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RESEARCH ARTICLE
Host-Microbe Biology

Comparison of Zoonotic Bacterial Agents in Fleas Collected from Small Mammals or Host-Seeking Fleas from a Ugandan Region Where Plague Is Endemic

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ABSTRACT Fleas (n = 407) were collected from small mammals trapped inside huts and surroundings of homesteads in five villages within the Arua and Zombo districts of Uganda. The most common flea species were *Dinopsyllus lypusus* (26%) and *Xenopsylla cheopis* (50%). Off-host fleas (n = 225) were collected inside huts by using Kilonzo flea traps. The majority of the off-host fleas were *Ctenocephalides felis* (80%). All fleas were examined for the presence of *Bartonella* spp., *Rickettsia* spp., and *Yersinia* spp. *Bartonella* DNA was detected in 91 fleas, with an overall prevalence of 14%. *Bartonella* prevalence was significantly higher in rodent or shrew fleas than in off-host fleas (22% versus 1%). The majority of *Bartonella*-positive fleas were of the species *D. lypusus* (61%), *X. cheopis* (20%), and *Ctenophthalmus calceatus* (14%). Sequencing analysis identified 12 *Bartonella* genetic variants, 9 of which belonged to the zoonotic pathogen *B. elizabethae* species complex. *Rickettsia* DNA was detected in 143 fleas, giving an overall prevalence of 23%, with a significantly higher prevalence in off-host fleas than in rodent or shrew fleas (56% versus 4%). The majority (88%) of *Rickettsia*-positive fleas were *C. felis* and were collected from Kilonzo traps, while a small portion (10%) were *X. cheopis* collected from rodents. Sequencing analysis identified six *Rickettsia* genogroups that belonged either to zoonotic *R. felis* or to the closely related "*Candidatus* Ricksettia asemboensis" and "*Candidatus* Rickettia sengalensis." *Yersinia* DNA was not detected in the fleas tested. These observations suggested that fleas in northwestern Uganda commonly carry the zoonotic agents *B. elizabethae* and *R. felis* and potentially play an important role in transmitting these infections to humans.

IMPORTANCE Fleas play critical roles in transmitting some infections among animals and from animals to humans. Detection of pathogens in fleas is important to determine human risks for flea-borne diseases and can help guide diagnosis and treatment. Our findings of high prevalence rates of *B. elizabethae* and *R. felis* in fleas in the Arua and Zombo districts of Uganda implicate these agents as potential causative agents of undiagnosed febrile illnesses in this area.

KEYWORDS *Bartonella*, *Rickettsia*, Uganda, *Yersinia*, fleas, off-host, rodents

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Fleas are well-known vectors that can transmit infectious pathogens to humans, including the etiologic agents of plague, bartonellosis, and rickettsiosis. Plague, caused by the highly pathogenic bacterium *Yersinia pestis*, was responsible for three great pandemics that killed millions of people around the world. Currently, plague is still endemic in Africa, Central Asia, and South and North America. According to WHO, more than 2,500 cases are reported each year from different regions, with nearly 80%

TABLE 1 Occurrence of *Bartonella* spp. and *Rickettsia* spp. in fleas collected from Kilonzo traps and from small mammals, 2002 to 2013, Uganda

Flea species	From Kilonzo traps						From small mammals				
		Bartonella			*Rickettsia*			*Bartonella*		*Rickettsia*	
	No. collected (% of total)	*n*	No. (%) positive	% prevalence	No. (%) positive	% prevalence	*n*	No. (%) positive	% prevalence	No. (%) positive	% prevalence
Ctenocephalides felis	182 (29)	181	1 (33)	0.6	125 (99)	69.1	1	0 (0)	0	1 (6)	100
Ctenophthalmus calceatus	44 (7)	0	0 (0)	NA[a]	0 (0)	NA	44	12 (14)	27	1 (6)	2
Dinopsyllus longifrons	2 (0)	0	0 (0)	NA	0 (0)	NA	2	2 (2)	100	0 (0)	0
Dinopsyllus lypusus	107 (17)	1	0 (0)	0	0 (0)	0	106	54 (61)	51	1 (6)	0
Echinophaga gallinaceae	30 (5)	30	0 (0)	0	0 (0)	0	0	0 (0)	NA	0 (0)	NA
Stivalius torvus	12 (2)	1	1 (33)	100	0 (0)	0	11	1 (1)	9	0 (0)	0
Tunga penetrans	3 (0)	3	0 (0)	0	0 (0)	0	0	0 (0)	NA	0 (0)	NA
Xenopsylla brasiliensis	43 (7)	3	0 (0)	0	0 (0)	0	40	1 (1)	3	0 (0)	0
Xenopsylla cheopis	208 (33)	6	1 (33)	16.7	1 (1)	16.7	202	18 (20)	9	14 (97)	7
Xenopsylla nubica	1 (0)	0	0 (0)	NA	0 (0)	NA	1	0 (0)	0	0 (0)	0
Total	632	225	3	1.3	126	56	407	88	22	17	4

[a]NA, not applicable.

of cases from Africa. Northwestern Uganda, often referred to as the West Nile region, is a region where plague is endemic, with more than 2,400 suspect plague cases reported between 1999 and 2011 (1). Multiple studies on fleas have been conducted in this area (2–5).

