THESIS

IDENTIFYING BLOOD MEALS IN CAT FLEAS (*CTENOCEPHALIDES FELIS*) FROM A PLAGUE-ENDEMIC REGION OF UGANDA USING A SYBR GREEN REAL-TIME POLYMERASE CHAIN REACTION-BASED ASSAY

Submitted by

Christine B. Graham

Department of Microbiology, Immunology and Pathology

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Advisor: William C. Black IV

Rebecca J. Eisen
RoxAnn R. Karkhoff-Schweizer
Kathryn P. Huyvaert

ABSTRACT

IDENTIFYING BLOOD MEALS IN CAT FLEAS (*CTENOCEPHALIDES FELIS*) FROM A PLAGUE-ENDEMIC REGION OF UGANDA USING A SYBR GREEN REAL-TIME POLYMERASE CHAIN REACTION-BASED ASSAY

A zoonotic disease that has killed millions over the course of at least three pandemics, plague remains a threat in regions where the etiologic agent, *Yersinia pestis*, persists in natural cycles involving small mammals and their fleas. Numerous flea species have been implicated as *Y. pestis* vectors, and some provide a "bridge" from zoonotic hosts to humans, particularly during the epizootics that decimate susceptible small mammal populations. In order to serve as a bridging vector, a flea species must be able to transmit *Y. pestis*, it must feed on infectious zoonotic hosts, and it must feed on humans. Identifying bridging vector species in plague-endemic regions can aid in the development of vector-control activities aimed at reducing the incidence of human plague.

The West Nile region is an established plague focus in northwest Uganda. Since 1999, more than 2400 suspect human plague cases have been reported from Vurra and Okoro counties. The most likely source of infection for humans in this region is the black rat, *Rattus rattus*, which commonly infests human habitations and is highly susceptible to *Y. pestis* infection. Other potential zoonotic hosts include other rodent and shrew species that predominate in the peridomestic environment and occasionally enter huts. Two rat flea species, *Xenopsylla cheopis* and *X. brasiliensis*, both among the most efficient flea vectors of *Y. pestis*, are very likely to serve as bridging vectors to humans in Vurra and Okoro counties. Recent investigations,

however, have found that the cat flea, *Ctenocephalides felis*, comprises more than 88% of host-seeking (off-host) fleas captured in huts in this region. Though an inefficient vector, this species is capable of transmitting *Y. pestis*. Given its dominance in human habitations and its catholic feeding habits in other regions, we hypothesized that *C. felis* might serve as a secondary bridging vector in Vurra and Okoro counties.

In order to address this hypothesis, we sought to determine what proportion of blood meals in off-host cat fleas collected in huts in this region come from humans, and what proportion come from potentially-infectious small mammal species. Blood meal assays have long been used to examine the feeding behavior of a wide variety of disease vectors, but existing blood meal assays were deemed inadequate for our purposes because they were either not sensitive enough to detect the very small amounts of host DNA in field-collected fleas, or they were unable to capture the wide range of potential cat flea hosts in the West Nile region. Therefore, we developed a blood meal assay that takes advantage of the exquisite sensitivity of SYBR Green I-based real-time polymerase chain reaction (PCR) and combines it with the specificity and flexibility afforded by sequencing. We found that this highly-sensitive assay was subject to human DNA contamination, so we analyzed vertebrate DNA detection in artificially-fed and unfed fleas to establish a threshold cycle (Ct) cutoff that would optimize specificity without completely sacrificing sensitivity. Specifically, we identified a Ct cutoff that maximized positive predictive value.

Using the established cutoff, our assay was 94% specific, detecting contaminating human DNA in 3 of 50 unfed fleas, and it detected and correctly identified the source of human and rat blood meals in 100% of artificially fed fleas held alive for up to 4 hours post feeding. Assay sensitivity declined as the time between feeding and collection increased, but we were able to

detect and identify human and rat DNA in a proportion of artificially-fed fleas held alive for up to 72 hours post feeding.

Using this assay, we detected and identified vertebrate DNA in 148 off-host *C. felis* collected in human habitations in Vurra and Okoro counties, none of it from wild rodents or shrews. Our findings indicate that cat fleas infesting huts in the West Nile region probably feed on humans, but the majority of off-host *C. felis* blood meals came from domesticated species that are unlikely to play a significant role in perpetuating transmission of *Y. pestis*. We concluded that *C. felis* is unlikely to serve as a bridging vector for *Y. pestis* in the West Nile region.

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This work would have been impossible without the hard work and guidance of many people. In addition to my committee members and all of the co-authors listed at the beginning of each chapter, I need like to thank Janae Stovall, Karen Boroughs and Heidi Brown for technical assistance and Jeannine Petersen for helpful discussions.

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CHAPTER 1: INTRODUCTION

PLAGUE

Historical Background: Three Pandemics. Plague has been responsible for millions of human deaths over the course of at least three historic pandemics. In the 6th century, a 50 to 60-year pandemic during the reign of Emperor Justinian claimed an estimated 100 million lives (1, 2). The Justinian plague likely spread from an endemic focus in Central Africa and ultimately affected North Africa, Europe, Central and Southern Asia, and Arabia (1, 2). A second pandemic emerged in the 14th century and ravaged Europe and Asia for four centuries. The "Black Death" had its origins in a Central Asiatic plague focus, and it is believed to have killed at least a quarter of Europe's population (1). While the incidence of human plague declined in the 17th century and disappeared from Europe by the mid 1800's, it persisted in small foci in Central Africa and Central Asia (1, 3). The third pandemic began in China's Yunnan Province and spread to Hong Kong and Canton in 1894 (1). From there it spread to every inhabited continent (2, 3). While the number of plague victims over the last century does not compare to the number of victims associated with the two previous pandemics, this fearsome disease remains a threat in parts of Africa, Asia and the Americas (Figure 1.1) (4, 5).

Early Evidence of a Deadly Triumvirate: *Yersinia pestis*, Rats and Fleas. It was during the Hong Kong epidemic that Alexandre Yersin isolated the etiologic agent of plague from the buboes, lymph nodes and – less often – the blood of infected patients (6). That gram-negative, non-motile, non-spore-forming coccobacillus now bears his name: *Yersinia pestis* (2, 7). Yersin also observed many dead rats in boroughs affected by plague and found that their tissues harbored numerous plague bacilli; he hypothesized that rats played a major role in propagation of the disease (6). This was not a shocking suggestion; people had long recognized

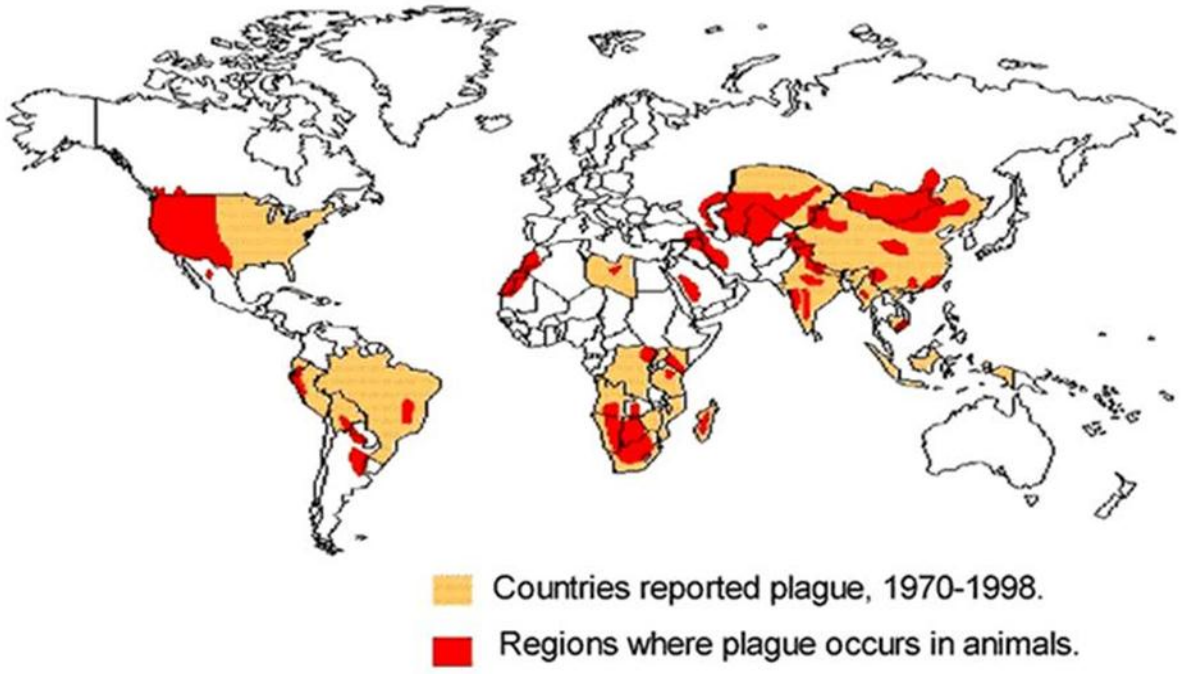

Figure 1.1. Worldwide distribution of plague-endemic areas and countries reporting human plague cases between 1970 and 1998 (From Centers for Disease Control and Prevention, 2005. Map: World Distribution of Plague, 1998. Available at http://www.cdc.gov/ncidod/dvbid/plague/index.htm. Accessed May 17, 2012).

that rat die-offs often preceded human plague outbreaks (8). A few years later, Simond noted that many plague patients in the early stages of the disease presented with bacteria-filled lesions consistent with insect bites, and that fleas removed from dead rats were replete with bacilli (9). Based on these observations, he suggested that fleas played a role in transmission of the plague bacillus (9). Simond and later Gauthier and Raybaud provided the first experimental evidence that fleas could transmit *Y. pestis* between rats (9-12). Liston (8) observed that the oriental rat flea, *Xenopsylla cheopis*, having fed on *Y. pestis*-infected rats, would infest non-rat hosts, including guinea pigs and humans, when their rat hosts had died. He further observed that guinea pigs and at least one person infested with infected rat fleas subsequently developed plague.

When the Indian government established a commission to investigate plague at the beginning of the 20th century, they began with the premise that plague was, "Essentially a rat disease in which human beings may participate" (11, p. 425). The Commission conducted experiments further demonstrating that the presence of fleas was required to instigate an epizootic among rats or guinea pigs, and that epizootics progressed faster when fleas were more abundant (11).

Transmission Cycles. Building upon the findings of the early 20th century, researchers have implicated a variety of mammalian hosts and flea species in natural cycles of plague (1, 13-16). Some have suggested that *Y. pestis* may persist long-term in infected mammalian tissues that are protected (e.g., bone marrow) or frozen (14), or in soil (17, 18). Others posit that infection by ingestion may play a role in maintaining the bacterium in rodent populations (19). Most researchers believe, however, that the bacterium persists in endemic foci primarily by circulating between rodents or, less often, lagomorphs or shrews, and their fleas (13, 14, 16, 20). Figure 1.1 illustrates the overlap between regions where plague occurs in animals and countries from which human cases were reported between 1970 and 1998.

The specific host/flea complexes involved in maintaining and amplifying *Y. pestis* vary from region to region. For example, there are ten different plague foci in China, each defined by its primary rodent reservoir species (15). In the focus reporting the majority of Chinese plague cases, a marmot (*Marmota himalayana*) is the primary reservoir species, and the principal flea vectors are *Callopsylla dolabris* and *Oropsylla silantiewi* (15). Epizootics in the southwestern region of the United States typically involve two different amplifying host/flea complexes: prairie dogs (primarily *Cynomys gunnisoni*) and their fleas (*Oropsylla hirsuta and O. tuberculatus*), and ground squirrels and their fleas (primarily *Oropsylla montana* and

Hoplopsylla anomalus), although *Y. pestis* also circulates among other rodents in the southwest plague focus (e.g., chipmunks, wood rats, and deer mice) and their fleas (15, 21-23).

Early transmission studies suggested that fleas would not develop a transmissible infection by feeding on an infected host until that animal was close to death (1, 24, 25). More recent work has provided further evidence that the host must achieve a very high septicemia ($\geq 10^6$ bacteria/ml in peripheral blood) in order to reliably infect feeding fleas (26, 27). In order to play a role in a natural plague cycle, a small mammal population must therefore include at least some individuals that succumb to infection. Some have proposed that the bacterium is maintained in partially resistant enzootic host species and occasionally spills over into highly-susceptible epizootic host populations, creating rapidly spreading epizootics (13-15, 23, 28). According to this model, a susceptible member of the enzootic host population occasionally succumbs to infection, providing infectious blood meals for feeding fleas. The fleas then survive by feeding primarily on resistant members of the population, but occasionally infect susceptible individuals. *Yersinia pestis* thus circulates in the enzootic host population at very low levels.

Others have argued that there is little evidence for distinct enzootic host species in at least some endemic foci (1, 16, 20). Alternatively, certain metapopulation dynamics might allow the bacterium to circulate long-term in a single, highly-susceptible species or in a population comprising several susceptible host species. According to this hypothesis, it is specific changes in environmental conditions, flea loads, or host population density, rather than a change in host species, that spark epizootics (16, 20).

Human Plague. Regardless of how the plague bacterium persists in endemic foci, humans – generally regarded as an accidental host – are most at risk for exposure to *Y. pestis* during epizootics (13, 14, 16). When large numbers of susceptible mammal species succumb to

Y. pestis infection, their fleas must seek out new hosts. If the fleas are inclined to feed on human blood, they may serve as a bridge from the zoonotic host to humans (8, 23). Studies in the United States indicate that human exposure occurs most often in the domestic or peridomestic environment (22, 29). In regions where rodents like *Rattus rattus* or *R. norvegicus* live in or in close proximity to human dwellings, human exposure to infectious fleas is most likely to result from epizootics among these commensal rodents (13, 23). The risk of human plague is often higher in areas where people sleep and store food in the same rooms, or where unsanitary conditions bring potentially infectious rodents into homes (30). Natural disasters, political unrest, and war may increase the risk of human plague outbreaks if they result in disorganization of public services and the accumulation of garbage around human habitations, or if they otherwise force people into close contact with potentially-infectious rodents and their fleas (2, 30, 31).

In North America, humans are more likely to be exposed to *Y. pestis* when peridomestic rodents or pets carry infectious wild rodent fleas into their homes, although they may also be exposed to infectious fleas when visiting rural or natural areas (14). Most human cases worldwide result from infectious flea bites, but a number of human cases in the U.S. have also been associated with infected domestic cats (14, 32). Humans may also become infected if bacteria from infected tissues or exudates enter an open skin lesion (e.g., while handling infected animals or carcasses) (14).

Bubonic plague is the most common form of the disease in humans (23). It is characterized by sudden onset of high fever, chills, weakness, headache, and painful regional lymphadenitis (buboes) (33). In some cases, an infection may develop in the deeper lymphatics, failing to produce a palpable bubo before it invades the bloodstream. Alternatively, *Y. pestis* may bypass the lymphatic system entirely and spread from the bite site via hematogenous extension.

Either scenario results in primary septicemic plague (13). Primary bubonic plague may also develop into secondary septicemic plague (33). Intermittent bacteremia may be observed in cases of bubonic plague, but a series of blood cultures (usually four) positive for *Y. pestis* indicates septicemic plague (14).

If secondary pulmonary involvement results from a bubonic, primary septicemic, or secondary septicemic *Y. pestis* infection, the patient may develop secondary pneumonic plague (33). Most patients with a secondary pulmonary *Y. pestis* infection, however, die without developing transmissible plague pneumonia (13). Primary pneumonic plague occurs when bacteria are inhaled directly into the lungs (33). This form of the disease is characterized by sudden onset of severe headache, chills and malaise, and, in the later stages of the disease, a cough and bloody sputum (34). Primary and secondary pneumonic plague can be transmitted between humans via respiratory droplets (13), but this type of transmission is relatively rare even among close contacts (35).

Approximately 50% of untreated bubonic plague patients recover, while septicemic and pneumonic plague are almost invariably fatal (33). Antibiotics including streptomycin, gentamicin, and tetracycline are effective against *Y. pestis* infections. Treatment early in the course of infection dramatically improves outcomes (33).

Flea Vectors of *Yersinia pestis*. While it was clear from observations at the turn of the century that fleas were involved in plague epidemiology, the mechanism by which they transmitted *Y. pestis* remained unclear. The Commission for the Investigation of Plague in India failed to find any bacilli outside the alimentary canal, indicating that the bacteria were not transmitted via infected salivary glands (10, 36). Indeed, later studies confirmed that *Y. pestis* infection is confined to the digestive tract (37). Early experiments demonstrated that it was

possible to infect rats by rubbing the cut surface of an infected spleen or an emulsion of plague bacilli into a rat's skin where it had been punctured by flea bites (10). Verjbitzki and the Commission for the Investigation of Plague in India suggested that transmission might occur when a host rubbed or scratched flea feces into the site of a recent bite (10, 11, 36). When repeated attempts to infect guinea pigs by rubbing feces from infected fleas into scarified skin failed, however, Eskey and Haas (24) later concluded that transmission via this route was highly unlikely. Bacot and Martin (10) hypothesized that while *Y. pestis* in flea feces might enter through the bite site, this was not the principal mode of transmission. In their seminal 1914 paper, they showed that two flea species, *Nosopsyllus fasciatus* and *X. cheopis*, could transmit the bacterium during the act of feeding, even when conditions precluded the possibility of transmission by infected feces. Moreover, they were the first to describe the "obstructed" or "blocked" flea phenomenon. Some of the fleas in their experiments developed a mass of *Y. pestis* in the proventriculus that prevented blood from entering the midgut. It did not prevent sucking, however, so the starving fleas fed repeatedly, causing the esophagus to become distended with blood. Bacot and Martin suggested that if the pharyngeal pump were to stop for a moment during feeding, some of the blood in the distended esophagus could be driven back into the bite wound, carrying the plague bacilli with it. Indeed, they found that blocked fleas, particularly *X. cheopis*, were more likely than their unblocked counterparts to transmit the bacterium to a host (10).

