Full Length Research Paper

Investigation of fleas as vectors in the transmission of plague during a quiescent period in North-Eastern, Tanzania

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Yersinia pestis, the etiologic agent of plague, is normally transmitted to animals by infective flea-bites. Fleas associated with rodents, cats, dogs and other small mammals are considered important for the maintenance and transmission of the bacterium. Therefore, a study was undertaken to investigate the presence of *Y. pestis* in fleas of North-Eastern Tanzania during a quiescent period. House rodents were trapped with box traps while field and forest rodents were trapped with Sherman live traps. Fleas were collected from rodents by brushing the animal using shoe-shiner brush. House dwelling fleas were trapped with light traps while fleas from cats, dogs, goats and pigs were collected by rubbing their fur with ether soaked cotton wool and brushing as for rodents. All collected fleas were identified to genus level and subjected to polymerase chain reaction (PCR) test for *Y. pestis* DNA. Chi square test was used for comparison of proportions and statistical significance and p value of less than 0.05 was considered statistically significant. A total of 340 rodents, the majority of which *Mastomys natalensis* (32.6%), *Rattus rattus* (26.7%), *Lophuromys flavopunctatus* (16.6%) and *Praomys delectorum* (16.3%) were captured. A total of 805 fleas (*Xenopsylla* spp., *Dinopsyllus* spp., *Ctenophthalmus* spp. and *Echidnophaga gallinacea*) were collected from rodents with an overall flea index of 2.4 fleas/rodent. Fleas from domestic animals were mostly *Ctenocephalides* spp. (>90%). A total of 270 house dwellings fleas with an overall index of 3.6 fleas per house were collected. *Pulex irritans, Xenopsylla* spp., *Tunga penetrans, E. gallinacea* and *Ctenophthalmus* spp. were dominant. All fleas were negative for *Y. pestis* DNA. This study has demonstrated a high flea abundance and high density indicating a high susceptibility of the study area to plague if and when other conditions are favorable, hence effective flea and rodent control measures should be put in place. The non-detection of *Y. pestis* in all fleas collected from rodents, domestic animals and domestic dwellings in the current study suggests that the ectoparasites do not normally harbor the bacterium during periods of quiescence. The findings of the present study further suggest that fleas should be tested for *Y. pestis* DNA during the active phase of plague outbreaks for confirmation of infection and during inter-epidemic periods to confirm disease quiescence or detect infection activity.

Key words: Plague, *Yersinia pests*, rodents, fleas, domestic animals, polymerase chain reaction (PCR).

INTRODUCTION

Flea-borne diseases are among the most important medical diseases of humans (Plague and murine typhus). Different types of parasites, eg.*Trypanosomatids* (Votýpka et al., 2013), *Yersinia pestis*, *Ricketsia felis* and *Bartonella henselae* are the most flea-transmitted pathogens. Rodents, dogs and cats may play an essential or an accidental role in the natural transmission cycle of flea-borne pathogens. They support the growth of some of the pathogens or they serve as transport vehicles for infected fleas between their natural reservoirs and humans. In United States, Gage et al. (2000) reported that exposure to cats infected with *Y.a pestis* is a risk for human plague. Fleas found on dogs originate from rodents, birds, insectivores and from other Carnivora. Dogs therefore may serve as ideal bridging hosts for the introduction of flea-borne diseases from nature to home. In addition to flea role as ectoparasites they cause nuisance for humans and animals and may be the cause for severe allergic reactions (Dobler and Pfeffer, 2011)

Plague is a highly infectious bacterial zoonotic disease. The plague bacillus causes a rapidly progressing, serious illness that in its bubonic form is likely to be fatal (40-70% mortality). Without prompt antibiotic treatment, a pneumonic and septicaemic plague is virtually always fatal. For these reasons *Y. pestis* is considered one of the most pathogenic bacteria for humans (Stenseth et al., 2008).

In Africa**,** plague remains a disease of major public health importance. More than 90% of all cases are now notified by African countries. Recent outbreaks have shown that plague may re-emerge in areas after a long period of silence. The African countries most affected are Democratic Republic of the Congo (DRC), Madagascar, Mozambique, Uganda and the United Republic of Tanzania. The DRC and Madagascar are the most endemic countries in the world. The average annual incidence in Madagascar is 900 cases, of which a third are laboratory confirmed (WHO, 2006). Plague has declined dramatically since the early part of the twentieth century, when outbreaks could cause tens of millions of deaths. This is due primarily to improvements in living standards and health services. However, a substantial number of countries continue to be affected by the disease, and case fatality rates remain high. In view of this, continuous vigilance is required, particularly in human populations living in or near natural plague foci.