The genus *Bartonella*, with more than 30 species identified, has drawn increased attention as more and more reported clinical illnesses have been associated with *Bartonella* infections (6–11). *Rickettsia felis*, the causative agent of flea-borne spotted fever, is considered an important neglected agent in sub-Saharan Africa (12). Both bartonelloses and rickettsioses are widely regarded as emerging/reemerging diseases (13). Many studies have reported *Bartonella* spp. in a variety of mammalian species and their ectoparasites, all over the world (14–20). In the Democratic Republic of Congo, Sackal et al. (21) reported the detection of *Bartonella* spp. and *R. felis* in *Ctenocephalides felis* and *Pulex irritans* collected from flea traps. Billeter et al. (22) identified *Bartonella* species in invasive and indigenous rodents and their fleas from Uganda. Surprisingly, the prevalence of *Bartonella* infections in invasive rats (*Rattus rattus*) reported in that study was very low (1.3%), a finding that stands in stark contrast to results of a study conducted in neighboring Kenya, in which the *Bartonella* prevalence was 13% in *Rattus* spp. (19). In addition, *R. felis* and "*Candidatus* Rickettsia asemboensis" were detected in fleas collected in Kenya, Tanzania, Ethiopia, and Uganda (13, 23–25).

Although *Bartonella* spp. and *Rickettsia* spp. have been reported in countries neighboring Uganda, information regarding the distribution of *Bartonella* spp., *Rickettsia* spp., and *Yersinia* spp. in fleas from Uganda is very limited and requires a better understanding, as each of these agents is likely to cause disease in humans. In this study, we evaluated the prevalence rates and characterized the genetic identities of *Bartonella* spp., *Rickettsia* spp., and *Yersinia* spp. in fleas collected from the West Nile region of Uganda.

RESULTS

Fleas. A total of 632 fleas belonging to 10 species were collected (Table 1). Of these, 407 fleas were collected from eight species of rodents (*Aethomys hindei*, *Aethomys kaiseri*, *Arvicanthis niloticus*, *Lophuromys sikapusi*, *Mastomys* spp., *Mus* spp., *Rattus rattus*, and *Tatera valida*) and from shrews (*Crocidura* spp.) (Table 2). The fleas belonged to eight species, including *Ctenocephalides felis* ($n = 1$), *Ctenophthalmus calceatus* ($n = 44$), *Dinopsyllus longifrons* ($n = 2$), *D. lypusus* ($n = 106$), *Stivalius torvus* ($n = 11$), *Xenopsylla brasiliensis* ($n = 40$), *X. cheopis* ($n = 202$), and *Xenopsylla nubica* ($n = 1$) (Table 1). The other 225 fleas were collected from Kilonzo traps and belonged to seven species, including *C. felis* ($n = 181$), *D. lypusus* ($n = 1$), *Echinophaga gallinacea* ($n = 30$), *S. torvus* ($n = 1$), *Tunga penetrans* ($n = 3$), *X. brasiliensis* ($n = 3$), and *X. cheopis* ($n = 6$) (Table 1).

TABLE 2 Flea species found on small mammals and detection of *Bartonella* spp. in the fleas

Order and species of small mammal	No. of fleas found (no. positive for *Bartonella*)						
	Total	*C. cabirus*	*D. lypusus*	*S. torvus*	*X. brasiliensis*	*X. cheopis*	Other species[a]
Order *Rodentia*							
Aethomys hindei	9 (6)		1 (1)			8 (5)	
Aethomys kaiseri	4 (2)		2 (1)			2 (1)	
Arvicanthis niloticus	72 (32)	28 (10)	35 (21)		2	6 (1)	1
Lophuromys sikapusi	3 (1)	3 (1)					
Mastomys natalensis	44 (26)	4	25 (17)	1	1 (1)	11 (6)	2 (2)
Mus spp.	1 (0)			1 (0)			
Rattus rattus	208 (10)	2	19 (6)		37	148 (3)	2 (1)
Tatera valida	9 (6)		8 (6)			1	
Order *Insectivora*							
Crocidura spp.	57 (5)	5	16 (2)	9 (1)		26 (2)	1
Total	407 (88)	42 (11)	106 (54)	11 (1)	40 (1)	202 (18)	6 (3)

[a]The other species included *C. felis*, *C. bacopus*, *D. longifrons*, and *X. nubica*. These were combined due to the very small number of each species collected.

