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Identification of Flea Blood Meals Using Multiplexed Real-Time Polymerase Chain Reaction Targeting Mitochondrial Gene Fragments

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Abstract. Human plague is found in the West Nile region of Uganda and Democratic Republic of the Congo where flea vectors are often found inhabiting homes. We have developed a multiplexed, real-time polymerase chain reaction assay targeting mitochondrial genes that is capable of detecting blood meal sources in fleas collected off-host in East Africa. Laboratory tests showed that the assay is specific for the intended targets and has a detection limit below one picogram of DNA. Testing of wild-caught fleas from the Democratic Republic of Congo suggests that humans are at significant risk from flea-borne disease and implicates domestic animals including cats, chickens, and the black rat as potential sources of human exposure to fleas and flea-borne diseases. Future application of the assay will help us better define the ecology of plague in East Africa to implement effective control measures to combat the spread of disease.

INTRODUCTION

Plague is a serious and potentially fatal zoonotic disease caused by the gram-negative bacterium *Yersinia pestis.* Plague has been responsible for three pandemics in human history, including the Black Death during the fourteenth century that killed an estimated one-third of Europe's population.[1] Plague exists naturally in rodent populations and is transmitted most often by the bite of an infected flea.[2] Flea-borne transmission leads to the bubonic form of the disease in humans and is often fatal, especially when treatment is delayed or insufficient. The majority of human plague cases currently occur in Africa, although the disease is found throughout the world, including the southwestern United States.[3,4] The rat fleas *Xenopsylla cheopis* and *Xenopsylla brasiliensis* are considered the primary vectors of plague in East Africa because of their propensity to feed on susceptible rodent hosts and humans, and their ability to transmit *Y. pestis* efficiently under laboratory conditions.[5,6] Recent evidence suggests that the human flea, *Pulex irritans*, could play an important role as a vector of human plague in Tanzania where it is the predominant species found in human habitations.[7] In the West Nile region plague focus of northwestern Uganda, the predominant species found in homes is the cat flea, *Ctenocephalides felis*, a species not considered previously to be an important vector of plague.[5] However, *C. felis* was demonstrated to be capable of transmitting *Y. pestis* through early-phase transmission, and is occasionally found on plague-susceptible hosts, including black rats and Nile grass rats.[8] Both rat species are found throughout East Africa, including northwestern Uganda and northeastern Democratic Republic of Congo.[9] Understanding host feeding preferences of host-seeking fleas collected in human habitations would be a valuable tool for assessing which vectors and hosts are implicated in transmission to humans.

Traditional methods for blood meal identification have been based on immunologic procedures such as precipitin tests and enzyme-linked immunosorbent assays (ELISA).[10,11] More recent molecular-based techniques have targeted mitochondrial DNA sequences.[12,13] Mitochondrial DNA is preferred to genomic DNA because of the high number of copies per cell and because of the level of divergence between species seen in genes, such as cytochrome *b* (*cytb*) and 12S ribosomal DNA (rDNA). Polymerase chain reaction (PCR) has been used to amplify species-specific sequences, although most described techniques are dependent on extensive post-reaction manipulation and/or are limited in sensitivity because of the requirement to visualize bands on agarose gels.

Here, we report the development and testing of a rapid assay for identifying host blood meal sources in fleas collected in regions of East Africa, including Uganda and the neighboring Democratic Republic of Congo. Laboratory evaluation using artificially fed fleas indicates that the assay is effective at detecting and identifying blood meals out to 72 hours post-feeding. Testing of field specimens demonstrated the effectiveness of the assay in identifying wild blood meals. Future studies using this assay will provide valuable information regarding the potential flea hosts present in and around human habitations in plague foci such as northwestern Uganda, and help us understand the level of human risk to diseases such as plague.

MATERIALS AND METHODS

Isolation of DNA. Anti-coagulated whole blood was either purchased from a commercial vendor (Bioreclamation, Jericho, NY) or collected from wild-trapped rodents via cardiac puncture and stored in EDTA. The DNA was extracted from 200 μL (mammal) or 20 μL (avian) of blood using the DNeasy Tissue Kit (Qiagen, Valencia, CA) and eluted in 200 μL of elution buffer. Purified DNA was stored at –20°C until analysis.

