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Research article

Potential Roles of Pigs, Small Ruminants, Rodents, and Their Flea Vectors in Plague Epidemiology in Sinda District, Eastern Zambia

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Abstract

A cross-sectional study was conducted in the Eastern part of Zambia that previously reported a plague outbreak. The aim of the study was to evaluate the potential role of pigs, goats, and sheep as sero-surveillance hosts for monitoring plague, and to investigate the flea vectors and potential reservoir hosts to establish the current status of plague endemicity in the district. Serum samples were collected from 96 rodents, 10 shrews, 245 domestic pigs, 232 goats, and 31 sheep, whereas 106 organs were eviscerated from rodents and shrews. As for fleas, 1,064 *Echidnophaga larina* Jordan & Rothschild, 7 *Xenopsylla cheopis* (Rothschild), and 382 *Echidnophaga gallinacea* (Westwood) were collected from these animals in 34 villages. Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) tests were performed on serum, and organs and fleas to determine IgG antibodies against Fraction 1 antigen and *pla gene* of *Yersinia pestis*, respectively. ELISA results showed that 2.83% (95% CI = 0.59–8.05) rodents, 9.0% (95% CI = 5.71–13.28) domestic pigs, 4.7% (95% CI = 2.39–8.33) goats, and 3.2% (95% CI = 0.08–16.70) sheep were positive for IgG antibodies against *Fra1* antigen of *Y. pestis*. On PCR, 8.4% (95% CI = 3.96–15.51) of the rodents were detected with *Y. pestis pla* gene, whereas all fleas were found negative. The common fleas identified were *E. larina* from pigs, whereas *X. cheopis* were the only fleas collected from rodents. The presence of sero-positive animals as well as the occurrence of *X. cheopis* on local rodents suggests that *Y. pestis* remains a risk in the district.

Key words: flea, *Yersinia pestis*, pig, small ruminant, plague

Plague is a reemerging flea-borne zoonotic disease caused by *Yersinia pestis*, a gram-negative coccobacillus bacterium. Plague has the ability to spread explosively under favorable conditions that contributed to three devastating global pandemics, which claimed millions of lives throughout the world. The Justinian plague occurred from 541 to 543 and affected the Middle east, and Africa across the Mediterranean basin into Europe (Wagner et al. 2014); the Black death occurred from 1347 to 1351, which affected the Middle east, Asia, Africa, and Europe, where 75% of population succumbed to the disease in Europe (Drancourt 2012, Harbeck et al. 2013); and the Oriental plague occurred in the 19th century, which probably started in 1884 in China, affecting the entire world except for Australia and New Zealand (Stenseth et al. 2008). The wide spread of the disease was facilitated by improved ship transport which carried different food stuffs from affected regions, especially from Far east, to plague-free countries via infected rats on steam ships (Sussman 2011, Lin et al. 2012, Vogler et al. 2013).

Plague is thought to persist for a long period of time in a very small prevalence in the enzootic cycles which cause some fatalities in the host and may involve partially resistant rodents (the enzootic or maintenance hosts; Ben Ari et al. 2011). This pattern shows that the disease can break out after a long time of quiescence. In some countries, the disease remains silent after the first outbreak for a long period of time (Bertherat et al. 2007). These quiescent periods are punctuated by occasional outburst of the disease outbreak among the hosts or epidemics, as described by Ben Ari et al. (2011). However, Eisen et al. (2015) have reported that fleas are thought to be responsible for interepizootic transmission, but that early phase transmission (this is when vectors transmit the pathogens without

the latter undergoing biological development) through the bite is essential during rapidly spreading epizootics and epidemics. In Zambia, plague was first reported in 1917 in the Luangwa valley (Low and Newman 1920) and has been reported in three zones, namely, in the North-western, Southern, and Eastern parts of Zambia. In the Eastern region, >75 villages were affected during the two plague outbreaks that were reported in 2001 and 2007, respectively (Ngulube et al. 2006, Ministry of Health 2007, Neerinckx et al. 2010).