Among all tested fleas, *X. cheopis*, *C. felis*, and *D. lypusus* were the most common species, accounting for 33% (208/632), 29% (182/632), and 17% (107/632), respectively. Both *X. cheopis* and *D. lypusus* fleas were mainly collected from small mammals (97% [202/208] and 99% [106/107], respectively) rather than from Kilonzo traps (3% [6/208] and 1% [1/107], respectively). The majority of *X. cheopis* fleas (73% [148/202]) were collected from *R. rattus*, while the *D. lypusus* fleas were mainly collected from *A. niloticus* (33% [35/106]), *Mastomys natalensis* (24% [25/106]), *R. rattus* (18% [19/106]), and *Crocidura* spp. (15% [16/106]). All but one *C. felis* flea (181 of 182) were collected from Kilonzo traps. All *C. calceatus* fleas ($n = 44$) were collected from multiple species of rodents, and all *E. gallinacea* fleas ($n = 30$) were collected from Kilonzo traps (Table 1).

***Bartonella* infections.** *Bartonella* DNA was detected in 91 of the 632 tested fleas, with an overall prevalence of 14%. Among the positive fleas, 88 (97%) were collected from small mammals; only 3 were collected from Kilonzo traps. The *Bartonella* prevalence was 22% (88/407) in fleas collected from small mammals and 1% (3/225) in fleas collected from Kilonzo traps, showing a significant difference between the two sources ($\chi^2 = 38.4$, $P < 0.01$). The *Bartonella*-infected fleas belonged to eight species (Table 1). Compared to other flea species, *D. lypusus* and *C. calceatus* fleas had a higher rate of *Bartonella* infection, with 51% (54/107) and 27% (12/44) of individuals infected, respectively. Of the most prevalent flea species found on rodents, *X. cheopis*, only 9% (18/202) of fleas of this species were found to be infected with *Bartonella* spp. Interestingly, only 3 of the 18 positive *X. cheopis* fleas were collected from black rats, which were the main hosts of *X. cheopis* in this study (Table 2). In other words, only 3 of 148 *X. cheopis* fleas (2%) collected from black rats were infected with *Bartonella* spp. *X. brasiliensis* fleas also had a very low infection rate for *Bartonella* species, with only 1 positive flea among 43 tested fleas (2%). The three *Bartonella*-positive fleas that were collected from Kilonzo traps were of the species *C. felis*, *S. torvus*, and *X. cheopis*. Notably, only 1 of 182 *C. felis* fleas was infected with *Bartonella*. None of the 30 *E. gallinacea* fleas, all of which were collected from Kilonzo traps, was infected (Table 1).

***Rickettsia* infections.** *Rickettsia* DNA was detected in 143 of the 632 tested fleas, giving an overall prevalence of 23%. Nearly all of the *Rickettsia*-infected fleas were of the species *C. felis* (88%; 126/143) or *X. cheopis* (10%; 14/143) (Table 1). In contrast to *Bartonella*-infected fleas, the majority (88%; 126/143) of *Rickettsia*-positive fleas were collected from Kilonzo traps. Only 17 of 173 *Rickettsia*-positive fleas (12%) were collected from rodents. The prevalence of *Rickettsia* in off-host fleas was 56% (126/225), whereas it was 4% (17/407) in fleas collected from rodents. The prevalence of *Rickettsia* in off-host fleas was significantly higher than that in fleas collected from rodents ($\chi^2 = 129.9$, $P < 0.01$). Of the 126 *Rickettsia*-positive fleas that were collected from Kilonzo traps, 125 were *C. felis*. The prevalence of *Rickettsia* in *C. felis* was also extremely high