Primer and probe design and real-time PCR. Primers and probes for real-time PCR were designed using Beacon Designer 7.0 software (Premier Biosoft, Palo Alto, CA). Cytochrome *b* or 12S sequences were obtained from the GenBank nucleotide sequence database for Nile grass rat (*Arvicanthis niloticus*), domestic dog (*Canis lupus familiaris*), goat (*Capra hircus*), human (*Homo sapiens*), cat (*Felis catus*), shrews (*Crocidura* spp.), black rat (*Rattus rattus),* and chicken (*Gallus gallus*). Two multiplexed panels were developed containing four targets in each panel. Each target-specific probe was tagged

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with CY5, HEX, ROX, or FAM as indicated. The PCR was performed in 25 μL reactions containing iQ Supermix (2X; Bio-Rad, Hercules, CA), 200 nM probe and 400 nM forward and reverse primers, and 4.5 μL of DNA template from each species in the two panels. Real-time PCR was performed on an Mx3005P (Stratagene, La Jolla, CA) detection platform with the following conditions: initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 30 s and 60°C for 1 min. Nuclease-free water was used as the no template control and DNA isolated from mouse (*Mus musculus*)-fed *Xenopsylla cheopis* was used as a negative control. All four channels were scanned simultaneously for increases in fluorescence following the annealing/extension stage of each cycle. The PCR software plots an amplification curve from a plot of relative fluorescence (ΔR_n) versus the cycle number. A threshold cycle (Ct) is determined for each sample at the point it crosses a threshold value, which was determined by the software. The lower the Ct value the higher the amount of starting template material. Samples that did not cross this threshold value before 40 cycles were determined to have no identifiable blood meal. Standard curves were generated to determine the overall effectiveness of each panel at detecting each individual target. The DNA isolated from whole blood was diluted ten-fold over 6 logs ranging from 450 pg to 4.5 fg of input DNA. The efficiency and R^2 value were used to determine the overall function of each primer/probe set within a panel. Efficiency of the standard curves was used to detect inhibition of particular targets by other components of a panel. The goodness of fit of each data point in the standard curve against a best-fit line is denoted by the R^2 value. Only those primer and probe sets demonstrating a mean efficiency (E) > 90% and $R^2 > 0.98$ when multiplexed together were chosen for further evaluation.

Artificial flea feeding and homogenization. A colony of *X. cheopis* was maintained by biweekly feedings on anesthetized Swiss-Webster mice at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention.[14] Before feeding on artificial blood sources, we collected ~1,000 adult fleas from the colony by vacuum aspiration and stored the fleas at 27°C for 1 week before feeding. Groups of ~200 fleas were allowed to feed for 1 hour on artificial feeders containing citrated whole blood of dog, cat, chicken, or human origin heated to 37°C. Fed fleas containing red blood in the proventriculus or midgut were separated from unfed fleas and stored in conical tubes containing filter paper at 27°C for up to 3 days following the feed. Three days was chosen as the cut-off for these experiments because this time point was previously shown to be the temporal limit of detection of blood meals in mosquitoes, and we also felt that this represented a realistic time frame between the last blood meal and collection and storage of the fleas in the field.[13] At the time points indicated in Table 3, the fleas were frozen at −80°C and stored until processing of the blood meal DNA. Template DNA for blood meal identification was acquired by homogenizing single fleas in 200 μL of Dulbecco's phosphate-buffered saline (Gibco, Carlsbad, CA) using a bead-beater set at 20,000 rpm for 3 min. The samples were then heated at 100°C for 15 min, clarified by centrifugation at 10,000 × g for 10 min, and the supernatants were frozen at −20°C until PCR analysis. A total volume of 4.5 μL of the homogenate was used at template for the PCR reactions. Pairwise comparisons of Ct values within blood types were made using Mann-Whitney U tests with a P value < 0.05 indicating statistical significance.

Testing of African flea blood meals. All wild-caught flea samples used in this work had previously been tested for the presence of *Bartonella* and *Rickettsia* spp. as reported by Sackal and others.[15] The fleas originated from the Ituri district, Democratic Republic of Congo, and were collected by light trap inside homes and stored in ethanol before species identification and DNA extraction. The DNA samples had previously been isolated from pools of up to 5 fleas, grouped by species, and stored at −20°C before analysis. Each sample was analyzed by adding 4.5 μL of DNA to each reaction. Each sample was analyzed with both panels and each sample was run in duplicate. Each panel consisted of 450 fg of DNA from each target species as positive controls, DNA from a mouse-fed adult *X. cheopis* as a negative control, and a no template reaction.

