ROLE OF POTENTIAL RESERVOIR HOSTS AND FLEA VECTORS IN PLAGUE EPIDEMIOLOGY IN EASTERN ZAMBIA

STANLEY SHABANI NYIRENDA

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

2017

EXTENDED ABSTRACT

This thesis is prepared according to "Published manuscripts" format of the Sokoine University of Agriculture. This thesis discusses the role of potential plague reservoirs and their flea-vectors in the plague epidemiology in eastern Zambia. The goal was to identify and describe roles domestic pigs, goats and sheep, and rodents with their fleas play in maintaining and transmitting the plague pathogen, *Yersinia pestis.* Plasminogen activator gene (*pla*) of *Y. pestis* was successfully identified in fleas and rodents, and Immunoglobulin G (IgG) antibodies against *Fraction* 1 *antigen* (*Fra1*)of *Y. pestis,* were detected in serum samples from the domestic animals using conventional Polymerase Chain reaction (c-PCR) and indirect Enzyme-linked Immunosorbent Assay (i-ELISA), respectively. Quantitative and qualitative approaches were applied using open and close ended questionnaires by individual respondents and focus group discussion (FGD) to obtain data on human and animal activities in relation to plague disease. The findings indicate that hunting, transportation and preparation or handling of infected animals or their materials predispose humans to plague pathogens and risk of acquiring the disease. The dissertation comprised five publications. The first paper published in the Journal of Medical Entomology, presents the roles domestic pigs, small ruminants, rodents and their fleas, play in the epidemiology of plague in Sinda district in eastern Zambia. This presents the first published literature on plague in pigs and small ruminants and demonstrated the naturally occurring IgG antibodies against Fra1 antigen. The second paper, published in Tropical Doctor presents the findings in Nyimba district after a suspected plague outbreak, where *pla* gene and IgG antibodies were detected in fleas, and pigs, goats and rodents respectively. The third paper published in the Journal of Zoonotic diseases is a review paper which identified factors that precipitated the spread of plague between 1914 and 2014 in Zambia. The fourth paper published in the American Journal of Tropical Medicine

and Hygiene and identified risk factors associated with plague epidemiology. The fifth paper has been submitted to BMC Microbiology Journal, revealed that *pla* gene of *Y. pestis* circulating among different hosts in the two districts were closely related to Antiqua (1.ANT) biovar.

DECLARATION

I, Stanley Shabani Nyirenda, do hereby declare, to the Senate of Sokoine University of Agriculture, that this thesis is my own original work and that it has neither been submitted nor being concurrently submitted for a degree award in any other institution.

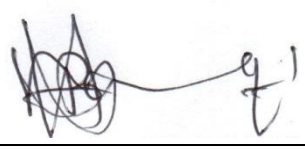
Stanley Shabani Nyirenda — Date

(PhD Candidate)

The above declaration confirmed by:

Prof. Robert S. Machang′u — Date

(Supervisor)



Prof. Bernard M. Hang′ombe — Date

(Supervisor)

COPYRIGHT

ACKNOWLEDGEMENTS

I am indebted to many individuals without whom the completion of this work would not have been possible. First and foremost, my heart is raised up to The Lord my God in Praise and Glory for all the Blessings and Guidance He has bestowed on me. Several institutions and individuals have directly or indirectly contributed towards the successful completion of this study. I wish to express my special thanks to the INTRA-ACPmobility scheme for the financial support throughout the course of the study. My particular thanks go to the Sokoine University of Agriculture (SUA), the University of Zambia (UNZA) and my employer at the Ministry of Fisheries and Livestock for giving me the opportunity to pursuepostgraduate studies. Also, my sincere appreciation goes to all individuals and organizations that assisted me in one way or another, in the completion of this dissertation. My sincere gratitude goes to my supervisors, Prof. B.S. Kilonzo (Retired), Prof Robert S. Machang′u and Prof. Bernard M. Hang′ombe for their professional guidance, encouragement and constructive criticisms during the course of this study.I am also indebted to all villagers in Luembe and Nyanje Chiefdoms in Nyimba and Sinda districts respectively,members of staff from Nyanje hospital and Chinambi clinic, Veterinary Assistants, Agricultural Assistants, community health workers and officials in Sinda and Nyimba districts for the data used in this work. I thank them for their time and support during the course of my research.Many thanks are also due to all academic and administrative members of staff of the Department of Veterinary Microbiology, Parasitology and Biotechnology in the College of Veterinary and Medical Sciences (CVMS) of the Sokoine University of Agriculture (SUA), and to my classmates for their advice and support in the preparation of this dissertation.Last, but notthe least, I would like to extend my deepest gratitude to my wife Faidess Enala Mhlanga and my children, Wrightwel Nyirenda, Martha Nyirenda, Stanley Nyirenda Jr. (late) MHSRIP and David

Nyirenda for their unconditional love and moral support during the whole course of my study. Grateful thanks go to my parents Rightwell Nyirenda and Tamanyauli Mhango for their love and prayers.

DEDICATION

This work is dedicated to my beloved wife Faidess Enala Mhlanga Nyirenda and my children, Wrightwel Nyirenda, Martha Nyirenda, Stanley Nyirenda Jr. (late) MHSRIP and David Nyirenda for their heartfelt love, care and sacrifice for my education.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS/ACRONYMS

BHI	Brain Heart Infusion
Caf1	Capsule antigen Fraction 1
CD	Calcium dependent
CDC	Centre for Disease Control and Prevention
CI	Confidence Interval
CIN	Cefsulodin, irgasan, novobiocin
DNA	Deoxyribonucleaic acid
DRC	Democratic Republic of Congo
EDTA	Ethylene Diamine Tetra-acetic acid
ELISA	Enzyme-linked Immunosorbent assay
FGD	Focus group discussion
Fra1	Fraction 1 gene
FSU	Former Soviet Union
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KAP	Knowledge Attitude and Practise
KIM	Kurdistan Iran Man
MHSRIP	May his soul rest in peace
MT	Murine toxin
NICD	National Institute for Communicable Diseases
ORF	Open reading frame
PCP	Plasminogen activator, coagulase, pesticin
PCR	Polymerase Chain Reaction
PHA	Passive Haemagglutination Assay

PII	Percentage incidence Index
Pla	Plasminogen activator gene
RCZ	Reformed Church in Zambia
SBA	Sheep blood agar
SFI	Specific flea index
SUA	Sokoine University of Agriculture
UK	United Kingdom
UNZA	The University of Zambia
Yop	*Yersinia pestis* outer membrane protein

CHAPTER ONE

1.0 General Introduction

1.1 Background

Yersinia pestis is a gram-negative coccobacillus, non-motile and non-spore-forming bacterium of the family*Enterobacteriaceae*. The bacterium causes plague disease, which is one of the most important re-emerging zoonotic diseases,which has claimed thousands of lives in world history. The most notable historical events are the Justinian plague pandemic in 541-543AD (Wagner *et al.,* 2014), the Black Death between 1347-1351 and the Oriental plague pandemic, which started in China during the middle of the 19th century and spread throughout the world (Stenseth *et al.,* 2008; Harbeck *et al.,* 2013). The disease occurred in Northern, Eastern, Southern, and Central Africa during the 19th to 20th centuries. The countries affected include: Libya, Algeria, Tanzania, Uganda, Kenya, Senegal, Republic of South Africa, Zimbabwe, Mozambique, DRC and Madagascar. Of these, Madagascar and Tanzania have experienced more severe, widespread and most recent outbreaks of the disease (Kilonzo, 1996, 1999; Ratovonjato*et al.*, 2014). In Zambia, there have been four plague outbreaks, in the past thirty (30) years. These occurred in North-western province (McClean, 1995), Southern province (Hang'ombe *et al.,* 2012), and two in Eastern province (Ngulube *et al*., 2006; Neerinckx *et al*., 2010) and involved substantial numbers of human cases and deaths.

