown FBSF due to infection with *R. felis* to be endemic to both the northeastern and western regions of Kenya (Richards et al. 2010, Maina et al. 2012). Risk factors of flea-borne rickettsioses in humans have not been fully characterized in those areas.

To identify the potential causative agent(s) of human flea-borne rickettsioses, we surveyed fleas collected from domestic and peridomestic animals and village homes for rickettsial agents. *R. felis* and a new rickettsia, *Candidatus Rickettsia asemboensis*, were identified among the fleas collected in Asembo, western Kenya.

Materials & Methods

Study site

The study was conducted in the Asembo division of Rarieda District, Nyanza Province in western Kenya. This rural site on the eastern shore of Lake Victoria falls within a health and demographic surveillance system (HDSS) run by the Kenya Medical Research Institute (KEMRI) and US Centers for Disease Control and Prevention (CDC) (Adazu et al. 2005). The Kenyan International Emerging Infections Program of KEMRI/CDC has conducted population-based infectious

disease surveillance (PBIDS) of the human population in Asembo since late 2005 (Feikin et al. 2011). The PBIDS population is approximately 25,000 people living in 33 villages. The Asembo area is approximately 225 km² and is culturally homogeneous, with 95% of people being ethnically Luo. It has a bimodal rainfall pattern, with rain seasons occurring from March to May and from September to November. The community lives in dispersed settlements, and houses are made of mud, cement, or brick, with roofs of iron sheet or thatch (Bigogo et al. 2010). The primary economic livelihood is subsistence farming and fishing. According to the demographic surveillance system (DSS) 2007/2008 census, the mean number of animals per compound was 2.6 cattle, 3.3 small ruminants, and 11 poultry (KEMRI/CDC, unpublished data).

Collection of fleas from domestic and peridomestic animals and human households

Three hundred livestock-owning compounds (LOC) were randomly selected from all known LOCs ($n=4528$) among the 33 PBIDS villages from January through May, 2009. The sampling frame of LOCs was compiled from livestock census data collected by the HDSS. A LOC was defined by ownership of 1 or more of the following animals—cattle, sheep, goats, and chickens. Where present, a maximum of 3 dogs and cats in each compound were surveyed for fleas. Fleas were also collected from 16 randomly selected houses from the 300 LOCs using light traps. The light traps were homemade and consisted of a hurricane lamp that was suspended a few inches above a tray containing water with dish soap and Vaseline smeared on the sides to prevent fleas from crawling out.

Additionally, peridomestic small mammal sampling was conducted in collaboration with the National Museums of Kenya using baited Sherman live traps over the period July 30 to August 7, 2009. Fifty compounds were randomly selected from the 300 LOCs. Informed consent for participation was obtained from household representatives prior to collection of animals. Five traps were placed within dwellings, in out-buildings, and outdoors in each compound for 4 consecutive nights, and the GPS locations of key points were recorded. Captured animals were euthanized using an overdose of halothane inhalant anesthesia (Rhodia Limited, Avonmouth, Bristol, BS119YF, UK). Fleas were collected from the animals by combing. Animal species identification was made on the basis of morphometric data at the National Museums of Kenya, where all specimens were submitted for archiving.

All fleas were preserved in 70% ethanol until delivery to the laboratory and then stored at -80°C . Fleas were identified using the entomological taxonomic keys of Segerman (1995), then pooled by individual host animal, method of collection, and flea species (49.3% of pools contained a single flea, the remainder contained 2–14 fleas per pool).

DNA extraction and rickettsiae detection from fleas

Fleas were washed in molecular-grade water and mechanically disrupted using a bead mill (QIAGEN TissueLyser LT, Valencia, CA). Genomic DNA was extracted using QIAamp blood and tissue kits (QIAGEN) according to the manufacturer's instructions, using a final elution volume of 50 μL .

A qPCR assay that amplifies and detects a 74-bp segment of the citrate synthase (*gltA*) gene (Stenos et al. 2005) was used to

screen flea specimens for the presence of rickettsial DNA. A second *Rickettsia* genus-specific qPCR assay that amplifies and detects a 115-bp segment of the 17-kD antigen gene (Rick17b) (Jiang et al. 2012) was used to confirm the results.

Identification of rickettsiae in fleas by species-specific qPCR assays

To identify which rickettsiae were present in the rickettsia-positive flea pools, DNA samples were subsequently tested by 2 species-specific qPCR assays: (1) The *Rfelis* qPCR assay, in which a 129-bp fragment of the *R. felis ompB* was amplified (Henry et al. 2007), and (2) the *Rtyph* qPCR assay, in which a 122-bp fragment of the *R. typhi ompB* was amplified (Henry et al. 2007).

Confirmation of rickettsial species and identification of new rickettsiae by multilocus sequence typing

To confirm the identity of the flea-borne rickettsiae, PCR and sequencing were performed on a subset of selected samples using the 17-kD antigen gene, *ompB* and *R. felis* plasmid genes pRF and pRF δ . At least one of these positive samples was randomly selected from each of the different arthropod vectors, hosts, and method of collection.

To identify novel rickettsiae, we conducted multilocus sequence typing (MLST) using segments of 5 rickettsial genes, *rrs*, *gltA*, *ompB*, *ompA*, and *sca4* (Fournier et al. 2003). Standard and nested PCR were carried out using primers and procedures previously described (Roux and Raoult 2000, Jiang et al. 2005) and new primers listed in Table 1. No positive controls were used in these PCR and nested PCR procedures to decrease chances of contamination; however, a negative control (molecular biology-grade water; Life Technologies) was run with the samples, and they were consistently negative in all runs.

