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Evidence of *Yersinia pestis* DNA in rodents in plague outbreak foci in Mbulu and Karatu Districts, northern Tanzania

MICHAEL H. ZIWA[1*], MECKY I. MATEE[1], BUKHETI S. KILONZO[2], BERNARD M. HANG'OMBE[3]
[1]*Muhimbili University of Health and Allied Sciences, P.O. Box 65001 Dar es Salaam, Tanzania*
[2]*Pest Management Centre, Sokoine University of Agriculture, P.O. Box 3110, Morogoro, Tanzania*
[3]*University of Zambia, P.O. Box 32379, Lusaka, Zambia*

Abstract: Human plague remains a public health concern in Tanzania despite its quiescence in most foci for years, considering the recurrence nature of the disease. Appreciable researches have involved serological screening of rodents, fleas and humans but none has involved molecular detection and hence proving the presence of *Yersinia pestis* in rodents in the most recent affected foci, Mbulu and Karatu districts in northern Tanzania. The objective of the current study was to employ a simple PCR to detect *Yersinia pestis* plasminogen activator (*pla*) gene in various potential mammalian hosts/reservoirs. The study was conducted in five villages in Mbulu and one in Karatu districts during the period of no disease outbreak. Rodents and small wild carnivores were captured, anaesthetized, identified, sexed and autopsied. Liver, spleen, heart and lung specimens were collected and DNA extracted after which PCR was used to detect the *Y. pestis pla* gene. A total of 517 small mammals were captured; of which, 493 (95.4%) were from Mbulu and 24 (4.6%) from Karatu. Two *Mastomys natalensis* (one from each district) and one *Gerbilliscus sp.* in Mbulu district were positive for *Y. pestis pla* gene. In conclusion, our results have provided a proof on the presence of *Y. pestis* in the two rodent species (*Mastomys natalensis* and *Gerbilliscus sp.*) and thus providing indicative evidence that the two are potential reservoirs of the pathogen and hence may be responsible for maintaining the same during periods of no disease outbreaks.

Key words: Human plague, *Yersinia pestis*, PCR, mammalian reservoirs, Tanzania

Introduction

Plague is an acute zoonotic bacterial disease caused by infection with *Yersinia pestis.* The bacterium is usually transmitted from one host to another through bites of infective fleas. Human infection usually occurs when these fleas escape from plague infected carcasses of domestic or wild animals especially rodents and bite humans (WHO, 2000; Neal, 2004). Humans can also contract the disease by droplet infection or direct contact with infected materials. In Africa, countries most affected include Madagascar, Democratic Republic of Congo, Mozambique, Uganda and Tanzania (Makundi *et al.,* 2008). In Tanzania, the disease is believed to have been introduced in 1886 (Msangi, 1968 cited in Kilonzo *et al.,* 2005). Since then outbreaks of the disease have occurred in various areas including Iringa, Bukoba, Musoma, Singida, Kondoa, Rombo, Hai, Arumeru, Mbulu, Same and Lushoto districts (Kilonzo *et al.,* 2006). All these foci have experienced several outbreaks of the disease involving varying numbers of human cases and high case-fatality rates (Kilonzo, 1992). During the period 1953 to 2003, a total of 8,956 plague cases with 731 (8.2%) deaths, were reported from ten districts in the country (Kilonzo *et al.,* 2005). Since 1980, however, only four districts (Lushoto, Singida, Karatu and Mbulu) have experienced outbreaks of the disease, involving 8,490 and 675 (8.0%) cases and deaths, respectively (Kilonzo *et al.,* 1997; 2006; Makundi *et al.,* 2008).

The latest outbreaks of plague in Tanzania were reported in Mbulu District in the northern part of the country. The outbreak first occurred between February and March 2007, involving 35

* Correspondence: Michael H. Ziwa; Email: michael.ziwa@sacids.org

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human cases with 6 (17.1%) recorded deaths (Makundi *et al.,* 2008), and with more recent outbreaks occurring in the same focus in 2010 (allAfrica.com, 2010). Repeated outbreaks of plague in Mbulu district strongly suggest that the focus is active and that wild animals especially rodents of the class Muridae (rats and mice), and some other types of small mammals are probably responsible for maintaining the bacterium between outbreaks. Indeed earlier studies in the district revealed that several species of these animals were seropositive for the disease (Kilonzo, 1992; Makundi *et al.,* 2008; Kilonzo & Mtoi, 1983). Elsewhere other types of small mammals including rabbits, marmots and chipmunks also maintain the plague organism in the wilderness (Gage & Kosoy, 2005).