RESULTS

Two independent, multiplexed panels targeting a total of eight unique species found in various regions of East Africa were developed to reliably identify whether fleas have fed on these blood meal sources. The first panel consisted of unique primers and probe sets targeting the *cytb* gene of Nile grass rat, dog, goat, and human. The second panel consisted of primers and probe sets targeting the *cytb* gene of cat, black rat, and chicken, and the 12S rDNA gene of shrews belonging to the genus *Crocidura*. Targeting the 12S rDNA gene proved to be more effective at identifying shrews at the genus level (data not shown). Table 1 lists the targets, the corresponding primer and probe sequences, and the location of the oligonucleotide sequences within the genes.

Each panel was tested against standard dilutions of target DNA to determine the efficiency with which each primer and probe set within a panel was able to detect its intended target. For both panels, each independent primer and probe set generated a standard curve with a minimum mean efficiency of 90% and a mean R^2 value of at least 0.98, as illustrated in Table 2. The slopes of all standard curves were close to the ideal value of −3.32, which represents the slope of a line fit to perfect 2-fold amplification per cycle. The most sensitive assay among all targets was for cat, with a limit of detection less than 4.5 fg of DNA. The least sensitive assay was for dog, with a limit of detection of between 450 fg and 45 fg of DNA. All other assays showed a limit of detection of between 45 fg and 4.5 fg of total input DNA.

To determine the temporal limitations of blood meal identification using our assay, we artificially fed adult *X. cheopis* fleas using glass feeders containing human, dog, cat, and chicken blood. Artificial blood meals were identifiable up to 72 hours post-feeding. The Ct values increased over time for each blood type (KW with χ^2 approximation; $\chi^2 \geq 36.42$, d.f. = 6, $P \leq 0.0001$). Results of pairwise comparisons are shown in Table 3. Correspondingly, the percent of detected blood meals decreased with time as Ct values exceeded the established thresholds. At 72 hours post-feeding we detected 100% of cat blood meals. Additionally, at 72 hours we detected 61% and 73% of human and chicken blood meals, respectively. Just over 25% of dog blood meals were detected at this same time point.

A total of 80 flea samples collected in the Democratic Republic of Congo were analyzed for the presence of blood meals. Samples were from pools of up to 5 fleas separated by flea species and collection date. *Pulex irritans* was by far the

TABLE 1
Primer and probe sequences grouped by panel

	Target		Primer/probe sequences	Location	Reference accession no.
Panel 1	Nile grass rat *cytB*	Forward	5′-CTACACATCAGATACAACAACAGC-3′	162	AF004572
		Reverse	5′-GCTCCGTTAGCATGTATGTATCG-3′	260	
		Probe	5′-Cy5-CCTCAGTAACACACATCTGCCGAGACG-BHQ-3′	191	
	Domestic dog *cytB*	Forward	5′-TTCTCCATCCTAATCTTGGCATTC-3′	886	NC002008
		Reverse	5′-ATTGAAGCGACTTGTCCGATAATG-3′	1,067	
		Probe	5′-HEX-CACTCCTCCACACATCTAAGCAACGCAGC-BHQ-3′	914	
	Goat *cytB*	Forward	5′-TCCTCCCATTCATCATCACAGC-3′	551	NC005044
		Reverse	5′-TGGTGTAGTAAGGGTGAAATGGG-3′	676	
		Probe	5′-ROX-CGCCATAGTCCACCTGCTCTTCCTCCA-BHQ-3′	576	
	Human *cytB*	Forward	5′-TCAATCGCCCACATCACTCG-3′	193	AC00021
		Reverse	5′-CAGGAGGATAATGCCGATGTTTC-3′	360	
		Probe	5′-FAM-TGGCTGAATCATCCGCTACCTTCACGC-BHQ-3′	225	
Panel 2	Domestic cat *cytB*	Forward	5′-ACCTGAATGATACTTCCTATTCGC-3′	807	NC001700
		Reverse	5′-TTTGGAGGTGTGGAGGATTGG-3′	933	
		Probe	5′-Cy5-ACGCAATTCTCCGATCCATCCCCAACA-BHQ-3′	833	
	Shrews *12S*	Forward	5′-TGGCGGTGCTTTATATCCAT-3′	82	AF441243
		Reverse	5′-GGCGGAATTAGCAAGAAGTG-3′	147	
		Probe	5′-HEX-GCCTGTTCTATAATCGATATACCCCGA-BHQ-3′	110	
	Black rat *cytB*	Forward	5′-CCACCACATATTAAGCCAGAATGG-3′	793	AB033702
		Reverse	5′-TTGGGTGATTGGGCGGAATG-3′	966	
		Probe	5′-ROX-TGCCTACGCTATTCTACGCTCCATCCC-BHQ-3′	828	
	Chicken *cytB*	Forward	5′-CCTCTACAAGGAAACCTCAAACAC-3′	254	NC001323
		Reverse	5′-GACTAGGGTGTGTCCAATGTAGG-3′	416	
		Probe	5′-FAM-AATCCTCCTCCTCACACTCATAGCCACCG-BHQ-3′	284	

