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Original Article

Biodiversity and distribution of flea (Siphonaptera), rodent (Rodentia), and Crocidura (Insectivora) species associated with plague epidemiology in eastern Zambia

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Summary

Fleas (Siphonaptera) are important vectors of several animal and human disease pathogens, while rodents are considered as reservoirs of most pathogens, including *Yersinia pestis* Factors that influence the parasitism rate of fleas, ecological aspects that modulate their distribution, and host-flea relationship in Eastern Zambia remain unknown. Furthermore, there is little information on the biodiversity and abundance of rodents and fleas in the study area. A total of 1212 mammals were sampled and examined. These included rodents (n=329), Crocidura (n=113), domestic pigs (n=254), small ruminants (n=346) and carnivores (n=168), and 1578 fleas, where five species were identified. There were nine genera and species of rodents with one genus of Crocidura captured. The results showed that 27(8.2%) and 19(5.8%) rodents and 8(7.0%) and 2(1.8%) Crocidura were positive for antibodies and *pla* gene for *Y. pestis*, respectively. *Echidnophaga larina* were the most mean abundant (MA=8.58), while *Xenopsylla cheopis* had the least mean abundant (MA=0.14), nevertheless it was the most infected with *Y. pestis*. *Mastomys. natalensis* was highest in plague positivity 31/56, followed by *Crocidura spp* 10/56 and *Rattus rattus* 6/56. The results indicated that three flea species were infected with *Yersinia pestis.* Shannon-Weiner (H) and dominance (D) indices of rodents were 1.5 and 0.2789, while the flea indices were 0.5310 and 0.8389, respectively. There was a strong association between richness of fleas and plague disease (p=0.01; x^2=65.3). It's established that rodents were more biodiversity than fleas while both were unevenly distributed. It's recommended that control measures of fleas be intensified and sustained to lessen the spread of their associated diseases.

Keywords: Biodiversity Crocidura, Fleas, Plague, Rodents

Introduction

Fleas (Insecta: Siphonaptera) encompasses at least 2500 species with 15 subspecies globally (Whiting et al., 2008). They are small, laterally flattened and wingless, and highly specialized arthropods. They are of great importance as

vectors of many viral, bacterial, and parasitic pathogens. Being hematophagous arthropods, fleas may transmit pathogens through soiled mouthparts (e.g., *Y. pestis*, and viral pathogens), regurgitation of gut contents (e.g., *Y. pestis*), and infectious saliva (e.g., *R. felis* in salivary glands) (Eisen et al., 2012). Also, transmission can occur through contaminated feces (e.g., *Rickettsia typhi*, *Bartonella,hanselae)* (Amatre et al., 2009) (Bitam et al., 2010). Fleas have complex life cycles, which include the production of non-parasitic omnivorous larvae and obligate-hematophagous imagoes (Sanchez and Lareschi, 2019). Once the flea emerges from the cocoon, it immediately seeks a potential host to get a blood meal. Most fleas prefer the host habitat (nest fleas), while others stay more or less permanently on the body of the host itself (body fleas) (Bitam et al., 2010).

Fleas have tremendous medical and economic importance because they are vectors of several causative agents of diseases in animals and humans. Such diseases as the cat scratch fever caused by *B. henselae*, Q fever *(Coxiella burnetii)*, murine typhus *(R. typhii)*, flea-borne spotted fever *(R. felis)*, and bubonic plague (*Y. pestis*) (Bitam et al., 2010). Due to the potential vector capacity of fleas, their distribution and biodiversity may have essential consequences for host survival and disease dynamics (Foottit and Galloway, 2018). Arthropod-transmitted diseases represented approximately 17% of all infectious diseases globally and are influenced by a complex and dynamic ecosystem that involves vectors, hosts, and infectious agents, as well as environmental factors(Young et al., 2015). Fleas are able to bite animals, suck blood and mechanically or biologically transmit disease pathogens such as *Yersinia pestis* to susceptible hosts, including humans and rodents (Ago et al., 2015). The abundance of fleas in an area may suggest an increased number or frequency of flea-borne diseases. This happens when fleas are numerous and consequently look for animals and humans to suck blood and subsequently transmit disease-causing pathogens (Nziza et al., 2019; Miarinjara and Boyer, 2016).