PCR products were purified using a QIAquick PCR purification kit (QIAGEN). Sequencing reactions were performed for both DNA strands using a Big Dye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Life Technologies) according to the manufacturer's instructions. After purification of sequenced products using Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD), sequencing was run in an ABI Prism 3130xl Genetic Analyzer (Life Technologies). Sequences were assembled with Vector NTI Advance 11 software (Life Technologies), and BLAST searches

were performed on the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/>).

Phylogenetic analyses were performed using MEGA version 5 (Tamura et al. 2011) based on the multialignment of *rrs*, *gltA*, *ompA*, *ompB*, and *sca4* sequences from the flea isolates and other rickettsial isolates from GenBank. The phylogenetic trees were constructed using the neighbor-joining method, and the bootstrap analyses were performed with 1000 replications.

Development of the agent-specific qPCR assay (Rasemb) for the new flea-borne rickettsia, *Candidatus R. aseboensis*

A 4311-bp fragment of *ompB* amplified from *Candidatus R. aseboensis* was aligned with the *ompB* from 28 different *Rickettsia* species obtained from GenBank using MEGA version 5; a unique 24-bp sequence fragment was identified to be the target of the probe. To insure the specificity of the assay, the probe Rasem2893BP (6-FAM-CCGCAGCTCCAATACCTTCGCCTAAGCCATATGCGG-BHQ-1) was designed as a molecular beacon with the first and the last 6-bp sequences as the stem. The primers Rasem2828F (5'-CACACTTAGCGGCGGTATTC) and Rasem2939R (5'-AAGTTGTTATAGTCTGTAGTAAACG) amplified a 112-bp fragment of *ompB*.

The concentrations of the primers, the probe, and MgCl₂, were optimized, as well as the annealing temperature using Platinum Quantitative PCR SuperMix-UDG (Life Technologies), and run on a StepOnePlus Real-Time PCR platform (Life Technologies). Each 25- μ L reaction contained 0.7 μ M of each forward and reverse primer, 0.3 μ M of probe, and 7 mM of MgCl₂. Two microliters of template DNA was used in each reaction. The cycle parameters included incubation for 2 min at 50°C, initial denaturation for 2 min at 95°C and 45 cycles of denaturation for 5 s at 95°C, and annealing/elongation for 30 s at 59°C.

The specificity of Rasemb assay was confirmed by testing a panel of bacterial nucleic acid preparations (Jiang et al. 2012) representing genetically closely and distantly related bacteria, including *R. felis* URRWXC2 and 19 other *Rickettsia* species and 12 nonrickettsial bacteria.

GenBank accession numbers

The sequences of the new rickettsial molecular isolates (F30, obtained from *Ctenocephalides canis*; F82, obtained from *C. felis*) have been deposited in GenBank with accession numbers JN315967 to JN315972 and JN315973 to JN315977 for *rrs*, *gltA*, 17-kD antigen, *sca4*, *ompA*, and *ompB*, respectively.

Ethical review

The collection of arthropod specimens from human dwellings and animals was approved by the KEMRI and CDC Animal Care and Use Committees (protocols nos. 1191 and 1562BRETBDX, respectively). Written informed consent was obtained from all animal owners before specimen collection.

Results

Vertebrate hosts and residences surveyed:

Of the 300 LOCs identified, 149 and 26 compounds owned at least 1 dog or a cat, respectively. Fleas were searched for on

TABLE 1. NEW PRIMERS USED FOR PCR AND SEQUENCING

Gene	Primer	Sequence (5' to 3')
<i>rrs</i>	16SU17F	AGAGTTTGATCCTGGCTCAG
	16sR34F	CAGAACGAACGCTATCGGTA
	16SU1592R	AGGAGGTRATCCAGCCGCA
	16sOR1198R	TTCCTATAGTTCCCCGGCATT
17 kD	R17k31F	GCTCTTGACGCTTCTATGTTACA
	R17k469R	ACTTGCCATTGTCCGTCAGGTTG
	Rf17k2608RN	CATTGTCCGTCAGGTTGGCG
<i>ompA</i>	RompAM50F	TTCGCTTATAACACTTTTTAAAGTGA
	RompA642R	ATTACCTATTGTTCCGTTAATGGCA
<i>ompB</i>	ompB1570R	TCGCCGGTAATTRTAGCACT
	Rf1524R	CACACCCGCAGTATTACCGTT

299 domestic dogs and 26 cats from a total of 149 compounds. Thirty-nine (17%) dogs and 9 (34.6%) cats were found to be infested with fleas. Other domestic animals noted but not surveyed for fleas at the 300 LOC included cattle ($n=463$), goats ($n=378$), sheep ($n=159$), and chickens ($n=760$).

One or more peridomestic small mammals were trapped at 27 of the 50 compounds (54%). Fifty-five rodents and shrews were trapped from a total of 1142 trap placements (4.8%) and 18 (32.7%) were found to host fleas. The 55 peridomestic mammals were identified as *Crocidura oliveiri* ($n=20$), *Rattus rattus* ($n=17$), *Mastomys natalensis* ($n=15$), *Lemniscomys striatus* ($n=2$), and *Mus minutoides* ($n=1$).

All of the 16 randomly selected LOC dwellings were infested with fleas. A total of 88 fleas were trapped, with an average of 5.5 fleas/home (range 1–26 fleas) during a single night's collection using a single light trap/household.