Since Bacot and Martin published their findings, most have regarded the oriental rat flea as the model *Y. pestis* vector, and studies of vector efficiency typically compare other flea species to *X. cheopis* (12, 13, 15, 23, 25, 26, 38, 39). This species is, indeed, widely distributed and has been implicated in countless human plague outbreaks (13, 15). *Xenopsylla cheopis* is not, however, the only flea species that vectors *Y. pestis*. Studies have found more than 150

different flea species naturally infected with the plague bacillus (23), and researchers have identified more than 30 species that are competent in the laboratory (1, 24, 25, 38-40). For the purposes of this thesis, a "competent" flea species is any species that can transmit *Y. pestis*, and "efficiency" refers to the proportion of infected fleas that transmit the bacterium to susceptible hosts.

Of course, not all flea species that are capable of transmitting the plague bacillus play a significant role in plague ecology or in the transmission of *Y. pestis* from rodents to humans. Early in the twentieth century, Hirst (12) observed that not all *Xenopsylla* species were equally efficient vectors. He attributed the absence of human plague in parts of India and Ceylon to the prevalence of a relatively inefficient vector, *Xenopsylla astia*, on commensal rats, while *X. cheopis* infested rats in areas that observed more human plague cases (12). He and others demonstrated that pools of *X. astia* fed on septicemic rodents were indeed less likely to transmit the bacterium than pools of *X. cheopis* fed on septicemic rodents (38, 41, 42). It is evident, however, that not every *X. cheopis* that feeds on a septicemic animal will then transmit *Y. pestis* to a susceptible host, whether it blocks or not (1, 10, 25-27, 38, 39, 41). Estimates of *X. cheopis* vector efficiency under laboratory conditions vary from 6.4 to 72% (40). This variability likely stems, at least in part, from methodological differences between studies (40). It is clear that a number of factors may impact transmission by any single species, including the temperature at which fleas are held (1, 43, 44), the source and concentration of infectious blood (24, 26, 45), the number and frequency of infectious and maintenance feeds (46) and the amount of time that passes between taking an infectious blood meal and feeding on a susceptible host (40, 47). Variability may also stem from differences between geographically distinct populations of a

single flea species (25), although this possibility needs to be further investigated using standardized experimental systems for assessing transmission efficiency (40).

Though the blocking phenomenon was rarely observed in some species known to be important vectors of *Y. pestis*, including *O. montana* (25, 26, 39, 48), blocking was long considered an essential component of vector potential (1, 23, 48, 49). The period required for a flea to develop a blockage varies between species, but the average for *X. cheopis* is between 12 and 16 days, and this species requires a minimum of 5 days to block (25, 26). It was observed, however, that some species feeding en masse could infect susceptible hosts within 36 hours of taking an infectious blood meal (25, 39). It was presumed that the mouth parts of fleas which had recently fed were contaminated with *Y. pestis* and that "mechanical" transmission thus occurred when large numbers of these fleas fed on a susceptible host (25). Burroughs (25) suggested that this might be the most important means of transmission during epizootics. A later study revealed, however, that *Y. pestis* does not survive on flea mouthparts for more than 3 hours (50).

When Engelthaler and others (26) investigated transmission rates and *Y. pestis* localization in laboratory-fed *X. cheopis* and *O. montana*, they found that both species developed infections in the midgut, but *X. cheopis* was more likely to have a concurrent infection in the proventriculus. Significantly, transmission rates did not correlate with blocking rates. In fact, none of the *O. montana* developed a proventricular blockage, but 4 of 196 *O. montana* transmitted *Y. pestis* to susceptible mice between 4 and 37 days after taking an infectious blood meal. Fifteen of 95 *X. cheopis* transmitted *Y. pestis* to susceptible mice, but only two of them were blocked; 7 additional blocked *X. cheopis* failed to transmit (26).

Within the last decade, Eisen and others have demonstrated that unblocked fleas belonging to several species can transmit *Y. pestis* in the first 4 days after taking an infectious

blood meal (47, 51-53). The mechanism for this phenomenon, termed "early-phase transmission" (47), remains unclear, but may involve regurgitation of the infectious remnants from a previous blood meal (47). Eisen and others (47, 51) found that early-phase transmission explains the rapid disease spread observed during epizootics, while the blocked-flea model does not, because transmission by unblocked fleas does not require a lengthy extrinsic incubation period (EIP), and unblocked fleas may remain infectious longer because they do not suffer block-induced mortality. This finding highlights the importance of distinguishing vector efficiency from vectorial capacity. Vector efficiency (also termed vector potential or transmission efficiency,) measures the proportion of infected fleas that transmit bacteria to susceptible hosts (40). But vector efficiency is only one element of vectorial capacity, which is the number of infective bites that will ultimately be delivered by all of the vectors feeding on a single host in one day (54). Macdonald's standard epidemiologic model of vector-borne disease dynamics is often used to define vectorial capacity,

$$V = (m \times a^2 \times p^n \times b)/-\ln p \qquad [1.1]$$

where m is vector density in relation to the host, a is the probability a vector feeds on a host in 1 day, b is vector efficiency, p is the probability the vector will survive one day, n is the duration of the EIP (in days), and $1/\ln p$ is the duration of the vector's life, in days, after surviving the EIP (54). According to this model, vectorial capacity is most sensitive to changes in daily survival rate, and it is also very sensitive to changes in the duration of the EIP and changes in the probability that a host feeds on a vector in one day (54). We will return to Macdonald's model later as it provides a useful framework for understanding our use of blood-meal analysis in an investigation of a possible *Y. pestis* bridging vector in a plague-endemic region of Uganda.

PLAGUE IN THE WEST NILE REGION OF UGANDA

History and Epidemiology. African countries accounted for only about 6% of human cases reported to the World Health Organization between 1958 and 1979 when a large proportion of the world's human cases occurred in Viet Nam as an indirect result of military operations there (31). The incidence of human plague in Africa began to increase dramatically in the early 1980s (55, 56). Between 1982 and 1996, Africa reported more than 17,000 cases, 66% of the world total (4, 56). African countries accounted for more than 90% of reported human plague cases every year between 1997 and 2009 (4). Since the 1980s, the majority of human plague cases have been reported from Madagascar and East African nations (4, 55, 56). The first records of human plague in Africa came from missionaries who observed cases in Uganda in 1877 (57). There is no doubt, however, that Ugandans suffered plague long before that; a number of ethnic groups were already familiar with the disease in 1877 and there were words for it in at least three traditional languages (58). Notably, this implies that *Y. pestis* was circulating in East Africa even before the third pandemic arrived at its shores. The third pandemic probably spread to ports on the east coast of Africa from India, and eventually spread into Uganda via construction of the Ugandan railway between 1896 and 1901. Between 1903 and 1908, most of the ports on Lake Victoria are reported to have suffered outbreaks, and plague was reportedly responsible for more than 61,000 deaths in central Uganda between 1910 and 1945 (1, 57, 59). All of the plague foci in central Uganda became quiescent by 1950 (Figure 1.2) (55, 58, 59). In the 1950s, outbreaks occurred in two villages located near the Uganda-Congo boarder, well outside the former endemic area. The origin of these outbreaks is unknown, but the villages were approximately forty miles from the Lake Edward plague focus in the Congo (now the DRC) (Figure 1.2B) (59).

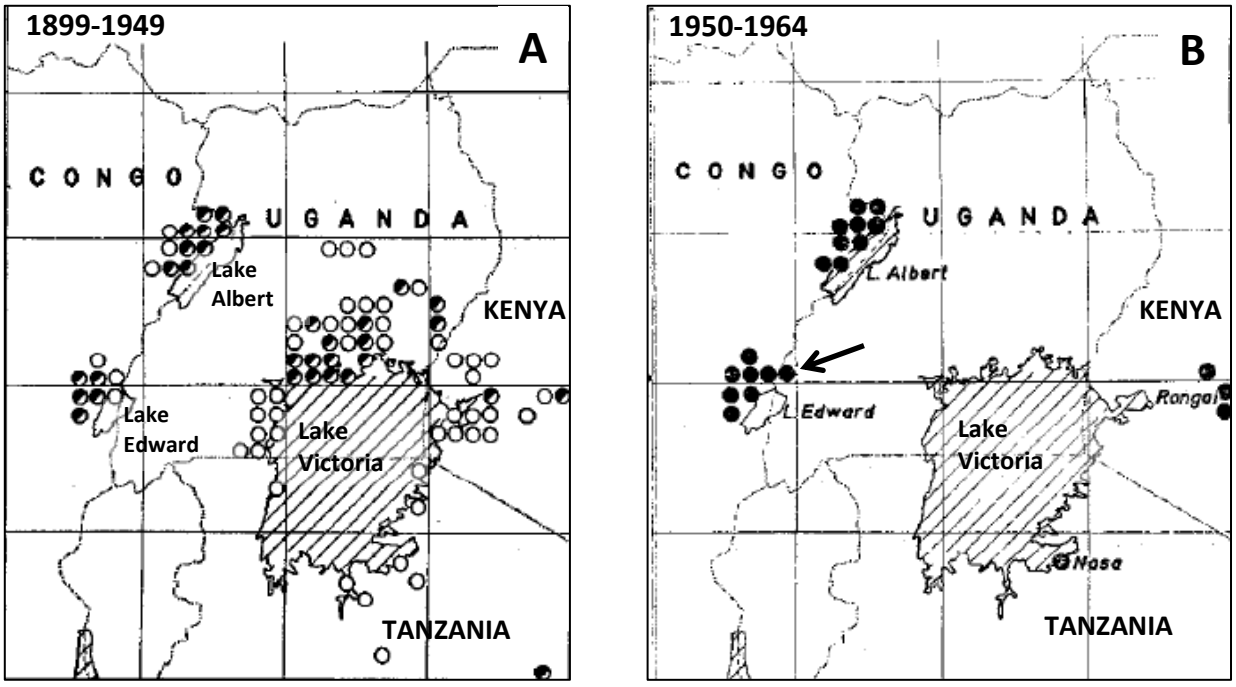

Figure 1.2. Human plague outbreak centers in Uganda between **A**, 1899 and 1949 and **B**, 1950 and 1964, as reported by D. H. S. Davis (59). Circles in **A** represent outbreak centers during the first half of the twentieth century. Half-moon symbols in **A** indicate that outbreaks continued to occur between 1940 and 1949. The arrow in **B** indicates the location of two villages where isolated outbreaks occurred in the 1950s.

In recent decades, the West Nile region of northwest Uganda has represented the primary epidemiological plague focus in Uganda (55, 58, 60). Plague has been endemic in Nebbi/Zombo District for at least 40 years, with most human cases occurring in its western-most county, Okoro, which lies on the Uganda-DRC boarder (Figure 1.3) (61, 62). (Note that Nebbi District was recently divided into two districts: Nebbi and Zombo. Okoro County now lies in Zombo District (63). Here, Nebbi/Zombo refers to Nebbi District before the boundaries were redrawn.) In 1998, the first human plague cases were reported from Arua District, immediately north of Nebbi/Zombo (61, 64). Human cases in Arua District are reported primarily from Vurra County,

which also lies on the Uganda-DRC border (Figure 1.3) (62). In both Vurra and Okoro counties, most cases are reported from villages located above 1,300 meters (62).

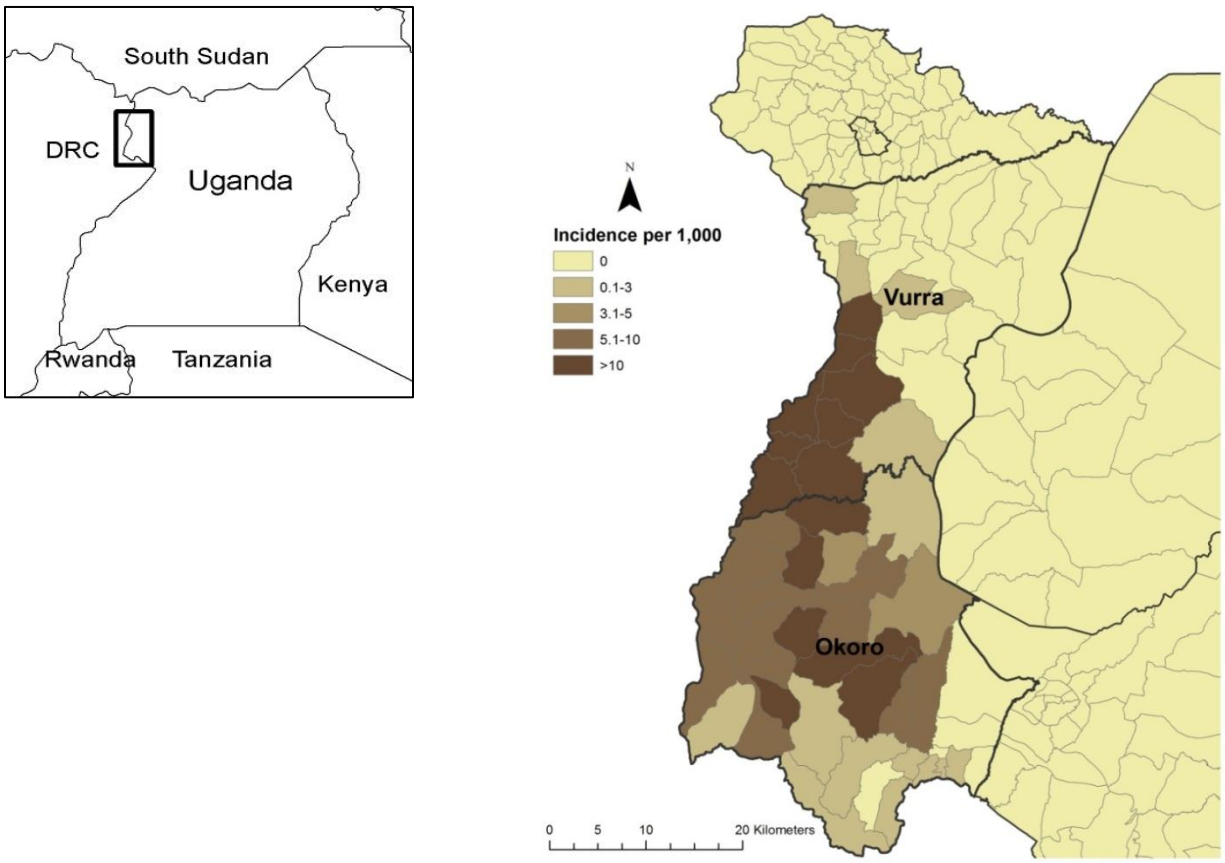

Figure 1.3. Reported cumulative human plague incidence per 1,000 population by parish (1999-2007) (62, CDC unpublished data).

A review of health records from clinics and hospitals indicated that more than 2,400 suspect human plague cases originated in Vurra and Okoro counties between January 1999 and January 2012 (Figure 1.4) (65, CDC unpublished data). Most human plague cases in the West Nile region occur between September and December, so a Uganda "plague year" may be defined as August of one year to July of the following year (65). Historically, cases were not laboratory-confirmed because of limited resources. Since 2008, however, the Centers for Disease Control

and Prevention has been working with the Uganda Virus Research Institute and the Uganda Ministry of Health to conduct research in the West Nile region and identify ways to reduce the incidence of human plague and plague-related mortality. This includes an effort to confirm plague diagnoses with laboratory testing. There were 3 laboratory-confirmed cases in early 2008, and of 169 suspect cases in the 2008-2009 plague year, 56 were laboratory confirmed. There were no laboratory-confirmed cases in either county between March of 2009 and October 2011. Three laboratory-confirmed cases were reported during the first half of the 2011-2012 plague year (Figure 1.4) (CDC unpublished data).

