MOLECULAR SURVEILLANCE OF *YERSINIA PESTIS* DNA FROM THE FLEAS AND RODENTS OF LUSHOTO DISTRICT OF TANZANIA

BY

BENEZETH LUTEGE

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University of Zambia

School of Veterinary Medicine

Department of Para-clinical Studies

Lusaka

DECLARATION

I, **Benezeth Lutege** do hereby declare to Senate of the University of Zambia, that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution**.**

Signature: _____ Date: _____

CERTIFICATE OF APPROVAL

This dissertation submitted by BENEZETH LUTEGE has been approved as fulfillment for the award of the degree of MASTERS OF SCIENCE IN ONE HEALTH ANALYTICAL EPIDEMIOLOGY at the University of Zambia.

Dr. Bernard M. Hang'ombe	…….	…….....
Supervisor	Signature	Date
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Examiner	Signature	Date
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Chairperson	Signature	Date
(Board of Examiner)		

ABSTRACT

Plague is a zoonotic disease, endemic throughout the world except Australia and Antarctica. The disease is highly infectious in humans. The causative agent is a gram-negative bacterium, *Yersinia pestis* (*Y. pestis*) which, primarily infects a wide range of rodents and is transmitted via flea vectors. The disease persists in many parts of the world with 90% of the plague cases being reported to the World Health Organization each year, come from Africa where public health and living conditions are poor. In Tanzania, plague has been endemic for many years in areas of Lushoto, Mbulu and Karatu districts. In Lushoto the disease has been quiescent for 10 years since 2003, when annual outbreaks were reported from 1980. In order to understand disease dynamics in the hosts and vectors of Lushoto district, this study was initiated. The study was designed to detect *Y. pestis* DNA in Lushoto district. In some areas of Mbulu and Karatu districts surveillance works revealed the presence of *Y. pestis* DNA despite the absence of plague outbreaks in the human population. This study was conducted in Lushoto district of Tanzania and involved four villages which had plague outbreaks previously. Live trapping of wild and commensal rodents was done after which rodent tissues and their fleas were collected for DNA extraction. The collected rodents and fleas were identified to genus and species levels. The extracted DNA was subjected to PCR for detection of *Y. pestis* DNA using primers targeting *Y. pestis* plasminogen activator gene. A total of 112 rodent tissues and 253 fleas were collected from Gologolo, Mavumo, Viti and Manolo villages in Lushoto district. There were nine species of rodents captured which are *Mastomys* (33%), *Rattus rattus*(25.9%), *Praomys* (14.3%), *Lophuromy*s (14.3%), *Grammomy*s (8%), *Beamys* (1.8%), *Arvicanths* (0.9%), *Croccidura* (1.8%) and *Mus* (0.9%), while five flea species were collected and these were *Xenopsylla* spp (39.5%), *Ceratophylla* (*Dinopsyllus*) spp (22.5%), *Ctenocephalides* spp (26.1%), *Leptopsylla* spp (6.3%) and *Echidnophaga* spp (5.5%). At the time of this study, no evidence of *Yersinia pestis* DNA was found neither in fleas nor in rodents, suggesting a quiescent period. During this study two rodents species (*Mastomys* and *Rattus*) were identified which have been observed to be plague bacteria reservoirs in other endemic areas. Also the oriental flea (*Xenopsylla cheopis*) which is the efficient vector in the propagation of *Y. pestis* was identified. Another flea of significance *Dinopsyllus spp* was observed.These findings are significant as they put Lushoto district in an re-emergence alert to encounter an outbreak in future due to the presence of important host reservoirs and the effective vectors for *Y. pestis*.

DEDICATIONS

This work is dedicated to my beloved wife Angela Gladstone Samanya Lutege and My Son Daniel Benezeth Lutege for their heartfelt love and care during my school time. I also dedicate this work to my Parents Mr. Bathromew Malinda Lutege and Mrs. Modester Nyeru Malinda Lutege for their parental guidance this far.

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LIST OF ABBREVVIATIONS

bp	Base Pair
caf1	Capsule antigen protein 1
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CSF	Cerebrospinal Fluid
DNA	Deoxyribose Nucleic Acid
F1	Fraction 1(One)
kbps	Kilo Base Pair
PCR	Polymerase Chain Reaction
pFra	Plasmid Fraction
pPCP1 or *pPst*	Pesticin Plasmid
pYV	Virulence plasmid
PII	Percentage incidence index
SFI	Specific Flea Index
spp	Species
WHO	World Health Organization
Yops	*Yersinia* Outer Membrane Protein
Yp-pla	*Yersinia Pestis* Plasminogen Activator Gene
Ysc	*Yersinia* Secretion

CHAPTER ONE

INTRODUCTION

Plague is a zoonotic disease, endemic throughout the world except Australia and Antarctica. The disease is highly infectious in humans. The causative agent is a gram-negative bacterium, *Yersinia pestis* (*Y. pestis*), which primarily infects a wide range of rodents and is transmitted via flea vectors, (Patrick *et al*., 2006). The disease persists in many parts of the world with 90% of the plague cases being reported to the World Health Organization each year coming from Africa where public health and living conditions are poor (WHO, 2000). The natural foci of plague are spread worldwide where plague is mainly maintained in the rodent and flea vector reservoirs. The disease is a public health threat in many parts of the world (Figure 1), especially in sub-Saharan Africa where many countries harbor endemic foci (WHO, 2006).

The disease can cause case fatality rates of 50 to 60% if left untreated. During the past decades however, most human cases were reported from East Africa and Madagascar (WHO, 2006). It is difficult to have an accurate estimate of plague incidence since several countries do not properly report their cases or have no laboratory skills for diagnosis. However, because of the acuteness and severity of the disease, clinically suspected cases are often treated without taking biological samples for bacteriological investigations. Plague remains the oldest infection in human history and was responsible for many deaths in the three great pandemic in Europe and Asia (Denis *et al*., 1999; WHO, 2000). The mode of transmission is mainly through a bite by an infective flea that is harboured by rodent reservoirs or from person-to-person through aerosols in case of

pneumonic form and by contact with infective fluids of infected animal or human (Lwihula and Kilonzo, 1996; Denis *et al.,* 1999).

The oriental rat flea *Xenopsylla cheopis* (*X. cheopis*) and the human flea *Pulex irritans* are thought to be important arthropod vectors in transmitting plague to humans (Laudisoit, 2007). *Xenopsylla cheopis* is an efficient vector because of its proventriculus, which creates a conducive location for growth of *Y. pestis*. The flea becomes blocked with *Y. pestis* and then it is unable to swallow a full blood meal (Butler, 2005; Hinnebusch, 2002). In an attempt by the flea to dislodge the blockage, the flea infects new mammalian hosts. There are other flea species implicated as primary vectors, but these may clear *Y. pestis* more quickly such that only excreta of the flea or the crushing of its body may infect the host in contact with the arthropod (Galdan *et al*., 2010; Stevenson *et al*, 2003).

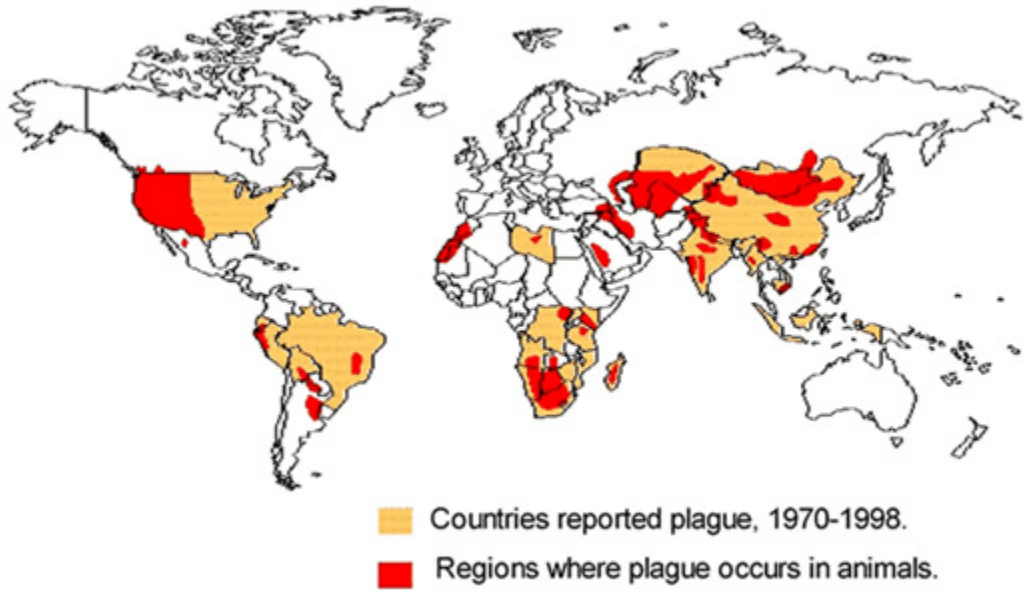

Figure 1: Global Distribution of Plague (CDC, 1998)

The rodents that are susceptible to *Y. pestis* infection include the roof rat (*Rattus rattus*), the multimammate mouse (*Mastomys natalensis*), the Nile rat (*Arvicanthis niloticu)*, gerbils (*Tatera* spp), groove-toothed rats (*Otomys* spp), and the striped grass mouse (*Lemniscomys striatus)*. The rodents are often infested with flea species that are capable of transmitting the plague bacteria belong to the following; *X. cheopis*, *X. brasiliensis, Dinopsyllus lypusus, Ctenophthalmus cabirus* and occasionally *Ctenocephalides felis* (Gratz *et al*., 1999).