In Mbulu and Karatu districts of Tanzania, available data on potential mammalian reservoirs of plague is based on serological studies using Enzyme Linked Immunosorbent Assay (ELISA), Passive Haemaglutination tests and Rapid Dipstick for detection of specific antibodies to *Y. pestis* Fraction I antigen (Kilonzo *et al.,* 2005; 2006; Makundi *et al.,* 2008). However, positive serological information suggests either current or previous infection of the animals with *Y. pestis*. The need to confirm the presence of the organisms in these potential reservoirs during non-epidemic periods is therefore desirable since such information is important in the control of plague. In this study Polymerase Chain Reaction (PCR) was used to detect *Y. pestis* DNA in potential mammalian hosts/reservoirs in two plague districts by amplifying the plasminogen activator/coagulase (*pla*) gene. This gene which is located on plasmid pPCP1, is incorporated into most *Y. pestis* PCR assays, and in several studies it has been used as the prime or sole marker (Riehm*et al.,* 2011; Adjemian *et al.,* 2008). Our reasons for including *pla* in our assay are its occurrence in multiple copies, its absence from closely related *Yersinia* species, and its role in *Y. pestis* virulence (Loïez *et al.,* 2003; Tomaso *et al.,* 2003).

Materials and Methods

Study area

The study was carried out in Mbulu and Karatu districts in northern Tanzania. Mbulu is located between latitudes 3.8° and 4.5° S, and between longitudes 35° and 36° E. The altitude ranges from 1000-2400m above sea level. The district contains areas having semi-arid and sub-humid climate that receive annual rainfall of greater than 400mm and less than1200mm, respectively. The 2012 National Census showed a population of 320,279 people comprised of 161,548 men and 158,731 women with an average of 6 people per household. Crop and livestock production are the most important economic activities, employing more than 90% of the total labour force (Ngowi *et al.,* 2010).

Karatu is located between latitudes 3° 10′ and 4° 00′S and at longitude 34° 47′E. According to the 2012 National Census the district had a population of 230,166 people comprised of 117,769 men and 112,397 women with an average of 5 people per household. The district has three physical-geographic zones namely upland, midland and lowland, with altitude ranging from 1000 to 1900m above sea level. Rainfall in the district is bimodal whereby short rains fall between October and December and long rains between March and June. Rainfall ranges from less than 400mm in the Eyasi basin to over 1000mm in the highlands with rainfall zones classified as semi-arid (300-700mm/year) and sub-humid (700-1200mm), (Karatu District Council, unpubl).

In Mbulu district, five villages, namely, Arri, Mongahay, Hayeseng, Boboa and Mangisa were involved, while in Karatu, the study was carried out in Slahhamo village. Selection of the villages for the study was based on recent reports of plague outbreaks and informed consent of villagers. The selection was also based on lists of households provided by village leaders and special consideration was given to households which had plague victims during the outbreaks.

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Specimen collection

Live trapping of wild, peridomestic and commensal rodents, as well as wild small carnivores was carried out from January to August 2012 in Mbulu and from late October to early November 2012 in Karatu. Ten randomly selected households in each village were involved. Sherman and locally made box traps baited with peanut butter were used for catching wild, peridomestic and domestic rodents. On the other hand, locally made wire cage traps baited with pieces of meat, ripe bananas and tomatoes were used for catching giant rodents and wild small carnivores. Trapping was simultaneously carried out in two villages for three nights consecutively, using 100 Sherman and 8 wire cage traps in the forest and peridomestic areas as well as 25 box traps in the selected houses in each village. Security of traps and proven activities of such animals were considered.

Traps were usually set in the afternoon and inspected in the morning and those with captures were taken to a central processing location, where each animal was carefully transferred to a cloth bag and then to a screw-capped museum jar/bucket containing pieces of cotton wool soaked in anaesthetic ether in order to anaesthetize the animal and collect its ectoparasites. The animals were then identified, sexed and autopsied after removing their ectoparasites. Sections of liver, spleen, heart and lungs were collected and preserved in a screw-capped micro-tube containing absolute ethanol and appropriately labeled for laboratory analysis.