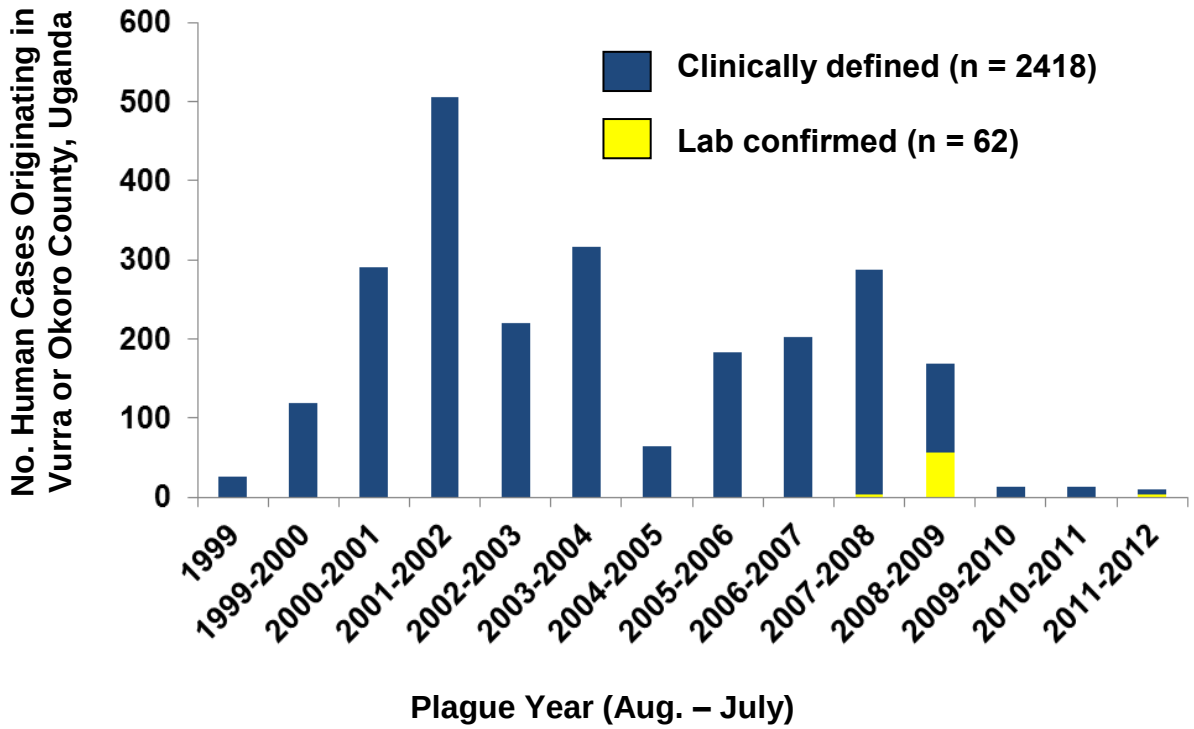

Figure 1.4. Annual cases of human plague in Vurra and Okoro counties between January 1999 and January 2012. Data compiled from a review of health records in clinics and hospitals (65, CDC unpublished data). A cases was defined as rapid onset of fever, chills, headache, severe malaise, and prostration with either (i) extremely painful swelling of lymph nodes in the arm-pits or inguinal area (bubonic plague), (ii) cough with blood-stained sputum, chest pain and difficulty breathing (pneumonic plague), or (iii) vomiting blood, bloody diarrhea (septicemic plague) (65).

The plague focus in northwestern Uganda is believed to be an extension of the Lake Albert focus in the DRC (1, 58). The first human cases were reported from the Lake Albert focus in 1928 (1), but there is evidence that *Y. pestis* has been established in East Africa since before Justinian's Plague in the 6th century. In the 1950s, Devignat identified three biochemical varieties (biotypes) of *Y. pestis* (59, 66). Both Orientalis and Antiqua reduce nitrate to nitrite, while Mediaevalis does not, and Orientalis is the only biotype that does not ferment glycerol (67). Devignat observed that all strains of *Y. pestis* isolated in northeast Congo (now the DRC) were biotype Antiqua, and he postulated that each of the three known pandemics was associated with one variety: biotype Antiqua with the Justinian Plague, Mediaevalis with the Black Death, and Orientalis with the third pandemic (59, 66). Analysis of seventy reference strains collected in Africa, Asia, North and South America, and Europe between 1908 and 1979 identified 16 ribotypes (rRNA gene restriction patterns) that could be organized into the three biotypes (3). This ribotype analysis also supported the hypothesis that *Y. pestis* originally evolved from the Central Asiatic plateau, but that one ribotype within biovar Antiqua arrived in Central Africa before Justinian's plague in the 6th century (3). The study classified the majority of twentieth-century isolates from Kenya and the Congo as biovar Antiqua (3). There is some recent evidence that *Y. pestis* strains most genetically similar to biotype Orientalis were responsible for all three pandemics (68), and one recent molecular study suggests that evolutionary branches of *Y. pestis* do not strictly correspond to the classic biovars, and that it is not possible to reliably determine the relationship between historical pandemics and modern isolates using existing data (69). This more recent study also indicates, however, that *Y. pestis* probably spread from Asia to Africa and other regions of the globe long before the 6th century (69). The West Nile region likely represents

part of an ancient plague focus in which the disease has recently emerged (or re-emerged) among humans.

Putative Host Species. It has been suggested that the recent incidence of human plague in the West Nile region can be attributed to the introduction of non-native *R. rattus* (58). It is believed that the black rat arrived in Uganda around 1900, and it quickly began to replace the multimammate mouse, *Mastomys natalensis*, as the dominant "hut rat" of central Uganda (57). Surveys conducted in 1937 and 1938 found that *R. rattus* had not yet invaded the West Nile region (57), but multiple studies over the past decade have confirmed that *R. rattus* is now the dominant domestic rat species in human habitations in the Arua and Nebbi/Zombo districts (52, 58, 60, 70). *Yersinia pestis* has been recovered from black rat carcasses collected in villages experiencing human plague outbreaks in this region (CDC unpublished data).

Mastomys natalensis, the Nile grass rat (*Arvicanthis niloticus*), and shrews (*Crocidura* spp.) also occasionally infest huts in the West Nile region and, along with *R. rattus*, account for the majority of peridomestic small mammals species (52, 58, 60). *Arvicanthis niloticus*, *M. natalensis* and *Crocidura* species also account for the majority of mammals collected in the "sylvatic" area between 20 and 300 meters away from villages (52). Recent serologic surveys have detected antibodies to *Y. pestis* in a small percentage of *A. niloticus* and *Crocidura* species trapped in the West Nile region (52, CDC unpublished data).

Hopkins (57) argued that *Y. pestis* circulated primarily among hut rats and their fleas, *Xenopsylla brasiliensis* and *X. cheopis*, in Uganda. While field rodents, including *Arvicanthis* species, might occasionally share fleas with the hut rats and thus become infected, Hopkins shared Roberts' conviction that field rodents did not play a significant role in maintaining the bacterium in endemic foci in East Africa (57, 71). Heisch and others (72) reported, however, that

during an outbreak in the Rongai area of Kenya, infected wild rodents were occasionally found around farms several months before *R. rattus* colonies became infected. They also found that wild rodents and their fleas still harbored the plague bacterium a year after plague had decimated the black rat population, and they could not find any evidence of infection in the few remaining black rats. They concluded that wild rodents, including *Arvicanthis* and *Mastomys* species, must serve as the primary *Y. pestis* reservoir in that region (72). *Arvicanthis* and *Mastomys* species have since been incriminated, along with other field rodents, as likely reservoirs for *Y. pestis* in other parts of Kenya (59), Tanzania (73, 74) and the DRC (15). *Mastomys* species have also been implicated as *Y. pestis* reservoirs in Zimbabwe and Mozambique (15).

Laboratory studies have demonstrated that while *A. niloticus* juveniles are somewhat susceptible to plague, adults are highly resistant (75). *Mastomys* populations in southern Africa are considered highly susceptible to *Y. pestis* (23, 59, 75), but Heisch and others (72) reported that both *Arvicanthis* and *Mastomys* populations in a plague-endemic region of Kenya were resistant. Isaacson and others (76) determined that *Mastomys natalensis* sensu lato is actually a species complex comprising two species with different karyotypes: the relatively resistant *M. natalensis* (2n = 32) and the highly-susceptible *Mastomys coucha* (2n = 36) (76). *Mastomys coucha* predominates in the drier habitats of southwestern Africa while *M. natalensis* predominates in southeastern regions where rainfall is more moderate (77). Shepherd and others (78) reported heterogeneity in the susceptibility of *M. natalensis* (2n = 32) from two different regions of eastern South Africa where plague was not endemic (78). Some level of resistance to *Y. pestis* infection would explain why serological surveys detect antibodies to *Y. pestis* in wild-caught *Arvicanthis* and *Mastomys* species (59, 73, 74), including within the West Nile region (CDC, unpublished data).

Borchert (58) proposed that both *A. niloticus* and *M. natalensis*, as relatively resistant species, may serve as enzootic hosts in the West Nile region of Uganda. Given that these species are abundant in the peridomestic setting, commonly harbor efficient vectors of *Y. pestis* (see discussion of flea vectors below), and are at least partially susceptible to *Y. pestis*, they may also serve as a potential source of infection to humans (52, 58). While *Crocidura* species have not been implicated as epizootic or enzootic hosts in any plague focus, another shrew (*Suncus murinus*) is believed to play a role in maintaining *Y. pestis* in Vietnam, Myanmar, Java and Madagascar (23, 79).

Putative Flea Vectors. Recent surveys have found that the black rats that likely serve as a source of *Y. pestis* infection for humans in the West Nile region are infested primarily with *X. cheopis* and *X. brasiliensis* (60, CDC unpublished data). Amatre (60) reported that these species accounted for more than 75% of fleas collected from black rats in Vurra and Okoro counties. *Rattus rattus* were also found infested with *Dinopsyllus lypusus*, *Echidnophaga gallinacea*, *Ctenophthalmus* species, and occasionally with *Ctenocephalides felis*. The majority of fleas collected from *A. niloticus* (73%), were *Dinopsyllus lypusus*, *Ctenophthalmus cabirus* and *Ctenophthalmus bacopus*, but the Nile rat also carried *Xenopsylla* spp. and – very rarely – *C. felis*. *Mastomys natalensis* and *Crocidura* species were also infested with *Xenopsylla* spp., *Dinopsyllus lypusus* and *Ctenophthalmus* species (60).

Xenopsylla brasiliensis, like *X. cheopis*, is believed to be among the most efficient vectors of the plague bacillus (1, 15, 38, 71, 80), and both species have been implicated as important *Y. pestis* vectors in other parts of East Africa (15, 57, 71, 80). *Dinopsyllus lypusus* and *C. cabirus* have also been shown to be capable of transmitting *Y. pestis*, and naturally-infected specimens have been collected in the DRC (80). *Dinopsyllus lypusus* is considered one of the

principal plague vectors in Tanzania and Kenya (15, 74). Given that *X. cheopis*, *X, brasiliensis*, *D. lypusus* and *C. cabirus* infest small mammal species in domestic, peridomestic and sylvatic settings, it is possible that one or all of these species may facilitate transmission of the plague bacillus within and between enzootic and peridomestic or domestic cycles in this region (52).

As noted above, human exposure to *Y. pestis*-infected fleas is most likely to occur in or near the home (22, 29). As the species that predominate on black rats, *X. cheopis* and *X. brasiliensis* are very likely to serve as *Y. pestis* bridging vectors to humans in the West Nile region (52). Therefore, vector-control efforts aimed at preventing human cases often target these species (70, 81). Recent investigations indicate, however, that the cat flea, *Ctenocephalides felis*, comprises more than 88% of off-host (host-seeking) fleas in huts in the West Nile region (52, 82). In plague-endemic regions of Tanzania and the DRC where the so-called human flea, *Pulex irritans*, is the most common off-host flea in human habitations, researchers have speculated that it may play a role in plague epidemiology (83, 84). Investigations in Africa and elsewhere suggest that *Pulex irritans* may serve as an anthroponotic vector, transmitting *Y. pestis* from septicemic to susceptible humans during outbreaks (18, 28, 85, 86). It is not known if *P. irritans* might play a role as a bridging vector in regions where it feeds on both rodents and humans (87). *Pulex irritans* rarely infests huts in Vurra and Okoro counties (52). Hopkins (88) described *C. felis* as "replacing" the so-called human flea in parts of East Africa (p. 150).

Naturally-infected *C. felis* have been found in the DRC (80) and Brazil (86), and while some previous studies had failed to demonstrate transmission by the cat flea (36, 80), Eisen and others (52) showed that *C. felis* is capable of transmitting *Y. pestis* during the early-phase period. Estimated early-phase transmission efficiency was only 0.57% (52), but a flea species that is a relatively poor vector under laboratory conditions may serve as an important vector under natural

conditions by virtue of its abundance (23, 48). Given their dominance in human habitations, we wondered if *C. felis* might serve as a secondary bridging vector in the West Nile region.

It should be noted here that cat fleas in East Africa are usually identified as *C. felis strongylus* based on morphological characteristics (52, 88, 89). Recent studies sought to determine some biological characteristics of *C. felis strongylus* and compare them to *C. felis felis* characteristics in the literature (90, 91). While these investigations indicated that *C. felis strongylus* might progress through some phases of development more slowly than *C. felis felis*, most characteristics, including hatch rate, the time required for larval development, and maximum lifespan away from the host both before and after the first blood meal, do not differ between the two alleged subspecies (90, 91). In addition, analysis of 8 different molecular markers has yielded no evidence for the existence of *C. felis* subspecies (92, 93). Therefore, it seems likely that *C. felis strongylus* shares important biological characteristics, including feeding behavior and transmission efficiency, with other *C. felis* subspecies, including *C. felis felis*. Except where indicated, references to cat fleas or *C. felis* in this thesis may refer to either subspecies.

Despite its common name, *C. felis* feeds on a wide range of hosts. Cat fleas have been found on dogs, cats, goats, pigs, sheep, cattle, poultry, and wild mammals including rabbits and rodents (94-100). In order to serve as a bridging vector, a flea species capable of transmitting *Y. pestis* must feed on a potentially-infected small mammal species and on humans (13, 23). As Macdonald's model indicates (equation [1.1]), the probability that a vector feeds on a host in one day, *a*, strongly affects vectorial capacity. Importantly, if a vector feeds on multiple species, *a* must be understood to incorporate not just the daily biting rate, but the host preference index, or the proportion of blood meals that a vector species takes from a specific host (54). The *a* term is

squared because the vector must feed twice to transmit a pathogen: once on an infected host and once on a susceptible host. In the context of a zoonotic pathogen like *Y. pestis*, a^2 must incorporate the probability that a vector will feed on a potentially-infected zoonotic host in one day, and the probability that it will feed on a human in one day. Admittedly, the Macdonald model, which was developed to describe the spread of *Plasmodium* from human to human by mosquitoes, may be somewhat limited in its ability to describe the capacity of zoonotic disease bridging vectors. It provides a useful framework, however, for understanding the important connection between host preference and vectorial capacity.

In order to determine if *C. felis* infesting human habitations in Vurra and Okoro counties might serve as a *Y. pestis* bridging vector, we sought to determine what proportion of these cat flea blood meals come from humans, and what proportion come from potentially-infectious small mammal species.

BLOOD MEAL IDENTIFICATION

Historical Perspective. Blood meal identification has been used for almost a century to determine the proportion of vector blood meals taken from host species of interest. In the early 1920s, King and Carroll (101) adapted the precipitin test to detect blood meals in mosquito populations associated with malaria in Louisiana. This technique, as described by Weitz (102), involves preparing antiserum (usually in rabbits) against proteins precipitated from the sera of potential host species. Antibodies in the antisera that cross react with sera from other species are then removed by absorption, although it often impossible to eliminate cross-reactivity with sera from closely-related genera. For example, a wide range of anti-bovid sera cross react with anti-ox or anti-sheep sera. The test is then conducted by examining the interaction between the antisera and a saline extract of the arthropod blood meal in a narrow tube. Formation of a white

precipitate indicates antigen-antibody binding and the presence of blood from the suspected host. In the 1950s, the World Health Organization and the Lister Institute of Preventative Medicine established a service to analyze mosquito blood meals using this method (103). In a 1964 paper (103), Garrett-Jones emphasized the value of blood meal identification for determining the "human blood index" needed to estimate case reproduction number and thus to assess the effectiveness of vector-control programs aimed at eradicating malaria.

The precipitin test remained the standard for blood meal identification for decades (104), but in the 1970s and '80s, researchers began to turn to more sensitive and specific serologic assays for blood meal identification, including passive hemagglutination inhibition (PHI) assays and the enzyme-linked immunosorbent assay (ELISA) (104, 105). PHI assays employ erythrocytes coated with host-specific antigen. In the presence of antisera produced against the host-specific antigen, antibody-antigen complexes are easily visualized as agglutination. If a blood meal extract contains sera from the same species, it will inhibit agglutination, while a blood meal that contains blood from a different species will not. PHI assays were therefore useful for differentiating blood meals from related species (e.g., humans, baboons and monkeys) (102). While this technique could be combined with the precipitin test to increase specificity, it was also labor-intensive and difficult (105, 106)

The ELISA involves coating microplates with blood smear eluates. As for the precipitin and hemagglutination tests, antisera are prepared against the sera from potential host species, but each antisera is then conjugated with an enzyme (e.g., horseradish peroxidase) that will act upon a substrate (e.g., ortho-phenylenediamine) to produce a color change. The conjugate is added to the coated microplate and a short incubation period allows for antibody binding to any corresponding antigen on the plate. The wells are then washed and the substrate added. A color

change indicates that the enzyme-linked antibodies are bound to antigen (from the blood meal eluate) on the plate (105, 107).

Serologic blood meal assays have provided invaluable insights into the blood feeding habits of a variety of disease vectors (104, 106). They can be sensitive and relatively simple to use, but cross-reactivity between antisera against related species can limit specificity (102, 105, 107-109). Serologic methods also require the production of antibodies against each host to be detected (105, 107), which precludes the possibility of detecting blood meals from unpredicted reservoirs.