most common flea species, accounting for 61 out of 80 different flea samples. *Ctenocephalides felis strongylus* was the next most common flea species ($N = 8$), followed by *Tunga penetrans* ($N = 7$), *Echidnophaga gallinacea* ($N = 3$), and *Xenopsylla brasiliensis* ($N = 1$), respectively.[15] Of 80 samples tested, 64 contained a blood meal(s) identified using our assay, and 16 of the 64 samples contained a second detectable blood meal. Humans were the most common blood meal source across all species of fleas as illustrated in Table 4, accounting for 63 of the 80 unique blood meals. The next most common blood meal across groups was chicken ($N = 10$), followed by cat ($N = 6$). One rat blood meal was detected in a single pool of *P. irritans*. Of the 16 mixed blood meals, 10 were a mix of human and chicken, and 6 were a mix of human and cat as shown in Table 5. All samples containing a cat or chicken blood meal were associated with cats and/or hens either inside or outside the household (data not shown).

DISCUSSION

We have developed a highly sensitive, multiplexed PCR assay to rapidly detect and correctly identify the source of blood meals in fleas. Using real-time PCR primers and probes tagged with multiple fluorophores, we amplified and detected unique gene fragments from *cytb* or 12S rDNA for 7 species-level targets and 1 genus-level target, respectively. Evaluation of the assay using template DNA isolated from whole blood showed that each primer and probe set efficiently amplified its intended target. We also successfully identified blood meals in fleas artificially fed on human, dog, cat, or chicken blood up to 72 hours post-feeding showing that the assay is functionally useful for analyzing blood meal sources for recently captured fleas. Recent human blood meals also were identified in the majority of flea specimens collected from inside domiciles in the Democratic Republic of Congo, showing that humans are at a significant risk of flea-borne diseases, including plague, *Bartonella*, and rickettsioses. The identification of black rat blood in the so-called human flea, *P. irritans*, suggests a possible mechanism other than the presence of infectious rat fleas for transferring plague from rat to human populations. This assay will be a useful tool for studying the ecology of flea-borne disease transmission in East Africa but will also fill a void during disease case studies when an evaluation of arthropod feeding behavior would be critical to identifying likely sources of infection and targeting the application of effective control measures.

Blood meal identification has been used extensively to study the ecology of many vector-borne diseases. The identification of blood meals in *Ixodes ricinus* ticks coupled with bacterial culture has identified, among others, the Red squirrel as a potential reservoir host of Lyme borreliosis.[16] Similarly, shifts in the feeding behavior of *C. pipiens*, the predominant vector

TABLE 2
R^2 values, efficiency (E), and slope of standard curves by blood type

	Target	R^2	E (%)	Slope	Detection limit
Panel 1	Nile grass rat	0.994 ± 0.003	98.47 ± 6.31	–3.36 ± 0.16	45–4.5 fg
	Dog	0.996 ± 0.0017	94.47 ± 8.69	–3.47 ± 0.22	450–45 fg
	Goat	0.998 ± 0.003	101.63 ± 5.99	–3.288 ± 0.14	45–4.5 fg
	Human	0.993 ± 0.007	98.33 ± 2.80	–3.364 ± 0.07	45–4.5 fg
Panel 2	Cat	0.996 ± 0.0046	102.87 ± 10.44	–3.269 ± 0.23	< 4.5 fg
	Shrew	0.98 ± 0.02	98.03 ± 8.92	–3.383 ± 0.22	45–4.5 fg
	Black rat	0.999 ± 0.001	98.97 ± 10.7	–3.366 ± 0.28	45–4.5 fg
	Chicken	0.987 ± 0.015	90.1 ± 5.3	–3.667 ± 0.16	45–4.5 fg

TABLE 3

Number of blood meals detected and threshold cycle (Ct) values of detected blood meals by time point and blood type