The disease is considered mainly to be transmitted by oriental rat fleas, *Xenopsylla cheopis* (Rothschild), from infected rodents during epizootic and enzootic periods (Eisen et al. 2015). The fleas are known to transmit *Y. pestis* from infected animals to other warm-blooded animals through biting, while other flea species which may also pose a risk of transmitting the disease include the *Echidnophaga spp*., *Pulex irritans* (L.), *Ctenocephalides canis* (Curtis), and *Ctenocephalides felis* (Bouché) (Laudisoit et al. 2007). In Madagascar, these fleas were considered to be potential vectors of disease, especially in an outbreak situation (Ratovonjato et al. 2014).

The disease occasionally could be transmitted by non flea-borne routes, such as direct contact with infected hosts and inhalation of infectious respiratory droplets especially in pneumonic cases (Ben Ari et al. 2011, Andrianaivoarimanana et al. 2013). Goel et al. (2014) reported that plague was directly transmitted by the contact of an index case with wild animals after hunting and deskinning (Goel et al. 2014). This scenario is very common in the study area because most people are in the habit of hunting wild animals, such as rodents. In some cases, the disease was directly transmitted to individuals via skinning of goats and consumption of camel meat (Christie et al. 1980, Bin Saeed et al. 2005). A wildlife biologist in the United States reportedly died after acquiring pneumonic plague as a result of doing a necropsy on the lion, which died on its own in the forest (Wong et al. 2009). Pigs also have been implicated in plague epidemiology as they have a prolonged period of high IgG antibody titers against *Fra1* antigen for >200 d when they were intentionally fed with infected meat contaminated with *Y. pestis*. The animals did not show signs of plague disease (Marshall and Harrison 1972) and are, therefore, considered to be the resistant hosts of the bacteria. This characteristic makes pigs be the good sentinel of plague (Nelson et al. 1985).

The study was conducted to determine antibodies against *Fra1* antigen in blood collected from domestic pigs, goats, sheep, and rodents, while PCR test was carried out to detect the presence of *pla* gene encoding a portion of the *Y. pestis* DNA in rodent organs, as well as in fleas collected from rodents and domestic animals after a plague outbreak.

Materials and Methods

Study Site and Sample Collection

This study was conducted in Sinda district of the Eastern region in Zambia (Fig. 1), where livestock such as domestic pigs and small domestic ruminants are reared under the free-range system. Sampling was conducted between March 2015 and June 2016. The animals live in the environment where they share water, pasture, and livestock pens. The study site had previously reported plague outbreaks in 2001 and 2007, where over 75 villages were affected (Ngulube et al. 2006, Ministry of Health 2007).

Animals were selected from 34 randomly selected villages using a systematic technique, where all domestic animals of the same species were identified by locating their households (Fig. 2). Animals that were <6-mo old and those that came from another village in the past 6 mo were excluded from the study.