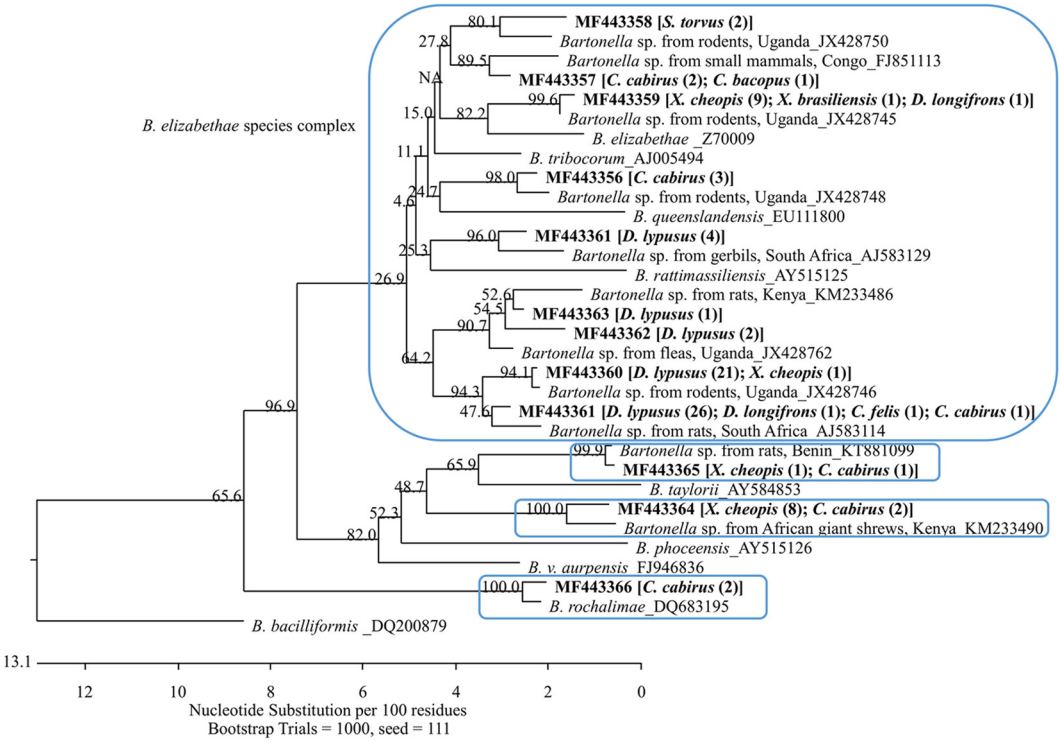

FIG 1 Phylogenetic relationships of the 12 *Bartonella* genetic variants, identified from 91 *Bartonella* sequences obtained from fleas collected from small mammals or Kilonzo traps in the West Nile region of Uganda, with other *Bartonella* species based on partial sequences of *gltA*. Each genetic variant is indicated by its GenBank accession number in boldface, followed by flea species and number of identical sequences obtained from the flea species in brackets and parentheses. The *Bartonella* variants formed four phylogroups (boxed clades). Nine of the 12 variants fell into the so-called *B. elizabethae* species complex. The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were calculated with 1,000 replicates.

(69%; 125/182). Of the 17 *Rickettsia*-positive fleas that were collected from small mammals, 14 were *X. cheopis*. None of the 30 *E. gallinacea* fleas collected from Kilonzo traps harbored *Rickettsia* DNA (Table 1).

***Yersinia* infections.** Neither *Y. pestis* nor *Y. pseudotuberculosis* DNA was detected in any of the fleas tested.

Genetic identification of *Bartonella* and *Rickettsia* species. Sequencing analyses were performed based on *gltA* sequences for both *Bartonella* and *Rickettsia*. The 91 *Bartonella*-positive DNA samples represented 12 distinct genetic variants that have been deposited in GenBank under accession numbers MF443355 to MF443366. These variants were clustered in four major phylogroups (Fig. 1). Nine variants (GenBank accession numbers MF443355 to MF443363) accounted for 77 of the 91 sequences identified, and all of these were relatively close to each other and similar to a few variants previously described in rodents from Uganda (22). These variants are clustered with the so-called *Bartonella elizabethae* species complex (26). Sequences belonging to this phylogroup were obtained from fleas of all species found positive, with the majority (70%; 54/77) coming from *D. lypusus* fleas. In fact, all sequences obtained from *D. lypusus* fleas belonged to this group, showing a specific relationship between this flea species and *Bartonella* species. Bartonella sequences obtained from the three positive fleas that were collected from Kilonzo traps also belonged to this phylogroup; one variant (GenBank accession number MF443364) was similar to variant KM233490, which was described in an African giant shrew (*Crocidura olivieri*) from Kenya (19). This variant contained 10 identical sequences that were obtained from *X. cheopis* ($n = 8$) and *C. calceatus* ($n = 2$) and formed a separate phylogroup. Another variant (GenBank accession number MF443365) was similar to the variant KT881099 from a *Mastomys* sp. rat in Benin (27). This variant contained only two sequences, which were obtained from