Plague foci are not fixed, and can change in response to shift in factors such as climate, landscape and rodent migrations. Natural foci of plague are found in all continents except Antarctica and Australia. In Tanzania plague has been an important health problem in various parts of the country since its introduction in the late 19th century and was first documented in 1886 in Iringa region (Kamugisha et al., 2007). Most foci were established in the South-Western, Northern, Central and North-Eastern zones of the country during the 20th century. These include Kagera, Karatu, Musoma, Singida, Kondoa, Rombo, Hai, Arumeru, Mbulu, Same and Lushoto (Kilonzo et al., 2006). Mbulu district experienced the most recent outbreaks which occurred in February to March 2007 (Makundi et al., 2008) and in December 2010 (Lyimo et al., 2010).

Y. pestis is commonly transmitted by the bite of infective fleas. Carnivores can be rarely infected with *Y. pestis* after consuming infected prey or being bitten by infective rodent fleas (Eisen et al., 2006). Studies have confirmed that *Y. pestis* is transmitted by at least 80 different flea species, but transmission efficiency is highly variable among competent vectors (Bitman et al., 2006). Likewise, the plague bacterium can infect a wide range of vertebrate hosts, but disease outbreaks are mainly associated with rodents (Keneth et al., 2005). Given the diversity of competent vectors and susceptible hosts, it is important to identify flea and rodent species responsible for driving enzootic transmission cycles of *Y. pestis* in order to elucidate on the local plague cycles and transmission dynamics. Such information is necessary for evaluating the most effective methods for the control and prevention of pathogen transmission.

Plague is transmitted between rodents and other animals primarily via wild rodent fleas (Robert and Fetherston, 1997). Wild plague exists in natural foci independent of human populations and their activity. Murine plague is intimately associated with rodents living with humans and can produce epidemics in both human and animal populations. Humans are extremely susceptible to plague and may be infected either directly or indirectly. Indirect transmission through the bite of a flea is the most common route of transmission between plague infected rodents and humans. Human infection rarely occurs within the natural foci of plague but occurs more frequently in human settlements when domestic rodents become infected following contact with infected wild rodents living in the surrounding areas. Humans who contract the disease may subsequently become infective to other people via the respiratory route (WHO, 2010).

Currently, knowledge of local plague transmission cycles in many parts of Africa where the majority of human cases have occurred in recent decades is limited or

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Abbreviations: DNA, Deoxyribose nucleic acid; **PCR,** polymerase chain reaction; **Pla,** plasminogen activator gene; **UV,** ultraviolet light; **WHO,** World Health Organization.

lacking. In many parts of East Africa including Tanzania rat fleas especially *Xenopsylla cheopis, Xenopsylla brasiliensis* and *Dinopsyllus lypusus* are believed to play crucial roles in plague epizootics and epidemics due to the fact that they commonly infest susceptible rodent hosts, which are usually abundant in endemic plague foci. *X. cheopis and X. brasiliensis* readily feed on humans when their natural hosts are not available (Tripp et al., 2009) and they can easily transmit the pathogen between the rodents and humans.

In Uganda, cat fleas (*Ctenocephalides felis*) have been reported as the most common fleas in the home environment, which is suspected to be a major exposure site for human plague in the country (Eisen et al., 2008). In Tanzania, the human flea (*Pulex irritans*) and the cat flea (*Ctenocephalides felis*), have been linked to be potential vectors of the disease based on the fact that the species are abundantly found in endemic foci of plague in the country and that they are involved in plague transmission elsewhere (Kilonzo et al., 1993). In Lushoto district, Tanzania for example, *P. irritans* has been observed to be more abundant in villages which experience plague outbreaks than in none or rarely plague affected villages (Laudiosoit et al., 2007). However, studies on comparative role of various flea species in transmitting *Y. pestis* in Tanzania are limited. The overall objective of the current study therefore was to determine the flea species and their distribution on different hosts, and to assess *Y. pestis* infection in such fleas using molecular techniques. Such information would substantially broaden the current knowledge of plague epidemiology and improve policies for management of the disease in the country.

MATERIALS AND METHODS

Study site

This was a cross sectional study conducted in Mbulu and Karatu districts, from 2012 to 2013. The two districts are located in the North-Eastern part of Tanzania and have registered occurrence of repeated outbreaks of plague in recent years and are considered active foci. Based on previously reported plague epidemics in the area, six villages were selected for inclusion in this study.