In Tanzania, human plague is an important public health problem since pre-colonial times, and was first documented in 1886 in Iringa region. Afterwards, several foci were identified in different parts of the country, but most of them have remained dormant for many years except for the Lushoto and Karatu Districts (Kilonzo *et al*., 1997). In this study, Lushoto district was considered as it had been involved in previous plague outbreak which was lastly on 2003. Furthermore, the need to determine the flea vector efficiency in plague transmission is very important to understand how plague is maintained in natural ecosystems as well as how it can be transmitted to humans. The study also aimed at understanding and determining hosts and vectors that may be responsible in the maintenance of plague. The knowledge on the existence of the flea species that may be vectors of *Y. pestis* is important in the establishment of a better understanding and of control program initiatives in Lushoto district.

1.1 Study Justification

Plague is an emerging and re-emerging neglected disease of poor communities in which poverty, and health service delivery is rampant and disproportionally offered, respectively (WHO, 2000). According to the Inter-regional meeting on Prevention and Control of Plague in Antananarivo,

Madagascar, 7–11 April 2006 (Anonymous, 2006) “the most heavily affected African countries are the Democratic Republic of Congo, Madagascar, Mozambique, Uganda and the United Republic of Tanzania”. In these regions, clinicians may be unfamiliar with plague leading to risk of misdiagnosis. Specific diagnostic tools are often not readily accessible in remote areas, where outbreaks are reported.

In Tanzania for the period commencing 1953 to 2003, a total of 8956 plague cases with 731 (8.2%) deaths, were reported from ten districts in the country (Kilonzo, 2003). Since 1980, however, only four districts (Lushoto, Singida, Karatu and Mbulu) have experienced outbreaks of the disease. In the four districts 2898 cases with 646 deaths (7.8%) were reported (Kilonzo, 2003). In Lushoto district, the outbreaks started from 1980 up to 2003 where 7907 cases with 640 deaths were reported (Table1). This study is therefore timely to ascertain the presence of plague in the suspected rodent reservoirs and vectors.

Table 1: Reported Plague cases in Lushoto from 1980 to 2003

Year	Recorded/affected villages	Suspected/reported cases	Number of reported deaths	Percentage Death
1980	2	49	11	22.4
1981	1	9	6	66.7
1982	9	76	18	23.7
1983	2	569	49	8.6
1984	11	603	41	6.8
1985	18	129	22	17.1
1986	23	360	57	15.8
1987	32	470	57	12.1
1988	34	452	13	2.9
1989	9	29	5	17.2
1990	28	459	58	12.6
1991	39	1203	68	5.7
1992	2	16	2	12.5
1993	3	18	0	0.0
1994	19	444	50	11.3
1995	14	831	74	8.9
1996	33	826	59	7.1
1997	30	499	30	6.0
1998	13	286	3	1.0
1999	15	364	11	3.0
2000	11	76	2	2.6
2001	1	4	0	0.0
2002	9	97	3	3.1
2003	5	38	1	2.6
TOTAL	48	7907	640	8.1

All these outbreaks of the disease have been recorded in the rural communities where poor standard of living and health system delivery are common. In order to enhance sustainable

development in these communities and improve the living standards of the people there is need to establish systems and protocols to verify the presence of the plague foci by using fleas which are the main vectors in humans. In Tanzania, *X. brasiliensis*, *X. cheopis* and *D. lypusus* are considered to be the most important vectors (Kilonzo *et al*., 1991; 1997).

However, the relative roles of each species of flea in the epidemiology of the disease in the country remain to be established. In our study fleas were targeted as they are easier and safer to handle than mammalian tissue (Hinnebusch and Schwan, 1993; 1998). There has been no such study carried out in Lushoto district, to verify whether the bacterium still interacts with animals which could be possible reservoirs. Therefore, the data collected and results obtained from this study will provide information that will contribute to disease management and preparedness for the future outbreaks.

1.2 Study objectives

1.2.1 General objective

To detect the presence of *Yersinia pestis* in fleas and rodents in Lushoto district of Tanzania.

1.2.2 Specific objectives

To achieve the aim of this study three specific objectives were designed and outlined as follows:

1. To determine the presence of *Yersinia pestis* DNA in fleas and rodents.

2. To determine and identify Rodent species that may be possible reservoirs of *Yersinia pestis* in Lushoto District.

3. To determine and identify flea species that may be possible reservoirs of *Yersinia pestis* in Lushoto District.

CHAPTER TWO

LITERATURE REVIEW

2.1 Characteristics of *Yersinia pestis* (*Y. pestis*)

Yersinia pestis is an aerobic, nonmotile, gram-negative bacillus belonging to the family *Enterobacteriaceae* (Josko, 2004). The genus *Yersinia* consists of 11 species, of which three are human pathogens which include *Y. pestis, Y. pseudotuberulosis* and *Y. enterocolitica* (Perry and Fetherston, 1997). *Yersinia pseudotuberculosis* and *Y. enterocolitica* are transmitted by fecal-oral routes and cause intestinal symptoms of moderate intensity rarely causing death (Gage, 1998; Stenseth *et al*., 2008). In case of *Y. pestis*, it survives only in mammals and anthropods such as fleas and is generally not found living free in the environment, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are viable for long periods of time in the soil and water (Hinnebusch, 1997).

The genome of *Y. pestis* has been decoded, showing that the organism evolved from *Y. pseudotuberculosis* 1,500 to 20,000 years ago (Achtman *et al*., 1999). The transmission of *Y. pestis* by fleabite is a recent evolutionary adaptation that distinguishes it from *Y. pseudotuberculosis* (Hinnebusch, 2005). The close genetic relationship between *Y. pestis* and *Y. pseudotuberculosis* indicates that just a few discrete changes were sufficient to give rise to flea-borne transmission and the *Y. pestis-Y. pseudotuberculosis* species complex provides an interesting case study in the evolution of arthropod-borne transmission (Hinnebusch, 2005).

2.2 Analysis of *Y. pestis* biovars

Yersinia pestis has been historically divided into three biovars: *antiqua*, *mediaevalis* and *orientalis*. This classification is based on the ability to ferment glycerol and arabinose and to reduce nitrate. *Yersinia pestis* strains can be assigned to one of three biovars: *antiqua* (glycerol positive, arabinose positive, and nitrate positive), *mediaevalis* (glycerol positive, arabinose positive, and nitrate negative), *orientalis* which can produce the following results glycerol negative, arabinose positive, and nitrate positive (Zhou *et al.*, 2004).

2.3 Plague Epidemiology

Yersinia pestis can be found circulating in rodent populations on five continents with the exception of Australia and Antarctica, (Antolin *et al*., 2010). In the five continents, epidemics still occur frequently especially in developing countries where plague is endemic and people live in unsanitary, commensal rat-infested environments. Formerly, outbreaks involving hundreds of human plague cases occurred in at least 14 countries, usually as a result of exposures to infectious rat fleas (Gage and Kosoy, 2005). In the past 20 years, the World Health Organization has reported 1,000 to 5,000 human cases of plague and 100 to 200 deaths per year in the world (Stenseth *et al*., 2008). However, because of poor diagnostic facilities and under-reporting, the number of cases is almost certainly much higher. Over the years there has been a major shift in cases from Asia to Africa, with more than 90% of all cases and deaths in the last five years occurring in Madagascar, Tanzania, Mozambique, Malawi, Uganda, and Democratic Republic of the Congo (DRC). Most are cases of bubonic plague contracted through contact with infected rodents and fleas (WHO, 2006).

Plague cannot be eradicated, since it is widespread in wildlife rodent reservoirs. Hence, there is a critical need to understand how human risks are affected by the dynamics of these wildlife reservoirs. For example, the likelihood of a plague outbreak in Northern American and Central Asian rodents, and the resulting risk to humans, is known to be affected by climate (Parmenter *et al.,* 1999; Enscore *et al.,* 2002).

