

Molecular, serological and epidemiological observations after a suspected outbreak of plague in Nyimba, eastern Zambia

Stanley S Nyirenda^{1,2}, Bernard M Hang'ombe³,
Bukheti S Kilonzo⁴, Mathews N Kabeta⁵,
Mundia Cornelli⁵ and Yona Sinkala⁵

Abstract

Plague is a re-emerging zoonotic disease caused by the bacterium *Yersinia pestis*. The disease has caused periodic global devastation since the first outbreak in the 6th century. Two months after a suspected plague outbreak in Nyimba district, samples were collected from 94 livestock (goats and pigs), 25 rodents, 6 shrews and 33 fleas. Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) techniques were used to investigate the presence of *Y. pestis*, which showed that 16.0% (4/25) of rodents, 16.7% (1/6) of shrews (*Crocidura spp*) and 6.0% (5/83) of goats were positive for IgG antibodies against Fraction I antigen of *Y. pestis*. Plasminogen activator (Pla) gene (DNA) of *Y. pestis* was detected in five pools containing 36.4% (12/33) fleas collected from pigs (n = 4), goats (n = 5) and rodents (n = 3). The detection of Pla gene in fleas and IgG antibodies against Fraction I antigen in rodents, shrews and goats suggest that *Y. pestis* had been present in the study area in the recent past.

Keywords

Domestic pigs, fleas, goats, plague, rodents, Zambia

Introduction

Plague is a re-emerging zoonotic disease mainly considered to be transmitted by an insect, the oriental rat flea *Xenopsylla spp.*, from infected rodents^{1,2} but occasionally it can be transmitted through direct contact and droplets in pneumonic cases. Other insects which may pose as potential vectors and risk transmitting the disease are *Echidnophaga gallinacea*, *Pulex irritans*, *Ctenocephalides canis* and *Ctenocephalides felis*. In Madagascar, these fleas were considered to be vectors of the disease, especially in an outbreak situation.³ Indiscriminate killing of rodents may facilitate migration of fleas from the dead carcasses to other animals passing nearby. Plague has been reported in some countries to be transmitted through direct contact with infected materials: in Libya, individuals were infected from skinning goat and camel meat and eating it,⁴ while in Afghanistan herdsmen were infected through contact with infected camel meat.⁵ In the USA, a wildlife biologist died after conducting a postmortem on an infected lion.

Domestic pigs fed on meat infected with *Yersinia pestis* have shown high levels of IgG antibodies against Fraction 1 antigen (Fra1) for > 200 days.⁶

Plague has caused three devastating global pandemics, claiming millions of lives. The Justinian plague (541–543) affected the Middle East;⁷ the Black death (1347–1351) affected the Middle East, Asia, Africa and Europe, killing 75% of the population;⁸ and the Oriental plague (probably started in 1855 in the Chinese province) affected the whole world except Australia and New Zealand.⁹ Spread of plague was facilitated by easier transportation of goods and

¹Research Scientist-CVRI, Central Veterinary Research Institute, Balmoral, Lusaka, Zambia

²Sokoine University of Agriculture, Department of Microbiology and Parasitology, Morogoro, Tanzania

³The University of Zambia, Department of Microbiology, Lusaka, Zambia

⁴Sokoine University of Agriculture, Pest Management Centre, Morogoro, Tanzania

⁵Department of Veterinary Services (DVS), Ministry of Fisheries and Livestock, Mulungushi House, Lusaka, Zambia

Corresponding author:

Bernard M Hang'ombe, The University of Zambia, School of Veterinary Medicine, Department of Microbiology, PO Box 32379, Lusaka 10101, Zambia.

Email: mudenda68@yahoo.com

movement of people.¹⁰ Notably, plague may have infected the Philistines in about 1000 BCE.¹¹

In Zambia, there have been sporadic plague outbreaks in the past century which included the eastern, northwestern and southern regions. In the east, three districts have reported cases since 1917, namely Chama (1917–1918 and 1956), Lundazi (1956) and Sinda (2001 and 2008).^{12–14}

Luembe in Nyimba district, located in the Luangwa valley (eastern district), had never reported human plague until March 2015, when cases were reported from Kaviyeni village. Three human fatalities and deaths of several domestic animals were reported. In May, two months after the suspected outbreak, epidemiological, molecular and serological investigations were undertaken to define both the potential animal reservoirs and their fleas in order to determine their association with *Y. pestis*.