Laboratory analysis

Approximately 10mg of material was cut from each of the tissues stored in absolute ethanol and placed in a microcentrifuge tube, in which 95µl water, 95µl 2X digestion buffer and 10µl proteinase K were added. The contents were mixed and incubated at 55^{o}C for 3hours. A total of 700µl of genomic lysis buffer was added and followed by vortexing and centrifugation at 10,000*xg* for one minute. The supernatant was transferred to a Zymo-Spin^{TM} IIC column in a collection tube and similarly centrifuged. Thereafter 200µl of DNA pre-wash buffer was added to the spin column in a new collection tube and similarly centrifuged. Then 400µl of g-DNA wash buffer was added to a spin column and similarly centrifuged. Finally, the spin column was transferred to a clean microcentrifuge tube and 100µl of DNA elution buffer was added and incubated at room temperature for five minutes and then centrifuged at 17,000*xg* for 30 seconds. The eluted DNA was finally stored at -20^{o}C until the time for assay.

DNA extraction and detection of Y. pestis

DNA was extracted using the DNA extraction kit (ZR genomic DNA^{TM}-Tissue MiniPrep Catalog No. D3051) following the manufacturer's instructions (Zymo Research Irvine, CA, USA). Detection of *Y. pestis* was accomplished using the conventional PCR technique. The *Y. pestis* specific primers (Hinnebusch & Schwan, 1993) were used to target the plasminogen activator (*pla*) gene of *Y. pestis* virulence plasmid pCP1. *Yp pla1*: (5'- ATC TTACTT 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 of the *pla* locus sequence, respectively. PCR was performed by using a PCR machine (GeneAmp PCR System 9700) in a total reaction volume of 25µl comprising of 0.125µl ExTaq, 2.5µl 10XPCR buffer, 1.5µl MgCl2, 2.0µl dNTP, 100µM of Forward and Reverse primers each, 14.875µl PCR water and 2.0µl of the Template DNA. Briefly the PCR test was accomplished as follows; initial denaturation at 94^{o}C for 1min, subsequently 35 cycles of denaturation at 94^{o}C for 20s, annealing at 55^{o}C for 15s, elongation at 72^{o}C for 30s and final elongation at 72^{o}C for 5min. The system was then allowed to cool and held at 4^{o}C. The correct sizes of the PCR products/bands were then confirmed in agarose gel electrophoresis and the UV illuminator.

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Ethical considerations

Ethical clearance Ref. No. MU/PGS/SAEC/Vol. VI/195 was granted by the Institutional Review Board of the Muhimbili University of Health and Allied Sciences. Permission to conduct the study in Mbulu and Karatu districts was granted by the respective District Executive Directors.

Results

Species and number of small mammals captured

A total of 517 small mammals were captured; of which, 154 (29.8%), 148 (28.6%), 85 (16.4%), 63 (12.2%) and 43 (8.3%) were from Mongahay, Arri, Boboa, Hayeseng and Mangisa villages, respectively, in Mbulu district, whereas 24 (4.6%) were from Slahhamo village in Karatu district (Table 1). All the mammals (100%) captured in Karatu were rodents while 476 (96.6%), 8 (1.6%) and 9 (1.8%) captured from Mbulu were rodents, wild small carnivores and shrews, respectively. Of the 517 mammals caught, 52.4% were males and 47.6% were females, respectively.

Table 1: Species and number of small mammals captured in Mbulu and Karatu districts

	Mbulu					Karatu	Total	%
Species/ Village	**Mongahay**	**Arri**	**Hayeseng**	**Boboa**	**Mangisa**	**Slahhamo**		
Mastomys natalensis	56	41	17	15	17	6	152	29.4
Lophuromys flavopunctatus	27	27	11	28	0	0	93	18.0
Rattus rattus	22	34	15	13	0	9	93	18.0
Praomys delectorum	30	21	12	17	0	0	80	15.5
Lemniscomys striatus	5	7	4	3	0	6	25	4.8
Aethomys sp.	0	0	0	3	15	0	18	3.5
Grammomys sp.	1	6	2	1	6	3	19	3.7
Croccidura sp.	4	3	0	2	0	0	9	1.7
Mus minutoides	2	3	1	1	1	0	8	1.5
Graphiurus murinus	6	1	0	0	0	0	7	1.4
Gerbilliscus sp.	0	0	0	0	1	0	1	0.2
Arvicanthis niloticus	0	0	0	0	1	0	1	0.2
Cricetomys gambianus	0	3	0	0	0	0	3	0.6
Herpestes sanguinea	0	2	1	1	0	0	4	0.8
Genetta genetta	1	0	0	1	2	0	4	0.8
Total	**154**	**148**	**63**	**85**	**43**	**24**	**517**	**100.0**
%	**29.8%**	**28.6%**	**12.2%**	**16.4%**	**8.3%**	**4.6%**		**100.0**