Recent analysis of data from Kazakhstan (Stenseth *et al*., 2006) shows that warmer springs and wetter summers increase the prevalence of plague in its main host, the great gerbil. Such environmental conditions also seem to have prevailed during the emergence of the second and third Pandemic (Kausrud *et al*., 2007; Treydte *et al*., 2006). These conditions might become more common in the future according to Intergovernmental Panel on Climate Change (Anonymous, 2001). Although the number of human plague cases is relatively low, it would be a mistake to overlook its threat to humanity, because of the disease's inherent communicability; rapid spread clinical course, and high mortality if left untreated. Outbreaks are usually tackled with a fire-fighting approach. Teams move into an infected area to kill fleas with insecticides, treat human cases and give chemoprophylaxis to exposed people. Many experts have argued that this crisis management approach is insufficient as the outbreak is likely to be on the wane by the time action is taken. Informed pre-emptive decision about plague management and prevention before outbreak occurs would certainly be more sustainable and cost-beneficial.

2.3.1 Life Cycle and Transmission of *Yersinia pestis*

Plague is a zoonosis, or an infectious disease that can be transmitted between humans and other animals. The natural reservoirs for the infectious agent, *Y. pestis*, are rodents and their fleas.

Humans play no role in the long-term survival of *Y. pestis* (Butler, 1983; Perry and Fetherston, 1997). Transmission between rodents is usually achieved by their associated fleas. Infection can also occur by direct contact, ingestion, and inhalation, but these routes do not normally play a role in the maintenance of *Y. pestis* in rodent populations. Fleas acquire *Y. pestis* from an infected blood meal and the maintenance of plague in nature is entirely dependent on cyclic transmission between fleas and mammals. The major known plague life cycle is from the classic vector for plague, the oriental rat flea (*Xenopsylla cheopis*), considered the most efficient vector for plague for almost a century (Eisen *et al*., 2007). *Xenopsylla cheopis* will typically ingest from 0.03 to 0.5µl of blood from its host (Perry and Fetherston, 1997). *Yersinia pestis* is cleared from some fleas, but multiplies in the midgut (stomach) of others. The digestive tract is made up of the foregut, which includes the pharynx, esophagus, and proventriculus, the midgut, or stomach of the flea; and the hindgut.

At the junction of the foregut and midgut lies a valve-like structure, the proventriculus (Vadyvaloo *et al*., 2007). The proventriculus is central to the mechanism of classic flea-borne transmission (Hinnebusch, 2005). Two days after an infected blood meal, the stomach shows clusters of brown specks containing *Y. pestis*, which develop into dark brown masses. Between days three and nine after ingesting the infected blood meal, the bacterial masses may completely block the proventriculus, extend into the esophagus, and prevent ingested blood from reaching the stomach. The flea will then repeatedly attempt to feed, and blood sucked from the host distends the esophagus, mixes with the bacilli, and is regurgitated into the host when the feeding attempt is terminated, (Perry and Fetherston, 1997) and hence introduce the bacteria into the host. Two basic disease cycles have been associated with *Y. pestis.* These include urban and sylvatic

cycles. The urban cycle is a pattern in which the pathogen is perpetuated by the house rat and domestic fleas. This is the principal cause of the massive epidemics of plague, characterized by an accompanying epizootic of plague among the rodents, particularly the black rat that lives in close proximity to human habitations.

An epizootic occurs when there is a sufficiently dense population of susceptible domestic rats, a highly index of parasitism with fleas capable of transmitting disease, adequate climatic conditions and introduction of disease in the population (Eisen *et al.,* 2012). However, urban plague cycle primarily involve transmission of *Y. pestis* among human-associated rodents, their fleas, and people. The commensal black or roof rat, (*Rattus rattus*), the sewer rat, (*R. norvegicus*), and their fleas (especially the oriental rat flea, *X. cheopis*) have been the principal sources of epidemics of plague and its pandemic spread (Dennis and Gage, 2004). Domestic animals living in close association with humans, including cats and dogs, may play important roles in transmission to people in an urban cycle (Rust *et al*., 1971).

Even if canines are thought to be largely resistant to plague, some do develop illness (Orloski and Eidsen, 1995). Cats that are allowed to roam outdoors may also introduce fleas into the human environment. Cats are also very sensitive to *Y. pestis* infection, and may transmit bacteria directly to people via respiratory secretions or, less likely, via direct contact with blood of bacteremic cats (Gage *et al*., 2000). Sylvatic cycles of plague can be much more complex than the urban cycle. Mostly, *Y. pestis* circulates within populations of wild rodents and their fleas in enzootic (quiescent or maintenance) cycles and periodic outbreaks or epizootic cycles. For reasons that are often unknown, *Y. pestis* will enter a population of host species and multiply or rapidly spread the

bacterium over wide areas causing an epizootic. The sylvatic cycle (Figure 2) is a portion of the natural transmission cycle of the pathogen occurring in a natural environment affecting all possible hosts. It is the fraction of the pathogen population's lifespan spent cycling between wild animals and vectors.

Naturally, the flea is essential for the perpetuation of plague in the sylvatic cycle as it is the only vehicle of disseminating the pathogen from one host to the other (Dennis and Gage, 2004; Gage, 1998). It should generally be mentioned that plague is endemic in variety of wildlife rodent species. This pattern leads to an interaction between the sylvatic reservoir and people. Various other animal-to-human transmission pathways have been documented.

Human plague may be contracted from being bitten by the fleas of wildlife rodent species in rural settings (e.g., in the south-western United States (Gage *et al*., 1995; Levy and Gage, 1999) or of commensal rodents that move freely between villages and the forest habitats occupied by reservoir hosts (e.g., in Tanzania); rodents' movements have become more frequent as human activity has fragmented the forest such as hunting, heading cattle and wild fruit and fire wood gathering (Kaoneka and Solberg, 1994). Another route is through eating infected animals such as guinea pigs in Peru and Ecuador (Gabastou *et al*., 2000; Ruiz, 2001) or camels that contract the disease from rodent fleas in Central Asia and the Middle East (Fedorov, 1960; Bin Saeed, 2005); or handling cats infected through the consumption of plague-infected rodents in Africa or the United States (Isaacson, 1973; Gage *et al*., 2000). Human-to-human transmission also occurs, either directly through respiratory droplets or indirectly via flea bites.