Rodents are small mammals of the order *Rodentia*, which comprises more than 2000 species and approximately 30 families. There are diverse biological and ecological differences among rodents in shape, size, weight, and habitat. The smallest rodents (*Mus minutoide*) may weigh 5 grams, while the largest (*American cabybard*) weighs more than 70 kg. Rodent's ecological requirements vary widely, which include domestic and semi-domestic field and forest habitats. These animals are potential reservoirs of infectious diseases of humans and other mammals and are also suitable hosts for flea and other ectoparasites (Esfandiari et al., 2017).

Shrews are insectivores and able to harbor disease pathogens, such as bacteria, virus, and protozoa. These disease pathogens could be transmitted to a suitable host and cause disease through the vector such as fleas (Moore et al., 2015).

The abundance of rodents and fleas depends on climatic and environmental conditions. Climate changes caused by global warming and human intervention have contributed to vicissitudes in the biological parameters and distribution ranges of vector/fleas and their hosts (Kausrud et al., 2007). These conditions are likely to affect the biodiversity and abundance or dominance of both rodents and their flea ectoparasites. At times of heavy rainfall and availability of food crops, there are corresponding large numbers of rodents and their fleas, contrary to times of adverse climate when the rodents and their fleas diminish in number. Rodents have the potential to transmit sylvatic plague and other zoonotic diseases directly to humans and other animals. Such diseases can be transmitted by contamination of foods with rodent fecal materials or urine, direct contacts with infected animals, droplets, or bites by appropriate insect vectors such as fleas in case of plague disease (Backhans and Fellström, 2012). These vectors then transmit the pathogens from one reservoir host to another susceptible animal, including humans, small carnivores, and other animals. In view of the aforegoing description of the relationship between rodents and fleas regarding plague epidemiology, it was felt desirable to investigate and establish the current status of biodiversity and abundance of these creatures in relation to plague epidemiology; therefore the aim of the study was to accomplish this desirability in the eastern region of Zambia.

Materials and methods

Area and time of study

This study took place in two districts, Nyimba and Sinda, both in the eastern province of Zambia from 2013 to 2017. The choice of these areas was based on their recent history of plague outbreaks, whereby the last human plague was detected in Nyimba and Sinda districts in 2015 and 2008, respectively. The eastern Zambia districts experience high rainfall between January and March of each year. The communities grow crops such as maize, groundnuts, cassava, which are all suitable for rodents and other animals.

Study design

A cross-sectional study design was conducted, where the abundance and biodiversity of the rodents, shrews, and flea ectoparasites were examined.

Sample collection

Domestic animals

Villages in the study area were randomly selected in which 20 or more domestic animals of the selected species, comprising pigs, dogs, cats, goats, and sheep were sampled. From each selected client, a verbal consent was acquired (as per approved Plague Research policy provided in Assurance No. FWA0000338). Each selected animal was assigned a unique identification number. The first animal was randomly selected, followed by a systematic technique (Systematic Random Sampling). The selected animal was immobilized and rested on the white plastic sheet for blood collection and inspection of fleas and other ectoparasites. The animal was groomed with cotton wool containing 90% diethyl ether to sedate the ectoparasites. The appropriate brush was used to scrub the animal to detach fleas and other ectoparasites from its fur and skin. A pair of forcepswas used to gently removed fleas that fell onto the white sheet and those which remained attached on the animal's fur/skin into vials with 70% ethanol. The study did not include animals that came from other villages in the past six months (Nyirenda et al., 2017).

Rodents and shrews trapping

The study villages were selected randomly and were alienated into six arbitrary sectors from where three zones were randomly nominated for trapping rodents and shrews. Sherman's live traps (50×65×157 mm) were baited with peanut butter mixed with soya beans flour and were placed at a distance of 10m apart in nearby bushes overnight. Wire cage traps (145×100×230 mm) (Hoga-lab, Kyoto, Japan), baited with fish, *Stolothrissa tanganicae* (Kapenta), and ripe tomatoes, were set in chosen houses in the zones. Inspection of traps was conducted the following morning. The captured animals were taken for flea ectoparasite inspection and collection in a mobile laboratory installed at a distant place away from the community to collect sera, organs, and fleas. Other processes included identification and enumeration of rodents and fleas were carried out in such a laboratory. The ensnaring process was continuous for three consecutive days in the same area as previously described (Kilonzo, 1976). Sera and organs were kept at -20^{o}C until needed for ELISA and PCR, respectively (Nyirenda et al., 2018).