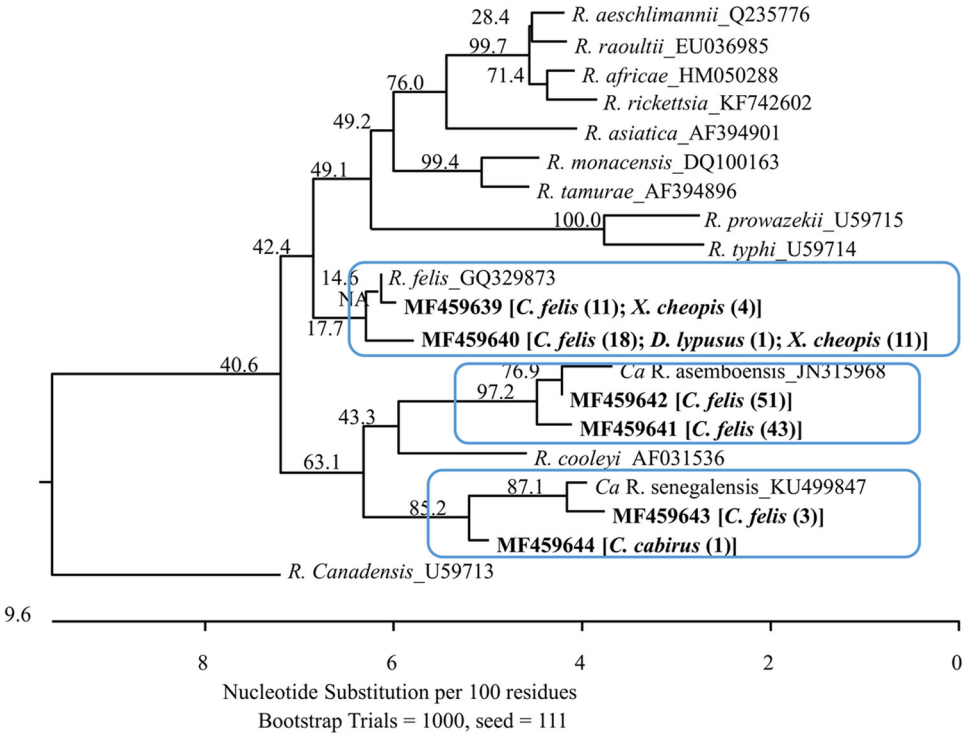

FIG 2 Phylogenetic relationships of the six *Rickettsia* genetic variants identified from 143 *Rickettsia* sequences, obtained from the fleas collected from small mammals or Kilonzo traps in the West Nile region of Uganda, with some other *Rickettsia* species based on partial sequences of *gltA*. Each genetic variant is indicated by its GenBank accession number in boldface, followed by the flea species and number of identical sequences obtained from the flea species in brackets and parentheses. The *Rickettsia* variants formed three phylogroups (boxed clades). Two variants fell into the cluster of *R. felis*; other variants clustered with either "*Candidatus* R. asemboensis" or "*Candidatus* R. sengalensis." The phylogenetic tree was constructed by the neighbor-joining method, and bootstrap values were calculated with 1,000 replicates.

one *C. calceatus* flea (collected from *A. niloticus*) and one *X. cheopis* flea (collected from *R. rattus*), respectively. They are also quite distant from other identified *Bartonella* genotypes and formed a separate phylogroup. The last variant (GenBank accession number MF443366) contained two sequences, both of which were obtained from *C. calceatus* fleas that were collected from African grass rats. This variant was similar to *Bartonella rochalimae* (Fig. 1).

The 143 *Rickettsia*-positive DNA sequences represented six distinct genetic variants and have been deposited in GenBank under accession numbers MF459639 to MF459644. The six variants were clustered into three phylogroups. Variants MF459639 and MF459640, containing 15 and 30 *Rickettsia* sequences, respectively, belonged to the zoonotic pathogen *R. felis*; variants MF459641 and MF459642 were the most common and comprised 43 and 51 of all identified *Rickettsia* sequences, respectively. These two variants were closely related to "*Candidatus* R. asemboensis"; the last two variants, MF459643 and MF459644, containing three and one sequence, respectively, were close to "*Candidatus* Rickettsia sengalensis" (Fig. 2).

DISCUSSION

Using molecular approaches, we estimated the presence of multiple potential zoonotic pathogens, including *Bartonella* spp., *Rickettsia* spp., and *Yersinia* spp., in fleas collected from small mammals and those that were host seeking in human habitations in northwestern Uganda, where plague is endemic and occasionally causes outbreaks in the local human population (28).