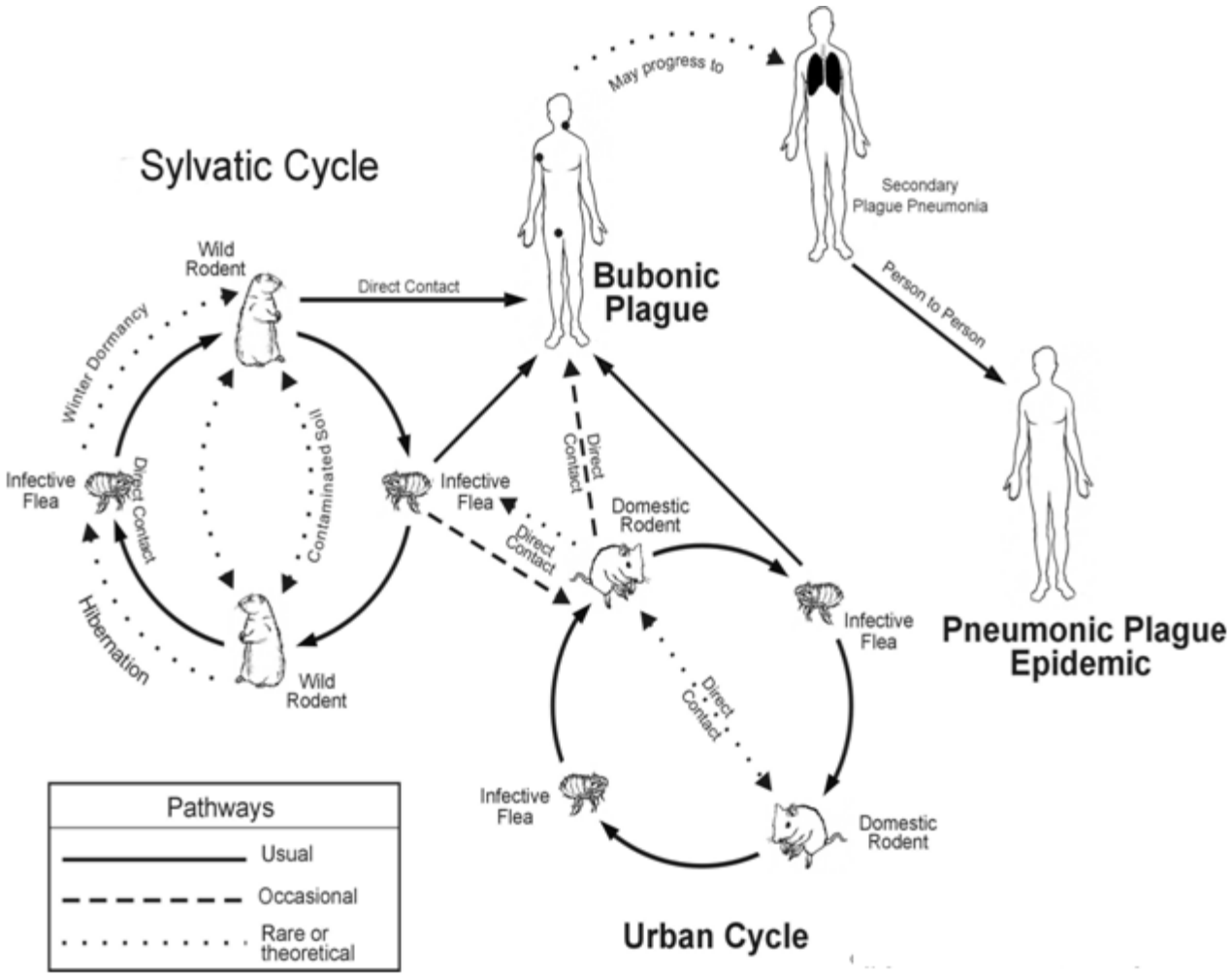

Figure 2: Maintenance cycle of *Yersinia pestis* in nature (Murray, 2002)

2.3.2 Reservoirs, Hosts and Vectors

In the rodent-flea-rodent plague infection cycle, rodent predators such as dogs, coyotes, cats and ferrets occasionally intrude and extend the radius of plague epizootic foci by mechanically carrying infected rodent fleas on their bodies or by transporting the infected carcasses from place to place. These predators typically acquire the infection through ingestion of infected rodents but occasionally through the bite of infected rodent fleas (Thomas and Hughes, 1992). In Tanzania,

X. brasiliensis, *X. cheopis* and *D. lypusus* are considered to be the most important vectors (Kilonzo and Msangi *et al*., 1991; 1997).

However, the relative roles of each species of flea in the epidemiology of the disease in the country remain to be established. Furthermore, this consideration is based on the facts that the fleas are most abundant on rodent hosts including those found positive with plague, that the first two species are ubiquitous feeders on both animals and humans and that elsewhere *Y. pestis* has been isolated from such fleas. Other species including *Pulex irritans* and *Ctenocephalides felis* have also been reported to be potential vectors of the disease in the country on the basis of observations elsewhere and their abundances in plague endemic areas (Kilonzo, 1984; Laudisoit *et al*., 2007).

2.4 Molecular Pathogenesis of Plague

Yersinia pestis encodes two antigenic molecules: Fraction 1 (F1) capsular antigen, and VW antigen. Both of these molecules are needed for pathogenicity, and are not expressed at temperatures lower than 37°C. This requirement is the main reason why *Yersinia* is not virulent in fleas, since their body temperature normally levels around 25°C. When placed at 37^{o}C, low-Ca^{2+} concentrations and in a nutrient rich environment, a plasmid (70-kbps called *pYV* or *pCD1*) encodes the *Yop* (*Yersinia* Outer membrane Proteins) virulon and a type III secretion apparatus called *Ysc* or *Yersinia* SeCretion.

There are 29 different *Ysc* proteins which assemble to form a pore in the inner and outer membrane of the bacteria. Once the bacterium makes contact with a eukaryotic cell, certain

translocator *Yops* will form a pore in the eukaryotic cell. Effector *Yops* then go across the channel formed through the bacterial and eukaryotic membranes and obtain access to the eukaryotic cell's cytoplasm. There are at least six different effector *Yops* which when transported into the eukaryotic cells inhibit phagocytosis, inflammation, and induce apoptosis of macrophages. This plasmid also encodes the V antigen that appears to also be involved in the type III secretion apparatus. The V antigen also appears to have immunosuppressive effects on the host's immune system (Simonet *et al.,* 1996).

Yersinia pestis has two plasmids not shared by other species of *Yersinia*, the 96.2kb plasmid *pFra* and the 9.5kb pesticin plasmid or *pPCP1*, also called *pPst* which are the plasminogen activators and are important for subcutaneous virulence (Ferber and Brubaker, 1981; Perry and Fetherston, 1997). The plasmid *pFra* also called *pMT1* (Tox plasmid) has been suggested to contribute to the transmission of the plague bacillus by the flea (Hinnebusch *et al*., 1998), and the plasmid *pPCP1* has been shown to contribute to the invasive character of the plague. The plasmid *pPCP1* encodes at least three proteins a bacteriocin termed pesticin, a protein conferring immunity to pesticin and an outer membrane protease termed *pla* (Sodeinde and Goguen, 1988). Furthermore, the genes in *pPCP1* have the virulence-enhancing properties that are encoded by the *pla* gene and the *pla* is essential for the invasive character of plague.

The *pla* of *Y. pestis* is a 292-amino-acid outer membrane protease and the exact mechanisms of how *pla* enhances invasiveness of *Y. pestis* remain to be elucidated, but *pla* is an efficient proteolytic activator of plasminogen (Sodeinde and Goguen, 1989), and analysis of the virulence of wild-type and *pla* knock-out *Y. pestis* in $plg^{+/+}$ and $plg^{-/-}$ mice have stressed the importance of

plasminogen activation in the virulence function of *pla* (Sodeinde *et al*., 1992; Goguen *et al*., 2000). The *pla* cleaves the C3 component of complement and thus suppresses migration of inflammatory cells to the infection site, which may reduce phagocytosis of the invading bacterium (Sodeinde *et al*., 1992). The *pla* also has weak coagulase activity, which is detectable only with rabbit plasma (Jawetz and Meyer, 1944). The *pla* contributes to the adhesion of eukaryotic cells (Kienle *et al*., 1992) and promotes adherence of *Y. pestis* to laminin of human and murine basement membrane (Lähteenmäki *et al*., 1998).

Yersinia pestis degrades laminin and subepithelial extracellular matrix through its capacity to generate plasmin (Lähteenmäki *et al*., 1998), which may enhance the spread of the bacterium through tissue barriers. The *pla*-generated plasmin also degrades fibrin (Beesley *et al*., 1967), enhancing bacterial migration by preventing entrapment of bacteria in fibrin clots (Sodeinde *et al*., 1992).

However most of the organisms are phagocytosed and killed by the polymorphonuclear leukocytes in the human host. A few bacilli are taken up by tissue macrophages and the macrophages are unable to kill *Yersinia pestis* because they produce plasmid fraction (*pFra*or *pMT1*) which enhances virulence. It contains the genes for the capsular protein (fraction 1) and a murine toxin. This capsule is believed to enhances resistance to phagocytosis by monocytes and provide a protected environment for the organisms to synthesize their virulence factors. The organisms then kill the macrophage and are released into the extracellular environment, where they resist phagocytosis by releasing *YopH* and *YopE* (*Yersinia* outer membrane protein) by the polymorphs. The *Y. pestis* quickly spread to the draining lymph nodes, which become hot,

swollen, tender, and hemorrhagic. This gives rise to the characteristic black buboes responsible for the name of this disease.

Within hours of the initial flea bite, the infection spills out into the bloodstream, leading to involvement of the liver, spleen, and lungs. The patient develops a severe bacterial pneumonia, exhaling large numbers of viable organisms into the air during coughing fits, fifty to sixty percent of untreated patients will die if untreated. As the epidemic of bubonic plague develops (especially under conditions of severe overcrowding, malnutrition, and heavy flea infestation), it eventually shifts into a predominately pneumonic form, which is far more difficult to control and which has 100 percent mortality (Simonet *et al.,* 1996).

2.5 Management of Plague

2.5.1 Diagnosis

2.5.1.1 Direct microscopic examination

Under microscope a presumptive diagnosis can be made by identifying the characteristic organisms in sputum, bronchial and tracheal washings, blood, lymph node (bubo) aspirates, cerebrospinal fluid (CSF) or postmortem tissue samples. *Yersinia pestis* is a gram-negative, facultative intracellular coccobacillus with bipolar staining. Bipolar staining (closed safety-pin) rod is particularly evident when Wright-Giemsa or Wayson stains are used.