Collection of flea ectoparasites from rodents and shrews

Fleas and other ectoparasites from the rodents and shrews were collected by introducing the latter into a plastic bag with a soaked cotton wool, in 90% diethyl ether to sedate both the animal and fleas. Once the animals were sedated, they were placed into a silver basin and brushed thoroughly with a hard toothbrush to disengage the ectoparasites. Fleas that dropped into the basin were gently picked

using either a pair of forceps or fine camel brush into vials with 70% ethanol as a preservative. Other ectoparasites were also collected into separate vials with the same concentration of ethanol (Nyirenda et al., 2018).

Rodent identification

All the captured rodents were identified to genus and/or specie level using the key features described by Kingdon (Kingdon, 1974). In addition, the body and tail lengths were measured and recorded. The carcasses were then counted, and numbers of each species were established.

Flea processing and identification

The fleas were preliminary identified and pooled (1–5) according to their species, location of the host, and species of its host. One to two (1–2) fleas were removed and processed from each pool to confirm the identity. The fleas were processed and identified to specie level using the method described by Kilonzo (Kilonzo, 1999), which basically involve drying the insects on the No. 1 filter paper, boiling them for 10 minutes in heat resistant tubes containing 10% Sodium Hydroxide. The fleas were then put in cold water for one hour and into acidified water (water + glacial acetic acid of equal volume) for 30 minutes to neutralize Sodium Hydroxide. After this, fleas were dehydrated through increasing strengths of ethanol (from 50%, 70%, 95%, and absolute alcohol) for one hour at each stage. After this process, the fleas were transferred into the vial with clove oil and incubated at room temperature overnight or until they were clear and transparent, after which they were mounted with Dibutylphthalate Polystyrene Xylene (DPX) on the glass slide with a coverslip for examination under the light microscope using dry objectives (x4, x10 or x40) magnification for detection of common taxonomic features (Kilonzo, 1999).

Identification of the fleas was based on main flea features such as pronotal combs, genal combs, and shape of the head and reproductive organs (spermathecae in females and penis plates in males (Nyirenda et al., 2018; Kilonzo, 1999; Pratt and Stojanovich, 1966; Traub, 1962).

DNA extraction from organs and fleas

The DNA from tissues of rodents was extracted using the DNA extraction kit (ZR genomic DNA-Tissue Mini-Prep Catalog No. D3051) following the manufacturer's instructions (Zymo Research Irvine, CA, USA). In the case of fleas, these were identified before preparing them for DNA extraction using the heat treatment method as described elsewhere (Nyirenda et al., 2017).

PCR Technique

PCR was performed using Phusion flash high fidelity master mix (Finnzymes Oy, Finland) in a highly PCR specialized laboratory. The

primers Yp pla1 (5'TGC TTT ATG ACG CAG AAA CAG G3') as the forward primer and Yp pla2 (5'CTG TAG CTG TCC AAC TGA AAC G3') as the reverse primer, that amplifies a 344-bp region spanning residues 425 to 769 of the plasminogen activator gene, were used (Sodeinde and Goguen, 1989). The PCR reactions were performed as described elsewhere (Nyirenda *et al.*, 2018) (Nyirenda *et al.*, 2017). The positive and negative controls, which came together with the kit, were also included in the test.

ELISA testing

The stored sera were removed from the freezer (−20 °C) and left at room temperature to thaw and processed using the protocol as previously described (Chu, 2000).

Data analysis

Data were entered in Microsoft Excel software and analyzed using Epi info™ 7.0.8.0, a computer statistical package from the Centre for Disease Control and Prevention (CDC), where confidence intervals (CI) and positive percentages were generated. The *p-value* and the chi-square (x^2) were also calculated.

Flea diversity was estimated by calculating the Specific richness (S=number of flea species) and Shannon diversity species index (H = -Σpi ln pi), where pi =proportion of each flea species in the sample). Flea evenness was estimated by calculating the Simpson dominance index ($D = \Sigma p^2 i$), where pi is the proportion of individuals found in species 'i' as calculated elsewhere (Sanchez and Lareschi, 2019; Ralaizafisoloarivony et al., 2014).