The disease affects warm-blooded animals, especially rodents, which are the natural hosts/reservoirs of the infectious agent. Other animals which are occasionally infected and serve as carriers and consequently serve as potential sources of infection to humans and other warm-blooded animals include small domestic and wild carnivores and camels (Saeed *et al*., 2005). Goats and sheep have been implicated in the epidemiology of plague

(Ministry of Health, 1978;Christie *et al*., 1980), while pigs have been implicated as maintenance hosts or reservoirs of the bacterium (Marshal *et al*., 1972).Specific antibodies against *Y. pestis* Fraction1 antigen (Fra1) were reportedly detectable in dogs for up to two years post-infection. Dogs are known to be efficient carriers of the disease and have been shown to be serologically positive for the same in Tanzania, Zimbabwe and elsewhere. However, not more than 2% of dogs are killed by the pathogen (Taylor *et al*., 1981;Kilonzo, *et al*., 2006).

1.2 Pathogenesis

The virulent factors of *Y. pestis* are contained in its three plasmids termed; Calcium dependents(pCD), Murine toxin(pMT)/Fraction (pFra) and Plasminogen activator gene, coagulase and pesticin (pPCP), (Rajanna *et al*., 2010). These virulent factors play a significant role in the pathogenesis of the bacterium. The Plasminogen activator gene (*pla*) products prevent the host blood from clotting and facilitate the spread of *Y. pestis* in the body, Murine toxin (MT),*Yersinia* outer membrane protein (*Yop*) and the structural gene for Fraction 1 (Fra 1) protein capsule, confers resistance to bacterium from being engulfed by the monocytes or macrophages. Murine Toxin has an additional function to protect the bacterium from being digested by the enzymes in flea gut, thus enabling it to colonise the flea's midgut and increase its transmission to the host (Rajanna *et al*., 2010).

1.3 Transmission

The bacterium is normally transmitted from one animal to another through a bite of an infective flea, usually but not always,*Xenopsylla cheopis* (Rothschild).Other potential arthropods such as flea vectors include *X. brasiliensis*(Baker),*Ctenocephalides* spp and *Pulex irritans*(Linnaeus), and occasionally lice vectors known as *Pediculus humanus*(L), may transmit the pathogen during the disease outbreak(Ratovonjato*et al*., 2014). The

disease can also be transmitted through direct contact with infected materials such as contaminated meat (Christie *et al*., 1980) and droplet infection, through inhalation of contaminated air dropletsoriginating from pneumonic plague cases.

Plague transmission by the rodent flea vector occurs after the flea leaves the infected host when the latter's body temperature falls following its death either in the house or in the bush due to disease or other causes including hunting, predators or poisoning as is the case in rodent control interventions. Transportation of dead rodents or other plague reservoirs by hunters such asin hunter's pockets or on shoulders or in carnivores' mouth facilitate jumping or crossing over of fleas to humans and other mammals in domestic environments. In some cases,rodents die of plague in their burrows or nests following which fleas leave the dead bodies and look for alternative hosts in the vicinity. These may include people working in farms or rodent hunters and predators looking for rodent preys. Farmers may be exposed to the fleas as they plough their fields using hoes or oxen plough. This scenario may facilitate the increase of fleas moving in search for an alternative host and consequent transmission of plague pathogens from the natural hosts infected with the disease (Fig. 1.1). People digging out rodent burrows to capture the animals for food canalso be infected by inhalation of dust from the burrows of rodents, which have recently died of plague as the *Y.pestis* is reportedly capable of surviving in soil for quite some time and can easily be airborne (Ayyadurai *et al*., 2008).

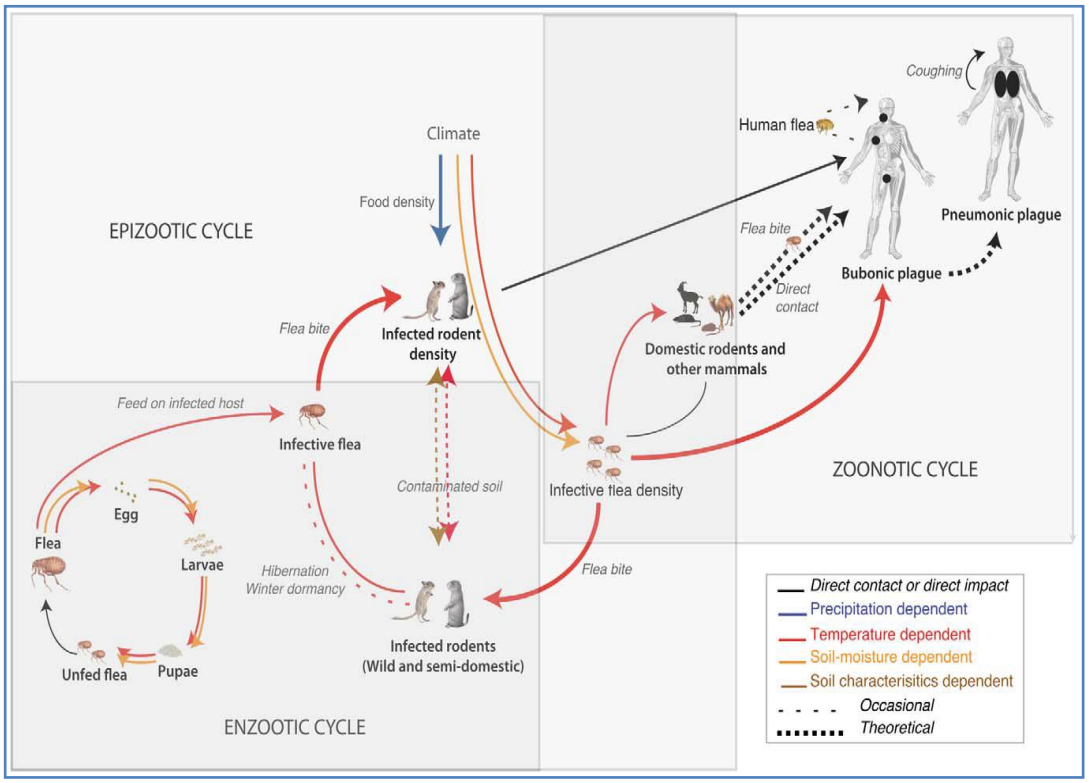


Figure 1.1: Transmission pattern of *Yersinia pestis* within the plague affected community