Not surprisingly, *Bartonella* spp. were prevalent in the fleas, with an overall prevalence of 14%. The prevalence in fleas collected from small mammals was much higher

than in off-host fleas (22% versus 1%). In fact, two of the three *Bartonella*-positive off-host fleas (*S. torvus* and *X. cheopis*) commonly parasitize small mammals (*Crocidura* sp. and *Rattus* spp. rats, respectively) and are likely to be found only temporarily off their mammal hosts. It is not clear whether these *Bartonella* spp. cause pathogenic damage in their animal hosts; it is less likely these fleas seek blood meals from humans. Given the context of assessing human risk, studies of vector competence by various flea species might be necessary. The off-host fleas were mostly comprised of *C. felis* (80%). In the Democratic Republic of the Congo, Sackal et al. (21) similarly found that only 3.6% of off-host fleas (mainly *C. felis* and *Pulex irritans*) were infected by *Bartonella* spp. The absence of *Bartonella* in the *C. felis* fleas in the present study might be related to their off-host origin. Bartonellae are known hemotropic parasites (29) and might not be able to survive in fleas that fail to take frequent blood meals, and starving fleas might be quickly cleared of any *Bartonella* infection. Another possible explanation is that these *C. felis* fleas may rarely become infected by *Bartonella* because of the low level of infection in their presumptive hosts. As common ectoparasites of cats and dogs, *C. felis* has been reported to carry *B. henselae* (the causative of Cat scratch disease), *B. clarridgeiae*, and other *Bartonella* species (30, 31). In northwestern Uganda, however, *C. felis* feeds primarily on people, goats, and occasionally on chickens, sheep, guinea pigs, or pigs (32). Previous studies have reported that *Bartonella bacterium* was not cultured in goats (33) and was not detected in chickens from Kenya (Y. Bai, unpublished data).

Among the fleas collected from rodents, *D. lypusus* demonstrated an extremely high prevalence (>50%) of *Bartonella* infection, suggesting that this species of flea may play a major role in transmitting *Bartonella* infection between rodents and potentially from rodents to humans. Again, the vector competence should be studied for this flea species. Although the prevalence of *Bartonella* in fleas collected from rodents was relatively high, the prevalence in *X. cheopis* fleas was quite low, with only 9% of this flea species being found infected. Notably, the *X. cheopis* fleas collected from black rats, which are the main hosts of the fleas, showed an even lower prevalence, with only 2% infected. Typically, high percentages (>40%) of *Rattus* spp. animals are infected by *Bartonella* spp. (14, 15). However, Billeter (22) reported that only 2% of *R. rattus* animals were infected with *Bartonella* spp. in another study in Uganda. *R. rattus* invaded the West Nile region of Uganda recently (34) and may have not had ample time to establish a niche in which *Bartonella bacterium* can amplify. The competence of *X. cheopis* is not clear, but field investigations have suggested that *Bartonella* infection is most likely transmitted between rats by *X. cheopis* (35). In our study, the low number of *X. cheopis* fleas that were found to be infected with *Bartonella* spp. might help explain the very low prevalence of this infection in *R. rattus* from the West Nile region, previously reported by Billeter (22).

Sequencing analysis demonstrated that at least four *Bartonella* species or phylogroups are circulating in the flea communities in the region of Uganda where plague is endemic. Most of the *Bartonella* sequences (85%) obtained from the fleas in this study belonged to the *B. elizabethae* species complex (26), and several species and strains of this complex have been linked to human illnesses recently (6–10). Importantly, *B. elizabethae* was found in all flea species that were positive for *Bartonella* in the present study. Our findings suggested that *B. elizabethae* might be the causative agent for undiagnosed illnesses in local residents, and it is recommended that further investigations be conducted to verify whether *B. elizabethae* is indeed a human pathogen in this region of Africa. Remarkably, *D. lypusus* fleas, a very common species found on rodents, were all infected with *B. elizabethae*, showing a very specific relationship between this flea species and the *Bartonella* species. *D. lypusus* is a known vector of *Y. pestis* in Kenya (36). Our results suggest that they may also transmit *B. elizabethae* to humans in this region. *B. rochalimae*, another human pathogen (11) which has been also reported in carnivorous species and their fleas (*Pulex simulans*) (37–39) and in rodents (40), was also detected in two fleas in this study, suggesting that the presence of this bacterium in local flea communities should not be ignored.

The other two *Bartonella* species or phylogroups (variants MF443364 and MF443365)

were found in small numbers of fleas. Interestingly, variant MF443364 was quite different from other identified genotypes in our study but was similar to the variant KM233490 previously described in an African giant shrew (*Crocidura olivieri*) from Kenya (12). This variant was found in 10 fleas which were collected from rodents (*A. niloticus*, *Mastomys* spp., and *R. rattus*) but not from shrews (*Crocidura* spp.) in the current study. Although *Bartonella* DNA was detected in fleas collected from four *Crocidura* in the present study, all of the *Bartonella* genotypes belonged to the *B. elizabethae* species complex. Further investigations are needed to illustrate the relationship between shrews and *Bartonella* spp. in Africa, but it should be noted that we have found shrews infested with *X. cheopis*, *X. brasiliensis*, *D. lypusus*, and *C. calceatus* fleas, providing a possible means for these animals to become infected with *B. elizabethae*-like bartonellae.