2.5.1.2 Serological tests

Plague serology have been based on the detection of antibody to whole cells and to the *Y. pestis*-specific Fraction antigen1 (Baker *et al.,* 1952; Chen and Meyer, 1954). The F1 envelope glycoprotein, sometimes referred to as the capsular antigen because of its location on the outer surface of the cell, is encoded by *caf1* gene complex located on the 110kb plasmid (Gaylov *et al.,* 1994), whereby a comparable study of the nucleotide sequence of the *caf1* shows that the gene is highly conserved and the envelope glycoprotein is consistently expressed (Chu, 2000). If a person or animal has been exposed to *Y. pestis*, a serum taken at the proper time after exposure will very likely contain anti-F1 antibodies. The presence of agglutination titer of 1:128 may be considered diagnostic for plague provided that the patient has not been previously exposed to *Y. pestis* by vaccination.

2.5.1.3 Molecular diagnosis

Molecular techniques are powerful tools that can be used to provide information about the etiological agent that cannot be obtained by traditional diagnostic methods. When standard microbiologic methods fail to yield a viable isolate, molecular-based tests may be the only means available to confirm the presence of *Y. pestis*. There are several molecular techniques which can be used but in this study the polymerase chain reaction (PCR) assays was used to detect nucleic acids as described by Hang'ombe *et al*., (2012b).

There are several primers which can be amplified by PCR in the molecular detection of *Yersinia pestis*. The primers include, chromosomal ferric iron uptake regulation (*fur*) gene. The PCR primers involved are *Ypfur1* (5'-GAAGTGTTGCAAAATCCTGCG-3') and *Ypfur*2 (5'-

AGTGACCGTATAAATACAGGC-3') corresponding to nucleotides 70-90 and 377-397, respectively, of the *fur* gene sequence reported by Staggs and Perry, (1992.) and yield a 328-basepair (bp) fragment in the PCR of *Y. pestis* DNA. Common to all of these species is the presence of a 70kb virulence plasmid that harbours a type III secretion system, and several secreted and translocated proteins called *Yersinia* outer proteins or *Yops* (Cornelis, 2002). The plasmid-encoded type III secretion system enables *Yersinia* to survive and proliferate extracellularly in host lymphatic tissues (Trülzsch *et al.*, 2004). Three of the *Yops – YopB*, *YopD* and *LcrV* are required for translocation of the others across the target-cell membrane (Viboud *et al.*, 2003). The hydrophobic *YopB* and *YopD* thus seem to be central for translocation of the effectors and for the formation of a channel in lipid membranes. The primer sequences are 5′-AAAAATGGCGGGGTGAGTT-3′ (forward) and 5′-AAAACTCGGCTCCTTTAGC-3′ reverse (Trülzsch *et al.*, 2004).

2.5.2 Clinical signs

Due to lack of diagnostic facilities in most health centers, clinical signs can be used to diagnose the disease. There are three predominant clinical manifestations of plague infection; bubonic, septicemic and pneumonic.

2.5.2.1 Bubonic Plague

Bubonic plague is the most common form of plague in all animals, and occurs in approximately 80% of human exposures (Gage, 1998). It is usually a result of a bite from an infected flea, and may also occur when plague-infected material enters an animal through a break in the skin. This form of plague is characterized by buboes, which are swollen lymph nodes that result from

infection and inflammation due to *Y. pestis*. Buboes are usually found in the groin, armpit, or neck area, and can range in size from a few millimeters to as large as a hen's egg (Gage, 1998). These buboes, which are often extremely tender and painful, usually develop in the lymph nodes, draining the initial site of infection. An inguinal bubo will likely appear in those people who are bitten on the leg by an infectious flea, whereas those who contract plague as a result of handling an infected animal are likely to develop an axillary bubo (in the armpit area) (Gage, 1998). In addition to swollen lymph nodes, a person infected with bubonic plague may experience mild to severe flu-like symptoms such as fever, headache, vomiting, nausea, chill, and exhaustion.

2.5.2.2 Septicemic Plague

Septicemic plague occurs when *Y. pestis* enters into and spreads through the body via the bloodstream causing signs and symptoms of gram-negative sepsis.

This is likely to occur in inadequately treated bubonic cases (secondary septicemic plague), but can also occur without prior evidence of lymphadenopathy (primary septicemic plague) (Sebbane *et al*., 2006).

The latter form can be dangerous because of the difficulties encountered in rapidly diagnosing human plague when there are no obvious buboes present. Septicemic plague can result in secondary plague pneumonia and occasionally meningitis, endophthalmitis (severe infections of the eye), and abscesses in the liver, spleen, kidneys, and lungs (Poland *et al*., 1994). Clinical signs of the septicemic form are similar to those associated with the bubonic form; however, they may become much more severe.

2.5.2.3 Pneumonic Plague

Pneumonic plague is the most dangerous form of plague. It is characterized by severe, life-threatening respiratory and systemic illness. Primary pneumonic plague is acquired through inhalation of infectious respiratory droplets containing *Y. pestis*. Secondary pneumonic plague occurs as a consequence of untreated bubonic or septicemic plague. Unlike bubonic and septicemic plague, pneumonic plague can potentially spread from person to person through infectious respiratory droplets (Gage, 1998; Poland and Dennis, 1999). Primary pneumonic exposure usually leads to clinical signs within 2 to 3 days. Symptoms of pneumonic plague can include high fever, chill, cough with bloody sputum and severe breathing difficulty. Mortality rates for pneumonic plague are very high and probably reach 100% for untreated cases (Poland and Barnes, 1979; Poland and Dennis, 1999).

2.5.3 Treatment

In the treatment of plague, Streptomycin is the most effective antibiotic against *Y. pestis* and the drug of choice particularly on the pneumonic form of plague, their efficacy is often limited if the symptoms have been present for more than 20 hours. Buboes are occasionally drained but usually resolve with antibiotic treatment. Generally *Yersinia pestis* is sensitive to easily available antibiotics, but in 1995 in Madagascar the Strain 17/95 isolate exhibited high-level resistance to eight antimicrobial agents, including not only those recommended for therapy (streptomycin, chloramphenicol and tetracycline) and prophylaxis (sulfonamide and tetracycline) of plague but also drugs that may have represented to classical therapy, such as ampicillin, kanamycin, and spectinomycin (Galimand *et al.*, 1997; Guiyoule *et al.*, 2011). This demonstration highlights the importance of antibiotic susceptibility tests of plague isolates from time to time. In addition to

that, Chloramphenicol is a suitable alternative to aminoglycosides in the treatment of bubonic or septicaemic plague and is the drug of choice for treatment of patients with *Y. pestis* invasion of tissue spaces into which other drugs pass poorly or not at all. Fatality rates for untreated bubonic plague cases are reported to be 50-60% (Poland *et al*., 1994; Levy and Gage, 1999; Eisen *et al*., 2007).

2.5.4. Prevention

Surveillance, education and environmental management are the cornerstones of plague prevention and control (Campbell and Dennis, 1995). Prevention measures should aim to educate the public regarding exposure sites, manifestations, diagnosis of disease, and what steps can be taken to protect individuals and their families (Poland and Barnes, 1979).

In endemic areas, rodents should be controlled around human homes, workplaces and recreational areas. Buildings should be rodent-proofed by providing with materials which may prevent rodents and their access to food sources. To prevent pets from serving as a link between wild animal hosts and humans, a good flea control program should be established for dogs and cats, and these animals should be kept from hunting or eating tissues from animals that may be infected.

Game meat, as well as tissues from domesticated animals that might be infected, should be cooked thoroughly. Die-offs of rodents should be reported and barrier precautions are necessary during examination and treatment, and suspected cases must be reported (CDC, 2006).

2.5.5 Re-emergence of Plague

The incidence of the disease has gradually decreased during the course of the third pandemic that started from Hong Kong in 1894. However, since the 1990's, human plague reappeared in countries where no cases had been reported for decades, and thus the plague is now categorized as a re-emerging disease (Schrag and Wiener, 1995). There are some remarkable human outbreaks which have occurred in recent years, the port of Mahajanga, Madagascar, in 1991 (Rasolomaharo *et al.*, 1995), where plague had disappeared from the coastal areas of Madagascar for over 60 years; it is the recent re-emerging focus with the highest number of human cases reported while at Beed and Surat in 1994 in India, a country that had not reported any human cases of plague since 1967 (Tikhomirov, 1999); this led to a massive human exodus from Surat. In Mozambique, after more than 15 years of silence the disease broke out in 1994 (Barreto *et al.*, 1995). This led to the spread of the epidemic to the neighboring countries of Zimbabwe and Malawi. At the end of 1996 in Zambia, after a period of more than 30 years of silence the disease resurfaced (WHO, 2000; 2001). The most surprising plague outbreak was in Oran, Algeria resurfaced in June 2003 after 50 years (WHO, 2003). During this outbreak a total of eleven confirmed and seven suspected cases of plague were reported from the same area (WHO, 2003).