Molecular Methods. Over the last two decades, researchers have adapted molecular methods to identify blood meals in hematophagous arthropods (108, 110). In addition to overcoming some of the limitations associated with serologic assays, a molecular assay also has the advantage of being able to incorporate polymerase chain reaction (PCR)-based detection of both host blood meal and pathogen DNA for studies that aim to identify potential reservoir species (111-115). Researchers have used a variety of molecular techniques including conventional PCR with host-specific primers (116, 117), dot blot hybridization (118), PCR-heteroduplex analysis (119), terminal restriction fragment length polymorphism analysis (120), and reverse line-blot hybridization (111, 112, 121, 122) to identify the source of arthropod blood meals to taxonomic levels ranging from order to species. At least two studies have employed real-time PCR with genus- or species-specific probes to detect and differentiate blood meals in disease vectors (87, 109). Like serologic assays, all of these methods require *a priori* knowledge of the host community. Primers or probes, for example, must be designed to target specific sequences associated with specific hosts. Some studies have achieved more flexibility by using conventional PCR with a "universal" primer set or sets to amplify a molecular target from a wide

variety of hosts, and then sequencing the amplicon to identify the blood meal source (114, 123-125).

One of the primary challenges associated with developing molecular methods to detect the tiny quantities of host DNA in blood-fed arthropods is achieving sufficient sensitivity. Many researchers address this challenge by designing blood meal assays that target mitochondrial DNA (110). Because each cell contains hundreds to thousands of copies of the mitochondrial genome, mitochondrial DNA targets are far more abundant than nuclear DNA targets (126). In addition, the mitochondrial genome evolves many times faster than the nuclear genome (127), and it is possible to differentiate taxonomic groups, including closely-related species, using relatively short mitochondrial sequences (128-130). Because inter-species and intra-species variability depend on the mitochondrial gene target, target selection depends on specific study goals (131), but popular blood meal assay targets include the cytochrome b, cytochrome c oxidase 1 and 12S rRNA genes (110).

There are a number of factors other than the molecular target that may impact blood meal assay sensitivity. Some researchers have reported that primers generating smaller amplicons are more likely to amplify DNA in old or degraded blood meals than primers generating relatively large amplicons (117, 132). Mota and others (113) found that the amount of blood consumed was the principle limiting factor for amplification of triatomine blood meal DNA by PCR. Kent and others (117) reported that it was more difficult to detect vertebrate DNA in blood-fed *Anopheles funestus* sensu lato than in other mosquito species because of their small body size. Others have reported, however, that there is no association between blood meal size and the rate of DNA amplification, whether comparing individuals that have consumed different volumes of blood (133) or comparing different species that take different-sized blood meals (134).

Not surprisingly, numerous studies in a variety of vectors have found that sensitivity is inversely related to the time between ingestion and collection; a molecular assay is most likely to detect host DNA in laboratory-fed arthropods collected immediately after they feed (111, 116, 124, 132, 133, 135, 136). Researchers also report that blood meal assays are less likely to amplify host DNA from field-collected arthropods containing more thoroughly digested blood meals as indicated by lower Sella scores which denote the relative amount of blood visible in mosquito abdomens (1 = unfed; 2-6 = partial to full blood meal; 7 = gravid; (137)) (114), or blood meal color in phlebotomine sand flies (124).

Vector-specific factors may also impact amplification success. Vertebrate DNA persists much longer in ticks than in hematophagous insects, for example, because ticks feed only once in a given life stage and digestion proceeds as a slow, intracellular process rather than in the lumen of the intestine (138). Using nested PCR to amplify a 95 base pair fragment, Kirstein and Gray (132) detected mouse DNA in all *Ixodes ricinus* nymphs examined 200 days after feeding (as larvae), but amplification succeeded in only 4 of 10 nymphal ticks 280 days post-engorgement, and DNA was no longer detectable 290 days post-engorgement. In contrast, Mukabana and others (133) detected human DNA in 90% of *Anopheles gambiae* held alive for 8 hours after feeding, but found that detection decreased significantly between 8 and 32 hours post-feeding. Ngo and Kramer (116) detected avian DNA in *Culex pipiens* up to 72 hours after feeding. Vertebrate DNA has been detected in tsetse flies and phlebotomine sand flies up to 96 hours post ingestion (136, 139).

Blood Meal Identification in Fleas. Relatively few blood meal assays have been developed to detect host DNA in fleas. Franklin and others (140) employed species-specific primers in a traditional PCR assay to detect DNA from prairie dogs, ground squirrels and

grasshopper mice in fleas collected directly from each of these hosts and in off-host fleas collected from prairie dog burrows. They used a "universal" primer set to try to detect vertebrate DNA in any sample that tested negative using all three host-specific primer sets. Their assay detected vertebrate DNA in 62% of fleas removed from vertebrate hosts, but they did not detect a vertebrate blood meal in any of the off-host fleas (n = 230), even though they only tested fleas with a visible blood meal in the abdomen. This suggests that blood meal identification in fleas collected off-host may demand a more sensitive detection method.

Real-time PCR is more sensitive than conventional PCR (141). Woods and others (87) designed a probe-based real-time PCR assay to identify blood meals from eight vertebrate hosts in off-host fleas collected in East Africa. The assay detected vertebrate DNA in artificially-fed fleas for at least 72 hour post feeding, although sensitivity varied between host species. Using the assay, they identified host DNA in 64 of 80 pools of field-collected off-host fleas. It was not possible, however, to determine if the fleas included in pools with no detectable vertebrate DNA had not fed at all, contained blood meals that were too old to detect, or contained blood from a host species not included in the test panels. For our study, we sought to develop an assay with sufficient sensitivity, specificity,, and flexibility to identify blood meals from a wide variety of vertebrate hosts in off-host cat fleas collected in the West Nile region.

HYPOTHESIS AND RESEARCH OBJECTIVES

We hypothesized that *C. felis* infesting human habitations in the West Nile region feed on humans and on potentially infectious rodents or shrews, and that they may therefore serve as a *Y. pestis* bridging vector in this plague-endemic region of Uganda. To test this hypothesis, we sought to determine what proportion of blood meals in these cat fleas come from humans, and what proportion come from potentially-infectious small mammal species, including *R. rattus*. In

order to identify blood meals in field-collected, off-host *C. felis* from the West Nile region, we developed a SYBR Green-I based real-time PCR assay using a single primer set to amplify vertebrate DNA for identification by sequencing. The assay thus capitalizes on the sensitivity of real-time PCR while allowing for identification of a wide variety of host species to the genus or species level. We used this assay to identify blood meals in off-host *C. felis* collected in huts in Vurra and Okoro counties.

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CHAPTER 2: COMBINING REAL-TIME POLYMERASE CHAIN REACTION USING SYBR GREEN I DETECTION AND SEQUENCING TO IDENTIFY VERTEBRATE BLOOD MEALS IN FLEAS[1]

SUMMARY

Programs that aim to control vector-borne zoonotic diseases require information on zoonotic hosts and on the feeding behavior of bridging vectors that are capable of transmitting pathogens from those hosts to humans. Here we describe an assay developed to identify blood meals in field-collected cat fleas (*Ctenocephalides felis*) to assess this species' potential role as a *Yersinia pestis* bridging vector in a plague-endemic region of Uganda. Our assay employs a single primer set and SYBR Green I-based real-time polymerase chain reaction (PCR) to amplify a segment of the 12S mitochondrial ribosomal RNA gene for identification by sequencing. The assay capitalizes on the sensitivity of real-time PCR and the specificity of sequencing and can be used to differentiate vertebrate blood meals to the genus or species level without *a priori* knowledge of the host community. Because real-time assays that detect vertebrate DNA are highly sensitive to human DNA contamination, we analyzed detection in artificially-fed and unfed fleas to establish a Ct cutoff that optimized specificity without completely sacrificing sensitivity. Using the established cutoff, our assay detected human and rat DNA in artificially-fed *C. felis* up to 72 hours post feeding.

INTRODUCTION

Programs that aim to control vector-borne zoonotic diseases require information on zoonotic hosts and on the feeding behavior of bridging vectors capable of transmitting pathogens

[1] Christine B. Graham, William C. Black IV, Karen A. Boegler, John A. Montenieri, Jennifer L. Holmes, Kenneth L. Gage and Rebecca J. Eisen contributed to the research described in Chapter 2.

from those hosts to humans (1-4). To better understand the feeding behavior of bridging vector species, researchers have developed various methodologies to identify blood meals in hematophagous arthropods.

For decades, researchers relied on serologic techniques, including precipitin tests, passive hemagglutination inhibition tests, and enzyme linked immunosorbent assays (ELISAs), to identify blood meals in arthropod vectors (5-7). These antigen-based methods have provided invaluable insights into the feeding behavior of many disease vectors, but they require the production of antisera against each potential blood meal source (7). Serologic tests thus require *a priori* knowledge of the host community; they cannot detect "unpredicted" hosts. In addition, their specificity is limited by the potential for cross-reactivity between related animal antisera (8, 9).

Over the last two decades, researchers have adapted PCR-based techniques to identify arthropod blood meals. Molecular assays have been used to identify blood meal sources from a variety of disease vectors, including mosquitoes (10, 11), ticks (12), sand flies (13), tsetse flies (4), kissing bugs (14), biting midges (15), and fleas (16, 17). Molecular methods for bloodmeal identification include amplification and analysis of microsatellites (tandemly-repeated 2-5 base pair DNA segments that vary in length according to the number of repeat units), minisatellites (tandemly-repeated DNA segments with more than 5 base pairs that vary in length according to the number of repeat units), short and long interspersed elements, heteroduplex analysis, reverse line-blot hybridization, restriction fragment length polymorphism (RFLP) and terminal RFLP analysis (18-20). PCR-based assays can be more specific than serologic tests, allowing identification to the species or even the individual level (4, 10). Some researchers have combined molecular methods with serologic techniques, using PCR, for example, to identify species that an

ELISA cannot reliably detect or differentiate (9). Others have developed highly specific stand-alone assays employing conventional PCR with species-specific primers, probes or RFLP analysis (4, 11, 12).

Real-time PCR is more sensitive than conventional PCR (21). A highly-sensitive assay is advantageous for detecting DNA from tiny blood meals that may be degraded due to digestion or storage conditions (18, 19). Researchers have developed probe-based real-time assays to identify blood meals in mosquitoes and fleas, but this technique requires knowledge of potential host species (9, 17). Using "universal primers" to amplify a molecular target from a group of species (e.g., vertebrates or mammals) and then sequencing the amplicon to identify the blood meal source allows for specific identification of both predicted and unpredicted hosts (13, 22). This is useful in situations when there is little or no data available on host abundance and diversity (13). Here, we describe a SYBR Green I-based real-time PCR assay using a single primer set to amplify vertebrate DNA for identification by sequencing.

Our assay capitalizes on the sensitivity of real time PCR and the specificity of sequencing. Like other sensitive molecular assays that amplify vertebrate DNA, real-time PCR is subject to human DNA contamination (23). Here, we discuss the utility of establishing an appropriate threshold cycle (Ct) cutoff value to dichotomize positive and negative results and thus reduce the risk of false positives. Having established an appropriate cutoff, we tested the assay's ability to detect vertebrate DNA in artificially-fed fleas up to 98 hours post feeding. We thus sought to verify that the assay could detect vertebrate DNA in partially-digested blood meals, and that it had similar sensitivity to human and rat blood meals taken under similar conditions.

We developed this method specifically to identify blood meals in field-collected cat fleas (*Ctenocephalides felis*) to assess this species' potential role as a bridging vector in the West Nile region of Uganda. Within this region, cat fleas are the predominant off-host species collected from human habitations, the presumed sites of human exposure to plague bacteria (24). A previous study demonstrated that *C. felis* is a competent vector of *Y. pestis* (24). However, cat flea feeding preferences in this region are largely unknown. Our aim was to determine what proportion of all cat flea blood meals come from small mammals that are susceptible to *Y. pestis* and might serve as a source of infection in this region, and what proportion of cat flea blood meals come from humans that could become infected by feeding cat fleas. It was essential to develop an assay that could be used to identify blood meals from all vertebrate hosts to establish the proportion of blood meals that could be attributed to any given species. Existing blood meal assays were deemed inadequate to assess the role of cat fleas as bridging vectors because they were either not sensitive enough to detect the very small amounts of DNA that are often observed in field-collected fleas or unable to capture the wide range of potential vertebrate hosts in this region.

MATERIALS AND METHODS

Primer Selection. Mitochondrial DNA is a commonly used and effective target for blood meal assays because of its high copy number and rapid rate of evolution relative to nuclear DNA (18). Optimal amplicon size for real-time assays using SYBR Green I as a fluorescent reporter is between 75 and 200 base pairs (bp) (25), and the ability to detect partially-digested blood meals via PCR appears to be inversely related to amplicon size (11, 26, 27). We therefore sought to identify primers that would amplify a relatively small mitochondrial gene fragment from vertebrates without amplifying flea DNA. Additional criteria for our molecular target included

low intra-species variability, sufficient inter-species variability to allow for differentiation at the species or genus level, and minimal primer degeneracy as this increases the risk of mispriming and may thus generate unintended amplification products and false positives in SYBR Green I-based assays (28). We found the mitochondrial gene encoding 12S ribosomal RNA best suited to our purposes.

Humair and others (12) previously identified two highly-conserved regions flanking a variable region within the 12S rRNA gene. We retrieved the corresponding nucleotide sequences from the GenBank database for *C. felis* (U73741.1) and for six species believed to be potential hosts for *C. felis* in the West Nile region of Uganda: cat (*Felis catus*; NC_001700.1), chicken (*Gallus gallus*; NC_001323.1), human (*Homo sapiens*; NC_012920.1), black rat (*Rattus rattus*; AJ005780.1), domestic dog (*Canis lupus familiaris*; NC_002008.4) and goat (*Capra hircus*; NC_005044.2). We aligned the sequences using ClustalW2 and designed a 2-fold degenerate forward primer, 12S_425F (5' – *TGT AAA ACG ACG GCC AGT* GGG ATT AGA TAC CCY ACT ATG C – 3'), that can be paired with the 12S_9R primer described by Humair and others (12) (5' – *CAG GAA ACA GCT ATG AC*A GAA CAG GCT CCT CTA G – 3') to amplify vertebrate DNA without amplifying *C. felis* DNA. Each primer included an M13 tag (shown in italics in primer sequences) to facilitate sequencing (29). When we compared real-time amplification of DNA from 3 different vertebrate species using primers with and without these tags, we found that they behaved similarly, although the tagged primers benefited from an increased annealing temperature (data not shown). Employing the tags allowed us to achieve longer, cleaner sequences, so we used the tagged primers for all experiments described here. The target amplicon length was 173-176 base pairs (bp).

DNA Extraction. Before extracting DNA from individual fleas, we removed surface contaminants by soaking each flea in 50% bleach (3.08% sodium hypochlorite) and rinsing in calcium- and magnesium-free Dulbecco's Phosphate Buffered Saline (DPBS). Linville and others (30) reported that bleach successfully eliminated vertebrate DNA from the surface of maggots without compromising analysis of mitochondrial DNA in the crop contents. A subsequent study (31) found that a 15-minute treatment with 50% bleach was sufficient to remove contaminating DNA from the surface of ancient skeletal remains handled with bare hands. Each flea was then homogenized in 100 µl DPBS using 3-mm glass beads and a Mixer Mill (Model MM300, Retsch, Hann, Germany) set at 20 beats per second for 10 minutes. We extracted DNA from the homogenate using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) per the manufacturer's instructions for purification of genomic DNA from small volumes of blood and eluted the DNA with 70 µl PCR-grade water (Roche Diagnostics, Indianapolis, IN). We extracted DNA from whole, citrated *Rattus norvegicus* blood (Bioreclamation, Westbury, NY) using the DNeasy Blood and Tissue Kit (Qiagen) and following the standard kit protocol. All DNA was stored at -80°C until PCR analysis.

Real-Time PCR. Each 25-µl real-time reaction contained 12.5 µl iQ SYBR Green Supermix (2X; Bio-Rad Laboratories, Hercules, CA), 100 nM forward and reverse primers and 10 µl DNA template. We performed all reactions in an Mx3005P thermal cycler (Stratagene, La Jolla, CA). A 5-minute initial denaturation at 95°C was followed by 38-40 amplification cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s. Bound SYBR Green I fluorescence was measured following each amplification cycle. The software set the background fluorescence for all samples at a common starting point (baseline correction), then plotted relative fluorescence (dR) versus cycle number to generate an amplification plot for each sample. The final

amplification cycle was immediately followed by a melting analysis cycle during which all products were melted at 95°C for 1 minute, annealed at 55°C for 30 s, and then subjected to a gradual rise in temperature to 95°C. The PCR software collected fluorescence data following each incremental temperature increase. The software then plotted the negative first derivative of the raw fluorescence data versus temperature to generate a melting peak. A single peak generally indicates the presence of a single PCR product in the corresponding well, and similar amplicons peak at similar temperatures (32).

Each real-time run included at least two no-template-control (NTC) wells (water in place of template DNA), and control DNA isolated from *R. norvegicus* whole blood, diluted to generate a single stock, and stored at -80°C in single-use aliquots. The *R. norvegicus* DNA served as a positive control and an inter-run calibrator (IRC). We ran the IRC in the same position on all plates. Following each run, we manually adjusted the threshold value such that the IRC amplification plot crossed the threshold at the same cycle. By keeping the IRC Ct value constant, we ensured equivalent threshold settings across runs. After we set the threshold, the software determined a Ct value for each sample.