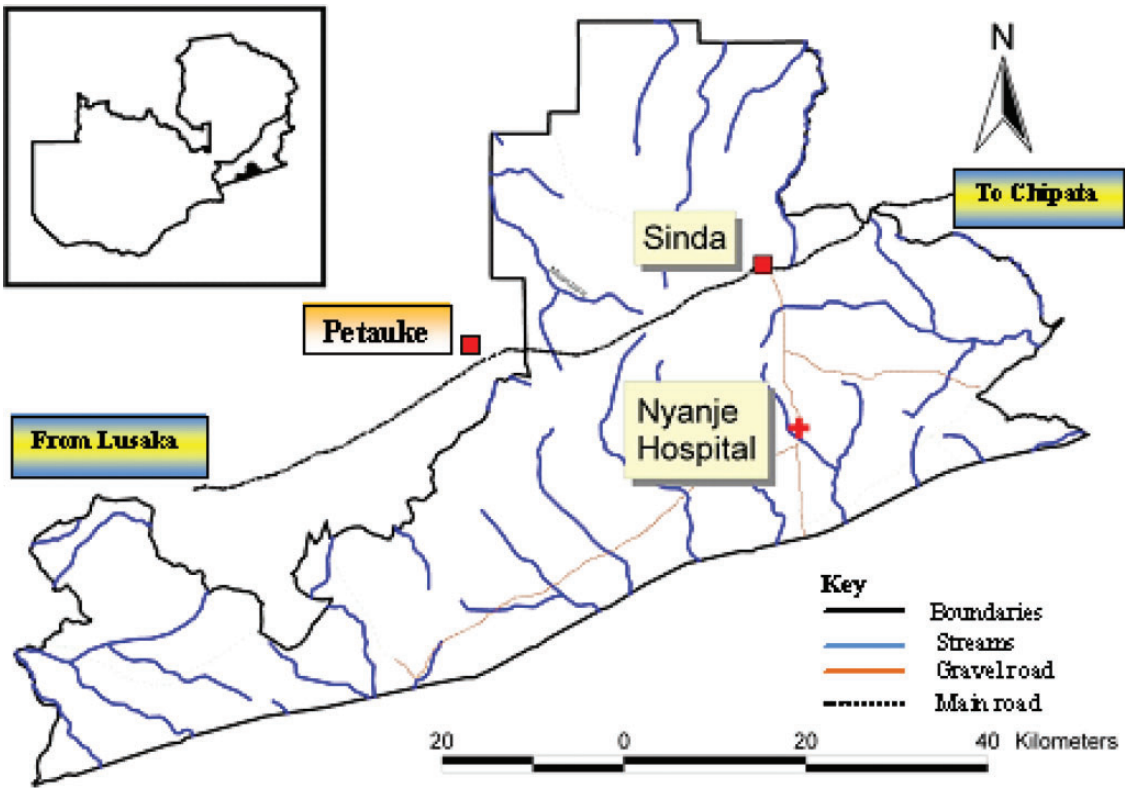

Fig. 1. Sinda district in Eastern Zambia.