Collection of rodents and fleas

House rodents were live trapped with box traps while field and forest rodents were live trapped with Sherman traps (H.P. Sherman Traps, Tallahassee, FL, USA) baited with peanut butter mixed with maize bran. All rodent traps were placed in houses, forest, crop fields and fallow in the selected villages. Choice of areas for trapping field and forest rodents were based on among other things, security of traps and proven activities of such animals. One hundred Sherman live traps were deployed per trapping night. Traps were usually set in late afternoon and inspected in the following morning and trapping was carried out for three consecutive nights at each selected location. Each captured rodent was carefully transferred to a white cloth bag and then to a screw-capped museum jar containing pieces of cotton wool soaked in

diethyl ether in order to anaesthetize both the animal and its ectoparasites. Anaesthetized rodents were transferred to a large aluminium pan and the fur was brushed from back to the front using shoe-shinning brush. Fleas falling into the pan were collected and preserved in absolute ethanol. Houses were randomly chosen and informed consent was sought from house owners prior to trap setting.

Domestic dwelling fleas were trapped by using light traps; ten to fifteen light traps were used in each village at a time. Fleas captured in light traps were collected by using fine pin/wooden peak/camel hair brush and carefully transferred to capped micro tubes containing absolute ethanol and taken to the laboratory for counting, identification and processing for *Y. pestis* DNA PCR. Small carnivores and other small ruminants were laid on white cloth sheet and their fur brushed with ether soaked cotton wool, and flea ectoparasites were removed by scrubbing the animals with shoe-shinning brush. Fleas from rodents' nests were collected using Barlese funnel. All fleas collected were preserved in absolute ethanol and taken to the SUA Pest Management Centre Laboratory for identification to genus level, using relevant taxonomic keys and other sources of information on fleas in Tanzania.

Detection of *Y. pestis* in fleas

Ethanol-preserved fleas were rinsed with distilled water for 10 min and dried on sterile filter paper in a laminar biosafety hood. All fleas collected from various sources were pooled in groups of 1-25 individuals according to their species, hosts and locality. They were crushed in sterile Eppendorf tubes with 100 µl of brain heart infusion broth (Oxoid Hampshire, England) as described in previous studies (Hinnebusch and Schwan, 1993; Stevenson et al., 2003; Hang'ombe et al., 2012). Crushed fleas were boiled at 95°C for 10 min, centrifuged at 10,000 xg for 10 s and 2 µl of the supernatant were used as template for *Y. pestis* DNA testing by PCR technique. Negative controls template employed were DNase free water and fleas collected from a non-endemic plague area. PCR was done using the Phusion™ flash high fidelity PCR master mix (Finnzymes Oy, Finland). The reactions were performed in a final volume of 10 µl containing 5 µl phusion flash PCR master mixes; 0.5 µM of primer sets in 1 µl volume of each (Forward and reverse) and 1 µl of PCR water. Protocol optimization was done by using positive control (Y. *pestis* DNA obtained by BHI boiling method from positive fleas) provided by the Department of Para Clinical Studies (Microbiology Laboratory) University of Zambia. PCR amplification was performed for the detection of the *Y. pestis* plasminogen activator gene using primers *Yp pla1* (5'-ATC TTA CTT TCC GTG AGA AG-3') and *Yp pla2* (5'-CTT GGA TGT TGA GCT TCC TA-3') corresponding to nucleotides 971 to 990 and 1431 to 1450, respectively, of the *pla* locus sequence reported by previous studies (Sodeinde et al., 1989). The primers amplify a 478 bp region of the *Y. pestis* plasminogen activator gene. The Piko™ thermal cycler (Finnzymes Instruments Oy, Finland) was part of plague surveillance (Hang'ombe et al., 2012; Hinnebusch et al., 1998) in areas where disease outbreaks are common in the human population. The Piko™ thermal cycler was programmed at 95°C for 10 s for initial denaturation, followed by 35 cycles consisting of 95°C for 1 s, 58°C for 5 s and 72°C for 15 s. Final extension was done at 72°C for 1 min. Specific *Y. pestis* detection was identified by the presence of a specific 478 bp DNA band on 1.5% agarose gel, stained with ethidium bromide and evaluated under UV trans illuminator. The estimation of the sizes of PCR products was done according to the migration pattern of a 100-bp DNA ladder.