We ran all samples, including the IRC, in duplicate. Following each run, we examined the results to ensure that replicates had similar Ct values and melting peaks. The software calculated a collective Ct value from each pair of replicates by averaging the fluorescence of both replicate wells at every cycle to generate a common amplification plot for the sample. We considered a sample positive at a given Ct cutoff value if the collective Ct value was less than or equal to the cutoff, at least one replicate crossed the threshold below the cutoff and the second replicate crossed no more than 2 cycles above the cutoff. We repeated any sample with dissimilar replicates. If a sample repeatedly yielded inconsistent replicates, or if a sample

repeatedly generated melting curves with multiple peaks, we concluded that the assay could not reliably identify a blood meal in that sample.

Sequencing. Following PCR, all amplicons were stored promptly at 4°C or -20°C. We chose a single replicate from each positive sample for sequencing and purified the amplicon using the QIAquick PCR Purification Kit (Qiagen). We eluted the purified product with 20 µl nuclease-free H_2O and estimated its concentration using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Purified products were used immediately or stored at -20°C until sequencing. We prepared forward and reverse sequence reactions for each sample. Each 20-µl sequencing reaction contained approximately 6 ng purified amplicon, 6.6 pmol M13 forward or reverse sequencing primer (29), and 8.0 µl BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Foster City, CA). Cycle sequencing comprised a 1 minute denaturation at 96°C followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. We used the BigDye Xterminator Kit (Applied Biosystems) to remove unincorporated dyes before analyzing the samples on an ABI 3130XL genetic analyzer. Using Lasergene software (DNASTAR, Inc., Madison, WI), we edited forward and reverse sequence traces and assembled a single sequence for each sample. We then manually removed the primer sequences from either end of the sequence and identified the source of the DNA using the Basic Local Alignment Search Tool (BLAST) to find matching mitochondrial sequences in the nucleotide collection database.

To minimize the risk of cross-contamination, we conducted DNA isolation, PCR set-up and amplicon processing in three separate rooms. We used certified DNA-free pipet tips and tubes for DNA isolation and PCR set-up. All surfaces and equipment were treated with 0.62% –

0.99% sodium hypochlorite or DNA Away (Molecular BioProducts, San Diego, CA) and rinsed with 70% reagent alcohol (Ricca Chemical Company, Arlington, TX) before and after use.

Establishing Ct Cutoff Value. *Blood***.** We validated the assay using rat and human blood because the assay was developed to assess the proportion of vertebrate *C. felis* blood meals taken from humans and the proportion taken from small mammals that might serve as a source of *Y. pestis* infection in the West Nile region of Uganda. The black rat (*Rattus rattus*) is the predominant small mammal species in human habitations in this region, is highly susceptible to *Y. pestis*, and develops the very high bacterial burden required to infect feeding fleas (33-37). We selected *Rattus norvegicus* Wistar Hannover as our rat blood source for this experiment because it is commercially available and the complete *R. norvegicus* 12S sequence is available from the GenBank database. We determined that the *R. norvegicus* Wistar Hanover sequence flanked by our primers differs from the *R. rattus* sequence by 3 nucleotides. We therefore concluded that *R. norvegicus* blood could serve as a reasonable proxy for *R. rattus* blood.

Fleas. To determine the assay's sensitivity and specificity at different Ct cutoff values, we used *Xenopsylla cheopis* Rothschild reared in colonies maintained by the Centers for Disease Control and Prevention Division of Vector-Borne Diseases (Fort Collins, CO). We starved adult fleas for 6 days before offering them a blood meal using artificial feeders containing citrated whole blood from either human or rat (Bioreclamation). After one hour, fleas were removed from the feeders, immobilized by chilling on ice and examined by light microscopy for the presence of an obvious red blood meal in the proventriculus or midgut. We stored fed fleas alive for 24 hours in flasks containing filter paper at 23°C and ~85% relative humidity. We then transferred all surviving fed fleas to petri dishes containing the solution used to trap off-host fleas in our field studies: 2% NaCl with 2 drops per liter Tween 80. The fleas died in the trap

solution, and we transferred them by hand to 95% ethanol. Because fleas are usually removed from traps in Uganda by hand without wearing gloves, we used the same method in this experiment so that our results would reflect any impact this might have on vertebrate DNA detection using our assay. We then determined the sex of each flea by light microscopy and stored individual fleas in tubes of 95% ethanol at -20°C until DNA isolation.

Unfed *X. cheopis* were collected by sifting pupae from the colony medium and holding the pupae in a conical tube for 2 weeks. We then shook the tube gently to encourage emergence and harvested teneral fleas by vacuum aspiration. We transferred the fleas to trap solution and processed them using the same method described above for fed fleas.

Calculating Optimal Ct Cutoff Value. We established a Ct cutoff value for our real-time assay using a modification of the "epidemiological approach" described by Caraguel and others (38). We expected DNA isolated from fed fleas to contain either human or rat DNA and DNA isolated from unfed fleas to be negative for vertebrate DNA. Using the real-time protocol described above with 40 amplification cycles, we tested each sample in duplicate and recorded either its collective Ct value or that it contained no detectable vertebrate DNA. To ensure that any differences between real-time runs would similarly affect all sample types, each run included rat blood-fed flea DNA, human blood-fed flea DNA, unfed flea DNA, and NTCs. We then determined whether each human and rat blood-fed flea would be classified as having a detectable blood meal at each whole Ct cutoff value between 34 and 40 cycles. From this we calculated assay sensitivity (the percentage of blood-fed fleas with detectable vertebrate DNA) at each Ct cutoff value. We followed the same procedure using the unfed flea data to determine assay specificity (the percentage of negative samples in which we could not detect vertebrate DNA). We sequenced amplicons from all false-positive samples to identify the source of contaminating

vertebrate DNA. We then calculated the positive predictive value (PPV, the percentage of samples with detectable rat DNA that came from rat blood-fed fleas and the percentage of samples with detectable human DNA that came from human blood-fed fleas) at each Ct cutoff value. We used these calculations to identify the Ct cutoff value that maximized PPV. That is, we selected the Ct cutoff that maximized the chances that a flea that tested positive for rat or human DNA had actually consumed a rat or human blood meal respectively. We sequenced all blood-fed flea samples with a Ct value below that cutoff to verify that we had amplified the expected sequence.

Determining Real-Time Assay Linear Dynamic Range, Limit of Detection and Variability. To verify that the established Ct cutoff fell within the dynamic range over which the real-time reaction is linear, we diluted *R. norvegicus* DNA 10-fold over 6 logs and ran 5 replicates of each dilution. We defined the limit of detection (LOD) for our assay as the lowest serial concentration at which all 5 replicates crossed the threshold before cycle 40 (38). We defined linear dynamic range (LDR) as the range of concentrations (highest to LOD) over which data from all replicates could be fit to a standard curve plot with an $R^2 \geq 0.985$. We analyzed intra-assay variation by calculating the standard deviation of Ct values at each concentration within the LDR (28).

Assessing Assay Sensitivity to Blood Meal DNA Over Time. To determine the effect of *C. felis* digestion on assay sensitivity over time, we allowed unfed, colony-reared *C. felis* (Heska Corporation, Loveland, CO) to feed on citrated human or rat blood using artificial feeders. Fed fleas were identified by light microscopy and held alive as described above. Surviving fleas were collected by vacuum aspiration and stored at -20°C in 95% ethanol at 7 time points post feed: 2 hours, 4 hours, 12 hours, 24 hours, 48 hours, 72 hours and 98 hours. We chose 98 hours as the

cutoff because *C. felis* generally die within 4 days of leaving their host (39). We tested each flea using our real-time assay with 38 amplification cycles, and a sample was considered positive only if it had a collective Ct value ≤ 36. Each real-time run included an IRC, NTCs and negative control DNA from unfed *C. felis*. Amplicons from all positive samples were sequenced to verify that we had amplified the correct vertebrate DNA (human or rat). We compared the percentage of positive samples between time points for each blood type and between blood types for each time point using contingency table analysis and Fisher's Exact tests, with a p-value < 0.05 indicating statistical significance. We conducted all statistical comparisons using JMP statistical software (SAS Institute, Cary, NC).

RESULTS

Ct Cutoff Value. We isolated DNA from individual, artificially-fed *X. cheopis* collected 24 hours post feed (n = 50 rat blood-fed and 50 human blood-fed fleas), and from unfed *X. cheopis* adults (n = 50). Using our real-time assay, we determined the Ct value for each sample. The DNA samples from 5 (10%) of the human blood-fed fleas, 6 (12%) of the rat blood-fed fleas and 44 (88%) of the unfed fleas had a Ct value greater than 40 or repeatedly yielded dissimilar replicates. Two additional rat blood-fed flea samples (4%) repeatedly yielded melting curves with multiple peaks. We classified all of these samples as having no detectable blood meal at any Ct cutoff value. All other samples were considered positive at a given Ct cutoff value if both replicates amplified no more than 2 cycles above the cutoff and had a collective Ct value less than or equal to the cutoff. Sensitivity to vertebrate DNA in rat blood-fed fleas and human blood-fed fleas decreased from 84% to 68% and from 90% to 20%, respectively, as we decreased the Ct cutoff from 40 to 34 (Table 2.1). Assay specificity (the proportion of unfed fleas correctly

Table 2.1. Assay performance using Ct cutoff values between 34 and 40 cycles.

Ct cutoff value	Se (%): RF (n = 50 fleas)	Se (%): HF (n = 50 fleas)	Sp (%) (n = 50 fleas)	PPV (%): RF vs UF	PPV (%): HF vs UF	% NTCs with no detectable vertebrate DNA (n = 110 wells)
40	84	90	88	100	≤ 88.2[a]	≥ 52.7[b]
39	84	90	90	100	≤ 90.0[a]	≥ 66.4[b]
38	84	90	90	100	≤ 90.0[a]	≥ 80.9[b]
37	78	76	92	100	≤ 90.5[a]	≥ 95.5[b]
36	74	64	94	100	91.4	100
35	72	40	96	100	90.9	100
34	68	20	96	100	83.3	100

Se: sensitivity, the percentage of rat blood-fed (RF) fleas or human blood-fed (HF) fleas with a detectable vertebrate blood meal; Sp: specificity, the percentage of unfed fleas without detectable vertebrate DNA; PPV: positive predictive value, the percentage of fleas with detectable rat DNA that had consumed rat blood (RF vs UF) or the percentage of fleas with detectable human DNA that had consumed human blood (HF vs UF); NTC: No Template Control

[a] Sequencing indicated that all unfed flea DNA isolates with Ct ≤ 40 contained human DNA. We sequenced amplicons from fed flea samples only if they had a Ct value ≤ 36. We calculated PPV assuming that all rat blood-fed fleas and human blood-fed fleas with a detectable vertebrate blood meal contained rat or human DNA respectively. The true PPV for with Ct cutoff values > 36 could therefore be lower.

[b] We did not attempt to sequence amplicons from NTCs with Ct > 36. Sequencing could lead us to re-classify some false-positive

NTCs as negative, increasing the % of NTCs with no detectable vertebrate DNA.

identified as having no detectable vertebrate blood meal), increased by 8%, from 88% to 96%, over the same range (Table 2.1). Via sequencing, we identified the DNA in all 6 false-positive samples (unfed flea isolates with detectable vertebrate DNA) as human. Because none of the false positives was identified as *R. norvegicus* DNA, the effective PPV of a sample identified as rat-fed was 100% regardless of the Ct cutoff value (100% of fleas identified as rat blood-fed were rat-blood fed; Table 2.1). We therefore focused on the PPV associated with samples containing human DNA. Specifically, we calculated the PPV associated with human blood-fed versus unfed fleas (the percentage of samples in which the assay detected human DNA that came from human blood-fed fleas). The maximum PPV occurs at a Ct cutoff value of 36 (Table 2.1). Using this cutoff value, our assay is 94% specific; we expect a 6% false positive rate.

Using a Ct cutoff value of 36, we did not detect a significant difference between the percentage of vertebrate DNA detected from rat blood-fed fleas (74.0%) and human blood-fed fleas (64.0%; two-tailed Fisher's Exact, $p = 0.3873$). This indicates that, using this cutoff, the assay is no more or less sensitive to rat DNA than to human DNA in artificially-fed *X. cheopis* collected 24 hours after feeding. We sequenced amplicons from all of our positive samples with a collective Ct value ≤ 36. Amplicon sequences from all positive samples isolated from human blood-fed fleas were most similar to the expected region of human 12S rDNA. Likewise, amplicon sequences from all positive samples isolated from rat blood-fed fleas were most similar to the expected region of *R. norvegicus* 12S rDNA.

We included a total of 110 no template controls (NTCs) in our real-time runs. Using a Ct cutoff value of 40, our assay yielded 52 (52.7%) false positives (Table 2.1). Only one NTC well had a Ct value ≤ 36, however, and repeated attempts to sequence the amplicon failed. Therefore,

we classified this NTC as containing no detectable vertebrate DNA. Thus, using a Ct cutoff value of 36, 100% of our NTCs were effectively negative (Table 2.1).

Linear Dynamic Range, Limit of Detection and Variability. Using 10-fold dilutions of *R. norvegicus* DNA, we established 100 fg (10^{-15} g) as the limit of detection for our real-time assay. The corresponding collective Ct value was 36.43. The highest concentration we tested had a collective Ct value of 21.73. All replicates between 100 fg and 1 ng could be fit to a linear standard curve plot ($R^2 = 0.990$). Thus, the Ct cutoff value we selected, 36, falls within the assay's linear dynamic range ($21.73 \leq Ct \leq 36.43$). As is typical in real-time assays, intra-assay variation increased as DNA concentration decreased (28). At the highest concentration tested, Ct standard deviation over 5 replicates was 0.09. At the limit of detection, the standard deviation for Ct variance over 5 replicates was 1.19.

Assay Sensitivity to Blood Meal DNA Over Time. To determine the effect of digestion on assay sensitivity in *C. felis*, we artificially fed *C. felis* either human or rat (*R. norvegicus*) blood and collected a subset of fed fleas at each of 7 time points. We considered a sample positive for vertebrate DNA only if it yielded similar replicates with a collective Ct value ≤ 36. Amplicon sequences from all positive samples isolated from human blood-fed fleas were most similar to the expected region of human 12S rDNA. Amplicon sequences from all positive samples isolated from rat blood-fed fleas except one were most similar to the expected region of *R. norvegicus* 12S rDNA. One rat blood-fed flea collected 98 hours after feeding tested positive for human DNA.

We determined the percentage of fed fleas in which the assay detected and correctly identified a blood meal at each time point. The results are summarized in Figure 2.1. We did not detect a significant difference between the percentage of human blood-fed fleas with an

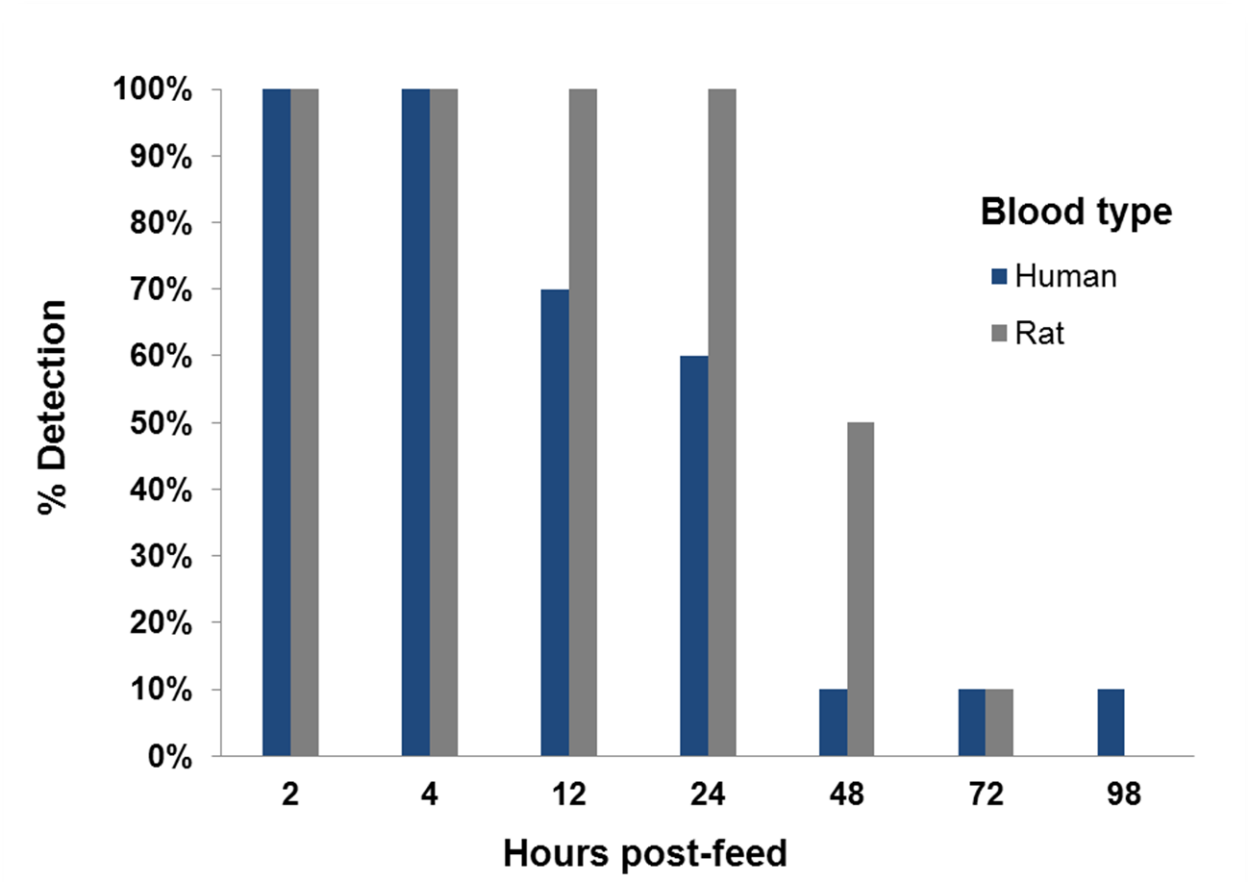

Figure 2.1. Percentage of blood meals detected and correctly identified in artificially-fed *C. felis* by time point and blood type (n = 10 fleas per blood type per time point).

identifiable blood meal and the percentage of rat blood-fed fleas with an identifiable blood meal at any single time point (two-tailed Fisher's Exact, $p \geq 0.0867$). As expected, detection of both blood types decreased over time. Comparing detection at the first time point (2 h post feeding, 100% detection) to detection at each subsequent time point for each blood type, we found a significant decrease in the percentage of fleas with a detectable human blood meal beginning at 24 hours (one-tailed Fisher's Exact, $p = 0.0433$), and a significant decrease in the percentage of fleas with a detectable rat blood meal beginning at 48 hours (one-tailed Fisher's Exact, $p = 0.0163$). We detected both human and rat DNA in fed fleas out to 72 hours. We detected human

DNA in a single human blood-fed flea at 98 hours, and we did not detect rat DNA in any of the rat blood-fed fleas at this final time point, but there was not a significant difference between the percent detection in human blood-fed versus rat blood-fed fleas at 98 hours post feeding (two-tailed Fisher's Exact, $p = 1.000$).