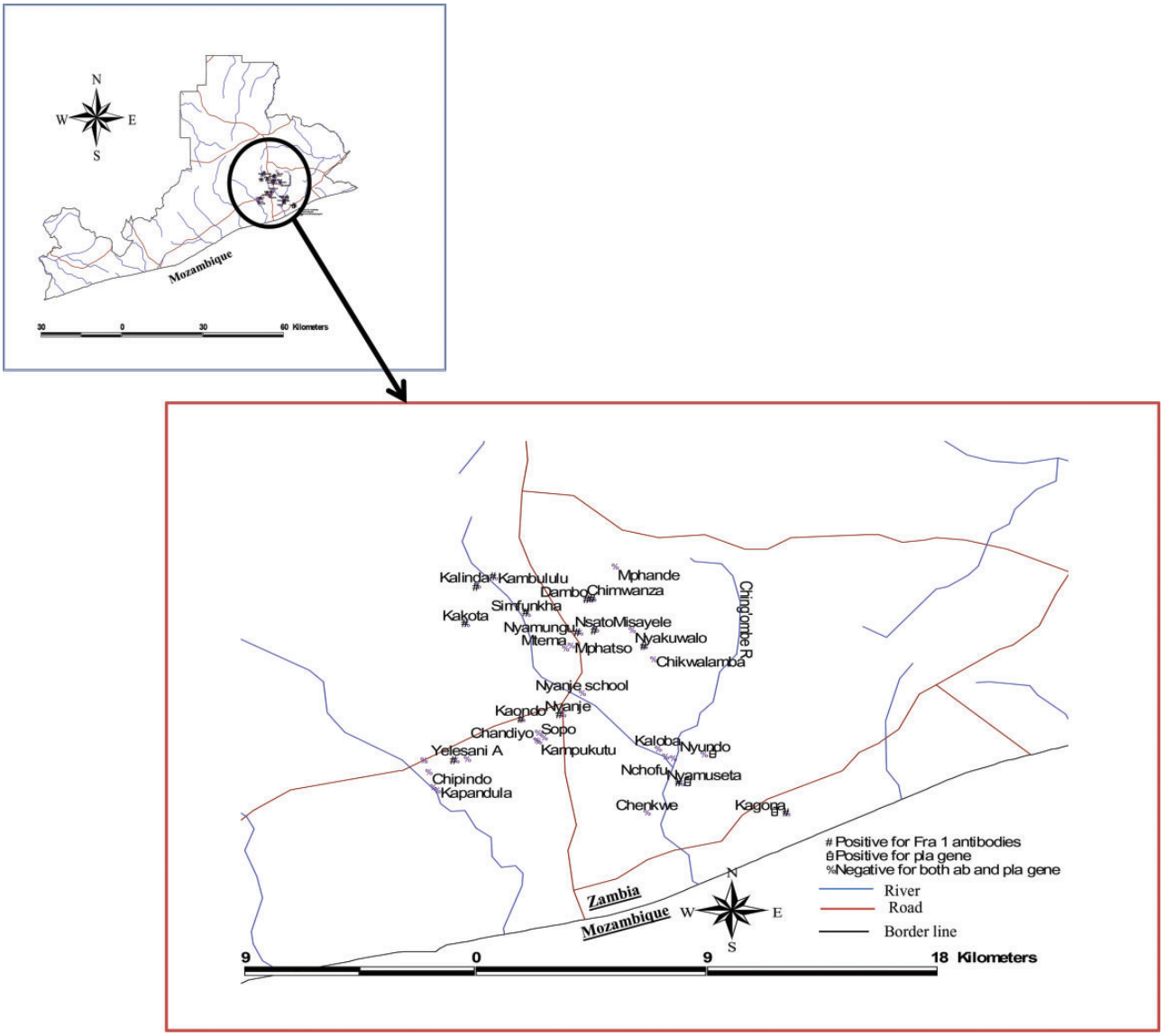

Fig. 2. Sampled villages and plague results.

Owners of the animals in the selected villages were identified and consulted for the consent through their district veterinary extension officers and village headmen.

The selected animals were restrained and about 2-ml blood was collected from the jugular vein into a sterile plain tube and left overnight for serum separation. The serum was transferred into sterile serum vials and stored at −20°C until required for use. Fleas were also collected from the restrained animal by lying the latter on a white sheet and brushing the host with a cotton wool soaked with 90% diethyl ether to anaesthetize the fleas, and brushed using the appropriate animal brush from head to tail. Fleas on the white sheet were collected into a small vial containing 70% ethanol. Fleas that remained attached to the animal skin and fur were gently removed with a pair of fine forceps into clean vials.

In the case of rodents, Shermans' live traps were used to trap rodents, and they were baited with peanut butter mixed with soya flour, set at a distance of 10 m apart and left overnight. Wire cage traps were also set in houses in the selected zones. Traps were inspected the following morning, and trapped rodents were subjected to sample collection of blood and organs (Nyirenda et al. 2016).

ELISA and DNA extraction were carried out according to the manufacturers' instructions for the kits.

The bacterium possesses two unique genes that contribute to its virulence, namely, Fraction 1 (*Fra1*) from MT1 plasmid and Plasminogen activator (*pla*) from the PCP1 plasmid, which are both specific to *Y. pestis*; and therefore, ELISA detects antibodies against *Fra1* gene while PCR detects *pla* gene, a section of *Y. pestis* DNA.

Flea Identification

Fleas were pooled (1–5) according to species and location, and from each pool, 1–2 fleas were removed, processed, and identified using main key features such as pronotal combs, genal combs, and shape of head and reproductive organs (spermathecae in females and penis plates in males; Pratt and Stojanovich 1966, Kilonzo 1999).