Statistical analysis

Assuming the data follows a normal distribution, comparison of

Table 1. Species and number of fleas collected from rodents of Mbulu and Karatu districts.

Rodent spp.	Echin.	Xen.	Ctenop.	Dinop.	Nosop.	Lepto	Ctenoce	Pulex	Strival.	Total
M.natalensis(94)	0	67	15	94	1	9	0	0	0	186
R. rattus.(81)	67	173	9	10	0	3	0	4	0	266
L.flavopuntatus (56)	0	7	121	64	15	7	1	0	1	216
P.delectorum(55)	0	9	43	53	11	0	0	0	4	120
Gramomys (4)	0	1	0	1	3	0	0	0	0	5
L.striatus (8*)*	0	2	9	1	0	0	0	0	0	12
Other spps (42)	0	0	0	0	0	0	0	0	0	0
Total (340)	67	259	197	223	30	19	1	4	5	805
Percentage of each flea sp.	8.2	32	24.5	27.7	3.7	2.4	0.1	0.5	0.6	100
Specific flea index	0.2	0.8	0.58	0.66	0.09	0.06	0.003	0.01	0.014	

Ctenop- Ctenophthalmus spp., *Xeno- Xenopsylla* spp. and *Dinop- Dinopsyllus* spp. Other host species are *Lemniscomys striatus, Crocidura* spp., *Mus minutoides and Genetta* spp. and *Crestomys gambianus.* Other flea spp. were *Echinophaga* spp*., Ctenocephalides* spp., *Pulex irritans, Strivalis* spp., *Nosopsyllus* spp*., and Leptopsyllus* spp.

RESULTS

During the study period, a total of 340 rodents were captured from five villages (Mongahay, Arri, Boboa, Hayseng and Slahamo). These comprised *Mastomy natalensis* (32.6%), *Rattus rattus* (26.7%), *Lophuromys flavopunctatus* (16.6%), *Praomys delectorum* (16.3%) and other species (*Gramomys dolichurus, Lemniscomys striatus* and *Mus munitoides*) (7.7%). A total of 805 fleas belonging to nine genera were collected from 57% of the captured rodents. Of these, 32.2% were *Xenopsylla* spp*.,* 27.7% were *Dinopsyllus* spp. and 24.5% were *Ctenophthalmus* spp. and 8.2% were *Echinophaga* spp*.* (Table 1). These flea species were found to be ectoparasitic on many rodent species except *Echidnophaga* spp*.* which was collected from *R. rattus* only. As summarized in Table 1, rodents were highly infested with an overall flea index (average number of fleas per animal) of 2.4 while the specific flea index (number of each flea species per animal) was 0.8 for *Xenopsylla* spp., 0.7 for *Dinopsyllus* spp. and 0.6 for *Ctenophthalmus* spp. (Table 1). Likewise, a total of 270 fleas were collected from 75 residential houses during the study (overall flea index was 3.6 fleas/house) (Table 2). Of the examined houses, 51% were infested by five flea species (*Pulex irritans, Xenopsylla* spp., *Tunga penetrans, Echidnophaga gallinacea* and *Ctenocephalides* spp.)*. Pulex irritans* and *Ctenocephalides* spp. were collected in all villages and their specific flea indices were 1.9 and 1.5, respectively. Also, 55 rodent nests were examined and 25 fleas collected from them (Table 2). These fleas comprised *Ctenophthalmus* spp., *Strivalius* spp. and *Dinopsyllus* spp. (68%, 28% and 4% respectively) (Table 2). *Ctenocephalides* spp. was the most abundant on cats (100%), goats (99.8%), dogs (99.5%) and pigs (91.5%).

Results of PCR tests for *Y. pestis* DNA in all fleas collected from various sources (rodents, domestic animals and human dwellings) were negative. Neither of the tested fleas was positive for the *Y. pestis* plasminogen activator gene.

DISCUSSION

The study investigated the various types of flea species and their distribution among different hosts in active plague foci in Tanzania. The fleas was also examined for possible *Y. pestis* infestation. *M. natalensis, R. rattus, L. flavopunctatus* and *P. delectorum* were observed to be the commonest rodent species in the districts.

These findings are consistent with previous obser-vations reported by other workers in the same districts (Kilonzo and Mtoi, 1983). *M. natalensis* and *R. rattus* were mostly found in human dwellings, cultivated and fallow fields, suggesting that if they harbor the plague pathogen, then they can pose substantial risk for plague transmission to human populations. Observations made during this study revealed that there is statistically significant interaction among domestic, peridomistic and wild rodent species *(R. rattus, M. natalensiss, L. flavopunctatus and P. delectorum).* Due to these positive rodent interactions, the risk of plague transmission from the forest to the domestic/human dwellings is substantial, and hence effective control measures targeted towards

Table 2. Species, population densities and indices of fleas collected from various sources in Mbulu and Karatu districts, Tanzania.