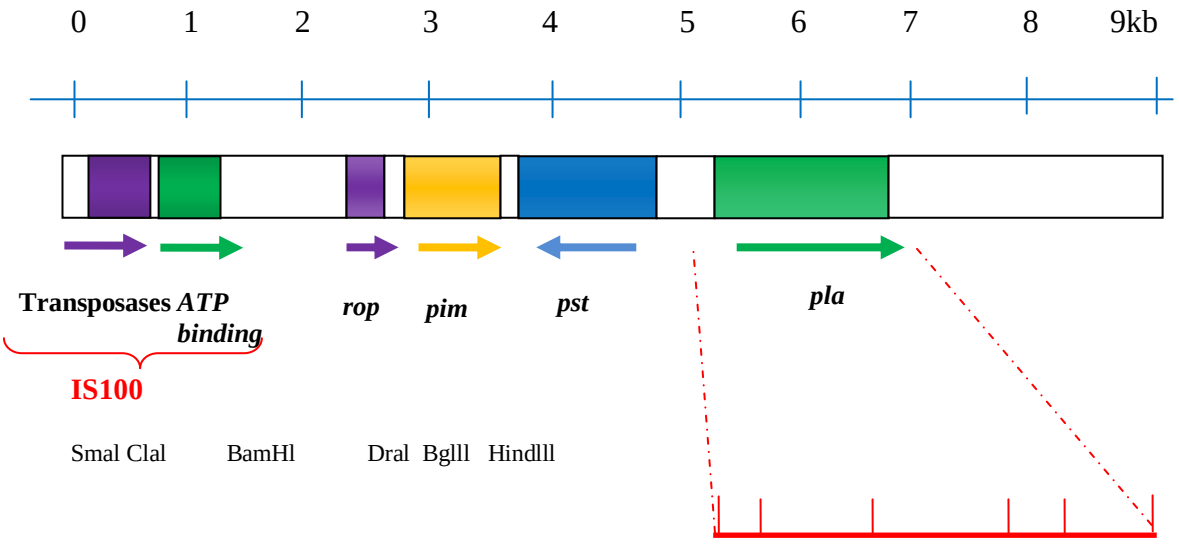
Source; Ben Ari *et al*., 2011

1.4 Diagnosis

1.4.1 Brief preamble

Yersinia pestis can be investigated in the laboratory byvarious methods including cultural, serological, biochemical, biological tests and molecular techniques (Perry and Fetherston, 1997). Molecular techniques are more precise, as the specific gene such as the Plasminogen activator gene (*pla*), which is located on plasmid pPCP incorporated into *Y. pestis*, is targeted.Plasmid PCP1 has 9.5 kb and is made up of fivedifferent genes: Pesticin (1,074 bp *pst*), Pesticin immunity- a transcriptional regulator gene (426 bp *pim*), the replication regulation proteins (195 bp rop), IS100 (made up of 1,02 kb transposase and 782 bp ATP-binding protein) and Plasminogen activator gene (939 bp *pla*gene). The *pla* gene, only found in *Y. pestis*, is 1.4 kb and has 936 bp open reading frame (ORF), is

responsible for cleaving fibrin deposits that trap the microorganisms consequently, helps to facilitate dissemination of the bacteria in the host (Fig 1.2)(Rajanna *et al.*, 2010). Investigations of the *pla* genein PCR assays are mainly based on the occurrence of the gene in multiple copies and its absence from closely related *Yersinia* species (*Y. pseudotuberculosis* and *Y.enterocolitica*)and its role in *Y. pestis* virulence (Jansen *et al.*, 2013).



Arrows show the direction of transcription of the genes

Figure 1.2: Physical and genetic maps of pPCP showing five genes IS100 (transposases and ATP binding), rop, plasminogen activator (*pla*), pesticin gene (*pst*) and pesticin immunity gene (*pim*)

Rajanna *et al*. 2010

In several studies, PCR assays are the prime or sole markers of a bacterium, which amplifies the targeted gene using appropriate forward and reverse primers. This technique has an advantage in that it can detect even small numbers of microorganisms present in a sample. The bacterium DNA can be detected in infected tissue samples such as liver,

spleen, lungs and whole blood preserved in Ethylene Diamine Tetra-acetic Acid (EDTA) and infected fleas. The anticoagulant has a higher affinity for divalent *ions* like Calcium (Ca^{2+}),Manganese (Mn^{2+}) and Magnesium (Mg^{2+}) which are co-factors for many active enzymes in the cells including DNAse, which breaks down DNA molecules. Once the cell is disrupted, during the process of DNA extraction, the nuclear envelope goes off and the nuclear content comes into contact with the cellular contents, which are rich in nucleases, and subsequently, the DNA is degraded. Thus, the disrupted cell treated with EDTA will chelate the cations making the nucleases lose their function and unable to disintegrate DNA and hence, results in a good yield of the DNA for PCR technique (Banfi *et al.*, 2007).

Serological tests are an important tool in detecting specific Immunoglobulin M (IgM) and Immunoglobulin G (IgG) against Fraction 1 gene (Fra1), a product which is part of *Y. pestis*. Fraction 1 gene is encoded by the capsular antigen fraction1 (*caf1*)gene located on the large 110 kb pFra plasmid, which is also unique to *Y. pestis*. It is responsible for resistance against phagocytosis by monocytes and protect*Y. pestis* from being digested in the flea midgut (Du *et al*., 2002). These antibodies against Fra1 are IgM for recent infection and IgG for chronic or past infection and are normally detected with serological tests such as Enzyme-Linked Immunosorbent Assay (ELISA) technique and Passive Hemagglutination assay (PHA).

Culture and biochemical tests are equally important in diagnosing *Y. pestis*. Collecting the suspected materials in Brain Heart Infusion broth (BHI) and sub-culturing the suspected material on sheep blood agar (SBA) or Mac Conkey agar or Cefsulodin-Irgasan-Novobiocin medium (CIN), allows the microorganisms to grow within 48-72 hours at room temperatures ($25^{o}C$-$28^{o}C$), thus differing from most Gram-negative bacteria which grow at $37^{o}C$ overnight. Inoculation of laboratory mice or guinea pigs with suspected

materials and careful monitoring for up to 21 days to observe plague symptoms in the experimental animals and complemented by the presence of bipolar staining bacilli in impression smears of the animals using Giemsa stain, is also a standard test for *Y. pestis* (Engelthaler *et al*., 1999).

1.4.2Clinical signs and symptoms

Plague disease has affected millions of people during the three most devastating pandemics of the world namely: Justinian plague, Black Death and Oriental plague. During disease outbreak, diagnosis of plague infection in humans is based on the patient's symptoms such as buboes, coughing with bloody sputum and fever, as well as exposure history, although the symptoms may be similar with other diseases (Perry and Fetherston, 1997). Plague manifest in three forms and are described as; Bubonic plague, Pneumonic plague and Septicaemic plague.

1.4.2.1 Bubonic plague

Bubonic plague is the classic form of the disease. Patients usually exhibit symptoms of fever, headache, chills, and swollen,extremely tender lymph nodes (buboes) (Fig. 1.3) within two to six days of contact with the bacteria. This can either be througha bite from an infective flea or by contact with open wounds to infected materials. Patients may also have gastrointestinal complaints such as nausea, vomiting, and diarrhoea. Skin lesions infrequently develop at the initial site of infection. Soreness in the affectedlymph nodes will sometimes precede swelling, and any of the lymph nodes can be involved, depending upon the siteof the initial infection. Buboes are typically found in the inguinal and femoral regions, but may also occur in other lymph nodes (Perry and Fetherston, 1997).

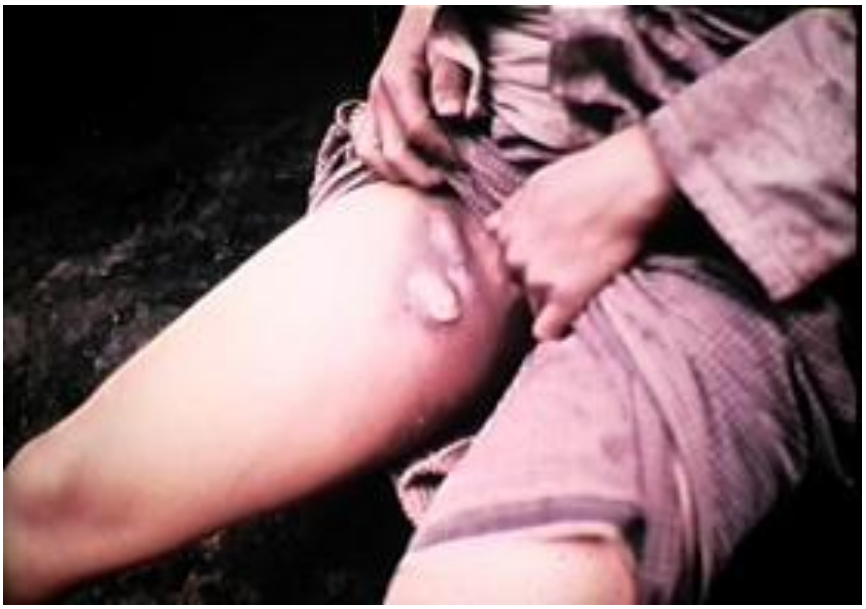


Figure 1.3: The buboes on a thigh of a boy

Source; CDC, 2009

1.4.2.2 Septicemic Plague

Primary septicaemic plague with no palpable lymphadenopathyis generally defined as occurring in a patient with positive blood cultures. Clinically, plague septicaemia resembles septicaemia caused by any other gram-negative bacteria. Patients are febrile,and most have chills, headache, malaise, and gastrointestinal disturbances. There is some evidence that patients with septicaemic plague have a higher incidence of abdominal pain than do bubonic plague patients. The mortality rate forpeople with septicaemic plague is fairly high, ranging from 30 to 50%, probably because of misdiagnosis by clinicians and the antibiotics generally used to treatundifferentiated sepsis not being effective against *Y. pestis* (Perry and Fetherston, 1997).