For each host species, the following indices and parameters were calculated: flea species richness (S=number of flea species), Simpson index (Mean Abundance (MA) = total number of individuals' of a parasite species on a host/total number of host species, including infested and non-infested species), and prevalence ([P = number of infested animals with one or more individuals of a parasite species/total number of examined mammals for that parasite species] multiplied by 100).

Results

In this study, 1212 mammals were examined for flea vectors, which comprised rodents (n=329), shrews (n=113), domestic pigs (n=254), small domestic ruminants (n=364), and domestic carnivores (n=168) were examined for fleas. A total of 1578 fleas were collected from such animals. The rodent population composed of nine genera and species, while all the shrews belonged to the genera of *Crocidura* spp. (n=113) The rodent species included: *Mastomys natalensis* (n=189), *Rattus rattus* (n=60), *Saccostomus* spp *(pouched mouse* (n=43), *Tatera* spp (n=2), *Graphiurus* spp (n=5), *Mus* spp (n=2), *Acomys* spp (n=1), *Gerbillurus (Gerbil)* spp (n=22) *and Steastomys parvus* (fat mouse) (n=5). On

ELISA and PCR, 27 (8.2%) and 19 (5.8%) were positive for *Yersinia pestis* antibodies and pla gene, respectively, while 8 (7.0%) and 2 (1.8%) shrews were also positive for *Yersinia pestis* antibodies and pla genes, respectively. Five species of fleas collected from the animals in the study area were; *X. cheopis* (n=59), *C. canis* (n=64), *E. gallinacea* (n=389), *E. larina* (n=1064), and *C. felis* (n=2). The results also showed *E. gallinacea* had the highest mean abundance (MA) of the (8.58), while *X. cheopis* had the lowest (MA=0.14) in both districts (Table 1). It was revealed that *Mastomys natalensis* (31/56) was the highest-ranking rodent species carrying *Y. pestis. Mus* spp, *Acomy* spp, and *Steatomys parvus* were negative for *Y. pestis* on both tests. Statistical analysis presented no significant statistical difference between the rodents and plague disease ($p = 0.347$; $x^2=10$) (Table 2).

Table 1. Types and numbers of animals sampled and fleas collected in Nyimba and Sinda districts

District	Host/source of fleas	No. sampled (n)	Species of fleas collected	No. fleas collected	Mean abundance (MA) of fleas
Nyimba	Rodents	120	*Xenopsylla cheopis*	15	0.12
	Shrews	17	-	0	0
	Pigs	2	*Ctenocephalides canis*	3	1.5
	Pigs	9	*Echidnophaga gallinacea*	7	0.78
	Goats	83	*Ctenocephalides canis*	16	0.19
Sinda	Pigs	121	*Echidnophaga gallinacea*	382	3.16
	Pigs	124	*Echidnophaga larina*	1064	8.58
	Dogs	165	*Ctenocephaalides canis*	45	0.27
	Goats	232	-	0	0
	Cats	3	*Ctenocephalides felis*	2	0.67
	Sheep	31	-	0	0
	Rodents	295	*Xenopsylla cheopis*	44	0.15
	Shrews	10	-	0	0
	Total	**1212**		**1578**	**1.3**

The study further revealed that three species, namely *X. cheopis*, *E. gallinacean*, and *C. canis*, were infected with the *Y. pestis* bacterium. The plague positive fleas were collected from rodents, pigs, and goats, respectively. Statistical analysis demonstrated a significant statistical difference between richness of fleas and plague disease ($p = 0.01$; $x^2=65$) (Table 3). Our results also showed that the biodiversity Shannon-Weiner (H) and dominance (D) of rodents were 1.5 and 0.2789 respectively in the study area (Table 4), while the biodiversity Shannon-Weiner (H) of the fleas showed 0.5310 and the dominance (D) showed 0.8389 (Table 5).