Invertebrate hosts and rickettsia detection

A total of 330 fleas were collected and identified as 5 flea species: *X. cheopis* ($n=77$), *C. canis* ($n=7$), *C. felis* ($n=193$), *Echidnophaga gallinacea* ($n=45$), and *Pulex irritans* ($n=8$). DNA preparations were obtained from 134 flea pools, of which 66 (49.3%) consisted of a single individual flea. Overall 89 of 134 (66.4%) flea pools were positive for rickettsiae by using *gltA*, Rick17b, and Rfelis qPCR assays, which included all 4 (100%) *C. canis* pools, 75 (100%) *C. felis* pools, 8 (22.9%) *X. cheopis* pools, 1(20%) *P. irritans* pool, and 1(6.7%) *E. gallinacea* pool (Table 2). Seventy-eight of the 89 rickettsia-positive pools were tested by the *R. typhi* qPCR assay, and all of those pools were negative.

Confirmation of rickettsial identification by 17-kD antigen gene, ompB, pRF, and pRF δ PCR sequencing

To confirm the identity of the rickettsiae, 12 of the 89 rickettsia-positive samples were selected and assessed by PCR

and sequencing of 17-kD antigen gene and/or *ompB*, as well as the plasmid genes pRF and pRF δ . A 390-bp fragment of 17-kD antigen gene and/or a 599-bp fragment of *ompB* were amplified from all 12 samples. The sequences of 17 kD and *ompB* from 1 of the 12 samples was 100% identical to *R. felis* URRWXCal2 (GenBank accession no. CP000053). The *R. felis*-positive sample was further tested for the presence of plasmids. The plasmid pRF, but not the pRF δ , was detected by PCR in this sample. The 429-bp pRF amplicon was sequenced and was determined to be 100% identical to that of *R. felis* URRWXCal2. The sequences from the other 11 samples were identical to each other for both genes. BLAST searches revealed a unique sequence of *ompB* that was different from all rickettsial *ompB* sequences in GenBank. However, the 371-bp fragment of the 17-kD gene sequence was identical (100%) to *Rickettsia* sp. SE313 isolated from *E. gallinacea* in Egypt. The plasmids pRF and pRF δ were not detected in any of these 11 samples.

MLST results (*rrs*, *gltA*, *ompB*, *ompA*, *sca4*)

To determine the identification of and to more fully characterize the novel flea-borne rickettsial agent, sequences for *rrs*, *gltA*, *ompA*, *ompB*, and *sca4*, from 6 of the 11 novel rickettsia-positive samples were determined and compared to those available on GenBank. The sequences of the 1395-bp fragment of *rrs* and 1130-bp of *gltA* from the new isolate were closely related to *R. felis* URRWXCal2 (99.5% and 98.0%, respectively) and *R. sp.* RF2125 (99.6% for *gltA* only), indicating that this new isolate belongs within the genus *Rickettsia*. A 1517-bp fragment of *ompA* (3' end) was amplified, providing evidence that the new molecular isolate is a member of the SFG of rickettsiae. The fragment of the 5' end of *ompA* was not amplified by a SFG universal primer set, which suggests that the gene might be truncated or the sequence was not recognized by 1 or both of the primers. The sequence of the 1517-bp fragment of *ompA* was most similar to *R. felis* URRWXCal2 (92.3%). The 4311-bp fragment of *ompB* sequence from the new agent was most similar to *R. felis* URRWXCal2 (94.8%), although the sequence of a 790-bp fragment was most similar to an *ompB* sequence (P. Parola, unpubl. data; GenBank #JX183538) from *Rickettsia* RF2125 (99.7%), suggesting a very close relationship between these 2 new flea-borne rickettsiae. The sequence of a 1034-bp fragment of *sca4* from the novel rickettsia was most similar to *R. felis* URRWXCal2 (95.5%). Because of the nucleotide dissimilarity between this new agent and the most homologous validated species *R. felis*, this novel agent should be considered for further studies to characterize it as a new *Rickettsia* species. Therefore, we propose this isolate be identified as *Candidatus R. asemboensis* until it is grown in culture and its biology is described more completely.

Phylogenetic relationships between *Candidatus R. asemboensis* and other validated rickettsial species were analyzed by constructing phylogenetic trees using *rrs* (1395 bp), *gltA* (1130 bp), *ompA* (1517 bp), *ompB* (1481 bp), and *sca4* (1034 bp). *Candidatus R. asemboensis* was either clustered with or placed closely to *R. felis* URRWXCal2 for all 5 genes (Fig. 1). *Candidatus R. asemboensis* closely related to *R. felis* matched up with the results from the blast searches.

Development of the Rasemb qPCR assay

In determining the specificity of the Rasemb qPCR assay, it was found that only the 6 MLST-identified *Candidatus*

TABLE 2. RICKETTSIA qPCR RESULTS BY FLEA SPECIES

	Number of pools	Number of positive <i>gltA</i> and Rick17 qPCR	Percent positive
Dogs ($n=39$ dogs) ^a			
<i>Ctenocephalides felis</i>	58	58	100
<i>Echidnophaga gallinacea</i>	3	0	0
Cats ($n=9$ cats)			
<i>Ctenocephalides canis</i>	1	1	100
<i>Ctenocephalides felis</i>	5	5	100
<i>Echidnophaga gallinacea</i>	8	1	12.5
Rodents ($n=18$ rodents)			
<i>Echidnophaga gallinacea</i>	2	0	0
<i>Xenopsylla cheopis</i>	26	7	26.9
Light trap ($n=16$ households)			
<i>Ctenocephalides canis</i>	3	3	100
<i>Ctenocephalides felis</i>	12	12	100
<i>Echidnophaga gallinacea</i>	2	0	0
<i>Pulex irritans</i>	5	1	20
<i>Xenopsylla cheopis</i>	9	1	11.1
Total	134	89	66.4

^aFor each species, n represents the number of animals or houses.