1.4.2.3 Pneumonic Plague

Primary pneumonic plague isa rare but deadly form of the disease that is spread via respiratory aerosols through close contact (a distance of 0.5 m to 1.5 m) with an infected individual. It progresses rapidly from a febrile flu-like illness to an invasive and

overwhelming pneumonia with coughing and the production of bloody sputum(haemoptysis). The incubation period for primary pneumonic plague is from 24 to 72 hours. The vast majority of the cases may be contracted from infected individuals following close contact with infected animals such as rodents, cats and dogs (Perry and Fetherston, 1997).

Appropriate samples such as bubo aspirates, blood and sputum, cerebrospinal fluids in patients with plague meningitis and scrapings from skin lesions are collected and taken to the laboratory for bacteriological confirmation of the disease. Tissue samples or bubo aspirates may also be subjected to PCR technique for the detection of *Y. pestis* DNA (Ziwa *et al*., 2013; Riehm *et al*., 2011). However, handling and working with live *Y. pestis*requires a laboratory with Biological Safety Level II (BSL-2) facilities and practices which include a laminar flow hood with eyes protected with safety glasses, eye shield and proper adherence to all standard measures recommended for such laboratory environments.

1.4.3 Bacterial culture and biochemical characterisation

Bacterial culturing followed by biochemical characterisation is an important tool for confirmation of *Y. pestis*. This is done by growing the suspected sample on the appropriate bacterial medium such as sheep blood agar (SBA), Cefsulodin, irgasan, novobiocin (CIN) medium or Mac Conkey agar (Sarovich *et al*., 2010). Gram stain is performed on the suspected and desirable colonies from cultures, which are further sub-cultured to obtain pure colonies that are then subjected to biochemical tests such as urease and oxidase reactions. Other biochemical tests may include catalase and indole as well as motility test at room temperature (25^{o}C). The three *Y. pestis* strains can be differentiated from each other by their ability to convert nitrates to nitrites and fermentation of glycerol. BiotypeAntiqua is positive for both characteristics, while Orientalis forms nitrite from

nitrates but does not ferment glycerolbecause of a 93 bp deletion in the *glycerol 3-phosphate dehydrogenase (glpD)* gene, andMedievalis ferments glycerol, but does not form nitrite fromnitratedue to a G to T nucleotides mutation that results in a stop codon in the *napA* gene (Haensch*et al*., 2010).Confirmation is done by PCR targeting conserved important genes of *Y. pestis* DNA such as plasminogen gene *(pla*) and *Y. pestis* outer proteins *(Yop)*(Hang'ombe *et al*., 2012; Cui *et al.,* 2013).

1.4.4 Laboratory animal inoculation

Yersinia pestis can also be diagnosed by inoculation of laboratory animals such as mice and guinea pigs. This is accomplished by inoculating the animals with suspected materials and observing the former for symptoms such as fever, anorexia, inactiveness and death within 21 days (Engelthaler *et al*., 1999). Although the method is the 'gold standard' for plague detection, it is laborious, costly and requires several days before the diagnosis is confirmed. After the inoculated animals show symptoms of plague, they are sacrificed and organs (especially spleen, heart, liver and lungs) macerates are cultured on appropriate medium as described above and incubated at 37^{o}C for 48 hours (Sarovich *et al*., 2010). Cefsulodin-Irgasan-Novobiocin medium (CIN) agar inhibitsthe growth of competitors and allows the differentiation of *Y. pestis* from commensal bacteria species. Single colonies suspected to be*Y. pestis* arefurther sub-cultured onto SBA and the resulting colonies used for DNA extraction byheat lysis for PCR confirmation (Rahalison*et al*., 2000).

1.4.5 Serology

A serological diagnosis is an important tool for surveillance of the disease as it reveals current or recent exposure of the hosts or reservoirs to the pathogen (Thullier *et al*., 2003). The test is based on the detection of specific plague antibodies in serum of animals or humans. IgM antibodies appear in the host animal sera between 7 and 14 days while the

IgG antibodies appear between 21 and 28 days of infection. Once the animal or person is exposed to*Y. pestis,* the body developsspecific antibodies against fraction 1 antigen (Fra1). The antibodies can be detected by using appropriate serological techniques such as ELISA and PHA (Makundi *et al.,* 2008;Nakamura *et al*., 2013;Nyirenda *et al*., 2017). Detection of IgM antibodies normally suggests current or recent infection while the presence of IgG indicates fairly past infection or chronic infection. The limitation of the tests is that sometimes the level of IgG antibodies in the animal may be low enough to be detected.

1.4.6 Molecular techniques

The use of PCR is the most precise way of diagnosing the *Y. pestis*(Riehm*et al*., 2011), although it is expensive. Organs from suspected animals or suspected bacterial culture or suspected fleas are subjected to PCR to detect the *Y. pestis* DNA plasminogen activator gene. DNA is extracted from the samples and run on PCR for amplification of the *pla* gene. For *pla* gene, there are three sets of primers which may be used i.e. Yp1 Pla1: 5'TGC TTT ATG ACG CAG AAA CAG G3';Yp1 Pla2:5'CTG TAG CTG TCC AAC TGA AAC G3', Yp2 Pla1: ATCTTACTTTCCGTGAGAAG3'; Yp2 Pla2: CTTGGATGTTGAGCTTCCTA3' and YP3 Pla1: 5'GAA AGG AGT GCG GGT AAT AGG TT3', Yp3 Pla2: 5'CCT GCA AGT CCA ATA TAT GGC ATA3' (Tsukano *et al.,* 1996).

Other structural genes of *Y. pestis,* whichmay be considered for molecular diagnosis includes; capsule antigen fraction 1 (*caf1*) using primers Yp *caf1*: 5′ATACTGCAGATGAAAAAAATCAGTTCC3′*Yp caf2*: 5′TAAAGCTTTTATTGGTTAGATACG3′GT3’ and *Yop* using primers Yp *yopM1*: 5′ATAACTCATCGGGGGCAAAAT3′ and Yp *yopM2*: 5′GCGTTATTTATCCGAATTTAGC3′ (Tsukano *et al*., 1996). The PCR of the DNA samples is run on an agarose gel which is impregnated with ethidium bromide and evaluated under UV transilluminator (Hang’ombe *et al.,* 2012). In positive samples, the bands are seen at an appropriate base pair depending on the primers used.

1.5 Prevention and Control of plague

Plague can be controlled by prompt treatment of infected patients with effective antibiotics and avoidance of contact with infected fleas and other materials. Regular and sustainable use of insecticides to control fleas, use of live rodent traps and rodenticides to maintain rodent population at low levels and minimising human-rodent contacts, as well as appropriate health education campaigns to communities regarding risks of acquiring the disease, as well as community-based preventive measures for the same, can reduce infection rates substantially (Eisen *et al*., 2014).

1.5.1 Prophylactic treatment with antibiotics

Antibiotics can be used to treat and prevent *Y. pestis* infections. Tetracyclines are popular antibiotics for plague prophylaxis (Perry and Fetherston, 1997). Usually, antibiotics are given as prophylactic measures only to close contacts of pneumonic plague patients.

1.5.2 Vaccination

There are two types of plague vaccines currently used in various parts of the world. The live vaccine is derived from an attenuated strain, usually related to EV76 strain, while the killed vaccine uses a formalin-fixed virulent strain of *Y. pestis* (Titball and Williamson, 2004). However, the use of vaccines is only recommended for people who are frequently exposed to plague-infected materials such as laboratory and field workers, since the vaccine is not 100% efficacious. In Tanzania, for example, a WHO produced vaccine prepared against strain EV76 was found to be effective in only 46% of the test population (Kilonzo, B.S. personal communication, 2013).