Like many previous reports, we found that *Rickettsia* infection is commonly circulating in the local flea community, with an overall prevalence of 23%. In contrast to *Bartonella*, *Rickettsia*-infected fleas were mainly *C. felis* fleas collected from Kilonzo traps. Only a very small portion of *Rickettsia*-infected fleas was obtained from rodents. Such results suggest that *Rickettsia* infections in the West Nile region are carried primarily by *C. felis* fleas.

Sequencing analysis demonstrated three *Rickettsia* species were circulating within the local flea community in the region of Uganda where plague is endemic. *R. felis*, a frequently identified zoonotic pathogen that causes cat-flea typhus in humans, was commonly detected in the *C. felis* fleas we tested. Due to its worldwide distribution, *R. felis* infection has been considered an emergent threat to human health (13). Because *C. felis* fleas in Uganda commonly feed on humans in the West Nile region and *C. felis* is a well-known competent vector of *R. felis* (41), our results suggest that humans might be exposed to cat flea-transmitted rickettsioses caused by *R. felis* or another closely related rickettsial species. The other two species identified in this study were "*Candidatus* R. asemboensis" and "*Candidatus* R. sengalensis." In fact, "*Candidatus* R. asemboensis" was the most common species detected in the study. Although it is not clear whether this species can cause disease in humans or animals, further investigations are needed regarding its wide distribution. The number of *E. gallinacea* fleas collected from Kilonzo traps was the second largest, after the number of *C. felis* fleas taken from these traps. Interestingly, neither *Rickettsia* DNA nor *Bartonella* DNA was detected in these fleas. Although these fleas occur on a wide range of bird and mammal hosts, they are commonly known as poultry fleas, a fact that could explain the absence of both bartonellae and rickettsiae in populations of these fleas.

Neither *Y. pestis* nor *Y. pseudotuberculosis* was detected in the present study. Notably, the villages tested were those lacking a history of plague cases, although all were within the portion of the West Nile region considered at risk for plague. Even in villages where plague has been reported repeatedly, it is very uncommon to find *Y. pestis* in fleas collected from rodents or to find this bacterium in years when there is no evidence of epizootic activity among rodent populations. Some researchers have suggested that *Y. pestis* circulates at very low and difficult-to-detect levels in fleas and rodents or is maintained in another reservoir, such as soil, during the interepizootic periods, but the true reservoir is yet to be determined (42, 43).

In summary, the relatively high prevalence rates of different genotypes of *Rickettsia* and *Bartonella* in fleas from the West Nile region where plague is endemic suggest that flea-borne diseases other than plague are likely present in this part of Uganda. The potential importance of these flea-borne diseases is also supported by the high abundance of certain species of fleas in this area, including *C. felis*, *X. cheopis*, and *X. brasiliensis*, each of which is known to bite people. Nevertheless, the vector competence of these flea species should be studied in order to gain understanding of whether these fleas pose a risk to humans. In addition, an animal host likely would not die from a *Bartonella* or *Rickettsia* infection; therefore, the relevant flea species may not leave their natural hosts. Further research is needed to determine the extent to which humans in this region become infected with not only *Y. pestis* but also with bacteria of

TABLE 3 Molecular detection of bacterial zoonotic agents in fleas from Uganda, 2012–2013

Agent	Target	PCR type	Primer and probe sequences	Reference
Bartonella spp.	*ssrA*	Real time	Forward: GCT ATG GTA ATA AAT GGA CAA TGA AAT AA Reverse: GCT TCT GTT GCC AGG TG Probe: ACC CCG CTT AAA CCT GCG ACG	50
	gltA (outer)	Nested conventional	Forward: GCT ATG TCT GCA TTC TAT CA Reverse: GAT CYT CAA TCA TTT CTT TCC A	18
	gltA (inner)	Nested conventional	Forward: GGG GAC CAG CTC ATG GTG G Reverse: AAT GCA AAA AGA ACA GTA AAC A	53
Rickettsia spp.	*gltA*	Real time	Forward: GAG AGA AAA TTA TAT CCA AAT GTT GAT Reverse: AGG GTC TTC GTG CAT TTC TT Probe: CAT TGT GCC ATC CAG CCT ACG GT	51
		Conventional	Forward: GGG GGC CTG CTC ACG GCG G Reverse: ATT GC AAA AAG TAC AGT GAA CA	52
Yersinia pestis	YPO2088	Real time	Forward: TCG GCA ACA GCT CAA CAC CT Reverse: ATG CAT TGG ACG GCA TCA CG Probe: CGC CCT CGA ATC GCT GGC CAA CTG C	This study
Yersinia pseudotuberculosis	*opgG*	Real time	Forward: ACG TGG GCG TGA ATT CTC TCA A Reverse: GCC GTT GGG ATC TCC ACC AA Probe: CCT GCG CCC AAG CGC GTG GG	This study

the genera *Rickettsia* and *Bartonella* and whether the people infected with these two arthropod-associated bacteria suffer clinically recognizable illnesses.