Laboratory observations from PCR tests

Two rodents (one *Mastomys natalensis* and one *Gerbilliscus sp.*) captured in Mbulu district and one *Mastomys natalensis* captured from Karatu district were found to be positive for *Y. pestis pla* gene (Table 2) where the expected 478bp segment of the gene was successfully amplified (Figure 1). The *Y. pestis* positive rodents in Mbulu district were captured from Mangisa village. Two (66.7%) of the three *Y. pestis* positive rodents were females with each one being captured from Mbulu and Karatu districts.

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Table 2: Species and number of small mammals tested for *Y. pestis pla* gene in Mbulu and Karatu districts

Species	Number tested	Plague positive	Percentage
Mastomys natalensis	152	2	1.3
Lophuromys flavopunctatus	93	0	0
Rattus rattus	93	0	0
Praomys delectorum	80	0	0
Lemniscomys striatus	25	0	0
Aethomys sp.	18	0	0
Grammomys sp.	19	0	0
Croccidura sp.	9	0	0
Mus minutoides	8	0	0
Graphiurus murinus	7	0	0
Gerbilliscus sp.	1	1	100
Arvicanthis niloticus	1	0	0
Cricetomys gambianus	3	0	0
Herpestes sanguinea	4	0	0
Genetta genetta	4	0	0
Total	**517**	**3**	**0.6**