1.5.3 Extensive surveillance of rodents and fleas

Appropriate control of plague should aim at reducing the numbers of human and animal plague cases and should involve the entire community, instituting a one health approach, i.e. involvement of all professional disciplines whenever an outbreak occurs. Control of plague can also be done by extensive surveys of rodents and fleas and the consequent identification of its source and level of activity (Meerburg *et al*., 2009). Serological surveys of wild and domestic carnivores, domestic pigs and small ruminants can also be used to monitor plague in the sylvatic or murine cycles. A surveillance network of people who report suspected plague oriented activities, such as the sudden disappearance of rodents, appearance of sick and/or dying animals, or an increase of flea populations in homes, should be established (Barnes, 1990).

1.5.4 Use of insecticides and rodenticides

Further approaches for plague control should include the use of insecticides and rodenticides to control fleas and rodents respectively.

1.5.4.1 Insecticides

Insecticides are pesticides that are formulated to kill, harm, repel or mitigate one or more species of insects. Insecticides work in different ways. Some disrupt the nervous system or damage their exoskeletons while others simply act as repellents. Insecticides can be packaged in various forms, including sprays, dusts, gels, and baits (Ford, 1993).

Types of insecticides

There are so many types of insecticides, but some of them may include:-

a) Organochlorines

The organochlorines are insecticides that contain carbon, hydrogen, and chlorine. They are also known as *chlorinated hydrocarbons, chlorinated organics, chlorinated insecticides,* and *chlorinated synthetics e.g. DDT*.(Kutz *et al.*, 1991).

b) Organophosphates

Organophosphates (OPs), a derivative of phosphoric acid, is the term that includes all insecticides containing phosphorus. The OPs work by inhibiting cholinesterase (ChE), an important enzyme of the nervous system. Examples of OP insecticides malathion, trichlorfon (Dylox®), monocrotophos (Azodrin®), dimethoate (Cygon®), dichlorvos (Vapona®), mevinphos (Phosdrin®)(Kwong, 2002).

c) Carbamates

The carbamate insecticides are derivatives of carbamic acid and their mode of action is that of inhibiting the vital enzyme *cholinesterase* (ChE). Examples of carbamate insecticides are methomyl (Lannate®), carbofuran (Furadan®), aldicarb (Temik®), oxamyl (Vydate®), thiodicarb and Larvin® (Ware and Whitacre, 2004).

1.5.4.2 Rodenticides

Rodenticides are pesticides that kill rodents. Rodents are mammals of the order Rodentia, which are characterised by a single pair of continuously growing incisors in each of the upper and lower jaws. These include not only rats and mice, but also squirrels, woodchucks, chipmunks, porcupines, nutria, and beavers. Rodenticides are usually

formulated as baits, which are designed to attract animals. Flavorings may include fish oil, molasses or peanut butter. Baits used in agriculture and natural areas may contain ground meat, vegetables, grains, or fruits. Rodenticides can be grouped together according to the mechanism of action on the host. All rodenticides can be toxic when eaten, inhaled and come into contact with skin. Many rodenticides inhibit blood clotting and these are called anticoagulants such as Bromadiolone, chlorophacinone, difethialone, brodifacoum, and warfarin (Campbell and Chapman, 2000).

There are a number of rodenticides that are not anticoagulants, and these works in different ways. For instance, Zinc phosphide, bromethalin, cholecalciferol, and strychnine (Table 1.1).

Zinc phosphide: Zinc phosphide was first registered in 1947. It changes into phosphine gas in the presence of water and acid. The phosphine gas is very toxic; it blocks the body's cells from making energy, and the cells die. Phosphine exposure is particularly damaging to the heart, brain, kidney and liver (Albretsen, 2006).

Bromethalin: Bromethalin prevents cells in the central nervous system from producing energy. The nerve cells swell, this puts pressure on the brain, paralysis and death soon follows. The major breakdown product of bromethalin is more toxic than bromethalin itself. The varying ability of different species to break down bromethalin may explain why it is more toxic to some animals than others. Bromethalin is considered a single-dose rodenticide (Dorman, 2006)

Cholecalciferol: Cholecalciferol is vitamin D3. Vitamin D helps the body maintain calcium balance by enhancing absorption of calcium *ions* from the gut and kidneys. Toxic

doses of cholecalciferol lead to too much calcium in the blood, which can affect the central nervous system, muscles, the gastrointestinal tract, cardiovascular system, and the kidneys. The body's ability to maintain proper calcium levels must be overwhelmed before cholecalciferol becomes toxic (Rumbeiha, 2006).

Strychnine: It can only be used below ground, and products with more than 0.5% strychnine are restricted. They are only sold to certified applicators. Strychnine comes from the seeds of certain plants, *Strychnos nux-vomica* and *Strychnosignatii*. It affects the cells in the spinal cord by causing nerve cells to fire more readily, which leads to muscle spasms. Depending on the dose, the spasms may be so severe which causes breathing paralysis and death (Talcott, 2006).

Table 1.1: Rodenticides and their mode of action

Rodenticide	**Type**	**Chemical Class**	**Days of feeding needed**
Warfarin	Anticoagulant	Hydroxycoumarin	multiple
Chlorophacinone	Anticoagulant	Indandione	multiple
Diphacinone	Anticoagulant	Indandione	multiple
Bromadilone	Anticoagulant	Hydroxycoumarin	single
Difethialone	Anticoagulant	Hydroxycoumarin	single
Brodifacoum	Anticoagulant	Hydroxycoumarin	single
Bromethalin	Non-anticoagulant	Other	single
Cholecalciferol	Non-anticoagulant	Vitamin D3	multiple
Zinc phosphide	Non-anticoagulant	Other	single
Strychnine	Non-anticoagulant	Other	single

(Campbell and Chapman, 2000)

However, elimination of both the host and the flea vectors using these toxic substances is not selective and may kill non target animals. Controlling the insects such as flea should be done concurrently with or precede the use of any rodenticide, this would reduce or prevent migration of fleas from dead rodents to new hosts, including humans, and consequently spread the bacterium.

Integrated Pest Management (IPM) strategy could also be used to control the pests. This is a combination of commonsense and scientific principles. It's a way of thinking about pest management that would reduce the number of rodents: 1) Using knowledge about the pest's habits, life cycle, needs and dislikes, 2) Using the least toxic methods first, up to and including pesticides, 3) Monitoring the pest's activity and adjusting methods over time, 4) Tolerating harmless pests, and 5) Setting a threshold to decide when it's time to act(Hendrichs*et al*., 2007).

Nevertheless, prolonged use of these chemicals on the rodents and insects may encourage the growth of some opposition to the rodenticides and insecticides respectively. This can be overcome by regular testing of drug resistance against the rodents and fleas.

1.5.5 Educational campaigns and community participation

Appropriate educational campaign in endemic areas is essential to raise awareness about the disease and how to reduce risks of exposure among residents. The best control measures are to eliminate habitats for plague-susceptible rodents and to rid domestic pets of flea infestation, using effective pesticides (Kilonzo, 1976; Mann *et al*., 1979). Furthermore, hygiene is paramount to prevent fleas from infesting humans and domestic animals such as dogs, goats, sheep, pigs and cats. This can be achieved by cleaning homestead surroundings to prevent rodents from coming near to human residences and granaries.Likewise, the purpose of rodent proof crop storages (e.g. pest guards on pillars)

on the granaries discourages rodents from climbing into the granaries to feed (Meerburg *et al*., 2009). Indeed, a community-based eco-health approach can effectively prevent and/or control outbreaks of plague as demonstrated in Lushoto district, Tanzania, where the disease has been breaking out yearly for more than two decades (Kilonzo *et al*., 1997).