MATERIALS AND METHODS

Study sites and flea collections. Study sites and flea and small mammal collection methods were described previously (3). Briefly, 15 villages, including 10 case villages and 5 control villages, were selected within portions of the Arua and Zombo districts that were considered to have increased plague risk, as classified by geographic information system-based statistical models that included variables for elevation (>1,300 m), precipitation, amounts of vegetative growth, and bare soil during the dry month of January (44). In other words, the study villages were believed to have conditions ecologically conducive for plague activity. Within each village, 10 homesteads were randomly selected for sampling. The fleas screened in this study were derived from the five control villages within which no human plague cases had been reported from 1999 to 2011. The fleas were collected either from small mammals that were trapped inside huts and surroundings of the selected homesteads or from modified Kilonzo flea traps (45) that were set inside huts. Collected fleas were stored in 70% ethanol and shipped to the CDC laboratory in Fort Collins, CO, for species identification based on morphological characteristics using appropriate taxonomic keys (46–49) and bacterial testing.

DNA extraction and PCR detection. DNA was extracted from individual fleas. The fleas were first homogenized using a Bullet Gold blender homogenizer (Next Advance, Averill Park, NY). The homogenates were then transferred to a KingFisher platform (Life Technologies, Inc., Grand Island, NY) for DNA extraction following the tissue protocol provided by the manufacturer. A blank well was included as a negative control to ensure no cross-contamination occurred during the extraction. The DNA was then analyzed for the presence of *Bartonella* spp., *Rickettsia* spp., *Yersinia pestis*, and *Y. pseudotuberculosis* by using molecular approaches. Real-time PCR was first performed in a CFX96 PCR detection system (Bio-Rad, Hercules, CA) that was set up to target *ssrA*, *gltA*, YPO2088, and *opgG* for *Bartonella* spp., *Rickettsia* spp., *Yersinia pestis*, and *Y. pseudotuberculosis*, respectively. *ssrA* for *Bartonella* spp. and *gltA* for *Rickettsia* spp. have been described previously and have been widely applied in many studies (50, 51). The YPO2088 and *opgG* assays, which were developed for detection of *Y. pestis* and *Y. pseudotuberculosis*, respectively, were applied for the first time in this study. Samples showing an amplification curve with a cycle threshold (C_T) value of <36 were considered positive. A one-step or nested conventional PCR was further performed in a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA) for real-time PCR-positive samples, targeting *Rickettsia*-specific *gltA* (52) and *Bartonella*-specific *gltA* (18, 53), respectively. The PCR products were analyzed for the presence of amplicons of the expected sizes via electrophoresis on 1.5% agarose gels containing GelGreen stain (Biotium, Hayward, CA). Positive and negative controls were included in each PCR to evaluate the presence of appropriately sized amplicons and to rule out potential contamination, respectively. The primers and probes used for the targets mentioned in this study are listed in Table 3.

Sequencing and phylogenetic analysis for *Bartonella* and *Rickettsia* spp. The PCR amplicons from conventional PCRs for *Bartonella* and *Rickettsia* were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions and then sequenced in both directions by using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were assembled using the SeqMan Pro program in Lasergene v.12 (DNASTAR, Madison, WI). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the GenBank database was

performed with all assembled sequences to confirm their *Bartonella* or *Rickettsia* identity. Positive sequences were compared between themselves and with *Bartonella* and *Rickettsia* reference sequences available in GenBank, after alignment using the Clustal algorithm in the MegAlign program in Lasergene. Phylogenetic trees were constructed for *Bartonella* or *Rickettsia* separately, using the neighbor-joining method. Branch support was estimated using 1,000 bootstrap replicates.

Statistical analyses. The chi-square test was performed to compare the prevalence of *Bartonella* and *Rickettsia* infections between fleas collected from small mammals and fleas collected from Kilonzo traps. Results were considered significant if P was <0.05.

Accession number(s). The novel *Bartonella* variants and *Rickettsia* variants obtained in the study have been deposited in GenBank under accession numbers MF443355 to MF443366 and MF459639 to MF459644, respectively.

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Y.B. and M.Y.K. designed the experiments, L.M.O. performed the experiments, R.J.E., L.A.A., J.T.M., K.A.B., R.E.E., and K.L.G. performed field work, Y.B. and L.M.O. analyzed the data, and Y.B., M.Y.K., R.J.E., K.L.G., and R.E.E. wrote the paper.

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