DISCUSSION

Since the precipitin test was first adapted almost a century ago to identify blood meals in vectors of malaria parasites, researchers have recognized vector feeding habits as a key factor in vector-borne disease transmission dynamics and blood meal assays as a powerful tool to help elucidate those feeding habits (40). Since then, researchers have developed and refined a range of serologic and molecular tools to improve blood meal assay sensitivity and specificity. Here we describe an assay that combines real-time PCR using SYBR Green I detection and sequencing to identify vertebrate blood meals in fleas. This method takes advantage of the sensitivity afforded by real-time PCR to overcome some of the challenges inherent in detecting tiny, often degraded blood meals in vectors. Because real-time assays that detect vertebrate DNA are highly sensitive to human DNA contamination, we analyzed detection in artificially-fed and unfed fleas to establish a Ct cutoff that optimized specificity without completely sacrificing sensitivity. Our assay employs sequencing to allow for specific identification of a wide range of vertebrate blood meal sources; it can be used to differentiate vertebrate blood meals to the genus or species level without *a priori* knowledge of the host community.

One of the primary challenges in developing a molecular blood meal assay is achieving sufficient sensitivity to detect DNA in tiny blood meals, often from species with enucleated erythrocytes (18). Franklin and others (16) employed species-specific primers in a conventional PCR assay to detect rodent DNA in field-collected fleas. Though they only tested fleas with a

visible blood meal in the abdomen, they did not detect a vertebrate blood meal in any of 230 off-host fleas. This suggests that blood meal identification in fleas collected off of hosts may demand a more sensitive detection method than conventional PCR, perhaps because some flea species take relatively small blood meals. While some studies have found that there is no association between blood meal size and the rate of DNA amplification (41), Mota and others (14) reported that the amount of blood consumed was the principle limiting factor for amplification of triatomine blood meal DNA by PCR. Haouas and others (13) suggested that the difficulty in detecting blood meals in sand flies may stem, in part, from their tiny size (0.5 – 1 µl). Flea blood meal size varies within and between species. *Ctenocephalides felis* tend to feed frequently, and actively reproducing females can consume an average of 13.6 µl per day when allowed to feed on a cat for 48 hours (42). It is not clear, however, exactly how much blood the *C. felis* gut contains at any given point. Adult fleas, particularly *C. felis*, begin to defecate soon after they begin feeding, and they excrete large amounts of fecal material (42, 43). Adult cat flea feces is an essential component of the larval flea diet, and studies of the protein content in adult cat flea feces suggest that adult fleas may digest blood less completely than other hematophagous arthropods to excrete more nutrients for larval development (43, 44). Digestion may therefore have less impact on the ability to detect a blood meal in fleas – particularly *C. felis* – than in some other hematophagous arthropods, but fleas may also excrete many components of the blood meal – including DNA – more quickly than other vectors.

To optimize assay sensitivity, we employed SYBR Green-I-based real-time PCR. Given that real-time PCR is exquisitely sensitive to contamination, however, we recognized the potential for our assay to yield false positive results, particularly since it detects human DNA. Forensic and anthropological studies highlight the risk of detecting contaminating human DNA

when using highly-sensitive molecular techniques to amplify DNA from tiny or degraded samples (45, 46). We took measures to eliminate contamination, and we sought to establish a Ct cutoff value for our real-time assay that would maximize specificity (minimize false positives) without completely compromising sensitivity. To this end, we conducted a laboratory study to determine the Ct cutoff value that would maximize PPV. We identified 36 as the Ct cutoff value below which real-time amplification of DNA isolated from a flea is most likely to indicate the presence of DNA from a blood meal (as opposed to contaminating human DNA). Significantly, selection of a Ct cutoff value based on maximum PPV is not prevalence dependent (38). Therefore, 36 should be an appropriate Ct cutoff regardless of the actual proportion of field-collected fleas that contain a detectable vertebrate blood meal. Ideally, we would have established the Ct cutoff using field-collected fleas from our target population, but this was not possible given that we were unable to verify the true status (fed vs. unfed) of our field-collected fleas from Uganda. Available flea samples were stored in ethanol and developed cloudy, gray midguts. This made it impossible to reliably distinguish blood-fed from unfed specimens via microscopy.

Using a Ct cutoff of 36, we detected contaminating human DNA in 6% of our unfed *X. cheopis*. Care must therefore be taken when interpreting the results from field-collected fleas that test positive for human DNA using this assay. Notably, we did not detect human DNA in any of the rat blood-fed *X. cheopis* with a Ct value less than or equal to 36, although 4 rat blood-fed *X. cheopis* were classified as having an undetectable blood meal at all Ct cutoff values because melting peak analysis of the real-time results indicated the presence of contaminants, which may have included human DNA, in some or all of the replicate wells. Only one of the 70 rat blood-fed *C. felis* we tested in the time-course experiment was misclassified as positive for human

DNA. We conclude that most false positives are likely to result from detecting contaminating human DNA in unfed fleas. It should therefore be possible to reduce the number of false positives by only testing vectors known to have taken a blood meal.

Having established a Ct cutoff of 36, we tested the assay's ability to detect rat and human DNA in artificially-fed *C. felis* out to 98 hours post-feeding. Our assay accurately classified all blood-fed fleas held up to 4 hours after feeding as positive for vertebrate DNA. As expected, our ability to detect human or rat DNA was inversely related to how long the fleas were held alive after feeding. Numerous studies in a variety of vectors have found a similar decline in sensitivity as the age of the blood meal increases (4, 9, 41, 47, 48). Because our aim is to accurately determine the proportion of blood meals from humans and small mammals in field-collected *C. felis*, we sought to develop an assay with similar sensitivity to blood from different species. Indeed, we did not detect significant differences between the assay's ability to detect rat and human blood at any time point. Using the Ct cutoff of 36, we also found no significant difference between the assay's ability to detect vertebrate DNA in rat blood-fed versus human blood-fed *X. cheopis* held for 24 hours after feeding.

Given that we only tested fleas that had fed on blood from two species, we cannot conclude that the assay has similar sensitivity to blood from all vertebrate species. It might, for example, be more sensitive to avian species which, unlike mammals, have nucleated erythrocytes (49). In addition, we found a significant decrease in detection beginning at 24 hours post-feed for human blood-fed fleas, while detection in rat blood-fed fleas did not decrease significantly until 48 hours post feed. This may indicate that *C. felis* excretes or digests rat blood more slowly than human blood, at least under our laboratory conditions. Other studies have demonstrated that host species can have a significant effect on the rate of blood digestion in fleas (50, 51), and a

difference in digestion rate might explain the impact of blood type on the ability of fleas to maintain a *Y. pestis* infection (52). Given these caveats, care must be taken when extrapolating flea feeding behavior based on the proportion of detected blood meals taken from any given species. Our results strongly suggest, however, that our assay is sufficiently sensitive to detect and accurately identify vertebrate DNA in fleas that have taken a recent blood meal from a rat or a human. This will allow us to confidently determine if a proportion of field-collected *C. felis* have fed on *R. rattus* or other small mammals that might serve as a source of *Y. pestis* infection and if this flea species might therefore serve as a bridging vector to humans in the West Nile region of Uganda.

We acknowledge several additional limitations associated with this type of assay. Unlike a probe-based real-time assay, this method requires post-reaction processing, and it does not allow for rapid identification of mixed blood meals. This method requires specialized equipment that may not be available in laboratories in developing countries. Also, the actual Ct value associated with any sample is likely to vary based on the specific real-time equipment and reagents used as well as on the calibrator used to set the threshold for each run. Any lab wishing to employ this assay would therefore have to conduct its own experiment to set an appropriate Ct cutoff value.

Despite these limitations, the assay described here will be useful for studies of flea-feeding behavior and plague ecology in the West Nile region of Uganda. It can be used to identify blood meals in off-host fleas collected from huts in these regions, and we are confident that it will detect rodent blood meals from potential reservoirs of *Y. pestis*, particularly *R. rattus*, if these fleas have taken a recent blood meal from such a host. With appropriate modifications, this assay could also be applied to research of other vector-borne diseases. Emerging zoonotic

infectious diseases with a wildlife origin represent a significant and growing threat to global health (53). As vector-borne zoonotic diseases emerge and re-emerge, they are most likely to originate in “hotspots” like tropical Africa, Latin America and Asia, precisely those regions where surveillance efforts are relatively weak (53). Control of these diseases will require identification and understanding of potential reservoir hosts and bridging vectors in regions where surveillance data may be absent or out of date. An assay combining real-time PCR with sequencing is sensitive enough to detect tiny, partially-digested blood meals, specific enough to identify the blood meal source to genus or species, and does not rely on *a priori* knowledge of host communities. This type of assay may therefore be particularly useful for investigating disease dynamics in the regions at greatest risk.

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CHAPTER 3: BLOOD MEAL IDENTIFICATION IN OFF-HOST CAT FLEAS (*CTENOCEPHALIDES FELIS*) FROM A PLAGUE-ENDEMIC REGION OF UGANDA[2]

SUMMARY

The West Nile region is an established plague focus in northwest Uganda. While rat fleas (*Xenospylla* spp.) are generally identified as the primary vectors for the plague bacterium, *Yersinia pestis*, in East Africa, recent investigations have found that the cat flea, *Ctenocephalides felis*, is the predominant off-host flea species in human habitations in the West Nile region. Recent laboratory studies have also demonstrated that while it is a less efficient vector than some other flea species, *C. felis* is capable of transmitting *Y. pestis*. A flea species must feed on potentially-infected zoonotic hosts and on humans in order to serve as a *Y. pestis* bridging vector. To determine if *C. felis* might serve as a bridging vector in the West Nile region, we employed a real-time PCR-based assay to estimate the proportion of off-host cat fleas collected in human habitations in Vurra and Okoro counties that had fed on humans and the proportion that had fed on potentially-infected zoonotic hosts. We also investigated *C. felis* infestation of small mammals trapped in the same huts. We trapped 944 black rats (*Rattus rattus*) in 765 of 2000 huts sampled for an overall capture rate of 38.3% per hut per sampling occasion. *Ctenocephalides felis* comprised only 2 of the 727 (0.3%) fleas collected from those rats. We detected and identified vertebrate DNA in 148 off-host *C. felis*, none of it from wild rodents or shrews. Our findings indicate that cat fleas in human habitations in the West Nile region are feeding primarily on domesticated species, especially goats. They appear to also be feeding on humans. We conclude that the cat flea is unlikely to serve as a bridging vector for *Y. pestis* in this region.

[2] Christine B. Graham, Jeff N. Borchert, William C. Black IV, Linda A. Atiku, Joseph T. Mpanga, Karen A. Boegler, Sean M. Moore, Kenneth L. Gage and Rebecca J. Eisen contributed to the research described in Chapter 3.

INTRODUCTION

Plague is a rare but highly virulent zoonotic disease (1). The etiologic agent, *Yersinia pestis*, circulates primarily in enzootic cycles between rodents or shrews and their fleas (2). Humans are most susceptible to infection during epizootics; when a large number of infected rodents die, their infectious fleas must seek new hosts, and these fleas may act as "bridging vectors" to humans (2). Control methods aimed at reducing the incidence of human plague often target these vectors (3). In order to serve as a bridging vector, a flea species must be capable of transmitting *Y. pestis*, it must feed on potentially-infected zoonotic hosts, and it must feed on humans.

The West Nile region is an established plague focus in northwest Uganda (4). More than 2,400 human suspect plague cases were reported in this region between 1999 and 2011 (5). While research has not determined exactly where humans are most likely to encounter infectious fleas in Uganda, studies in other regions suggests that human exposure occurs most often in the domestic or peridomestic environment (6, 7). Researchers generally identify rat flea species, *Xenopsylla brasiliensis*, *Xenopsylla cheopis*, and *Dinopsyllus lypusus*, as the primary bridging vectors for *Y. pestis* in East Africa (8-10). Some studies, however, have implicated the human flea, *Pulex irritans*, as a potentially important *Y. pestis* vector in regions where it is the predominant off-host flea species in human habitations, although it is unclear to what extent its postulated role in human plague outbreaks derives from an ability to spread the bacterium from one person to another versus an ability to serve as a bridging vector from rodent hosts to humans (10-14). Recent investigations have found that the cat flea, *Ctenocephalides felis*, comprises more than 88% of host-seeking (off-host) fleas captured in huts in the West Nile region (15, 16), but relatively little is known about its potential role as a *Y. pestis* vector in this plague focus. Cat

fleas in East Africa are most often identified as *C. felis strongylus* based on morphological characteristics (15, 17, 18). Recent molecular studies, however, have found no evidence for the existence of *C. felis* subspecies (19, 20). *Ctenocephalides felis strongylus* may therefore share biological characteristics, including feeding behavior and transmission efficiency, with other *C. felis* subspecies, including *C. felis felis*.

Worldwide, *C. felis* is associated with a wide variety of hosts including cats, dogs, livestock and wild mammals, including rodents (21-27). The cat flea also feeds readily on humans (26, 28-30). In the West Nile region, this species occasionally infests rodents, including the black rat (*Rattus rattus*), which is abundant in the domestic environment and susceptible to *Y. pestis* infection (8, 15, 31). Further implicating the cat flea as a potential bridging vector, Eisen and others (15) recently demonstrated that *C. felis* is capable of early-phase transmission of *Y. pestis*.

Here, we sought to investigate the potential for *C. felis* to serve as a *Y. pestis* bridging vector in the West Nile region. Specifically, we aimed to identify vertebrate blood meals in off-host (host-seeking) *C. felis* collected in huts in this region as a means to determine the proportion of meals taken from humans and the proportion taken from potential rodent or shrew reservoirs of *Y. pestis*. We also sought to determine what flea species infested small mammals captured in the same huts.