Each pool containing the remaining fleas were put in the Eppendorf Tubes and 100 µl of Brain-heart infusion broth (Oxoid, Hampshire, England) was added and triturated with sterile pipettes. The triturated samples were then boiled at 95°C for 10 min and centrifuged at 10,000 × *g* for 10 s, after which 10 µl of each sample was

collected in a clean Eppendorf Tube and subjected to PCR testing as previously reported (Engelthaler et al. 1999, Hang'ombe et al. 2012).

PCR Technique

PCR was done by Phusion flash high fidelity master mix (Finnzymes Oy, Finland) in a highly PCR specialized laboratory using *Yp pla1* (5′TGC TTT ATG ACG CAG AAA CAG G3′) as forward primer and *Yp pla2* (5′CTG TAG CTG TCC AAC TGA AAC G′3) as the reverse primer, that amplifies a 344-bp region spanning residues 425 to 769 of the *pla gene* (Sodeinde and Goguen 1989). The reactions were performed in a final volume of 10 µl, i.e., 5-µl phusion flash PCR master mix, primer sets in 1 µl of each, 1 µl of the template, and 2 µl of PCR water. The Piko thermal cycler (Finnzymes instruments Oy, Finland) was programmed at 98°C for 10 s for denaturation. This was followed by 35 cycles consisting of 98°C for 1 s, 58°C for 5 s, 72°C for 15 s, 72°C for 2 min, and 4°C for infinity. One microliter of amplified DNA was mixed with 2-µl loading dye and loaded into 1.5% agarose gel for each sample, run in electrophoresis, and viewed under UV transilluminator (Hang'ombe et al. 2012). The positive and negative controls, which came together with the kit, were included in the test.

Data Analysis

Data was entered in Excel Microsoft software and analyzed using Epi info 7.0.8.0, a computer statistical package (CDC, GA, USA) from the Centre for Disease Control and Prevention (CDC).

Results

In total, 106 sera and organs from 96 rodents and 10 shrews, 245 sera from domestic pigs, 232 sera from goats, and 31 sera from sheep were collected and analyzed. As for fleas, 1,064 *E. larina*, 7 *X. cheopis*, and 382 *E. gallinacea* fleas were collected from 34 villages in the district (Table 1). In addition to fleas, there were 48 tick ectoparasites collected from rodents. The largest number of rodents captured was *Mastomys natalensis* Smith (52), followed by the *Gerbils* spp. (22), *Rattus rattus* (19), and *Saccostomus* spp. (3; Table 2). In all, 22 (9.0%) pigs, 11 (4.7%) goats, 1 (3.2%) sheep, and 3 (3.1%) rodents were positive for antibodies against *Fra1* antigen of

Table 1. Animals and their fleas sampled in villages in Sinda district

Village	Pigs		Goats		Sheep		Rodents	
	No. of samples collected	No. of fleas collected	No. of samples collected	No. of fleas collected	No. of samples collected	No. of fleas collected	No. of samples collected	No. of fleas collected
Kalinda	9 (5)	25	7	0	0	0	0	0
Simfunkha	12 (2)	22	12	0	0	0	0	0
Nyanje	20 (1)	174	13	0	0	0	0	0
Yelesani A	11	0	20	0	0	0	0	0
Chimwanza	12 (1)	71	24 (1)	0	0	0	0	0
Dambo	14 (3)	19	8 (1)	0	6 (1)	0	0	0
Nsato	11 (6)	33	2	0	0	0	0	0
Kakota	9 (2)	38	0	0	0	0	0	0
Kambululu	8 (1)	21	0	0	0	0	0	0
Yelesani B	0	0	20 (1)	0	0	0	0	0
Kaondo	0	0	4 (1)	0	0	0	0	0
Mphande	0	0	0	0	25	0	0	0
Chipindo	16	137	23	0	0	0	0	0
Kapandula	6	74	7	0	0	0	0	0
Kampukutu	4	47	10	0	0	0	0	0
Chimtima	7	92	0	0	0	0	0	0
Sopo	2	16	0	0	0	0	0	0
Chandiyo	5	36	1	0	0	0	0	0
Kaloba	24	156	3	0	0	0	10	0
Nchofu	7	60	2	0	0	0	0	0
Tundubala	0	0	2	0	0	0	0	0
Nyamuseta	10	48	15 (1)	0	0	0	51 (2)	3
Chenkwe	11	55	4	0	0	0	0	0
Chikwalamba	8	100	14	0	0	0	0	0
Nyakuwalo	2	7	10 (6)	0	0	0	0	0
Misayele	14	65	16	0	0	0	0	0
Nyamungu	12 (1)	73	9	0	0	0	0	0
Mtema	0	0	3	0	0	0	0	0
Zomba	5	70	0	0	0	0	0	0
Mphatso	6	7	0	0	0	0	0	0
Chimpango	0	0	3	0	0	0	0	0
Kagona	0	0	0	0	0	0	14 (1)	2
Nyundo	0	0	0	0	0	0	30	2
Nyanje school	0	0	0	0	0	0	1	0
Total	245 (22)	1,446	232 (11)	0	31 (1)	0	106 (3)	7