Table 2. Current status of plague endemicity among rodents and shrews in Nyimba and Sinda districts

Species of animals	Sinda district			Nyimba district			Grand total		
	No. sampled	No. pos for *Y. pestis*		No. sampled	No. pos for *Y. pestis*		Total sampled	Total +ve (%)	X^2; p-value
		ELISA	PCR		ELISA	PCR			
Mastomys natalensis	124	14	8	65	4	5	189	31(55.4)	
Rattus rattus	35	2	1	25	2	1	60	6(10.7)	
Saccostomus spp	18	2	1	25	0	0	43	3(5.6)	X^2=10.0 p-value =0.347
Tatera	2	1	0	0	0	0	2	1(1.8)	
Graphiurus spp	5	1	0	0	0	0	5	1(1.8)	df=9
Crocidura spp	96	7	1	17	1	1	113	10(17.8)	
Mus spp	2	0	0	0	0	0	2	0	
Acomys spp	1	0	0	0	0	0	1	0	
Gerbil spp	22	1	3	0	0	0	22	4(7.1)	
Steatomys parvus	0	0	0	5	0	0	5	0	
Total	**305**	28	14	**137**	7	7	**442**	**56(100)**	

Table 3. Status of *Yersinia pestis* in fleas collected from animals in the study area

Species	Host (Name)	Sinda district		Nyimba district		Grand total		
		No. fleas sampled	No. +ve *Y.pestis*	No. fleas sampled	No. +ve *Y. pestis*	No. sampled	No. +ve (%)	X^2; p-value
Xenopsylla cheopis	Rodent	44	0	15	3	59	3(25)	
Ctenocephalides canis	Pig	0	0	3	0	3	0	
Ctenocephalides canis	Goat	0	0	16	5	16	5(41.7)	X^2=65
Ctenocephalides canis	Dog	45	0	-		45	0	p-value =0.01
Ctenocephalides felis	Cat	2	0	-		2	0	
Echidnophaga gallinacea	Pig	382	0	7	4	389	4(33.3)	
Echidnophaga larina	Pig	1064	0	0		1064	0	
TOTAL		**1537**	**0**	**41**	**12**	**1578**	**12**	

The result is significant at $p < 0.05$.

Table 4. Shannon-Weiner and dominance index for rodents and shrews

	Category (Animal spp)	Value	x	x^2	-x ln(x)
1	*Mastomys natalensis*	189	42.8%	0.183	0.363
2	*Rattus rattus*	60	13.6%	0.018	0.271
3	*Saccostomus* spp	43	9.7%	0.009	0.227
4	*Tatera* spp	2	0.5%	0.000	0.024
5	*Graphiurus* spp	5	1.1%	0.000	0.051
6	*Crocidura* spp	113	25.6%	0.065	0.349
7	*Mus* spp	2	0.5%	0.000	0.024
8	*Acomys* spp	1	0.2%	0.000	0.014
9	*Gerbil* spp	22	5.0%	0.002	0.149
9	*Steatomys parvu*	5	1.1%	0.000	0.051
R1	**Simpson Dominance (D)**			**0.2789**	
R2	**Shannon Entropy (H)**				**1.5231**

Table 5. Shannon-Weiner and Dominance index for the fleas collected from the study area

No	Species	No. of fleas	x	x^2	-x ln(x)
1	*Xenopsylla cheopis*	59	3.7%	0.001	0.092
2	*Ctenocephalides canis*	64	4.1%	0.002	0.131
3	*Ctenocephalides felis*	2	0.1%	0.000	0.009
4	*Echidnophaga gallinacea*	389	25.0%	0.062	0.346
5	*Echidnophaga larina*	1064	68.3%	0.466	0.260
R1	**Simpson Dominance (D)**			**0.5310**	
R2	**Shannon Entropy (H)**				**0.8389**

Discussion

Rodents

The current observations that *M. natalensis* is the most abundant rodent species in the study area, followed by *R. rattus*, and that the two species had the highest infection rates with *Yersinia pestis* (Table 2). This suggests that the plague organisms are endemically in the area and that the rodent species in question are the most suitable reservoirs of the plague disease in the area. These observations are consistent with earlier findings described by Kilonzo (Kilonzo, 1999). In view of their ecological nature and close association with humans in houses and the fields, these rodents could easily transmit *Y. pestis* to humans and other animals as they were diverse and abundant in the study area. The observation that the two species are abundant and the fact that they are domestic and/or semi-domestic suggest that they can destroy substantial food and cash crops, thus causing hunger and

poverty in the area. Furthermore, the current results indicate that two rodent species, as well as shrews were plague positive in both Sinda and Nyimba districts. Detection of *Yersinia pestis* antibodies and the pla gene in the *Crocidura* spp suggest that these small mammals were in contact with the bacterium and could serve as the reservoir host for the disease in the area(Moore et al., 2015). Despite the detection of plague in these animals in both ELISA and PCR, there was no outbreak during our study. This is at least partially consistent with observation elsewhere that rodents are merely reservoirs of *Yersinia pestis*, and the presence of suitable vectors alone could not stimulate the outbreak of the disease.