MATERIALS AND METHODS

Study Area. We collected fleas and small mammals in Vurra and Okoro, contiguous counties located along the Democratic Republic of Congo (DRC) border in the West Nile region of Uganda (Figure 3.1). The Rift Valley escarpment roughly bisects these counties, and most human plague cases are reported from villages located west of the escarpment at elevations

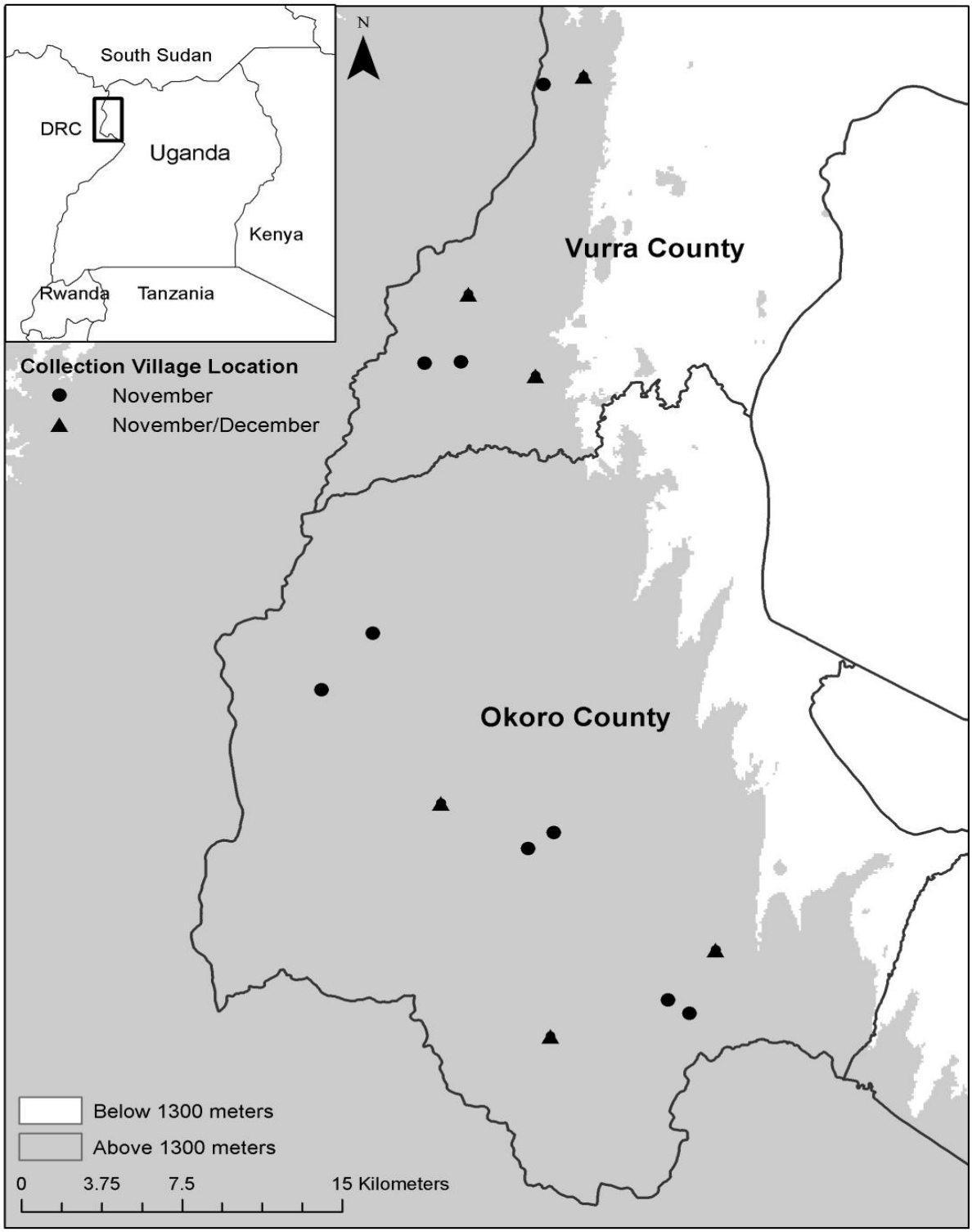

Figure 3.1. Study area within a plague-endemic region of Uganda. Points indicate village locations where fleas and rodents were collected from huts in November 2009 (circles) or November and December 2009 (triangles) for this study.

above 1300 meters (32). The western highlands are characterized by lush vegetation, fertile soil, numerous water sources, and highly-fragmented land use associated with subsistence farming (32, 33). This sub-humid region experiences heavy, reliable rains from late August through November and a less-reliable rainy season between March and June (32, 33). We randomly selected one hundred huts in each of 15 villages west of the escarpment for flea and small mammal collections in November 2009. We conducted a second collection 22 days later in 5 of the villages (Figure 3.1). Although most human plague cases are reported from this region between September and January (5, 32), our study occurred during an inter-epizootic period; there were no confirmed human plague cases in Vurra or Okoro county between March 2009 and October 2011 (CDC unpublished data). We collected all fleas and rodents inside homes typical of this area: square or round huts constructed of mud bricks and waddle with thatched grass roofs. Residents had often smeared a mixture of mud and bovine feces on their dirt floors and walls which had dried to create a hard surface (33).

Off-Host Flea Collection. We set one modified Kilonzo light trap (34) to collect photosensitive fleas in each hut. The trap consisted of a flashlight suspended over a metal pan (25.4 cm diameter) containing 2% saline with a surfactant (1 drop/liter Tween 80 detergent) to force trapped fleas to sink. We put fresh batteries in flashlights for each night of collection. We applied petroleum jelly to the rim of the pan to prevent fleas from escaping. Residents turned the flashlights on at night and left them on overnight. The following day, we collected the fleas from each trap and stored them in 70% ethanol.

Small Mammal and On-Host Flea Collection. Small mammals and their fleas were collected in the same huts on the same nights we collected off-host fleas as part of a previously-described study (35). We placed two traps (48.3 x 17.1 x 17.1 cm, Tomahawk Trap Co.,

Tomahawk, WI) against the inside wall of each hut. The following day we sedated trapped animals by halothane inhalation and identified captures to species based on morphological measurements (e.g., length of body, tail, right hind foot and ear) (36). We combed each animal to recover on-host fleas and stored the fleas in 70% ethanol. Rodents were then released at the point of capture.

We identified all fleas to species using published keys (17, 37, 38). We differentiated *C. felis strongylus* from the morphologically similar *C. felis felis* based on the relative lengths of the first two spines of the genal comb (26).

Blood Meal Identification. *Real-Time PCR and Sequencing.* We employed a SYBR-Green I-based real-time PCR assay using a single primer set to amplify vertebrate DNA for identification by sequencing. The assay was described in detail in Chapter 2. Briefly, we used primers targeting regions of the 12S mitochondrial RNA gene that are conserved across vertebrate species but differ in *C. felis*. The primers flank a variable region of approximately 100 base pairs (bp), and each includes an M13 tag to facilitate sequencing (39). We ran all reactions in an Mx3005P thermal cycler (Stratagene, La Jolla, CA). A 5-minute initial denaturation was followed by 38 amplification cycles, and the final amplification cycle was immediately followed by a melting analysis cycle. We stored all PCR products at 4°C or -20°C until purification. Selected amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced immediately or stored at -20°C. We generated forward and reverse sequences as previously described (Chapter 2), and we used DNASTAR Lasergene software to edit sequences and generate a single sequence for each sample. We manually removed the primer sequences from either end of the sequence for final analysis.

Analysis of DNA from Known Vertebrates. To test the assay's ability to detect and differentiate potential vertebrate host species from the study area, we purchased citrated human (*Homo sapiens*), cat (*Felis catus*), dog (*Canis lupus familiaris*), goat (*Capra hircus*), and chicken (*Gallus gallus*) whole blood from a commercial vendor (Bioreclamation, Westbury, NY). These are all species previously observed in or within 10 meters of huts in the West Nile region (CDC unpublished data). We collected tail tissue or whole blood from the 4 small mammal species that predominate in domestic and peridomestic environments in our study area: black rat, Nile rat (*Arvicanthis niloticus*), multimammate mouse (*Mastomys natalensis*), and shrew (*Crocidura* sp.) (15, 31). Mammals were trapped in the West Nile region and identified to genus or species based on morphological measurements (36). Tail tissue was stored in 70% ethanol. Whole blood was absorbed on Nobuto strips (Advantec, Toyo Roshi Kaisha, Ltd., Japan) made of cellulose paper. The absorbed blood (approximately 100 ul per strip) was air dried and the strips were stored at ambient temperature. We extracted DNA from citrated whole blood and tail tissue using the DNeasy Blood and Tissue Kit and from dried blood samples using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) per the manufacturer's protocols. All DNA was stored at -80°C until PCR analysis. We subjected each sample to amplification and sequencing as described above and aligned the 9 sequences to verify that no two sequences were identical. We also used the Basic Local Alignment Search Tool (BLAST) to identify similar mitochondrial sequences in the nucleotide database.

All animal-handling procedures were approved by the Animal Care and Use Committee at the Division of Vector Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. Voucher specimens for small mammals were previously submitted to Makerere University (Kampala, Uganda) for verification of species identification.

Blood Meal Identification in Off-Host Fleas. We removed surface contaminants from each Kilonzo-trapped flea, homogenized it in calcium- and magnesium-free Dulbecco's Phosphate Buffered Saline (DPBS), and extracted DNA as previously described (Chapter 2). Each set of extractions included a teneral (unfed), colony-reared *C. felis* (Heska Corporation, Loveland, CO) as a negative control. We used 10 µl eluted DNA per real-time PCR. Each real-time run included at least two no-template-control (NTC) wells (water in place of template DNA). *Rattus norvegicus* DNA from a single stock stored at -80°C in single-use aliquots served as a positive control and an inter-run calibrator (IRC) as previously described (Chapter 2). We ran all samples in duplicate. We considered a flea sample positive for vertebrate DNA if both replicates crossed the threshold within 38 cycles and had similar dissociation curves and a collective Ct value less than or equal to our previously established cutoff of 36 (Chapter 2). We classified a sample as having no detectable vertebrate DNA if both replicates had Ct values greater than 36 or if the sample generated similar replicates with a collective Ct value greater than 36. We repeated any sample with dissimilar replicates. If a sample repeatedly yielded dissimilar replicates, we concluded that the assay could not reliably detect a blood meal in that sample. We chose a single replicate from each positive sample for sequencing and used BLAST to identify the blood meal source.

Estimating the True Number of Human Blood-Fed Fleas and Statistical Analyses.

To determine if the distribution of fleas per hut and fleas per rat was consistent with a Poisson distribution, we compared actual and expected values for the number of rats and the number of huts infested with 0, 1, 2, 3, and 4 or more fleas and conducted a chi-square goodness-of-fit test.

Given that our assay previously detected human DNA in 3 of 50 unfed fleas (Chapter 2), we assumed that the total number of fleas with detectable human DNA in our study, *h*, included fleas that had actually consumed human blood, *x*, and samples that contained no detectable blood meal but had been contaminated with human DNA, *c*:

$$h = x + c \quad [3.1]$$

We estimated that approximately 6% of fleas with no detectable blood meal, *n*, tested positive for contaminating human DNA:

$$0.06n = c \quad [3.2]$$

The total number of fleas tested, *t*, included those with no detectable blood meal, those with a detectable human blood meal, and those with a detectable blood meal from a non-human vertebrate species, *b*:

$$t = n + x + b \quad [3.3]$$

Combining equations [3.1] through [3.3] and solving for *x*:

$$x = (h + 0.06(b - t))/0.94 \quad [3.4]$$

We solved equation [3.4] to estimate the number of field-collected fleas that had taken a human blood meal.

We used JMP software (SAS Institute Inc., Cary, NC) to calculate a Wilson score confidence interval (40) for the percentage of *C. felis* with detectable vertebrate blood meals that had fed on wild rodents or shrews.

RESULTS

Small Mammal and Flea Collections. *Rattus rattus* (n = 944) was the only small mammal species collected in the huts during the 4000 trap nights included in this study. We trapped at least one *R. rattus* in 577 of the 1500 huts sampled in November and in 188 of the 500 huts sampled in December for an overall capture rate of 38.3% per hut per night. We collected a

total of 727 fleas from *R. rattus*, of which the majority (91.1%) were *X. cheopis* or *X. brasiliensis* (Table 3.1). By contrast, the majority (86.9%) of the 839 off-host fleas collected from modified Kilonzo light traps were *C. felis* (Table 3.1). All off-host *C. felis* were identified to subspecies as *C. felis strongylus*. The number of fleas per *R. rattus* ranged from 0 to 27, but more than 90% of the captures were infested with two or fewer fleas (Figure 3.2A). Among sampled huts that yielded off-host fleas (n = 455 of 2000), the number ranged from 1 to 23 fleas per hut per sampling occasion, although most Kilonzo traps yielded no more than 1 flea (Figure 3.2B). Chi-square goodness-of-fit analysis revealed that neither the distribution of fleas per rat ($n = 944$, $\lambda = 0.77$) nor the distribution of fleas per hut ($n = 2000$, $\lambda = 0.42$) was consistent with a Poisson distribution ($p < 0.001$). The number of rats and huts with zero fleas and the number of rats and huts with more than four fleas were higher than the expected Poisson values. The number of rats and huts with one flea were lower than the expected Poisson values.

Table 3.1. Summary of on- and off-host flea captures, by species, in human habitations in Vurra and Okoro counties. Fleas were collected using modified Kilonzo light traps (off-host) or by combing *R. rattus* trapped in huts.

	Number of fleas (% of all fleas collected from the same source)					
Flea source	*C. felis*	*X. brasiliensis /X. cheopis*	*E. gallinacea*	*D. lypusus*	Other[a]	Unknown[b]
Kilonzo traps (off-host)	729 (86.9%)	32 (3.8%)	68 (8.1%)	0 (0.0%)	10 (1.2%)	0 (0.0%)
Rattus rattus[c]	2 (0.3%)	662 (91.1%)	0 (0.0%)	17 (2.3%)	23 (3.2%)	23 (3.2%)

[a]Other off-host flea species: *Tunga penetrans*, *Ctenocephalides* spp. too damaged to identify to species. Other flea species collected from *R. rattus*: *Xenopsylla* spp. too damaged to identify to species, *Ctenophthalmus calceatus cabirus*, *Afristivalius torvus* Rothschild (syn. *Stivalius torvus* in Hopkins 1947 (17)).
[b]"Unknown" includes fleas that were lost or too damaged to identify to genus.
[c]Borchert and others (35) previously reported flea species collected from *R. rattus* as part of a larger data set.

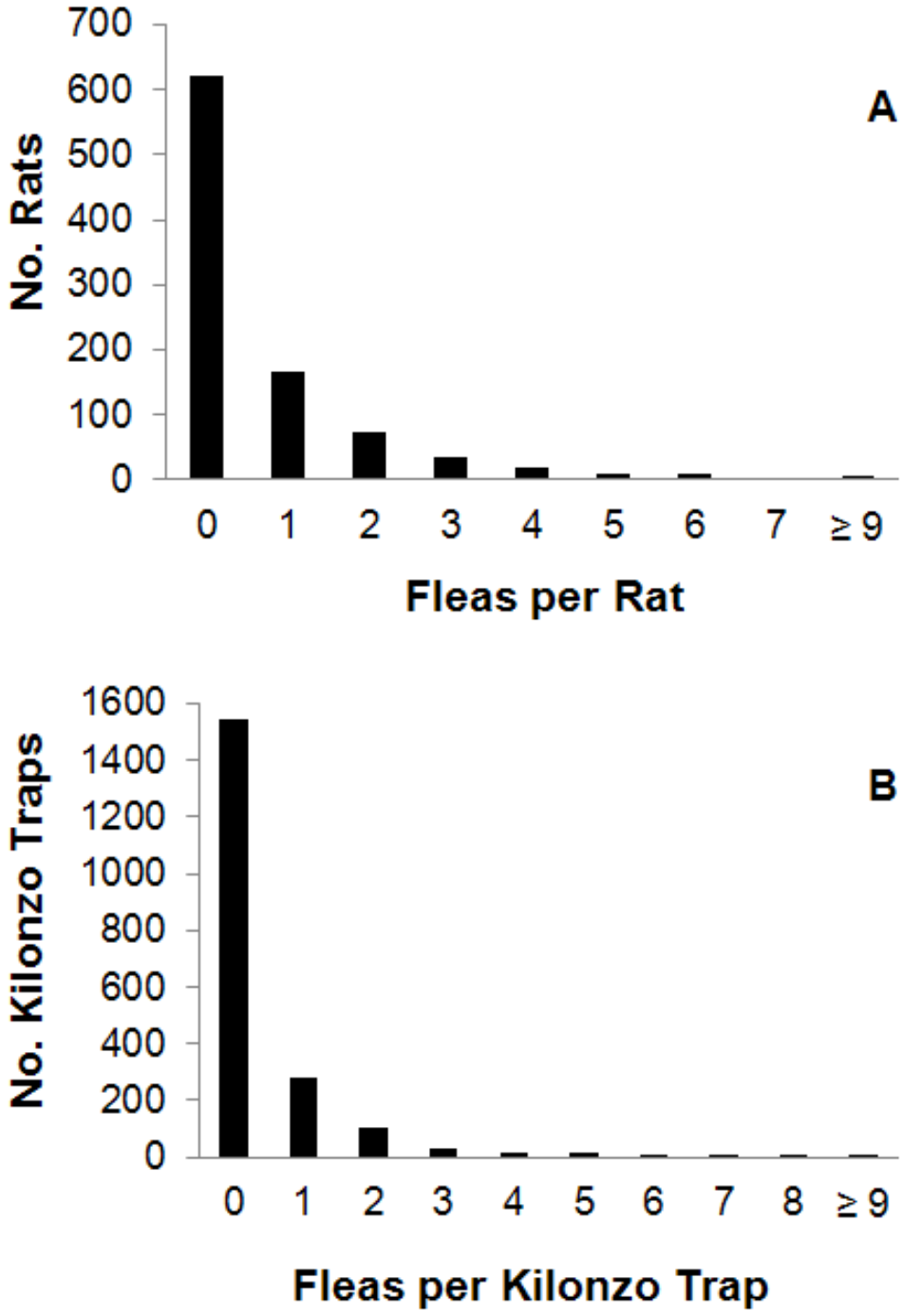

Figure 3.2. Distribution of fleas collected inside huts in Vurra and Okoro counties per **A**, *Rattus rattus* and **B**, modified Kilonzo light trap.