1.6 Statement of the problem and Justification of the study

There have been two Plague disease outbreaks in Sinda district and one in Nyimba district of Eastern province of Zambia in 2001 and 2007, and 2015 respectively (Ngulube *et al*., 2006;Phiri, 2015; Sinyange *et al*., 2016; Nyirenda *et al*., 2017). Due to the above outbreaks and recent demonstration of *Y. pestis* DNA *(pla)* among rodents and *Crocidura* spp in the area (Nyirenda *et al.*, unpublished report, 2013), it was felt desirable to carry out investigations on the presence of the bacteria among potential and susceptible hosts such as domestic pigs and domestic small ruminants, which are closely associated with human dwellings. In the study area, pigs are kept at free range methods and thus, they move around the villages scavenging, and eating waste and carcasses, including dead rodents. In so doing, they may be exposed to and bitten by infective fleas from dead rodents and become carriers or maintenance hosts (Marshal *et al*., 1972). Likewise, sheep and goats are abundant in the study area and move freely and thus are exposed to infected fleas. Dogs and cats in the study areas may be exposed to infective fleas when they predate on rodents in bushes, fields or homesteads.

In view of the foregoing facts and the close association of these animals with humans in the study area, the need to investigate their involvement in the epidemiology of plague in the foci is justifiable. The desire to undertake the study was enforced by the recent studies in the Sinda district in which *Y. pestis* DNA *(pla)* was documented in peri-urban and domestic rodents and *Crocidura* spp in the area.This study also demonstrated high levels of

antibodies against *Y. pestis*(Fra1) in rodents and dogs, indicating that they have been recently or currently exposed to the bacterium. Results from the current study will assist health personnel and policy makers to appreciate the status of plague endemicity in the area and consequently take appropriate measures to prevent future outbreaks of the disease.

1.7 Objectives of the study

1.7.1 General objective

To investigate and establish the role of potential plague reservoir hosts and flea vectors in the epidemiology of plague in Eastern Zambia.

1.7.2 Specific objectives

i. To establish the presence of *Y. pestis* in domestic small ruminants, domestic pigs andwild rodents.
ii. To determine the flea species on the study animals and infection rates of the fleas with *Y. pestis.*
iii. To establish the predisposing social, cultural, biological and environmental factors associated with plague transmission in the area.
iv. To determine the biotypes or strains of *Y. pestis* present in Eastern Zambia.

1.8 Research questions

i) Do domestic pigs and domestic small ruminants complement rodents as reservoirs of *Y. pestis?*

ii) Does the same biotype or biovar of *Y. pestis* circulate among these reservoir hosts?

iii) What are the most important flea vectors ofplague in the area?

iv) What are the major predisposing human behaviouralfactors contributing to plague in Eastern Zambia?

1.8.1 Sample size

The minimum sample size required was extracted from the formula described by Charan and Biswas, 2013 to achieve the first and second specific objectives.

$$n = \frac{Z^2 p(1-p)}{d^2}$$

Where:-

n = required sample size, **Z** = Is standard normal variate (at 5% type 1 error **(P<0.05)** it is 1.96), **P** = expected proportion based on previous studies (20% wild animals and 10% small ruminants, 5% pigs), **(1-P)** = the probability of having no disease and **d** = absolute error or desired precision (5%).Therefore, using the above formula the sample size was established as: 276 small wild animals (wild rodents and small carnivores), 276 domestic small ruminants and 246 domestic pigs, Total 798 samples.

1.9 References

Albretsen, J. C. (2004).Zinc phosphide. In:Clinical Veterinary Toxicology.(Edited by Plumlee, K. H.)Mosby Inc.: St. Louis, MO. pp.456-459.

Ayyadurai, S., Houhamdi, L., Lepidi, H., Nappez, C., Raoult, D. and Drancourt, M. (2008).Long-term persistence of virulent *Yersinia pestis* in soil.*Microbiology* 154:2865-2871.

Banfi, G., Salvagno, G. and Lippi, G. (2007).The role of ethylenediamine tetraacetic acid (EDTA) as in vitro anticoagulant for diagnostic purposes. *Clinical Chemical Laboratory Medicine* 45(5):565-576.

Barnes, A. M. (1990). Plague in the U.S.: Present and future. In:Proceedings of the 14th Vertebrate Pest Conference.(Davis, L. R. and Marsh, R. E.).March 1990,University of California, Davis, California, pp.43–46.

Ben Ari, T., Neerinckx, S., Gage, K.L., Katharina, K., Laudisoit, A., Leirs, H. and Stenseth, N.C.(2011). Plague and Climate: Scales Matter. *PLoS Pathogen* 7(9):1-6.

Campbell, A. and Chapman, M. (2000).Anticoagulant rodenticides. Handbook of poisoning in dogs and cats; Blackwell Science: Oxford, England. pp.64-73.

CDC (2009). Public Health Library (PHIL No. 2047), GA. USA.[http://phil.cdc.gov] 21 September, 2017.

Charan, J. and Biswas, T. (2013). How to calculate sample size for different study designs in medical research? *Indian Journal of Psychological Medicine* 35(2):121-126.

Christie, A.B., Chen, T.H. and Elberg, S.S. (1980). Plague in camels and goats: Their role in human epidemics.*Journal of Infectious Diseases* 141:724-726.

Cui, Y., Yu, C., Yan, Y., Li, D., Li, Y., Jombart, T., Weinert, L.A., Wang, Z., Guo, Z., Xu, L. and Zhang, Y. (2013). Historical variations in mutation rate in an epidemic pathogen, *Yersinia pestis*. *Proceedings of the National Academy of Sciences* 110(2):577-582.

Dorman, D. C. (2006).Bromethalin. In:Small Animal Toxicology.(Edited byPeterson, M. E. andTalcott, P. A.) Elsevier Saunders: St. Louis, MO. pp.609-618.

Du, Y., Rosqvist, R., and Forsberg, A. (2002).Role of fraction 1 antigen of *Yersinia pestis* in inhibition of phagocytosis. *Infection and immunity* 70(3):1453-1460.

Eisen, R. J., MacMillan, K., Atiku, L. A., Mpanga, J. T., Zielinski-Gutierrez, E., Graham, C. B. and Gage, K. L. (2014). Identification of risk factors for plague in the West Nile region of Uganda.*American Journal of Tropical Medicine and Hygiene*90(6):1047-1058.

Engelthaler, D.M., Gage, K.L., Montenieri, J.A., Chu, M. and Carter, L.G. (1999). PCR Detection of *Yersinia pestis* in Fleas: Comparison with Mouse Inoculation. *Journal of Clinical Microbiology* 37:1980–1984.

Ford, M. (1993). Insecticides and pesticides.In:Handbook of Medical Toxicology.(Edited byViccellio, P.)Little, Brown and Company.Boston, MA.pp.303-314.

Haensch, S., Bianucci, R., Signoli, M., Rajerison, M., Schultz, M., Kacki, S., Vermunt, M., Weston, D.A., Hurst, D., Achtman, M., Carniel, E.and Bramanti, B. (2010). Distinct clones of *Yersinia pestis* caused the black death. *PLoSPathogens* 6(10):1-8.

Hang'ombe, B.M., Nakamura, I., Samui, K.L., Kaile, D., Mweene, A.S., Kilonzo, B.S., Sawa, H., Sugimoto, C. and Wren, B.W. (2012).Evidence of *Yersinia pestis* DNA from fleas in an endemic plague area of Zambia.*BMC Research Notes* 5:72-81.

Harbeck, M., Seifert, L., Hänsch, S., Wagner, D.M., Birdsell, D., Parise, K.L, Wiechmann, I., Grupe, G., Thomas, A., Keim, P., Zoller, L., Bramanti, B., Riehm, J.M. and Scholz, H.C. (2013). *Yersinia pestis* DNA from skeletal remains from the 6th century AD reveals insights into Justinianic Plague. *PLoS Pathogen* 9(5):1-8.