Blood meal source	Time point (h)	No. positive/no. tested	Median Ct (min–max)
Human	1	18/18[f]	26.33 (23.93–29.44)[a]
	5	18/18[f]	29.75 (25.71–36.92)[b]
	24	18/18[f]	32.08 (25.51–37.97)[b,c]
	36	15/18[g]	35.4 (23.96–37.38)[c,d]
	48	14/18[g]	34.45 (23.73–38.73)[b,d]
	60	14/18[g]	36.76 (23.97–39.62)[d]
	72	11/18[g]	34.81 (23.79–39.29)[c,d]
Dog	1	14/15[f]	26.72 (23.93–31.18)[a]
	5	12/15[f]	28.75 (26.76–34.28)[b]
	24	11/15[f]	33.35 (30.19–38.39)[c]
	36	12/15[f]	33.83 (29.74–39.97)[c]
	48	6/15[g]	33.86 (31.85–39.14)[c]
	60	5/15[g]	34.91 (26.27–39.38)[b,c]
	72	4/15[g]	35.74 (35.18–39.32)[c]
Cat	1	8/8[f]	24.44 (22.15–26.34)[a]
	5	6/8[f]	23.05 (22.37–24.86)[a]
	24	7/8[f]	27.07 (23.86–28.53)[b]
	36	8/8[f]	27.68 (25.04–36.34)[b,c]
	48	7/8[f]	31.58 (26.75–32.83)[c]
	60	8/8[f]	30.42 (27.9–37.43)[c]
	72	8/8[f]	31.25 (29.71–34.76)[c]
Chicken	1	16/16[f]	26.11 (24.81–27.78)[a]
	5	16/16[f]	25.79 (24.01–27.98)[a]
	24	16/16[f]	29.68 (25.96–33.7)[b]
	36	15/16[f]	30.01 (24.0–34.62)[b,c]
	48	12/16[g]	32.83 (24.59–37.35)[c,d]
	60	11/16[g]	36.53 (26.38–39.51)[e]
	72	11/15[g]	35.64 (27.22–37.17)[d,e]

[a–e] Unique individual letters indicate groups that are statistically significant ($P \leq 0.05$) from another within blood types as determined by Mann-Whitney *U* tests.
[f–g] Unique individual letters indicate groups that are statistically significant ($P \leq 0.05$) from another within blood types as determined by contingency table analyses.

of West Nile virus (WNV) in the northeast United States, have been shown to coincide with increases in cases of human infection. The American robin appeared to be responsible for the majority of WNV-positive mosquitoes but seasonal migration of the birds led to increased feeding on humans because of the lack of available preferred hosts.[17,18] Diseases such as plague have lacked this kind of in-depth analysis of vector feeding habits, which has limited our understanding of plague ecology and human risk.

This newly developed assay is both highly sensitive and highly specific and a significant upgrade from older immunoglobulin-based assays. Laboratory evaluation of artificially fed fleas showed that the number of detected blood meals decreased with time, and likewise the Ct value of detected blood meals increased with time. This increase in Ct value we expect is a result of digestion of the blood meal by the flea. Since mixed-age, adult fleas were used in these studies the size of the blood meals was highly variable because of blood meal remnants from past maintenance feedings. This may explain some of the variability seen in Ct values, but the overall trend for all blood types was the same. It is also important

TABLE 5

Number of flea pools containing a mixed blood meal by species and blood types

Blood meal sources	Flea species (No. detected/no. tested)				
	Pulex irritans	*Ctenocephalides felis strongylus*	*Tunga penetrans*	*Echidnophaga gallinacea*	*Xenopsylla brasiliensis*
Human/chicken	9/61	0/8	1/7	0/3	0/1
Human/cat	2/61	3/8	0/7	1/3	0/1