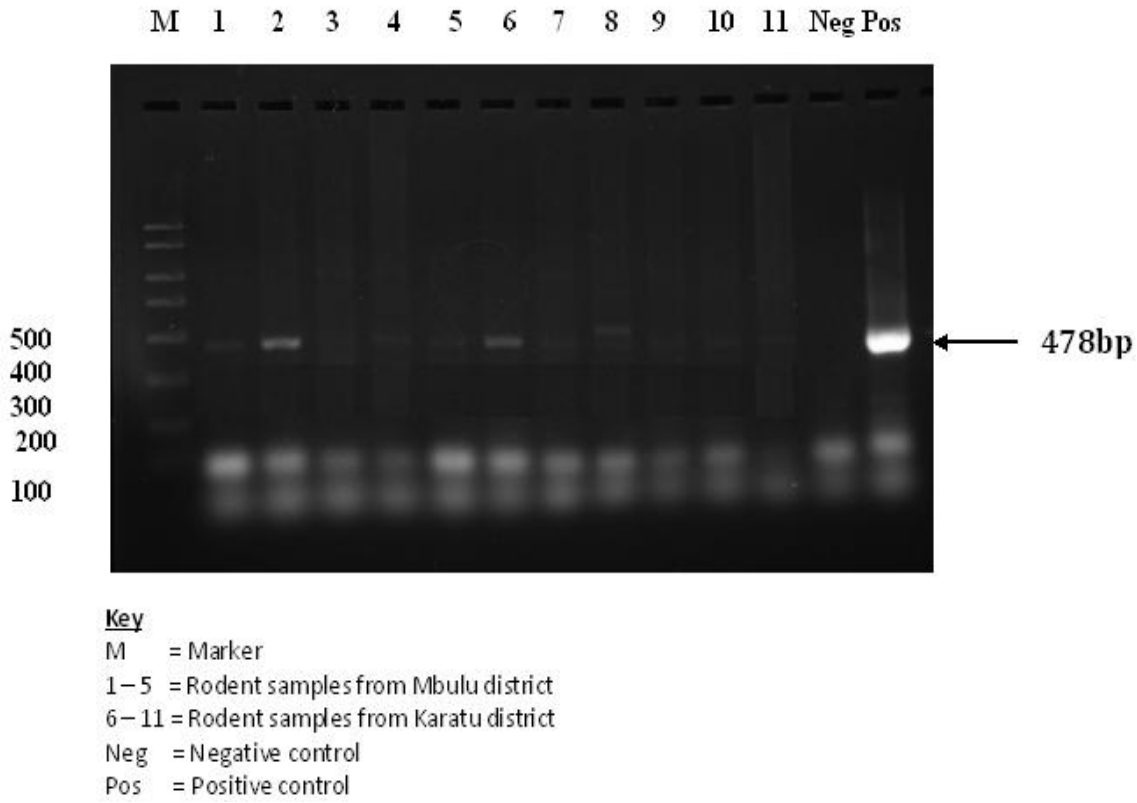

Figure 1: PCR detection of *Y. pestis pla* gene in rodents captured in Mbulu and Karatu districts

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Discussion

This study employed PCR to detect *Y. pestis* by targeting the plasmid encoded plasminogen activator (*pla*) gene, which is responsible for two activities of virulent *Y. pestis*. The virulence attributes of this gene include; plasminogen activation that result in lysis of fibrin clots, and weak coagulase activity. The gene is unique to and highly conserved in *Y. pestis* (Hinnebusch & Schwan, 1993). The PCR assay targeting this gene has been used by various researchers in similar studies (Stevenson *et al.,* 2003; Griffin *et al.,* 2010; Hang'ombe *et al.,* 2012). In this study *Y. pestis* was detected in three (0.6%) rodents (two *Mastomys natalensis* and one *Gerbilliscus sp.*); of these two were females; thus providing a direct evidence on the presence of *Y. pestis* in potential host mammals. These results further provide indicative evidence on the involvement of these animal species as reservoirs of the bacterium in the two districts and possibly in other plague endemic foci in Tanzania as previously described (Kilonzo & Mtoi, 1983; Kilonzo *et al.,* 2005; Makundi *et al.,* 2008). In a recent study by Haule *et al.* (2013) *Mastomys natalensis* was identified as one of the most abundant rodent species and that it was highly infested by fleas.

The fact that neither of the positive rodents was infested with fleas, that the positive animals had no clinical signs for plague and the disease was quiescent during the time of specimen collection suggest that the pathogen is circulating between various hosts in the areas and that these hosts are relatively resistant to the lethal effects of the pathogen and are presumably responsible for maintenance of the pathogen in the sylvatic cycle (Neal, 2004; Davis *et al.,* 2006). Indeed plague outbreaks are seasonal and they most often occur when the necessary conditions prevail and are primarily effected via the bites of infective fleas (Eisen *et al.,* 2007; Hang'ombe *et al.,* 2012).

The overall infection rate (0.6%) obtained in this study was relatively low when compared with results from similar studies from other areas (Riehm *et al.,* 2011; Hang'ombe *et al.,* 2012). In their studies, Riehm and Hang'ombe independently, got overall infection rates of 5.3% and 6.0%, respectively. Such differences in infection rates between our and previous similar studies could be attributable, at partly, by the fact that we conducted our study during the period of disease quiescence and possibly also due to the geographical-related differences and seasonality. In fact plague transmission in Tanzania and elsewhere is usually seasonal and in some foci, outbreaks normally occur in November to March and peaks are observed between December and February with strong inter-annual variations (WHO, 2006; Hang'ombe *et al.,* 2012). On the other hand, according to our findings the infection rate was relatively higher in female rodents (66.7%) than in males. Such findings could have an implication on the disease dynamics due to the fact that, when a female rodent bears offspring she lives together with the later in the burrow/nest hence they could become infested with *Y. pestis* infected fleas. If that happens and when this new generation grows up and start their lives they become modes of plague pathogen reservoirs and can transfer infected fleas from one area to another.

On the basis of these results we conclude that *Y. pestis* is circulating among others in *Mastomys natalensis* and *Gerbilliscus sp*. in Mbulu and Karatu districts; that the two rodent species are likely to be responsible for maintaining the pathogen during periods of no disease outbreak. Presence of the pathogen during the period of disease quiescence implies that continuing outbreaks should be expected whenever conditions become favorable and hence necessary preventive measures and emergency preparedness plans should be put in place. We noted that female rodents have a higher infection rate, a fact that may be taken into consideration when prioritizing infection control measures.

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