Hendrichs, J., Kenmore, P., Robinson, A.S. and Vreysen, M.J.B. (2007).Area-wide integrated pest management (AW-IPM): Principles, practice and prospects. In: Area-Wide Control of Insect Pests: From research to field implementation'.(Edited by Vreysen, M.J.B, Robinson, A.S., and Hendrichs, J.) Springer: AA Dordrecht, The Netherlands. pp.3-33.

Jansen, I., Hamidjaja, A.R. and Reusken, C. (2013).*Yersinia pestis* Plasminogen activator gene homology in rat tissues.*Emerging Infectious Diseases* 19(2):342-344

Kilonzo, B.S. (1976): A survey of rodents and their flea ectoparasites in north-eastern Tanzania. *East African Journal of Medical Research* 3:117-126.

Kilonzo, B.S. (1996). Plague in Africa; Epidemiology and Control. *Africa Health*19:14-16.

Kilonzo, B.S. (1999). Plague epidemiology and control in eastern and southernAfrica during the period 1978 to 1997. *Central African Journal of Medicine*35:70-76.

Kilonzo, B.S., Mvena, Z.S.K., Machang′u, R.S. and Mbise, T.J. (1997). Preliminary observations on factors responsible for long persistence and continued outbreaks of plague in Lushoto district, Tanzania.*Acta Tropica*68(2):215-227.

Kilonzo, B. S., Mbise, T. J., Mwalimu, D. C. and Kindamba, L. (2006).Observations on the endemicity of plague in Karatu and Ngorongoro, northern Tanzania. *Tanzania Journal of Health Research* 8(1):1-6.

Kutz, F.W., Wood, P.H., and Bottimore, D.P. (1991).Organochlorine pesticides and polychlorinated biphenyls in human adipose tissue.In: Reviews of environmental contamination and toxicology(Edited by Kutz, F.W., Wood, P.H., and Bottimore, D.P.)Springer New York pp.1-82.

Kwong, T.C. (2002).Organophosphate pesticides: Biochemistry and clinical toxicology. *Therapeutic drug monitoring*24(1):144-149.

Makundi, R.H., Massawe, A.W., Mulungu, L.S., Katakweba, A., Mbise, T.J. and Mgode, G. (2008). Potential mammalian reservoirs in a bubonic plague outbreak focus in Mbulu District, Northern Tanzania, in 2007. *Mammalia* 72:253-257.

Mann, J.M., Martone, W. J. Boyce, J.M., Kaufmann, A.F., Barnes, A.M. and Weber, N.S. (1979). Endemic human plague in New Mexico: Risk factors associated with infection. *Journal of Infectious Diseases*140:397–401.

Marshall, J.D., Harrison, D.N., Murr, J.A. and Cavanaugh, D.C. (1972).The role of domestic animals in the epidemiology of plague. III. Experimental infection of Swine. *Journal of Infectious Diseases* 125:556-559.

McClean, K.L. (1995). An outbreak of plague in North-Western province, Zambia.*Clinical Infectious Disease* 21:650-652.

Meerburg, B.G., Singleton, G.R. and Kijlstra, A. (2009).Rodent-borne diseases and their risks for public health.*Critical Review Microbiology*35:221–270.

Ministry of Health, (1978). Plague in Kenya. Health information Bulletin, Communicable disease control, Nairobi, Kenya. 28pp.

Nakamura, I., Hang'ombe, B.M., Sawa, H., Kobayashi, S., Orba, Y., Ishii, A., Thomas, Y., Isozumi, R., Yoshimatsu, K., Mweene, A.S., Takada, A., Sugimoto, C. and Arikawa, J. (2013). Cross reactivity of secondary antibodies against African rodents and application for sero-surveillance. *Journal for Veterinary Medicine Science* 75: 819-825.

Neerinckx, S., Bertherat, E. and Leirs, H. (2010). Human plague occurrences in Africa: an overview from 1877 to 2008. *Transactions of the Royal Society of Tropical Medicine Hygiene* 104(2):97–103.

Ngulube, T.J., Mwanza, K., Njobvu, C.A. and Muula, A.S. (2006).Knowledge, attitudes and health responses towards plague in Petauke Zambia.*Tropical Doctor* 36:223-225.

Nyirenda, S.S., Hang'ombe, B.M., Kilonzo, B.S., Kabeta, M.N,Cornellius, M.and Sinkala, Y. (2017).Molecular, serological and epidemiological observations after a suspected outbreak of plague in Nyimba, eastern Zambia.*Tropical Doctor* 47(1):38–43.

Perry, R.D. and Fetherston, J.D. (1997).*Yersinia pestis*-Etiologic Agent of Plague.*Clinical Microbiology Reviews* 10:35-66.

Phiri, J. (2015). Bubonic plague kills 3 in Nyimba. *Times of Zambia*. Lusaka Apr 16.

Rahalison, L., Vololonirina, E., Ratsitorahina, M., and Chanteau, S. (2000).Diagnosis of bubonic plague by PCR in Madagascar under field conditions.*Journal of Clinical Microbiology*38(1):260-263.

Rajanna, C., Revazishvili, T., Rashid, M.H., Chubinidze, S., Bakanidze, L., Tsanava, S., Imnadze, P., Bishop-Lilly, K.A., Sozhamannan, S., Gibbons, H.S., Morris, Jr., J.G. and Sulakvelidze, A. (2010). Characterization of pPCP1 plasmids in *Yersinia pestis* strains isolated from the Former Soviet Union. International Journal of Microbiology 9:1-10.

Ratovonjato, J., Boyer, S., Rajerison, M. and Raheliniria, S. (2014).*Yersinia pestis* in Pulex *irritans* fleas during plague outbreak, Madagascar. *Emerging Infectious Disease*

20(3):1414-1415.

Riehm, J.M., Rahalison, L., Scholz, H.C., Thoma, B., Pfeffer M., Razanakoto, L.M., Dahouk, S.A., Neubauer, H. and Tomaso, H. (2011).Detection of *Yersinia pestis* using real-time PCR in patients with suspected bubonic plague.*Molecular and Cellular Probes* 25:8-12.

Rumbeiha, W. K. (2006). Cholecalciferol. In:Small Animal Toxicology, 2nd ed.(Edited byPeterson, M. E. and Talcott, P. A.) Elsevier Saunders: St. Louis, MO. pp.629-642.

Saeed, A.A.B., Al-hamdan, N.A. and Fontaine, R.E. (2005). Plague from eating raw camel liver. *Emerging Infectious Diseases* 11:1456-1457.

Sarovich, D.S., Colman, R.E., Price, E.P., Chung, W.K., Lee, J., Schuppo, J.M., Alexander, J., Keim, P. and Wagner, D.M. (2010). Selective isolation of *Yersiniapestis* from plague infected fleas. *Journal for Microbial Methods* 82:95-97.

Sinyange,N., Kumar,R., Inambao,A., Moonde,L., Chama,J., Banda,M., Tembo,E., Nsonga,B., Mwaba, J., Fwoloshi,S., Musokotwane,K., Chizema,E., Kapin'a,M., Hang'ombe, B.M, Baggett, H.C. and Hachaambwa, L. (2016).Outbreak of Plague in a High Malaria Endemic Region-Nyimba District, Zambia, March–May 2015. *Morbidity and Mortality Weekly Report*65:807-811.

Stenseth, N.C., Atshabar, B.B., Begon, M., Belmain, S.R., Bertherat, E., Carniel, E. and Rahalison, L. (2008). Plague: past, present and future. *PLoS Medicine5*(1):9-13.

Talcott, P. A.(2006). Strychnine. In:Small Animal Toxicology, 2nd ed.(Edited by, Peterson, M. E.,and Talcott, P. A.) Elsevier Saunders: St. Louis, MO.pp.1076-1082.

Taylor, P., Gordon, D.H and Isaacson, M. (1981).The status of plague in Zimbabwe.*Annals of Tropical Medicine and Parasitology* 75:165-173.