Libya, a neighbouring country to the west of Egypt, experienced several plague outbreaks during the period from 1913 to 1920, the largest of which resulted in 1449 deaths in Benghazi in 1917. Other epidemics of lower amplitude occurred in 1972, 1976, and 1977. More cases of bubonic plague were diagnosed in 1984 in two locations; 25 km from Tobruk where plague foci had been noted between 1976 and 1977 (Anonymous, 1984). After an apparent absence for 25 years, plague cases recurred in 2009 near Tobruk. Environmental factors favorable for plague re-

emergence might exist in this area and lead to reactivation of organisms in other ancient foci (Cabanel *et al*., 2013). The city is very close to the border with Egypt, thus the danger of plague transmission from its natural foci in Libya to Egypt should be highlighted.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Lushoto District (Figure 3) is situated in Tanga region, in the West Usambara Mountains, a part of the Eastern Arc Mountains. With an elevation ranging from 900 to 2,250 m above sea level, Lushoto District (04°22′–05°08′S, 038°05′–038°38′E) covers a surface area of 3,500 km^2, of which 2,000 km^2 are arable land and 340 km^2 are forest reserve. Soils are mainly low-pH loams, rich in iron, manganese, and magnesium. Agriculture is the major economic activity, on which more than 90% of the population depends (Tenge, 2006; Lyamchai *et al.,* 1998). The temperate climate is characterized by a short rainy season during November–December and a longer one during March–May. The region is the most densely populated area in Tanzania, with an annual growth rate of 2.8% and 102 inhabitants per square kilometer. Inhabitants belong to one of the three major tribes: Wasambaa (80%), Wambugu (10%), and Wapare (5%); the remaining 5% are immigrants from diverse other regions (Vainio-Matilla, 2000). Most Lushoto residents (70%) keep livestock in close proximity with their houses, but cats and dogs are usually kept outside (Kilonzo *et al*., 1997).

The research was conducted in four villages of Gologolo, Mavumo, Viti and Manolo in Lushoto District. The villages were selected on the basis of their involvement in the past and recent outbreaks of the disease and co-cooperativeness of the village leaders and villagers. The houses

were randomly selected from a list of villages involved in the past and recent outbreaks and informed consent of house owners was sought prior to setting traps.

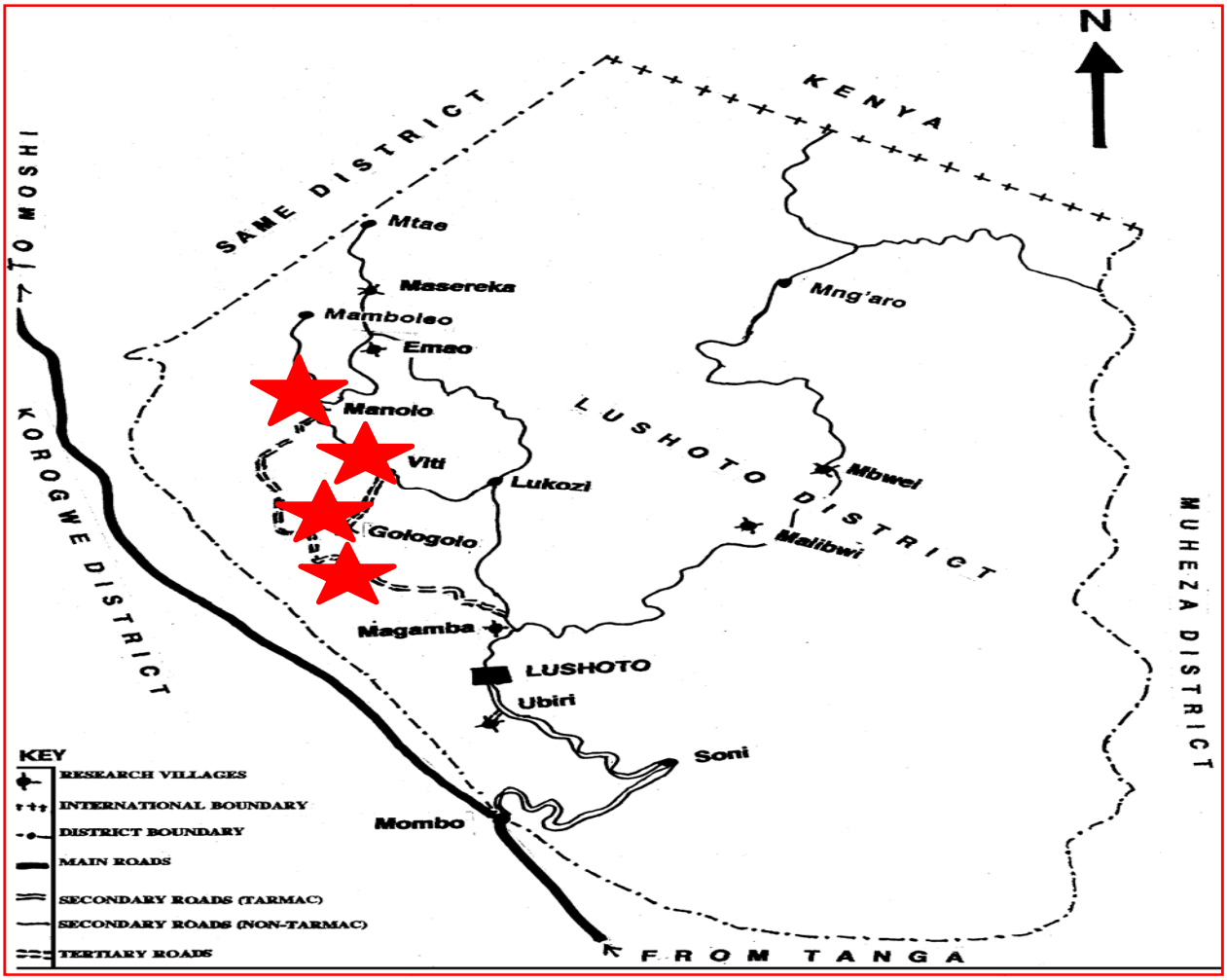

Figure 3: Map of the study area, indicating areas of sampling denoted by ★ in Lushoto district

3.1.1 Study design

A cross sectional study design was used in this study which involved visitation of the trapping site three days consecutively.

3.1.2 Sample size

The sample was estimated by using the formula described by Cochran (1963).

$n=Z^2 P (1-P)/d^2$

Where:

n = required sample size

Z = multiplier from normal distribution 95% CI (1.96)

P = estimated prevalence 10% rodents

(1-p) = the probability of having no infected rodents or fleas

d = desired precision (5%)

In this study, it was anticipated that the sample size will be 140 rodents and all ectoparasites would be collected.

3.1.3 Sampling technique

The sampling procedure was purely purposive because the villages were selected by being involved in the past outbreaks, and also the population of rodents and fleas are unknown.

3.2 Procedures

Live trapping of wild and commensal rodents was done by using Sherman traps baited with peanut butter. The total of 150 Sherman traps baited with peanut butter were used to trap rodents in the forest, forest edge in farms and nearby peridomestic areas. The process of trapping was accomplished in three consecutive nights as described by Kilonzo (1977).

An average of 110 samples (rodent's tissues, liver, spleen and lungs) and two *Croccidura spp* was aseptically collected while 253 fleas were collected from rodents. The rodents were transferred into a bag cloth wherein piece of cotton soaked in diethyl ether so as to anaesthetize both rodents and fleas for safe handling and collection.

Following application of diethyl ether, the insects were brushed on a white cloth for visibility and easy collection. The collected fleas were preserved in 70% ethanol till analysis. Thereafter, fleas were identified to genus level and placed in pools of one to10 individuals (corresponding to the same animal host and flea species) and then tested for the presence of *Y. pestis* DNA according to Hang'ombe *et al* (2012a). In this study other ectoparasite (mites and ticks) found on the body of the rodents were collected and similarly detected for the presence of *Yersinia pestis*. Rodents were aseptically dissected in a well-equipped mobile clinic so as to obtain tissues which are liver, spleen and lung. All tissues were stored in 70% alcohol until analysis.

3.2.1 Identification of fleas

Fleas were identified to genus level using a field manual on the basic procedures for collecting, processing and identification of common fleas by Kilonzo (1999). The preserved fleas were placed on a microscopic slide then physiological saline was added and covered by the microscopic slide cover and finally observed by the aid of the electronic microscope.

3.2.2 Identification of Rodents

Rodents were identified to specie level using the Guide to the identification and collection of New Zealand rodents, the information which was taken during identification were, Head-Body

Length, tail Length, right hind foot and right ear, habitat and color (fur on belly and fur on back) of the rodents (Twigg, 1975).