DNA Amplification and Sequencing from Known Vertebrate Species. Using our real-time PCR-based assay, we successfully amplified and sequenced the 12S rDNA molecular marker from 9 vertebrate species found within the domestic or peridomestic environment in our study area. With the primer sequences trimmed from either end of the amplicon, the resulting sequence was 99-102 bp long. Using BLAST, we confirmed that seven of the test sequences were identical to mitochondrial sequences in GenBank from the expected species (*A. niloticus*, AF141259.2:3-101; *C. hircus*, HM623880.1:25-124; *C. lupus familiaris*, AB499817.1 :513-611; *F. catus*, D28892.1:3-103; *G. gallus*, GU261719.1:1760-1861; *H. sapiens*, HQ700378.2:1097-1196; *R. rattus*, EU273707.1:514-613). GenBank did not contain the target sequence from *M. natalensis*, which was identified in our study using morphological characters found in taxonomic keys for this region of Africa (36). Our *M. natalensis* sequence (99 nt) shared 99% identity with corresponding sequences from two species in the same monophyletic group: *M. huberti* (AF141282.2:3-101; 26C>T) and *M. erythroleucus* (X85952.1:443-541; 465G>A) (41). We isolated *Crocidura* sp. DNA from a shrew that had not been identified to species. The target sequence (99 nt) was not identical to any sequence in the GenBank database; it differed by one nucleotide from the corresponding sequence for *Crocidura gueldenstaedtii* (AF434825.1:447-545; 493A>T). Aligning the sequences from the 9 species we tested revealed that each pair differed by at least 7 nucleotides. None of the BLAST results from these 9 species indicated that a different species from our study area had an identical target sequence.

Blood Meal Identification in Off-Host Fleas. Of the 729 *C. felis* collected in modified Kilonzo light traps, 694 undamaged fleas were tested. We detected vertebrate DNA in 151 (21.8%). Three samples contained vertebrate DNA from multiple sources as indicated by multiple melting peaks and/or sequence traces with overlapping fluorograms. In one case,

repeated attempts to obtain reverse sequence for a mixed DNA sample failed, so we generated and analyzed a 75-nt consensus sequence from two forward sequence reactions. In the other two cases we were able to assemble 100-nt sequences from overlapping forward and reverse sequences. We aligned the mixed-sample sequences, including ambiguous base calls where overlapping fluorograms resulted in double peaks, with the target sequences from the *Crocidura* sp. trapped in our study area and from the three predominant domestic and peridomestic rodent species in our study area: black rat, Nile rat and multimammate mouse (15, 31). The mixed-sample sequences differed from the rodent sequences by ≥ 5 bp and from the *Crocidura* sp. sequence by ≥ 3 bp. We then used the PHASE algorithm in DnaSP (42) to infer haplotypes for each of the three mixed samples from the sequence data associated with all 151 samples containing detectable vertebrate DNA. BLAST analysis of the inferred haplotypes suggested that two of the samples contained a combination of goat and human DNA, and the third sample contained DNA from a combination of human and cow (*Bos* sp.). Because these data indicated that these fleas had not fed on a rodent or shrew host, we did not pursue positive identification of each vertebrate DNA source. We did not include these three samples in our statistical analyses.

We identified a single vertebrate DNA source in 148 of the off-host *C. felis* samples. Ct values associated with these samples ranged from 24.81 to 36.00, but samples tended to yield Ct values closer to rather than farther from the limit of detection (Figure 3.3). Seventy samples contained human DNA. The remaining samples contained DNA from various domesticated animals (Table 3.2). Using equation [3.4] and the values listed in Table 3.3, we estimated that approximately 35 of the 70 field-collected *C. felis* samples in which our assay detected human DNA had actually taken a blood meal from a human host.

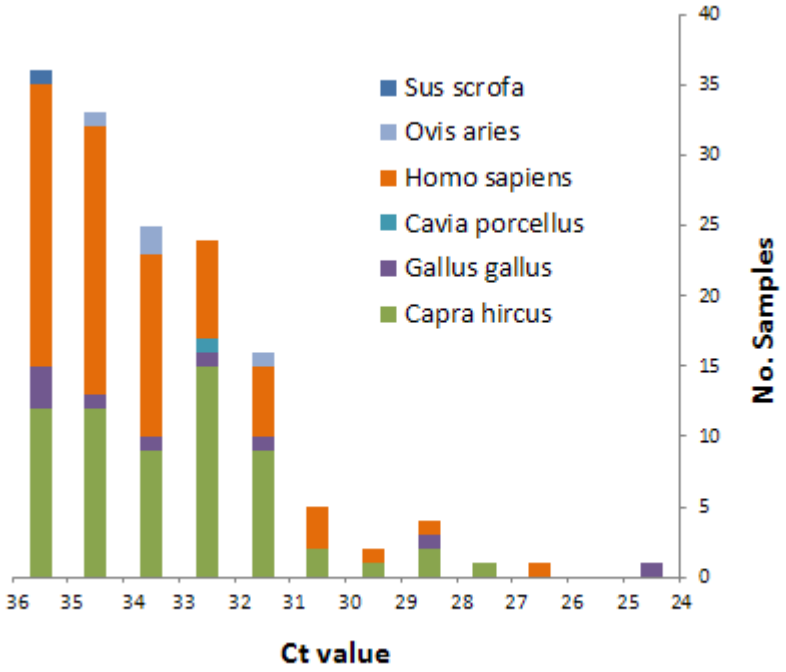

Figure 3.3. Distribution of Ct values associated with field-collected *Ctenocephalides felis* in which our real-time PCR assay detected vertebrate DNA.

Table 3.2. Vertebrate DNA identified in off-host *C. felis* collected inside huts in Vurra and Okoro counties.

Vertebrate (species)	No. Detected (% of total with detectable vertebrate DNA)
Human (*Homo sapiens*)	70[a] (47%)
Goat (*Capra hircus*)	63 (43%)
Chicken (*Gallus gallus*)	9 (6%)
Sheep (*Ovis aries*)	4 (3%)
Guinea Pig (*Cavia porcellus*)	1 (<1%)
Pig (*Sus scrofa*)	1 (<1%)

[a]Our assay has a 6% false positive rate for human DNA (Chapter 2). Using equation [3.4] and the values listed in Table 3.3, we estimated that approximately 35 of the samples with detectable human DNA represent human blood-fed fleas.

Table 3.3. Terms used in equation [3.4] to estimate the number of fleas testing positive for human DNA that had actually consumed human blood.

Variable	Definition	Value[a]
h	No. fleas testing positive for human DNA	70
b	No. fleas containing detectable DNA from non-human vertebrates	78
t	Total number of fleas tested	691

[a]Values do not include three samples containing mixed DNA.

We detected non-human vertebrate DNA in 78 samples. None of them contained DNA from a rat or shrew species. Thus, even if all of the samples testing positive for human DNA contained contaminating DNA, the proportion of detectable *C. felis* blood meals taken from rat species was 0/78. From this we estimate that no more than 4.7% (upper 95% confidence limit) of off-host *C. felis* infesting huts in Vurra and Okoro counties during the collection period had fed on rats or other wild rodent or shrew species.

DISCUSSION

Consistent with previous studies (15, 16), we found that *C. felis* is the predominant off-host flea species in human habitations in the West Nile region of Uganda. Laboratory studies have demonstrated that this species is capable of transmitting *Y. pestis*, but estimated transmission efficiency is very low (0.57%) (15). To determine if *C. felis* might serve as a bridging vector in this region, we investigated *C. felis* infestation of small mammals trapped in human habitations in Vurra and Okoro counties, and we estimated the proportion of off-host cat fleas with a detectable blood meal that had fed on potentially-infectious wild hosts. Consistent with previous studies (15, 31), we observed very low *C. felis* infestation rates on rats trapped in huts (< 0.5%). Results from our blood meal analysis indicate that off-host fleas are not feeding

on potentially-infected zoonotic hosts; they are feeding primarily on non-rodent domesticated species.

We verified that our SYBR Green real-time PCR-based assay allows detection and differentiation of vertebrate DNA from at least nine different species found in or near huts in the West Nile region. This includes black rats, Nile grass rats, multimammate mice and shrews, the predominant domestic and peridomestic small mammal species in our study area (15, 31). One limitation associated with identifying the vertebrate source of arthropod blood meals by searching for matching sequences using BLAST is that the nucleotide database does not include the target sequence from every vertebrate species. It is therefore possible that amplifying vertebrate DNA from a field-collected flea may yield an amplicon sequence for which no match is available in the nucleotide database, but we did not encounter that problem in the course of this study.

Using our real time PCR-based assay, we detected vertebrate DNA in approximately one fifth of off-host *C. felis*. We may have observed this low proportion because many off-host fleas had either not taken a blood meal or had not fed recently and had only thoroughly-digested remnants of blood meals in their guts. Using microscopy, we sought to determine if field-collected fleas had fed. However, the flea samples were stored in ethanol and had developed cloudy, gray midguts, which made it impossible to reliably distinguish blood-fed from unfed specimens. In some cases, a flea may have taken a blood meal that was too old to detect. In laboratory studies, our assay detected both human and rat DNA in artificially-fed *C. felis* held alive up to 72 hours post feeding, but detection decreased as the time between blood feeding and collection increased (Chapter 2). A previous study found that the amount of blood consumed was the principle limiting factor for amplification of triatomine blood meal DNA by PCR (43) so it is

possible that we failed to detect vertebrate DNA in smaller fleas that had taken smaller blood meals. A study in *Anopheles gambiae* (44) found, however, that there was no significant difference in blood meal detection in mosquitos held for 0-32 hours post-feeding that had consumed blood meals categorized as either small or large based on gravimetric analysis.

Because we were unable to distinguish blood-fed from unfed specimens via microscopy, we tested every off-host flea for vertebrate DNA using our real-time PCR assay. Given that our assay may fail to detect vertebrate DNA in some blood-fed fleas, however, we cannot conclude that fleas with no detectable vertebrate DNA were unfed. For future studies, it might be beneficial to adapt a catalytic assay like the phenolphthalein (Kastle-Meyer) test to detect heme in homogenized samples (45). Only samples that tested positive for heme, indicating that they had consumed a blood meal, would be subjected to real-time PCR analysis. Not only would this reduce the number of real-time PCR reactions required, it would reduce the number of false positives associated with unfed fleas contaminated with human DNA. This would, in turn, increase the probability that fleas testing positive for human DNA had actually consumed human blood.

While we cannot make any conclusions about the proportion of field-collected fleas that had actually taken a blood meal, our assay was designed to minimize bias toward detection of any one species. Others have reported that amplicon size can affect the sensitivity of PCR-based blood meal assays (46, 47). By using a single primer set that targets highly-conserved regions of the 12S mitochondrial gene and thus generates similar amplicons from all vertebrate species, we hoped to achieve similar sensitivity to blood from different hosts. There is evidence that fleas with catholic feeding habits take similarly-sized blood meals from different vertebrate species, even if they prefer one species over another (48), so differences in blood meal size should not

bias our assay toward detection of any particular vertebrate, although host species may affect the rate of blood digestion (49). Laboratory studies found no significant difference in our assay's sensitivity to human versus rat blood at any of 7 time points post feed (Chapter 2). Therefore, we used data from those samples with detectable vertebrate DNA to estimate the proportion of all off-host *C. felis* blood meals from each host.

A significant proportion of the off-host *C. felis* had fed on domesticated animals, particularly goats and, to a lesser extent, chickens. This is consistent with a 2006 observational survey in the West Nile region which found that these were the two domesticated species most likely to be present inside or within 10 meters of human habitations (CDC unpublished data). Previous studies in other parts of East Africa have found domesticated species including goats, pigs, sheep, cats, dogs and cows infested with *C. felis* (17, 21). None of the non-rodent domesticated species we detected is likely to serve as a source of *Y. pestis* infection. In order to infect a feeding flea, a host must generally develop an overwhelming bacteremia ($>10^6$ cfu/ml), which is often fatal to the host (50, 51). Chickens are resistant to *Y. pestis* infection (52). Pigs are susceptible to infection but do not show any obvious signs of disease (53); they are highly unlikely to develop the high bacteremia required to infect feeding fleas. The German Plague Commission found evidence of active infection in experimentally-infected sheep and goats, but the animals all recovered (54). Others have found that sheep succumb to *Y. pestis* infection when inoculated intravenously (55). There is limited evidence, however, of naturally-acquired infection or plague-associated deaths in domesticated sheep or goats. Based on serological observations and flea infestation data from domesticated animals in Tanzanian villages where plague was active, Kilonzo (25) concluded that goats and sheep were not involved in plague epidemiology in that region. One report from Libya suggested that a small number of human

plague cases may have been associated with killing or skinning sick goats and sheep, but there was no indication of transmission from these species by fleas. Even in this case the authors, "Regard the goat as a sentinel animal . . . not necessarily the immediate cause of cases in humans" (56).

Guinea pigs are highly susceptible to *Y. pestis* infection; they have been used in laboratory studies of plague since the early 20th century (57). Contact with infected guinea pigs or their fleas has been cited as a potential source of human *Y. pestis* infection at a zoo in India (58) and in Ecuador (59, 60). Guinea pigs were observed in less than 2% of huts included in a 2006 survey of the West Nile region (CDC unpublished data). We did not collect data on the percentage of huts included in our study that were raising guinea pigs, however, so we cannot determine the extent to which guinea pig abundance contributed to the low proportion of guinea pig blood meals detected in off-host *C. felis*. Given the cat flea's low transmission efficiency, transmission by this species from an infected guinea pig to a human is likely only if *C. felis* feed frequently on guinea pigs in human habitations where this domesticated species is abundant. In addition, guinea pigs are unlikely to become a source of infection unless they are infested with flea species that feed regularly on rats and could thus transmit *Y. pestis* from a zoonotic host. Kilonzo (21) observed that domesticated guinea pigs were poor hosts of fleas in the Lushoto district of Tanzania and unlikely to play a large role in plague epidemiology there. Liston (58) noted, however, that while *X. cheopis* rarely feeds on guinea pigs, some guinea pigs kept at the zoological gardens of Bombay were heavily infested with *X. cheopis* a few days after dead rats were found near their cage. The infested guinea pigs died of plague. Further study is needed to determine what role guinea pigs might play in plague epidemiology in the West Nile region.

Our results indicate that *C. felis* in human habitations in the West Nile region feed very infrequently on potential zoonotic hosts of *Y. pestis*. Though we trapped at least one *R. rattus* in 38.3% of huts, we did not detect *R. rattus* DNA in any of the off-host *C. felis* collected concurrently. Only 2 of the 727 fleas collected from the rats were *C. felis*. Other studies have indicated that other small mammals including Nile rats, multimammate mice and shrews inhabit the domestic and peridomestic environments in this region (15, 31), but we did not detect DNA from any of these species in off-host *C. felis* either. We estimate that not more than 4.7% of off-host *C. felis* infesting huts during the collection period had fed on *R. rattus* or other potential zoonotic hosts of *Y. pestis*.

Using a modification of a plague model based on Macdonald's equation (50, 61), Eisen and others (15) estimated that at least 20 cat fleas per host would be required for a focal infection to give rise to a secondary infection. This estimate assumes that all cat fleas are feeding on a single host. In order to serve as a bridging vector, a flea must first feed on a potentially infectious zoonotic host and then on a human host. If not more than 4.7% of off-host *C. felis* infesting huts feed on a potentially infectious zoonotic host, the estimated number of cat fleas required for a focal infection (in a rodent or shrew) to give rise to a secondary infection increases more than 20-fold. Given that our assay detects contaminating human DNA in approximately 6% of samples tested, we estimate that approximately 35 of the off-host *C. felis* in which we detected human DNA had fed on a human. Notably, this is less than the number of goat blood meals we detected. Our data thus suggest that while cat fleas may be feeding on humans in this region, they take the majority of their blood meals from domesticated animals that are unlikely to play a significant role in perpetuating transmission of *Y. pestis*. This finding further increases the estimated number of cat fleas required for a focal infection in a zoonotic host to give rise to a

secondary infection in a human. We conclude that *C. felis* is highly unlikely to serve as a bridging vector for *Y. pestis* in the West Nile region.

It should be noted that our study was conducted within a single two-month period, and flea prevalence may vary between seasons. Amatre and others (31) reported significant differences between flea loads on some wild rodent species, including *R. rattus*, trapped in the West Nile region during different two-month collection periods between January and August of 2006. Regardless of season, however, a tiny proportion of on-host fleas were *C. felis* (31). While the number of *C. felis* infesting huts might vary, we would not expect to find that a significantly higher proportion of off-host *C. felis* had fed on wild rodent hosts during different seasons.

Our findings suggest that efforts to control *C. felis* are unlikely to impact *Y. pestis* transmission to humans in Vurra and Okoro counties. Efforts to prevent human plague cases in this plague-endemic region should remain focused on controlling the fleas that feed on potential zoonotic hosts, particularly *X. cheopis and X. brasiliensis*. During inter-epizootic periods, like the period during which we conducted this study, these fleas are most likely to be found on rodent hosts, so flea-control measures that target on-rodent fleas are more likely to limit human exposure to *Y. pestis* than measures targeting only off-host fleas.

Our study does suggest, however, that the off-host *C. felis* found in huts in the West Nile region are biting humans. This is consistent with previous reports that cat fleas readily feed on people (26, 28-30). This flea species may transmit pathogens other than *Y. pestis*, including *Rickettsia typhi, R. felis* and *Bartonella* species (62-65). Further study is required to determine whether cat fleas are likely to transmit these pathogens to humans in Uganda. In addition, insofar as *C. felis* is a nuisance biter in this region, vector control efforts, including those that target

plague, may be more appealing to residents of the West Nile region if they eliminate *C. felis* in addition to those species most likely to transmit pathogens. Cat flea larvae have been found infesting bedding material in huts in the West Nile region (CDC, unpublished data), so control methods that target these breeding sites may reduce the number of flea bites that humans receive in their huts.

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