R. asemboensis DNA preparations were positive, whereas *R. felis* and 19 near-neighbor rickettsial DNA preparations (Jiang et al. 2012) were negative.

Subsequently the Rasemb qPCR assay was used to assess all 134 DNA preparations. All 45 of the rickettsia-negative preparations were also negative for the Rasemb qPCR assay. Of the 89 rickettsia-positive samples, 81 samples (which include the 6 MLST-confirmed *Candidatus R. asemboensis* DNA preparations and 2 pools that had both *Candidatus R. asemboensis* and *R. felis*) were positive (Table 3) and 8 were negative. The identities of the 8 negative samples were determined by PCR and sequencing a 599-bp fragment of *ompB*. Seven of the 8 were determined to be 100% identical to *R. felis*. One sample's *ompB* sequence (from a DNA preparation of F28-a pool of 13 *X. cheopis*), was determined to be a unique rickettsial sequence that had only 98.3% similarity to *R. felis* and 92.0% similarity to *Candidatus R. asemboensis* (GenBank # JX183537). Thus, in addition to our near-neighbor panel of DNA preparations, the Rasemb qPCR assay was shown to be specific to *Candidatus R. asemboensis* using this study's sample of flea DNA preparations.

Discussion

With the recently determined risk of FBSF to citizens of and travelers to western Kenya (Maina et al. 2012), we investigated the breadth, diversity, and prevalence of flea vectors containing *R. felis* in Asembo, because effective mitigation measures would certainly depend upon informed preventive medicine decisions. Of 300 LOCs selected, 149 had 1 or more dogs and cats, vertebrate hosts to *C. felis*, *C. canis*, and *P. irritans*, fleas known to be infected with *R. felis* (Venzal et al. 2006). Due to the ubiquitous nature of peridomestic rodents also known to harbor fleas infected with *R. felis* (Jiang et al. 2006), we included them in our investigation. Therefore, we surveyed from 300 randomly selected LOCs 50 compounds for fleas from domestic and peridomestic mammals that might harbor fleas infected with *R. felis*. From these locations 299 dogs, 26 cats, and 55 rodents were obtained and searched for fleas. In addition, 16 randomly selected residences were assessed for the presence of rickettsia-containing fleas by use of a light trap. Results showed that, 17% dogs (39/299), 34.6% cats (9/26), 32.7% rodents (18/55), and 100% homes (16/16)