The general picture is that rodents were biodiverse in the area, and they were unevenly distributed, thus resulting in sporadic numbers in some areas. This scenario is probably attributable to climate and environmental changes(Morand, 2011)(Eisen et al., 2015). In view of the current observations, establishment and adherence to regular surveillance services for plague and other flea/rodent zoonoses is desirable.

Fleas

The observation in the current study that *Echidnophaga larina* collected from domestic pigs were the most abundant fleas species in the study area, followed by *Echidnophaga gallinacea* collected from the same animals, probably suggest that the animals (pigs) were not regularly dipped in or spray/dusted with appropriate pesticides and their houses are unhygienic. The presence of *Yersinia pestis* antigen in 1% *E. gallinacea* collected from pigs and 31.3% of *C. canis* collected from goats in Nyimba districts suggests that the two flea species are suitable reservoirs/carriers of pathogen. However, the two flea species are potential, but not efficient vectors of the disease. On the other hand, the presence of *Y. pestis* in 5.1% *X. cheopis* collected from rodents trapped in Nyimba district suggests the potential plague outbreak of human plague in the area if and when conditions become favorable, since the flea is known to be the efficient vector of the disease globally (Miarinjara and Boyer, 2016).

Furthermore, the current observation showed that the fleas were neither biodiverse nor evenly distributed, thus suggesting that they are seasonal as reported elsewhere in Argentina, Tanzania, and Uganda (Sanchez and Lareschi, 2019;Young et al., 2015; Eisen et al., 2012; Kilonzo et al. 2006; Njunwa et al., 1989). During the hot season, fleas can breed more efficiently, and their survival rate is higher than in other seasons. Flea density dynamic is high and is influenced by several factors, including the host, altitude, temperature, and relative humidity (RH) (Eisen et al., 2015b). The latter is the most

appropriate condition for vectors to breed and transmit *Y. pestis* and thus facilitate disease outbreaks(Eisen et al., 2015a).

Besides transmitting disease pathogens, fleas also inflict and injure the skin of their host, thus allowing opportunistic bacteria to infect the host open wounds (Krasnov *et al.*, 2019). The current observation of the large number of fleas on domestic pigs, therefore, suggests that such animals are at risk of getting severe skin problems or infection caused by skin-penetrating opportunistic organisms. The low flea index of *X. cheopis* (efficient vectors) on rodents (suitable vectors) suggests a low plague outbreak at the moment. However, monitoring of these ectoparasites in the disease quiescent period is essential to assess seasonal increase and subsequently predict the plague outbreak in the area (Ralaizafisoloarivony *et al.*, 2014). The significant association (p=0.01) between flea numbers and plague is a further indication that plague depends on the bites from infected fleas.

Conclusion and recommendations

It can be justifiably concluded from the observations in this study that rodents are more bio-diverse than fleas while both of them are unevenly distributed in the study area. It can also be concluded that *Echidnophaga larina* was the most abundant domestic animal flea species in the area and it is most hosted by domestic pigs. Likewise, it is conclusive from the study a well-known efficient vector of plague was the only rodent flea species in the area and its population density (flea per rodent) was too low to maintain transmission of plague at the time of the study. It can further be concluded from the study that a thorough understanding of the flea-host association and rodent and flea distribution and diversity may be useful in epidemiological studies of plague disease and could provide a reliable base for prediction and surveillance of plague and other related re-emerging zoonotic diseases. It is, therefore, recommended that management of flea ectoparasites should be strengthened and surveillance services are maintained to forecast and consequently prevent or minimize the outbreaks of such diseases.

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Ethical Approval

Ethical approval to conduct this study was sought the Biomedical Research Ethics Committee (BREC), Zambia (Assurance No. is FWA00000338). Verbal consent to sample the animals was granted by the livestock farmers from the study area.

Conflict of Interest Statement

The authors have declared that no competing interests exist.

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