The bacterium possesses two unique genes which contribute to its virulence namely; Fraction 1 (Fra1), from MT1 plasmid, and Plasminogen activator (Pla), from PCP1 plasmid, are both specific to *Y. pestis* and are therefore targeted by PCR and ELISA detection techniques, respectively.

Materials and methods

Our study was done to investigate potential reservoir hosts and their vectors, and was conducted in Nyimba district in eastern Zambia, where there was a suspected

plague outbreak (Figure 1). In March 2015, two months after the reporting of three human fatalities likely to have been caused by plague, samples were collected from livestock, rodents and their fleas in the area. There had been heavy rainfall in villages around Chinambi Health Clinic in Luembe, where villagers experienced a higher infestation of rodents and fleas in fields and houses.¹⁶

A cross-sectional study design was employed and samples were collected from domestic pigs, goats and peri-urban and domestic rodents. These animals share grazing and water sources and so share fleas.

Written or verbal consent from camp veterinary assistants and village headmen was sought before the sampling was done. Research was conducted in compliance with the Animal Welfare Act and the Guidelines for the care and use of laboratory animals of the country. Animals were chosen from selected villages using a systematic random technique, where all animals of the same species were specified by locating their households and assigning them a unique number. Animals which were less than six months old and those which came from another village in the previous six months were excluded from the study. Each selected animal was physically restrained and blood was aseptically collected from a jugular vein into a sterile plain vacutainer tube. The blood was left at room temperature overnight to facilitate blood clotting and the separated serum was transferred into serum vials.

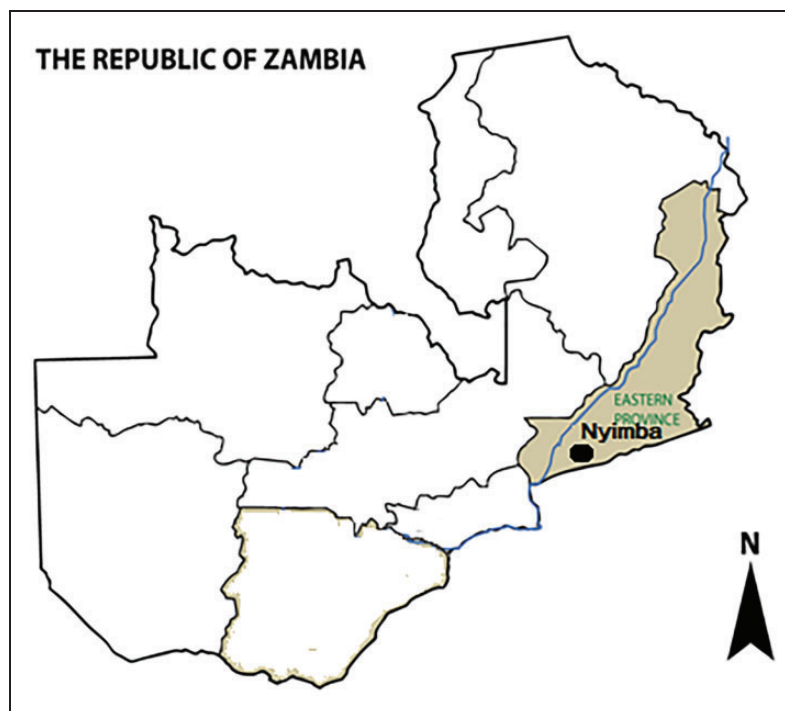


Figure 1. Nyimba district in eastern Zambia, the study area.

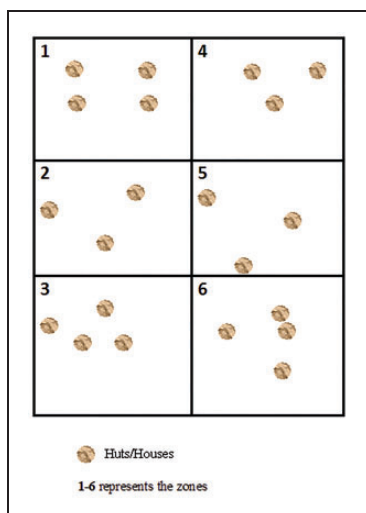


Figure 2. Zones in a village where trapping of rodents and fleas were done.

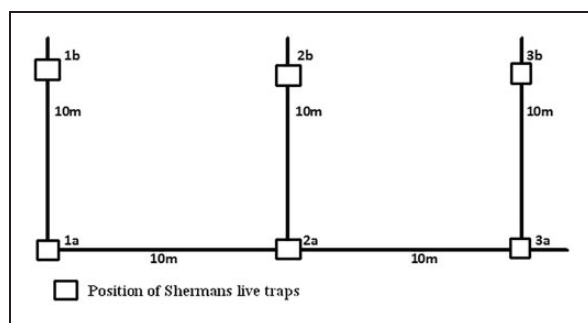


Figure 3. The position of Sherman's live traps set at a distance of 10 m apart in the bush near the houses.