3.3 DNA extraction from Fleas

The ethanol preserved fleas were dried on sterile filter paper in a laminar biosafety hood and the fleas were placed in Eppendorf tubes with 100µl of brain-heart infusion broth (Oxoid, Hampshire, England). Therefore, the fleas sample were triturated with sterile pipette while the tubes were placed in ice packs, triturated samples were boiled at 95ºC for 10min then centrifugation was done for 10 seconds at 10,000*xg*, then 1µl was used as template for PCR. Negative control template was employed which are brain-heart infusion broth only, and fleas collected from non-endemic plague area. Thereafter PCR amplification was done specifically for fleas samples to detect *Y. pestis* DNA.

3.3.1 DNA extraction from Rodents tissues

The tissues were homogenized mechanically using tissue homogenizer up to 25mg of frozen tissue in 500µl of Genomic Lysis buffer and then centrifuged the lysate at top speed (10,000*xg*) for 5min. Insuring that the pellet debris were not disturbed the supernatant were transferred to a Zymo-Spin™ column in a collection Tube and centrifuged at 10,000*xg* for one minute. Then the collection tubes were discarded with the flow through. During this process Zymo-spin™ column were transferred to a new collection tube where 200µl of DNA Pre-Wash Buffer was added and centrifuged at 10,000*xg* for one minute, before 500µl of g-DNA Wash Buffer added to the spin column and centrifuged at 10,000*xg* for one minute. The process continued by transferring the spin column to a clean microcentrifuge tube, followed by addition of greater than or equal to 50µl

DNA Elution Buffer to the spin column. The mixture was incubated for two to five minutes at room temperature and then centrifuged at top speed for 30 seconds to elute the DNA. Finally, the eluted DNA was used immediately for molecular based applications.

3.3.2 Sample analysis

The extracted DNA samples from the rodents and fleas were analyzed using the PCR. This was accomplished using PCR technique as described by Hang'ombe *et al* (2012b). Briefly, Polymerase Chain Reaction amplification was performed for the detection of the *Y. pestis* plasminogen activator gene using primers *Yp pla1* (5'TGC TTT ATG ACG CAG AAA CAG G3') and *Yp pla2* (5'CTG TAG CTG TCC AAC TGA AAC G3'). The primers amplify a 344 bp region of the *Y. pestis* plasminogen gene. Polymerase Chain Reaction was done using the Phusion™ flash high fidelity PCR master mix (Finnzymes Oy, Finland).

The reactions was performed in a final volume of 10 µl containing consisting 5 µl Phusion flash PCR master mix, 0.5 µM of primer sets in 1 µl volume of each, 1 µl of template DNA and 2 µl of PCR water. The template DNA consisted of the rodent DNA and the flea DNA lysates. The Piko™ thermal cycler (Finnzymes Instruments Oy, Finland) was programmed at 95°C for 10 sec for initial denaturation, followed by 35 cycles consisting of 95°C for 1 sec, 58°C for 5 sec and 72°C for 15 sec. Final extension was given 72°C for 1 min. Specific *Y. pestis* detection would have been identified by the presence of a specific 344 bp DNA band on 1.5% agarose gel, stained with ethidium bromide and evaluated under UV transilluminator. The estimation of the sizes of PCR products was done according to the migration pattern of a 100-bp DNA ladder. In this

analysis, a positive control was used so as to standardize the protocol before analysis of the Lushoto collected samples from fleas and rodents.

CHAPTER FOUR

RESULTS

4.1 Rodents

A total of 112 rodents representing nine species were trapped from five different sites which are forest, forest edge, in farms, along water streams and peridomestic areas (Table 2). Most of the rodents trapped were in normal condition except one and two *Rattus rattus* trapped from Forest edge and peridomestic areas which had splenomegaly respectively, one *Mastomys* trapped from Farm and peridomestic areas each also had splenomegaly, while two *Rattus rattus* and one *Mastomys* trapped from peridomestic areas had liver abscesses.

Two species *Mastomys* (33%) and *Rattus rattus* (25.9%) were trapped in all sampled areas, followed by *Lophuromys* (14.3%), *Praomys* (14.3%) and *Grammomys* (8.0%) which were trapped in three villages except in Viti village, while *Beamys* (1.8%), *Mus* (0.9%) and *Arvicanthis* (0.9%) at Mavumo, *Croccidura* (1.8%) at Viti (Table 3). This study also observed that there was random mixing of rodent's species in the different sites where the rodents were trapped.

Table 2: Trapping areas and their Rodent's Species

Site of Trapping	Number of Rodents	Rodents/Species	Species of Rodents
Forest	24	12(50%)	*Praomys*
		7(29.16%)	*Lophuromys*
		3(12.5%)	*Grammomys*
		2(8.33%)	*Beamys*
Forest Edge	14	2(14.28%)	*Rattus*
		3(21.42%)	*Praomys*
		6(42.85%)	*Grammomys*
		3(21.42%)	*Mastomys*
Farms	28	24(85.71%)	*Mastomys*
		2(7.14%)	*Grammomys*
		2(7.14%)	*Mus*
Peridomestic Area	36	2(5.56%)	*Croccidura*
		11 (30.56%)	*Mastomys*
		22(61.11%)	*Rattus*
		1(0.027%)	*Arvicanthis*
Along Streams	10	8(80%)	*Lophuromys*
		2(20%)	*Praomys*

Table 3: Rodents and shrews according to villages and species trapped.

Species	Villages				Total	Percentage
	Gologolo	Mavumo	Viti	Manolo		
Mastomys	2	10	12	13	37	33.0
Rattus	4	13	5	7	29	25.9
Praomys	7	2	0	7	16	14.3
Lophuromys	9	6	0	1	16	14.3
Grammomys	2	2	0	5	9	8.0
Beamys	0	1	0	0	1	0.9
Arvicanthis	0	1	0	0	1	0.9
Croccidura	0	0	2	0	2	1.8
Mus	0	1	0	0	1	0.9
Total	24	36	19	33	112	100.0

Figure 4: *Rattus rattus* species the house rodent

Figure 5: *Arvicanthis nairobae* species

4.2 Fleas and flea indices

A total of 253 fleas which representing five species were collected from various rodent species as shown in table 4 below. Most of the rodents trapped were co-infested by more than one flea species, and also the study observed that there were high interactions of various rodent species in the area. The most observed flea species were the *Xenopsylla* species (39.5%), followed by *Ctenocephalides* species (26%) and the *Ceratophylla* species *(Dinopsyllus spp)* (22.5%). All of

these species were collected in all villages while *Leptopsylla spp* (6.3%) and *Echidnophaga spp* (5.5%) were collected in only one village Manolo.

Figure 6: (a) *Xenopsylla* species (b) *Dinopsyllus* species

Table 4: Summary of Fleas species caught in Lushoto district

Species	Villages				Total	Percentage
	Gologolo	Mavumo	Viti	Manolo		
Xenopsylla spp	10	42	22	26	100	39.5
Ceratophylla spp	25	15	1	16	57	22.5
Ctenocephalides spp	43	17	4	2	66	26.1
Leptopsylla spp	0	0	0	16	16	6.3
Echidnophaga spp	0	0	0	14	14	5.5
Total	78	74	27	74	253	100.0

Table 5: Shows the Specific Flea Index (SFI) and Percentage Incidence Index (PII) in Lushoto District.

Rodent spp	Total number Caught	Total number Infested	Total number of fleas	PII	SFI
Mastomys spp	37	22	100	59.5	2.7
Rattus spp	29	24	57	82.8	1.97
Praomys spp	16	10	66	62.5	4.1
Lophuromys spp	16	7	16	43.8	1
Grammomys spp	9	8	14	88.9	1.6
Beamys spp	1	0	0	0	0
Arvicanthis spp	1	0	0	0	0
Croccidura spp	2	0	0	0	0
Mus spp	1	0	0	0	0

From Table 5 above it shows that the flea–rat indices of each category of rodents are above 0.5, which is the standard given by World Health Organization (1999).

Table 6: Rodent co-infestation with flea at Lushoto district 2014

Rodent Species	Flea Species	Number of Fleas
Mastomys	*Ceratophylla spp*	21
	Xenopsylla spp	22
	Ctenocephalides spp	56
Rattus rattus	*Ceratophylla spp*	1
	Xenopsylla spp	72
	Ctenocephalides spp	1
Praomys	*Ceratophylla spp*	5
	Xenopsylla spp	1
	Ctenocephalides spp	0
Grammomys	*Ceratophylla spp*	20
	Xenopsylla spp	1
	Ctenocephalides spp	0
Lophuromys	*Ceratophylla spp*	2
	Xenopsylla spp	0
	Ctenocephalides spp	9

4.3 PCR Results

The process started by standardizing the protocol by using previous positive samples and the was as follows

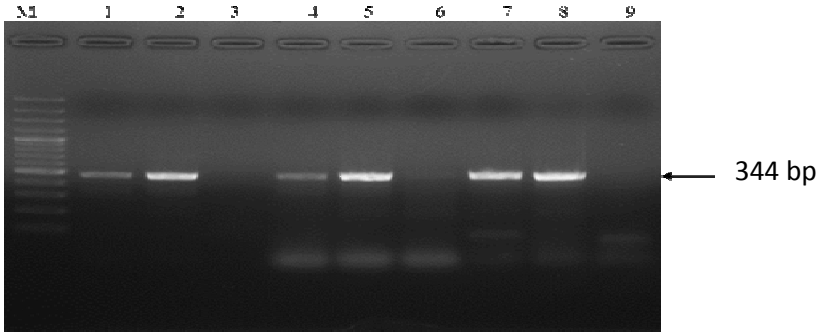

Figure 7: Standard PCR using positive control samples. **M** is the 100 bp Marker, 1, 2, 4, 5, 7 and 8 are positive samples while 3, 6 and 9 are negative samples.