The numbers in brackets shows the animals which were positive for IgG antibodies against F1 antigen of *Yersinia pestis*.

Y. pestis (Table 3). The *Y. pestis* plasminogen activator gene (DNA) was detected in nine (9.4%) rodents, whereas there was no *Y. pestis* DNA detected from fleas. Immunoglobulin G (IgG) antibodies against *Fra1* antigen were detected in animals collected and captured from 14 villages in the district, while in three villages, *Y. pestis* pla gene from the rodents was detected (Tables 1 and 3, Fig. 2). The specific flea index (SFI) for X. *cheopis* on rodents was 0.067 while the percentage incidence index (PII) was 2.8%. The SFI and PII for individual rodents were as follows: *M. natalensis* (0.06; 2%), *Gerbil* (0.09; 5%), and *Saccostomus spp.* (0.67; 33%), respectively (Table 4).

Discussion

The detection of IgG antibodies against *Fra1* antigen of *Y. pestis* in pigs in this region of Zambia can be interpreted to indicate that these animals were previously or currently exposed to the bacterium.

Table 2. PCR and ELISA results of individual species of rodents and shrews

Rodent and shrew spp.	No. collected	Results	
		ELISA	PCR
M. natalensis	52	2	5
Gerbil	22	1	3
R. rattus	19	0	0
Crocidura spp.	10	0	0
Saccostomus spp.	3	0	1
Total	106	3 (3.1%) 95% CI (0.65–8.86%)	9 (9.4%) 95% CI (3.4–15.5%)

Table 3. ELISA results from domestic pigs, small domestic ruminants, and rodents

Animal	Total	Positive	% Positive
Pigs	245	22	9.0 (95% CI = 5.7–13.3
Goats	232	11	4.7 (95% CI = 2.39–8.33)
Sheep	31	1	3.2 (95% CI = 0.08–16.7)
Rodents	96	3	3.1 (95% CI = 0.65–8.86)
Shrews	10	0	0%
ND	614	37	ND

ND = No data, CI = Confidence Interval.

The table shows the total number of animals sampled and the positive samples using Enzyme Linked Immunosorbent Assay (ELISA). It also shows the 95% Confidence interval in each animal species in the positive samples.

Pigs can remain infected for about 200 d or more days (Marshall and Harrison 1972). The possible exposure may be either by bites from infected fleas or by consuming carcasses infected with the bacterium, as pigs are not confined but rather are allowed to roam freely within and around the villages and come back to their shelters in the evenings. This exposes them to fleas from various animals like the rodents, which are known reservoirs of plague (Goel et al. 2014). The results indicate that the animals may act as potential sentinel hosts for plague in the area (Nelson et al. 1985). Furthermore, the area could indeed be a plague hotspot.