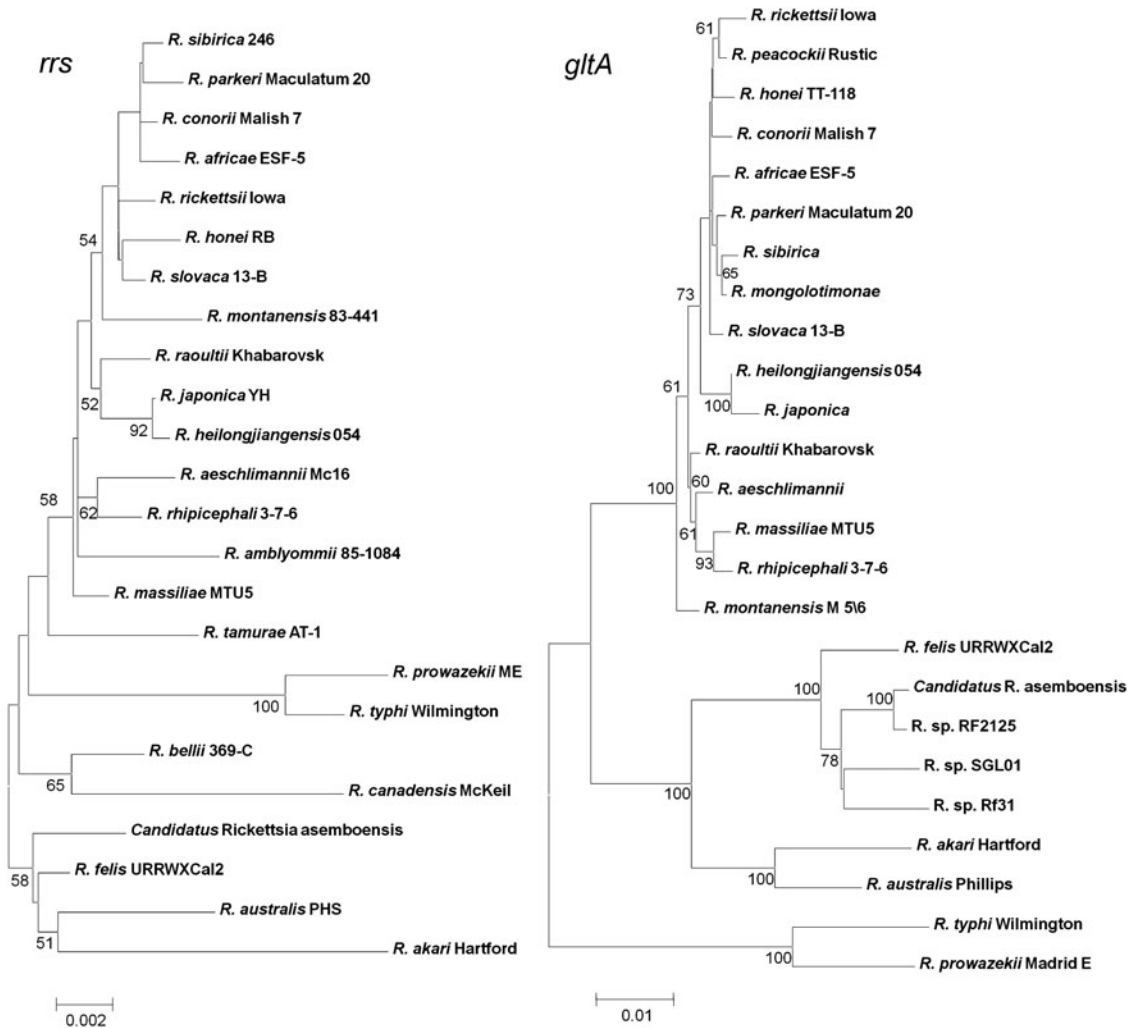


FIG. 1. Phylogenetic trees from 5 genes. Phylogenetic relationships: *Candidatus Rickettsia asemboensis* with other rickettsia; trees were constructed for *rrs* (1395 bp), *gltA* (1130 bp), *ompB* (1481 bp), *ompA* (1517 bp), and *sca4* (1034 bp) using maximum composite likelihood model, neighbor-joining method (MEGA software).

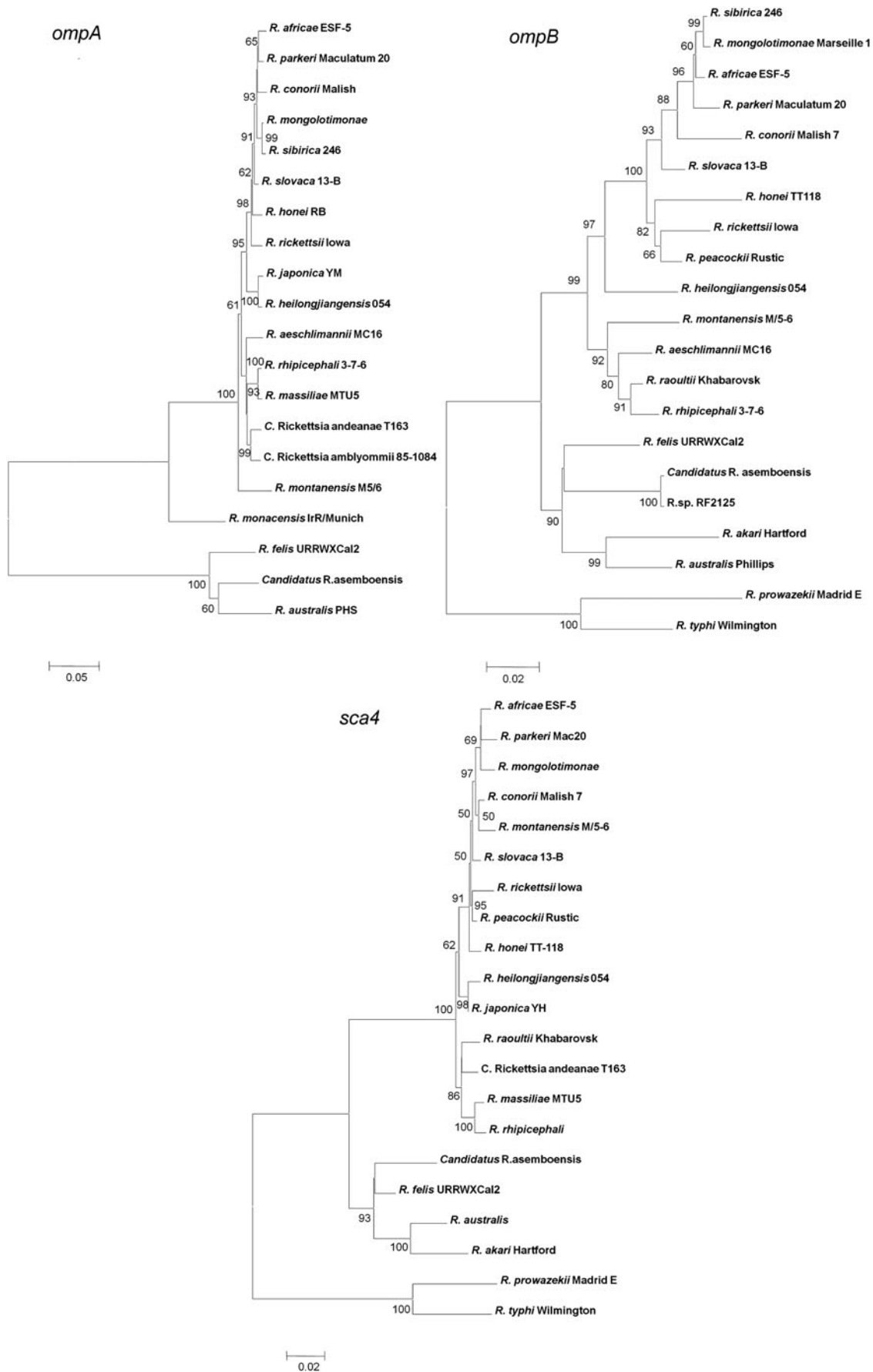


FIG. 1. (Continued)