Each restrained animal was laid on a white cloth and inspected for flea infestation.

The animal was brushed with cotton wool soaked in 90% diethyl ether and scrubbed with an appropriate animal brush from tail to head to remove fleas and other ectoparasites. Fleas which dropped onto the white cloth were collected into small vials containing 70% ethanol. Fleas which remained attached to the animal's skin or fur were gently removed with fine forceps.

Each selected village was divided into six arbitrary zones and three such zones were selected at random (Figure 2). Sherman's live traps were baited with peanut butter mixed with soya flour and set at a distance of 10 m apart and left overnight (Figure 3). Wire cage traps were also set in the selected houses in the selected zones.

Traps were inspected in the following morning and trapped rodents were taken to the mobile laboratory for sample collection and trapping continued for three consecutive days. Each rodent was put in a plastic bag containing cotton wool soaked with 90% diethyl ether

to anaesthetise both host and ectoparasites. The rodents were transferred to a silver basin where they were brushed with a tooth brush to remove fleas and other ectoparasites. Fleas which dropped into the basin were collected into small vials containing 70% ethanol.

Cardiac blood was collected from each rodent or shrew using a 2 mL syringe and a 21G needle. Serum was separated from collected blood as above, and preserved at -20°C until required for use.

Each rodent or shrew was dissected and organs (spleen, liver, lung, kidney and heart) were collected, divided into two parts and put in two separate vials.

Light traps, as previously described,¹⁷ were set in the selected houses in the evening and inspected the following morning. Trapped fleas were collected using a camel hair brush and transferred to small vial containing 70% ethanol.

PCR, ELISA and DNA extraction were carried out according to manufacturers' instructions.

Fleas were identified using the key features described^{18,19} and pooled ($n=1-5$) according to the location, host source and species: 12 pools were created (see Table 1).

Each pool containing 1–5 fleas was put in an Eppendorf tube and 100 μL of brain–heart infusion broth (Oxoid, Hampshire, UK) was added and triturated with sterile pipettes tips. The triturated samples were 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.^{20,21}

Data were entered in Microsoft Excel software and analysed using Epi infoTM 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 flea population density on the sampled animals was measured using specific flea index (SFI), calculated by dividing the number of fleas collected by the total number of animals sampled (number of fleas per individual animal species).^{22,23}

Results

A total of 25 rodents (18 *Mastomys natalensis*, five *Rattus rattus* and two *Saccostomus spp.*) and six shrews were captured, from which six fleas were collected. Among 83 goats and 11 pigs sampled, 26 fleas were collected, while one flea was captured from inside a house (Table 2).

Of the total 33 fleas, one *Ctenocephalides canis* was captured from a house, six *Xenopsylla spp.* from rodents, three *Ctenocephalides canis* and seven *Echidnophaga gallinacea* from pigs, 16 *Ctenocephalides canis* from goats (Table 3).

Table 1. Pooled flea samples and the PCR results.

Pool	Village	Flea species	Host of fleas	Fleas collected (n)	PCR results
1	Kaviyeni	<i>Echidnophaga gallinacea</i>	Pig	3	Negative for <i>Y. pestis</i> pla gene
2	Kaviyeni	<i>Echidnophaga gallinacea</i>	Pig	4	Positive for <i>Y. pestis</i> Pla gene
3	Kaviyeni	<i>Ctenocephalides canis</i>	Pig	3	Negative for <i>Y. pestis</i> pla gene
4	Kaviyeni	<i>Ctenocephalides canis</i>	House	1	Negative for <i>Y. pestis</i> pla gene
5	Kaviyeni*	<i>Xenopsylla spp</i>	Rodent	1	Positive for <i>Y. pestis</i> Pla gene
6	Kaviyeni*	<i>Xenopsylla spp</i>	Rodent	3	Negative for <i>Y. pestis</i> pla gene
7	Kaviyeni*	<i>Xenopsylla spp</i>	Rodent	1	Positive for <i>Y. pestis</i> Pla gene
8	Kaviyeni*	<i>Xenopsylla spp</i>	Rodent	1	Positive for <i>Y. pestis</i> Pla gene
9	Katanta*	<i>Ctenocephalides canis</i>	Goat	5	Negative for <i>Y. pestis</i> pla gene
10	Wilson	<i>Ctenocephalides canis</i>	Goat	1	Negative for <i>Y. pestis</i> pla gene
11	Katanta*	<i>Ctenocephalides canis</i>	Goat	5	Negative for <i>Y. pestis</i> pla gene
12	Chikaneni	<i>Ctenocephalides canis</i>	Goat	5	Positive for <i>Y. pestis</i> Pla gene
Total				33	

*Samples collected from different zones of the same village.