to note that in all fleas tested, we detected no additional blood types besides the experimental blood meal source. We chose 72 hours as the cut-off for laboratory testing because we felt that to be an accurate snapshot of recent feeding behavior in field-trapped fleas. In fact, we had very good success detecting blood meals in fleas at that time point for human, chicken, dog, and cat blood sources. Likewise, previous reports using artificially fed mosquitoes indicated a lack of detectable blood meals after 72 hours, although our assay may potentially be useful for time points beyond 3 days.[12,13] In comparison to previous immunoglobulin-based assays such as ELISA and precipitin tests our assay is significantly more sensitive and specific.[19] It should be noted that a rapid evaluation of feeding behavior using this assay is only achievable when there is a firm understanding of animal fauna for a particular area, such as in northwestern Uganda. Likewise, this technique requires specific equipment that might not be available in certain developing countries. Another potential drawback to a multiplexed, real-time PCR assay is that we are limited in the number of targets we can identify. Genetic sequencing of blood meals has been used to bypass this problem, however given the size of flea blood meals and the temporal detection limits previously established for sequence identification of nucleated, avian blood meals in mosquitoes, it is unlikely that we could accurately identify flea blood meals with the sensitivity provided using our assay.[17] Still, given that we have flea samples with no identifiable blood meal, it begs the question of whether a blood meal was absent, degraded, or present but from another host type. Additional panels can be created to expand the breadth of diversity while still applying the same level of sensitivity and specificity achievable using real-time PCR.

Of the wild fleas tested using our assay, the vast majority of them were *P. irritans*. Previous work showed a correlation between the density of *P. irritans*, a predominately human-biting flea, and the appearance of plague in villages in the Usambara Mountains of Tanzania.[7] In these studies *P. irritans* accounted for almost 75% of the fleas found in human homes and the occurrence of this flea was twice as high in villages with a high frequency of plague compared with low-frequency villages. Our data confirms that *P. irritans* will readily feed on humans and occasionally on black rats, which could have important implications in the control of plague in that region.

TABLE 4

Number of flea pools with a specific blood meal by species and blood meal type

Blood meal source	Flea species (No. detected/no. tested [%])				
	Pulex irritans	*Ctenocephalides felis strongylus*	*Tunga penetrans*	*Echidnophaga gallinacea*	*Xenopsylla brasiliensis*
Human	47/61 (77%)	7/8 (87.5%)	5/7 (71.4%)	3/3 (100%)	1/1 (100%)
Chicken	9/61 (14.8%)	0/8 (0%)	1/7 (14.3%)	0/3 (0%)	0/1 (0%)
Cat	2/61 (3.3%)	3/8 (37.5%)	0/7 (0%)	1/3 (33.3%)	0/1 (0%)
Rat	1/61 (1.6%)	0/8 (0%)	0/7 (0%)	0/3 (0%)	0/1 (0%)

In northwestern Uganda the primary flea infesting homes is the cat flea, *C. felis*, which has recently been shown to be a competent early phase vector of plague.[8] Across 9 villages in the Arua and Nebbi districts of Uganda, *C. felis* represented 85% of the host-seeking fleas. In the Democratic Republic of Congo, domestic flea populations were dominated by *P. irritans* (55%) but *C. felis* still accounted for 19% of the fleas found in human homes (Laudisoit A, unpublished data). Evidence from our analysis of *C. felis* collected in the Democratic Republic of Congo shows that the primary feeding hosts of these fleas were humans and cats. Cats are highly susceptible to plague infection most often associated with exposure because of predatory hunting of rodents. Cases of human plague in the United States and a few other countries have been attributed to direct exposure to cats either through scratch, bite, or exposure to aerosolized droplets.[20–22] Our data suggest that domestic cats are a common blood meal source for fleas in the Democratic Republic of Congo.

As stated previously, plague is most commonly associated with endemic rodent populations. Recent work in Arua and Nebbi Districts in Uganda showed that the two predominant species in the domestic and peridomestic setting were black rats and Nile grass rats, accounting for 70% and 88% of captures, respectively.[8] Both species are known to be susceptible to plague and harbor diverse flea assemblages.[23] We detected only one flea (*P. irritans*) containing a blood meal acquired from black rats. This is perhaps not surprising given the relatively low numbers of fleas commonly associated with rats. All fleas caught in our study were collected from off-host populations and it is important to consider that flea feeding behavior most likely changes depending on the availability of hosts, which means that some of the fleas might have been more likely to take blood meals from acceptable hosts rather than their most preferred hosts. For example, host-switching is likely to occur during a plague epizootic because of a drop in the rodent population due to death of the highly susceptible black rats. This could then trigger important ecologic shifts involving multiple potential hosts including other rodent species, predatory domestic cats, and most importantly humans.

Future studies using this assay will address the feeding behavior of fleas collected off-host in plague-endemic areas such as Uganda and the Democratic Republic of Congo to determine the importance of *C. felis* as a human-biting flea. We also expect to determine the frequency with which rodents and other animals are responsible for introducing fleas into homes. This data will allow us to determine the primary hosts responsible for human plague risk, enabling us to institute effective control measures to limit the spread of disease.

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