TABLE 3. *RICKETTSIA* DETECTED IN 5 FLEA SPECIES FROM ASEMO

Flea species	Female				Male				No. pool	Sum Positive %
	No. pool	<i>R. felis</i>	<i>Candidatus R. aseboensis</i>	<i>R. felis and R. aseboensis</i>	No. pool	<i>R. felis</i>	<i>Candidatus R. aseboensis</i>	<i>R. spp F28</i>		
<i>Ctenocephalides canis</i>	1		1		3		3		4	100
<i>Ctenocephalides felis</i>	43		42	1	32	3	29		75	100
<i>Echidnophaga gallinacea</i>	10	1			5				1	6.7
<i>Xenopsylla cheopis</i>	20	2	3	1	15	1		1	8	22.9
<i>Pulex irritans</i>	3				2		1		1	20
Total	77	3	46	2	57	4	33	1	89	66.4

contained arthropod vectors known to transmit flea-borne rickettsiae.

The prevalence of rickettsial infections within the vectors was determined by 2 genus-specific qPCR assays and 1 presumed species-specific qPCR assay, *Rfelis* (Henry et al. 2007). Most notable was the high prevalence of rickettsiae found in dog (100%) and cat (97.3%) fleas (*Ctenocephalides* spp.). When combined with the frequent detection of these species on domestic dogs and cats, and within households, it is clear that the potential for human exposure to rickettsiae in these communities is very high.

When identities of the rickettsiae were confirmed by sequencing small fragments of the 17-kD antigen gene and *ompB* gene, it was determined that of the 12 samples assessed, only 1 was *R. felis*; the other 11 samples contained DNA of novel rickettsial agents. Further characterization by MLST (Fournier et al. 2003) of this unique agent determined that it was most closely related to the validated species *R. felis*. However, it did not have enough nucleotide homology to *R. felis* to be considered to be that species. Therefore, by molecular techniques, this agent was found to represent a new rickettsial species that has some nucleotide similarity to members of a recently described, but poorly characterized, rickettsial *R. felis*-like genotype.

The *R. felis*-like genotype group includes molecular isolates with partial sequences of a few conserved rickettsial genes (17-kD gene, *rrs*, and *gltA*) and have been found not only among various fleas species, but in mites, ladybird (*Coccidulla rufa*), soft ticks (*Ornithodoros moubata*), and tsetse flies (Khaldi et al. 2012, Mediannikov et al. 2012, Roucher et al. 2012). The rickettsiae of this *R. felis*-like genotype group were initially identified in the 2000s and included *Rickettsia* RF2125 and *Rickettsia* RF31, which were detected in *C. canis* and *C. felis* (Parola et al. 2003) following a survey in Kanchanaburi Province, Thailand, near the Myanmar border. *Rickettsia* RF2125 and *Rickettsia* RF31 were distinct from other rickettsiae but most similar to *R. felis* (97.9 and 97.4%, respectively, for *gltA*). Subsequently, Reeves et al. (2005) identified, using molecular techniques, these 2 rickettsiae in *C. felis* collected from animals killed by automobiles in the Piedmont and foothills of South Carolina. A similar agent named *Rickettsia* sp. SE313, similar to RF2125, was also discovered in 12 of 12 *Echidnophaga gallinacea* (sticktight fleas) and in 7 of 120 (5.8%) pools of *Ornithonyssus bacoti* (tropical rat mites) obtained from rats live-trapped in Egypt (Loftis et al. 2006, Reeves et al. 2007). In a survey of ectoparasites and associated pathogens of free-roaming and captive animals in zoos of South Carolina, *Rickettsia* RF2125 was found in *C. felis* removed from a zoo-

keeper and a grizzly bear (Nelder et al. 2009). In addition, *Rickettsia* RF2125 was identified in *P. irritans* (human flea) obtained from a dog in Hungary (Hornok et al. 2010). Most recently, an agent similar to RF2125 (99.7% identical sequence to a 728-bp portion of the conserved *gltA* gene) has been identified in *Synosternus pallidus* fleas from Dielmo village, Senegal (Roucher et al. 2012). Collectively, these references indicate that other flea-borne rickettsiae exist throughout the world and that a much more thorough characterization of these agents by molecular and biological means is needed, especially in regard to pathogenicity.

The high prevalence of *Candidatus R. aseboensis* among fleas known to bite humans and to transmit agents that cause human disease raises the potential that this newly identified rickettsia could be an important cause of human illness. However, in the same area of Asembo where 50 of 699 (7.2%) fever patients were determined to have FBSF, 21 of 21 molecular isolates from the 50 positive blood samples had sequence-confirmed *R. felis* DNA (Maina et al. 2012). Similarly, in a study of non-malaria fever patients, 6 of 163 (3.7%) patients had evidence of FBSF, and all 6 samples were sequence verified to have *R. felis* DNA in the serum samples (Richards et al. 2010). *Candidatus R. aseboensis* was not detected in any patients' samples from those areas. However, before concluding that *Candidatus R. aseboensis* is not pathogenic for humans, screening of specimens from additional febrile patients will be done while ensuring that illnesses from a distribution of age groups and during different months of the year and different years will be needed.