Goats are known to sero-convert to *Y. pestis*, and they also have been suspected in some instances of becoming ill with and actually dying of plague. However, this cannot be confirmed because samples from the sick or dead goats were not tested by culture to determine if an active *Y. pestis* infection was present at the time of their illness or when they died (Kenya, Communicable Disease Centre, Ministry of Health 1978, Christie et al. 1980). The demonstration of *Y. pestis* DNA plasminogen activator gene in five *Mastomys spp.*, three *Gerbils*, and one *Saccostomus spp.* indicates that these animals were infected with the bacterium either through the common flea bites or rarely from soil contamination, as the bacterium can persist in the soil for substantial periods of time (Ayyadurai et al. 2008, Boegler et al. 2012). In addition to these modes of transmission, animals can also be infected through consuming infected materials or part of the infected carcasses (Wang et al. 2011). The fact that *Mastomys spp.* had the highest infection rate as compared with other rodents is consistent with observations reported by Kilonzo et al. (2005) that *M. natalensis* was the major natural reservoir host of plague in Tanzania's Lushoto district, playing an important role in maintaining the disease in the study area (Kilonzo et al. 2005).

The absence of *Y. pestis pla* gene in all the collected fleas from rodents and pigs suggested that during quiescent periods, the flea ectoparasites do not harbor the bacterium. It has been previously reported that highly susceptible rodents with active *Y. pestis* infections are very rare during interepizootic periods, and if flea numbers are very low at such times, it is also unlikely that sufficient numbers of fleas will become infected to maintain transmission at substantial rates. This can also be supported by the low population density of *X. cheopis*, the known natural vector of plague, as measured by SFI (0.067) and a PII (2.8%). These findings in the recent study are consistent with the previous studies (Hinnebusch et al. 1998, Wimsatt and Bigginsb 2009, Haule et al. 2013). 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 interepidemic periods to confirm disease quiescence or detect infection activity. Ratovonjato et al. (2014) suggested that most species of fleas can at least briefly carry the plague bacterium during outbreaks and some are capable of

Table 4. Arthropods collected from rodents and PCR results

Rodent & shrew spp.	Number collected	No. infested	Fleas collected	SFI	PII (%)	PCR results	Ticks collected & prevalence (%)[a]
M. natalensis	52	1	3	0.06	2	No *pla* gene detected	22 (42.3%)
Gerbil	22	1	2	0.09	5	No *pla* gene detected	20 (90.9%)
R. rattus	19	0	0	0	0	0	6 (31.6%)
Crocidura spp.	10	0	0	0	0	0	0
Saccostomus spp.	3	1	2	0.67	33	No *pla* gene detected	0
Total	106	3	7	0.067	2.8		48 (45.3%)

[a] PCR was not done on ticks.

SFI = Specific flea Index, PII = Percentage Incidence Index.

transmitting the disease to other mammals at varying rates, depending on the flea species involved. At the time of this study, no plague cases were being reported, in agreement with low population of *X. cheopis* and fleas being negative for *Y. pestis* bacterium. Nondetection of plasminogen activator gene (*pla*) of *Y. pestis* in all fleas collected from rodents and domestic animals suggests that flea ectoparasites do not normally harbor the bacterium during the period of serenity.

Detection of specific anti *Y. pestis* antibodies against *Fra1* antigen in pigs, goats, rodents and sheep, and the *Y. pestis* DNA *pla* gene in rodents gives the vivid picture that the pathogen of plague are still in circulation; hence, if and when environmental factors become favourable, plague can breakout. The results also revealed that these animals may be considered as sentinel of the plague endemic area. Therefore, the result is important in providing control and management of plague, as such policy makers will reinforce the application of preventive measures in the affected communities, such as reducing flea infestation and contact with plague-borne natural potential reservoirs, the rodents.

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Note: Animal experiment: Research was conducted in compliance with the Animal Welfare Act and the Guidelines for the care and use of laboratory animals of the country.

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