Table 2. Samples collected from different villages in Nyimba district.

Name of village	Pig		Goat		Rodent and <i>Crocidura spp.</i>		
	Samples collected (n)	Fleas collected (n)	Samples collected (n)	Fleas collected (n)	Organs collected (n)	Blood collected (n)	Fleas collected (n)
Wilson	0	0	12	1	0	0	0
Sangu	0	0	12	0	0	0	0
Katanta	0	0	4	10	0	0	0
Kanita	0	0	2	0	0	0	0
Chikaneni	0	0	5	5	0	0	0
Mwaluka	0	0	3	0	0	0	0
Nyalungwe	0	0	12	0	0	0	0
Kaviyeni	11	10	33	0	31	31	6
Total	11	10	83	16	31	31	6

Table 3. Results of PCR and Specific Flea Index (SFI) on pooled fleas.

Host/Source of fleas	Hosts sampled (n)	Fleas collected (n)	Species of fleas collected	SFI	Positive (PCR) (n)	Positive (%)
Pigs	2	3	<i>Ct. canis</i>	1.5%	0	0
Pigs	9	7	<i>E. gallinacea</i>	0.8%	4	57.1
Goats	83	16	<i>Ct. canis</i>	0.19%	5	31.2
Rodents	31	6	<i>Xenopsylla spp</i>	0.19%	3	50.0
Houses	10	1	<i>Ct. canis</i>	0.1%	0	0
		33			12	36.4% (95% CI 20.4–54.9)

Table 4. Results of ELISA and PCR on various rodents and *Crocidura* spp in Nyimba.

Species captured	Total animals captured (n)	ELISA results	PCR results
<i>Crocidura</i> spp. (Shrew)	06	1 (3.2%)	0
<i>Mastomys natalensis</i>	18	3(9.6%)	0
<i>Rattus rattus</i>	05	1 (3.2%)	0
<i>Saccostomus</i> spp.	02	0 (0.0%)	0
	31	5 (16.12%) 95% CI 5.45–33.73%	0

ELISA results showed that 5/31 (16.12%; 95% CI 5.45–33.73%) rodents and shrews were positive for Fra1 antibodies; of these *Mastomys natalensis* had the highest number of positives (9.6%) while *Crocidura* spp. and *Rattus rattus* had 3.2% positives (Table 4). No antibodies against Fra1 were found in pigs, while 6% (5/83) goats were positive. PCR results showed five pooled samples (2, 5, 7, 8 and 12) were positive for the plasminogen activator (pla) gene of *Y. pestis*, detected at 344 base pair on the agarose gel (Tables 1, 3).

Discussion

The role of rodents and the shrew as natural reservoirs of *Y. pestis* in the study area was clearly demonstrated.^{25,26} Detection of such antibodies in 9.6% (3/31) and 6.5% (2/31) of the animals when anti-mouse antibody and anti-rat antibody, respectively, were used as secondary antibodies, suggests that anti-mouse IgG is more cross-reactive to the heterologous antibodies in the rodent and shrew population as it gives a higher percentage of positive results.²⁴ Further, *Mastomys natalensis* appears to be the most important reservoir host of plague in the area.²⁵

Goats were shown to have been exposed to *Y. pestis*, possibly through the bite of an infective flea. Thus goats may also be potential reservoir hosts of plague.^{4,27}

The demonstration of *Y. pestis* DNA *pla* gene in five pools containing fleas from different animals indicates that these arthropods were infected with the plague bacterium from an infected animal.²⁸ As the rodents from where the fleas came were PCR-negative, the insects must have fed on infected animals prior to infesting their current host animals. The findings that positively infected fleas were from different species is also an indication that any flea can acquire *Y. pestis* from infected animals and serve as a potential vector, host or carrier. These results are consistent with findings from Madagascar.³ Moreover, our findings suggest

that *Y. pestis* is still in circulation and so further outbreaks of plague may break out whenever environmental conditions become favourable.

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Declaration of conflicting interests

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