Source		Flea species collected								Index
		Pulex.	*Echin*	*Ctece*	*Xeno*	*Dino*	*Strive.*	*Tung.*	Total	
Houses (n=75)	n	139	13	109	2	0	0	7	270	3.6
	%	51.5	4.8	40.4	0.7	0	0	2.6	100	
Burrows (n=55)	n	0	0	17	0	1	7	0	25	0.45
	%	0	0	68	0	4	28	0	100	
Dogs (n=17)	n	0	0	402	0	0	0	2	404	23.8
	%	0	0	99.5	0	0	0	0.5	100	
Goats (n=25)	n	1	0	565	0	0	0	0	566	22.6
	%	0.2	0	99.8	0	0	0	0	100	
Cats (n=11)	n	0	0	28	0	0	0	0	28	2.6
	%	0	0	100	0	0	0	0	100	
Pigs (n=12)	n	4	5	195	0	0	0	9	213	17.8
	%	1.9	2.3	91.5	0	0	0	4.2	100	

n- numbers, %- percentage*, Pull- Pulex.*, *Echini- Echidnophaga spp., Ctece- Ctenocephalides* spp.*, Striv-Strivarius* spp.*, Tunga spp. Xeno- Xenopsylla* spp..

rodent control can result in some positive effects on plague prevention in the districts.

Xenopsylla spp., *Dinopsyllus* spp. and *Ctenophthalmus* spp. were the most common rodent flea species in Mbulu and Karatu districts. Among the other domestic species collected, *Ctenocephalides* spp. are commonly found on domestic animals (cats, dogs, pigs and goats) in Karatu and Mbulu Districts. These species are non efficient (transmit plague at low rate) plague vectors but can be pestiferous, as observed in Democratic Republic of Congo (Devignat, 1949). *T. penetrans* status as plague vector is unknown. The females of this species are embedded in the host epidermis (humans, dog, rat, pigs and cats), but males are free hematophagous ectoparasites (Witt et al., 2004). *E. gallinacea* is frequent in human homes where hens are kept. It has been found to be infected with *Y. pestis* in the field (Wheeler et al., 1941) but is considered a poor plague vector due to its "stick tight" behavior (Burroughs, 1947). In addition, *Pulex irritans* and *Ctenocephalides* spp. were previously reported to be potential vectors of *Y. pestis* in the area (Kilonzo and Mtoi, 1983). These findings are also similar to those of Amatre (2009) in Uganda. *Ctenocephalides* spp.*,* especially *Ctenocephalides felis* (cat flea) are the most abundant flea ectoparasites of domestic animals and their potential role in maintaining plague during inter-epizootic periods and ability of transmitting *Y. pestis* at low rates could not be ruled out since some of these animals, especially dogs, harbor plague organisms in the area and elsewhere (Kilonzo et al., 2005). These previous findings together with those from the present study underscore the need for effective control strategies of these flea species in plague control initiatives.

The current absence of *Y. pestis* DNA in all the collected, processed and PCR tested fleas suggest that during quiescent periods the ectoparasites (fleas) do not harbor the plague pathogen. It has been previously reported that during periods of quiescence, rodent hosts may not have enough *Y. pestis* to infect the fleas (Hinnebusch et al., 1998). These findings are similar to previous studies (Wimsott and Biggnsb, 2009; Cully et al., 2000; Thiagarajan et al., 2008). The findings of the present study further suggest that fleas should be tested for *Y. pestis* DNA during the active phase of plague outbreaks for confirmation of infection and during inter-epidemic periods to confirm disease quiescence or detect infection activity.

Conclusions and recommendation

This study has demonstrated a high flea abundance and high density indicating a high susceptibility of the study area to plague outbreak, hence effective flea and rodent control measures should be put in place. The non-detection of *Y. pestis* in all fleas collected from rodents, domestic animals and domestic dwelling in the current study suggests that the ectoparasites rarely harbor the bacterium during periods of quiescence. It is recommended that during outbreaks of plague rodent, fleas and human samples should be collected at the same time for *Y. pestis* detection in order to study the dynamics of *Y. pestis* transmission comprehensively.

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