The following primers *Yersinia pestis* plasminogen gene1 (5'TGC TTT ATG ACG CAG AAA CAG G3') and *Y. pestis* plasminogen gene 2 (5'CTG TAG CTG TCC AAC TGA AAC G3') were used, to amplify a specific *Y. pestis* 344 bp plasminogen gene. The amplified gene is a very well conserved gene for the *Y. pestis* bacterium which is easily detected by using PCR technique in very short time hence being useful in diagnostic purposes.

CHAPTER FIVE

DISCUSSION

Plague has been the scourge of mankind for many years. In Lushoto district the first outbreak was recorded in 1980. The disease persisted for 23 years. During this period, 7907 peoples were suspected to have been infected by the disease. About 640 deaths equivalent to 8.1% mortality rate were reported. After 2003, the disease regressed almost 10 years due to employment of different control measures and enforcement of environmental sanitation laws. This study was initiated to monitor the hosts and vectors for the evidence of plague. The results showed that *Yersinia pestis* bacterium is not currently in the rodents and fleas because there was no evidence of *Y.pestis* DNA after PCR detection. Although the study observed that all potential hosts and efficient vectors for the transmission of the disease were available. In this study the rodents were trapped in five different areas which are forest, forest edge, in the farms, along water streams and peri-domestic areas.

Rodent hosts suspected to be involved in the past outbreaks (Table 3) namely; *Mastomys natalensis* (33.0%), *Rattus rattus* (25.9%), *Mus musculus* (0.9%) and *Arvicanthis nairobae* (0.9%) were trapped. During the study period *Mastomys* and *Rattus* species were found to be more abundant than other species trapped. These results shows similar situation as mentioned by (Kilonzo *et al.,* 1997), that the sylvatic reservoir(s) for plague in Lushoto have not been clearly identified.

While serological studies have confirmed infection in a number of species it is not known which true reservoir species are and which provide a route for the bacteria to spread from the wildlife reservoir to commensal rodents and humans. Small mammal species that have been implicated in maintenance and transmission of plague in the Lushoto district are *Rattus rattus*, *Mastomys natalensis*, *Arvicanthis nairobae*, *Lophuromys flavopunctatus* and *Otomys* spp. Some observations made in plague endemic areas in the country revealed that *Mastomys natalensis* was the most frequent natural reservoir of plague and that it played an important role in maintaining the disease as it is partly refractory to the infection, and hence, it is not eradicated during plague epizootics (Guggisberg, 1966; Hubbard, 1973). Many other wild rodent species including, *Grammomys dolichurus;* have been suggested as suitable reservoir of the disease (Kilonzo *et al*., 1997).

These species are commonly trapped in and around plague-affected villages. As described in (Table 6) all rodents trapped during this study were co-infested by more than one flea species. This kind of infestation shows that there is high rate of mixing or interaction between various rodent species and their vectors. The interaction mentioned above is the best indicator of facilitating transmission of the infection in the sylvatic as well as in the human population immediately when the bacterium happens to invade the hosts and vectors of the disease. The study also found that *Mastomys* species were highly infested by fleas followed by *Rattus* species and then *Lophuromys* species.

The flea vectors suspected to be involved in the past outbreaks were also identified as *Xenopsylla cheopis* (39.5%) and *Ceratophylla* also called *Dinopsyllus spp* (22.5%). Of the identified vectors

Xenopsylla species were found to be abundant followed by the *Dinopsyllus* species. Among the other flea species collected, *Ctenocephalides*, *Leptopsylla* and *Echidnophaga gallinacea* are commonly found on rodents in Lushoto. Table 5 shows a very high flea-rat index in Lushoto District which indicates that the high flea population may lead to fleas spilling out. When fleas are too many the usual host may no longer be sufficient to provide them with a meal and then find alternative hosts which may be other animals or human beings and hence transmit the infection. According to World Health Organization report rat-flea index of greater than 1 represents an increased potential dangerous situation with respect to plague risk for humans (WHO, 1999). The general rat-flea index was 2.26 during the study period, suggesting important information in the surveillance of plague, and indicators of plague transmission. According to Hirst (1927), specific flea index (SFI) of 0.5 to 1 is considered sufficient to maintain plague in a locality.

The same hosts and vectors observed in the study area has been observed in other plague endemic areas within Tanzania and other countries, which are plague endemic like Zambia and Madagascar (Bertherat *et al.,* 2007; Hang'ombe *et al.,* 2012a). Most of the rodents captured and fleas collected from the study area are the same with those observed within other active plague foci in Tanzania and in other countries which are plague endemic for example Madagascar, Uganda and Brazil. Despite the foci being silent for some years now we cannot rule out the presence of the disease because it has been observed that the bacteria can also survive in soils, small mammals, rodents' burrows and nests (Eisen *et al.,* 2009). The information showed that the flea population was very high despite no outbreak. This could be attributed to the fleas not being infected by the bacterium, therefore reducing the chances of the fleas transmitting the disease

from one rodent to another or from rodent to humans. Two species of rodents trapped in the study area *Mastomys spp* and *Rattus spp* were also observed to have evidence of *Yersinia pestis* DNA in Zambia (Hang'ombe *et al.,* 2012b). This indicates that they are the most important hosts for maintaining the bacterium and may serve as vessel in the transmission of the bacterium into human population especially if there is an outbreak.

Despite the absence of the bacterium in both fleas and rodents we cannot rule out the absence of the disease in the study area because the sampling population might have been small. Also the research was conducted in the period of time where there was no outbreak for more than ten (10) years. Along with the information above the community awareness about plague is higher as at the same time people are employing environmental sanitation program such as plastering their houses frequently as mentioned by Kilonzo *et al* (1997). The other reason of not recovering the bacteria may be that, the bacterium is circulating at very low levels in the reservoirs to the extent that it is not able to infect the vectors and hence infect the human population.

The presence of natural foci of plague plays a serious role in re-emergence of the disease. *Yersinia pestis* bacteria may have been maintained circulating at low levels in the rodent populations without diagnosis of any human cases (Duplantier, 2005). Also, *Y. pestis* can survive in the soil under laboratory conditions, possibly providing the opportunity for rodents to be infected and promoting re-emergence of the disease (Eisen, 2008; Ayyadurai *et al*., 2008). The new settlements and urbanization may help in re-emergence of plague from potential natural foci. The only measure to assist in avoiding the re-emergence of plague is through surveillance to monitor the presence of potential natural foci and reservoir habitats.

CHAPTER SIX

CONCLUSION

1) This study has shown that at the moment there is no evidence of the bacterium in the rodents and flea population in Lushoto district.

2) The rodents captured in the study areas which are important plague hosts are *Rattus rattus*, *Mus musculus, Mastomys natalensis* and *Arvicanthis nairobae.*

3) The flea vectors belonging to *Xenopsylla spp* and *Dinopsyllus spp* were identified and these fleas are efficient plague vectors.

4) The flea-rat index of 2.26 is very high which suggests the possibility of an outbreak if the bacterium shall invade the population at any time.

5) The presence of high interaction between various rodent species and co-infestation with different flea species is an indicator for the efficient transmission dynamics for the disease.

6). The study also shows that *Leptopsylla* and *Ctenocephalides species* are commonly found on rodents in Lushoto.

CHAPTER SEVEN

RECOMMENDATION

For the purpose of prevention and control of future outbreaks or sporadic outbreaks of plague in Tanzania, the following measures that could assist are recommended:

1) The study suggests that regardless of the absence of the bacteria in rodents and fleas at the moment frequent surveillance must be done to ensure preparedness.

2) The government should ensure the continuation of environmental sanitation law enforcement in addition to rodent and flea controls in the area to cut off the flea-rat cycle.

3) The research must be conducted in other hypothesized reservoir sites such as soils, rodent burrows and nests, small mammals and wild animals.

4) Immediate intervention must be done on the control of flea population as the rat-flea index was very high.

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