In contrast to the high prevalence of *Candidatus R. aseboensis* in arthropods obtained from animals and human dwellings was the low prevalence of *R. felis* (5.97%), a demonstrated human pathogen, among the same arthropods. This low prevalence of *R. felis* seems contrary to the 7.2% prevalence of FBSF among fever patients in Asembo (Maina et al. 2012) and the 4.4% and 3.7% prevalence among malaria-negative febrile patients in Senegal (Socolovschi et al. 2010) and northeastern Kenya (Richards et al. 2010), but more in line with the sporadic nature of this disease reported from non-sub-Saharan locations (only 68 cases from 20 countries on 6 continents; Parola 2011).

In conclusion, we report our findings that fleas collected from Asembo, Kenya, contain in addition to the rickettsial pathogen *R. felis* (the causative agent of flea-borne spotted fever), a new rickettsia, similar to the previously described *Rickettsia* RF2125 from Thailand, South Carolina, Egypt, Hungary, and Senegal. This new rickettsia, provisionally referred to as *Candidatus R. aseboensis*, is commonly found

within fleas known to vector *R. felis* in western Kenya where flea-borne spotted fever is endemic. Although we did not find evidence of human infection with this new rickettsia, more testing on human specimens will be needed to determine whether the organism is pathogenic for humans.

Acknowledgments

We would like to acknowledge and thank the data team Allan Audi and Linus Ochieng; the lab team Terryson Yator, Sylvia Omul'o, and Fredrick Ade; and the entire CDC-Kenya Integrated Human and Animal Health Program (IHAHP) field team. We would also like to thank Bernard Risky Agwanda and his team at the National Museums of Kenya for their assistance with small mammal trapping.

This work is supported by the Global Emerging Infections Surveillance and Response System, a Division of the Armed Forces Health Surveillance Center; work unit number 0000188M.0931.001.A0074 and the Wellcome Trust (grant no. 81828).

Author Disclosure Statement

The views expressed in this article are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government. As an employee of the US Government this work was prepared as part of my official duties and therefore under Title 17 USC paragraph 105 copyright protection is not available.

References

- Adams JR, Schmidtman ET, Azad AF. Infection of colonized cat fleas, *Ctenocephalides felis* (Bouché), with a rickettsia-like microorganism. *Am J Trop Med Hyg* 1990; 43:400–409.
- Adazu K, Lindblade KA, Rosen DH, Odhiambo F, Ofware P, Kwach J, et al. Health and demographic surveillance in rural western Kenya: A platform for evaluating interventions to reduce morbidity and mortality from infectious diseases. *Am J Trop Med Hyg* 2005; 73:1151–1158.
- Azad AF. Epidemiology of murine typhus. *Ann Rev Entomol* 1990; 35:553–569.
- Azad AF, Sacchi JB Jr, Nelson WM, Dasch GA, Schmidtman ET, Carl M. Genetic characterization and transovarial transmission of a typhus-like rickettsia in cat fleas. *Proc Natl Acad Sci USA* 1992; 89:43–46.
- Bigogo G, Audi A, Aura B, Aol G, Breiman RF, Feikin DR. Health seeking patterns among participants of population-based morbidity surveillance in rural western Kenya: Implications for calculating disease rates. *Int J Infect Dis* 2010; 14:967–973.
- Feikin DR, Olack B, Bigogo GM, Audi A, Cosmas L, Aura B, et al. The burden of common infectious disease syndromes at the clinic and household level from population-based surveillance in rural and urban Kenya. *PLoS One* 2011; 6:e16085.
- Fournier PE, Dumler S, Greub G, Zhang J, Wu Y, Raoult D. Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. Nov. *J Clin Microbiol*. 2003; 41:5456–5465.
- Gillespie JJ, Beier MS, Rahman MS, Ammerman NC, Shallom JM, Purkayastha A, et al. Plasmids and rickettsial evolution; insight from *Rickettsia felis*. *PLoS One* 2007; 2:e266.
- Heisch RB. Urban *Rattus* as the main reservoir of murine typhus in Kenya. *J Trop Med Hyg* 1969; 72:195–196.
- Heisch RB, Harvey AEC. Rickettsioses in Kenya: Serological reactions of wild rodents and inoculated guinea pigs. *East Afr Med* 1959; 36:116–118.
- Heisch RB, Grainger WE, Harvey AEC, Lister G. Feral aspects of rickettsial infections in Kenya. *Trans R Soc Trop Med Hyg* 1962; 56:272–286.
- Henry KM, Jiang J, Rozmajzl PJ, Azad AF, Macaluso KR, Richards AL. Development of quantitative real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of murine typhus and flea-borne spotted fever. *Mol Cell Probes* 2007; 21:17–23.
- Hornok S, Meli ML, Perreten A, Farkas R, Willi B, Beugnet F, et al. Molecular investigation of hard ticks (Acari: Ixodidae) and fleas (Siphonaptera: Pulicidae) as potential vectors of rickettsial and mycoplasmal agents. *Vet Microbiol* 2010; 140:98–104.
- Jiang J, Blair PJ, Felices V, Moron C, Cespedes M, Anaya E, Schoeler GB, Sumner JW, Olson JG, Richards AL. Phylogenetic analysis of a novel molecular isolate of spotted fever group rickettsiae from northern Peru: *Candidatus Rickettsia andeanae*. *Ann NY Acad Sci* 2005; 1063:337–342.
- Jiang J, Soeatmadji DW, Henry KM, Ratiwayanto S, Bangs MJ, Richards AL. *Rickettsia felis* in *Xenopsylla cheopis*, Java, Indonesia. *Emerg Infect Dis* 2006; 12:1281–1283.
- Jiang J, Stromdahl EY, Miller M, Richards AL. Detection of *Rickettsia parkeri* and *Rickettsia andeanae* in *Amblyomma maculatum* Gulf Coast ticks collected from humans, USA. *Vector Borne Zoonotic Dis* 2012; 12:175–182.
- Kelly DJ, Richards AL, Temenak JJ, Strickman D, Dasch GA. The past and present threat of rickettsial diseases to military medicine and international public health. *Clin Infect Dis* 2002; 34(Suppl 4):s145–s169.
- Khaldi M, Socolovschi C, Benyettou M, Barech G, Biche M, Kernif T, Raoult D, Parola P. Rickettsiae in arthropods collected from the North African Hedgehog (*Atelerix algirus*) and the desert hedgehog (*Paraechinus aethiopicus*) in Algeria. *Comp Immunol Microbiol Infect Dis* 2012; 35: 117–122.
- Loftis AD, Reeves WK, Szumlas DE, Abbassy MM, Helmy IM, Moriarity JR, et al. Surveillance of Egyptian fleas for agents of public health significance: *Anaplasma*, *Bartonella*, *Coxiella*, *Ehrlichia*, *Rickettsia*, and *Yersinia pestis*. *Am J Trop Med Hyg* 2006; 75:41–48.
- Maina A, Knobel D, Jiang J, Halliday JEB, Feikin DR, Cleveland S, et al. *Rickettsia felis* infection in febrile patients western Kenya, 2007–2010. *Emerg Infect Dis* 2012; 18:328–331.
- Mediannikov O, Audoly G, Diatta G, Trape JF, Raoult D. New *Rickettsia* sp. in tsetse flies from Senegal. *Comp Immunol Microbiol Infect Dis* 2012; 35:145–150.
- Nelder MP, Reeves WK, Adler PH, Wozniak A, Wills W. Ectoparasites and associated pathogens of free-roaming and captive animals in zoos of South Carolina. *Vector Borne Zoonotic Dis* 2009; 9:469–477.
- Parola P. *Rickettsia felis*: From a rare disease in the USA to a common cause of fever in sub-Saharan Africa. *Clin Microbiol Infect* 2011; 17:996–1000.
- Parola P, Sanogo OY, Lerdthusnee K, Zeaiter Z, Chauvancy G, Gonzalez JP, et al. Identification of *Rickettsia* spp. and *Bartonella* spp. in fleas from the Thai–Myanmar border. *Ann NY Acad Sci* 2003; 990:173–181.
- Raoult D, La Scola B, Enea M, Fournier PE, Roux V, Fenollar F, et al. A flea-associated rickettsia pathogenic for humans. *Emerg Infect Dis* 2001; 7:73–81.

- Reeves WK, Nelder MP, Korecki JA. *Bartonella* and *Rickettsia* in fleas and lice from mammals in South Carolina, USA. *J Vector Ecol* 2005; 30:310–315.
- Reeves WK, Loftis AD, Szumlas DE, Abbassy MM, Helmy IM, Hanafi HA, et al. Rickettsial pathogens in the tropical rat mite *Ornithonyssus bacoti* (Acari: Macronyssidae) from Egyptian rats (*Rattus* spp.). *Exp Apply Acarol* 2007; 41:101–107.
- Richards AL, Jiang J, Omulo S, Dare R, Abdirahman K, Ali A, et al. Human infections with *Rickettsia felis*, Kenya. *Emerg Infect Dis* 2010; 16:1081–1086.
- Roucher C, Mediannikov O, Diatta G, Trape JF, Raoult D. A new *Rickettsia* species found in fleas collected from human dwellings and from domestic cats and dogs in Senegal. *Vector Borne Zoonotic Dis* 2012; 12:360–365.
- Roux V, Raoult D. Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer membrane protein rompB (*ompB*). *Int J Syst Evol Microbiol* 2000; 50:1449–1455.
- Schriefer ME, Sacci JBJr, Dumler JS, Bullen MG, Azad AF. Identification of a novel rickettsial infection in a patient diagnosed with murine typhus. *J Clin Microbiol* 1994; 32: 949–954.
- Segerman J. Siphonaptera of southern Africa. In: *Handbook for Identification of Fleas. Publication of the South African Institute of Medical Research*. No 57. Johannesburg, South Africa: South African Institute for Medical Research, 1995.
- Socolovschi C, Mediannikov O, Sokhna C, Tall A, Diatta G, Bassene H, et al. *Rickettsia felis*- associated unruptive fever, Senegal. *Emerg Infect Dis* 2010; 16:1140–1142.
- Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group rickettsiae. *Am J Trop Med Hyg* 2005; 73:1083–1085.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; MEGA5.
- Venzal JM, Perez-Martinez L, Felix ML, Portillo A, Blanco JR, Oteo JA. Prevalence of *Rickettsia felis* in *Ctenocephalides felis* and *Ctenocephalides canis* from Uruguay. *Ann NY Acad Sci* 2006; 1078:305–308.

Address correspondence to:

Allen L. Richards, PhD

Research Coordinator

Rickettsial Diseases Research Program

Viral & Rickettsial Diseases Department

Naval Medical Research Center

503 Robert Grant Avenue

Silver Spring, MD 20910

E-mail: allen.richards@med.navy.mil