Thullier, P., Guglielmo, V., Rajerison, M. and Chanteau, S. (2003). Short report: Serodiagnosis of plague in humans and rats using rapid test. *American Journal of Tropical MedicineandHygiene* 4:450-451.

Titball, R.W. and Williamson, E.D. (2004).*Yersinia pestis* (plague) vaccines. *Expert opinion on biological therapy* 4(6):965-973.

Tsukano, H., Itoh, K.I, Suzuki, S. and Watanabe, H. (1996). Detection and Identification of *Yersinia pestis* byPolymerase Chain Reaction (PCR) UsingMultiplex Primers.*Microbiology and Immunology* 40(10):773-775.

Wagner, D.M., Klunk, J., Harbeck, M., Devault, A., Waglechner, N., Sahl, J.W., Enk, J., Birdsell, D.N., Kuch, M., Lumibao, C., Poinar, D., Pearson, T., Fourment, M., Golding, B., Riehm, J.M., Earn, D.J.D., Dewitte, S., Rouillard, J-M., Grupe, G., Wiechmann, I., Bliska, J.B., Keim, P.S., Scholz, H.C., Holmes, E.C. and Poinar, H.(2014). *Yersinia pestis* and the Plague of Justinian 541–543 AD : a genomic analysis. *Lancet. Infectious Disease*14(4):319-326.

Ware, G.W. and Whitacre, D.M., (2004).An introduction to insecticides. *The Pesticide Book.*Meister Pub. Willoughby, Ohio. 23pp.

Ziwa, M.H., Matee, M.I., Kilonzo, B.S. and Hang'ombe, B.M. (2013).Evidence of *Yersinia pestis* DNA in rodents in plague outbreak foci in Mbulu and Karatu Districts, northern Tanzania. *Tanzania Journal of Health Research* 15(3):1-8.

CHAPTER TWO

2.0 PAPER I: Potential roles of pigs, small ruminants, rodents and their flea vectors in plague epidemiology in Sinda district, Eastern Zambia

Stanley S. Nyirenda,[1,2,3] Bernard M. Hang′ombe,[4] Bukheti S. Kilonzo,[5] Henry L. Kangwa,[1] Evans Mulenga,[4] and Ladslav Moonga[4]

[1]Central Veterinary Research Institute, P.O. Box 33980, Balmoral, Lusaka, Zambia (stanleynyirenda@yahoo.co.uk;henrylombekangwa@yahoo.com,)

[2]Department of Microbiology and Parasitology, Sokoine University of Agriculture, Box 3019,Morogoro, Tanzania,

[3]Corresponding author, e-mail: stanleynyirenda@yahoo.co.uk,

[4]Department of Clinical Microbiology, TheUniversity of Zambia, P.O. Box 32379, Lusaka, Zambia (mudenda68@yahoo.com; ntongo2004@yahoo.co.uk; ladslavm@yahoo.com,) and

[5]Pest Management Centre Sokoine University of Agriculture, P.O. Box 3010, Morogoro, Tanzania (bskilonzo@yahoo.com)

Publication status Received 19 April 2016; Editorial decision 23 November 2016

Journal of Medical Entomology, Accepted for publication on November 24, 2016 as doi:10.1093/jme/tjw220

Citation: Nyirenda, SS., Hang′ombe,BM.,Kilonzo,BS., Kangwa, HL.,Mulenga, E and Moonga L. (2017). **Potential roles of pigs, small ruminants, rodents and their flea vectors in plague epidemiology in Sinda district, Eastern Zambia**.*Journal Medical Entomology***1-7. Doi:10.1093/jme/tjw220**

CHAPTER THREE

3.0 PAPER II: Molecular, serological and epidemiological observations after a suspected outbreak of plague in Nyimba, Eastern Zambia

Stanley S Nyirenda[1,2], *Bernard M Hang′ombe[3], Bukheti S Kilonzo[4], Mathews N. Kabeta[5], Mundia Cornellius[5] and Yona Sinkala[5]

[1]Central Veterinary Research Institute, Balmoral, Lusaka, Zambia

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

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

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

[5]Ministry of Fisheries and Livestock, Mulungushi House, Lusaka, Zambia

Corresponding author:

Prof. Hang′ombe M. Bernard, The University of Zambia, School of Veterinary Medicine, Department of Microbiology, P.O. BOX 32379, Lusaka 10101, Zambia

Email: mudenda68@yahoo.com

Publication status: Published in **Tropical Doctor,** volume 47(1):38–43

Citation: Nyirenda, SS., Hang'ombe, BM., Kilonzo, BS.,Kabeta, MN,Cornellius, Mand Sinkala, Y. (2017).Molecular, serological and epidemiological observations after a suspected outbreak of plague in Nyimba, eastern Zambia.***Tropical Doctor*** **47(1):38–43.**

3.1 Supplementary data on the techniques

3.1.1 Indirect ELISA testing

The antigen, supplied by National Institute for Communicable Diseases (NICD), Johannesburg, South Africa,was diluted 1:100 with Phosphate Buffered Saline (PBS) and 100µl was dispersed on Nunc ELISA plates and incubated at 37^{o}C overnight to dry the plates. Following fixation of the antigen onto the plates, blocking was done by adding 100µl of PBS-tween 20 (0.1%) with 1% Bovine Serum Albumin (BSA) and incubated at room temperature for 60 minutes. The plates were washed four times with Phosphate Buffered Saline-tween 20 (0.1%). The test sera were diluted with PBS-tween 20 (0.1%) with 1% BSA at the rate of 1:100 and 100µl of diluted sera were added to each well on the plates and incubated at room temperature for 60 minutes. The plates were washed four times with PBS-tween 20 (0.1%) and 50µl of diluted secondary antibody with PBS-tween 20 (0.1%) with 1% BSA at the rate of 1:1000 for rodents and pigs while 1:5000 for goats was added to the plates respectively and incubated at room temperature for 60 minutes.

The plates were washed four times with PBS-tween 20 (0.1%). The colour was developed by the addition of 50µl ABTS (2, 2'-Azino Bis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate (Roche Diagnostic, Mannheim, Germany) and the plates were incubated for 30 minutes at room temperature. Each plate contained two replicates of a negative control serum sample and two replicates of a positive control serum sample.Optical density was measured at 405nm in the ELISA reader.

Note: *For rodent sera, anti-rat IgG and anti-mouse IgG (Sigma Chemical MO, USA) were used, for pigs sera, anti-pig IgG sera (ICN, Biomedical, Aurora) and for goat sera, rabbit anti-goat IgG (Novus Biotechne, USA) were used as secondary antibody respectively.*

3.1.2 DNA extraction-Organs

About 10µg pieces of spleen and liver from each animal were put into the tube containing beads and 750µl lysis solution was added. The tubes were secured in the bead beater (Biospec Products, Bartlesville, USA) fitted with 2ml tube holder assembly and processed at maximum speed for 10 minutes. The tubes were centrifuged at 10,000 x *g* for 1 minute, after which 400µl of supernatant were transferred to a Zymo-Spin™IV Spin filter in a collection tube and further centrifuged at 7,000 x *g* for one minute. After centrifugation, 1,200µl of Genomic lysis buffer was added to filtrate in the collection tube, where 800µl of the mixture were transferred to the Zymo Spin™IC Column in a collection tube and further centrifuged at 10,000 x *g* for one minute. The flow was discarded through the collection tube. This was repeated and 200µl of DNA Pre-Wash buffer was added to Zymo Spin™IC Column and centrifuged at 10,000 x *g* for one minute. About 500µl of g-DNA wash buffer was added to the Zymo-Spin™IC column and centrifuged at 10,000 x *g* for one minute. The Zymo-spin™IC Column was then transferred to a clean 1.5ml microcentrifuge tube and 10µl DNA elution buffer was added directly to the column matrix. This was finally centrifuged at 10,000 x *g* for 30 seconds to collect the purified DNA.

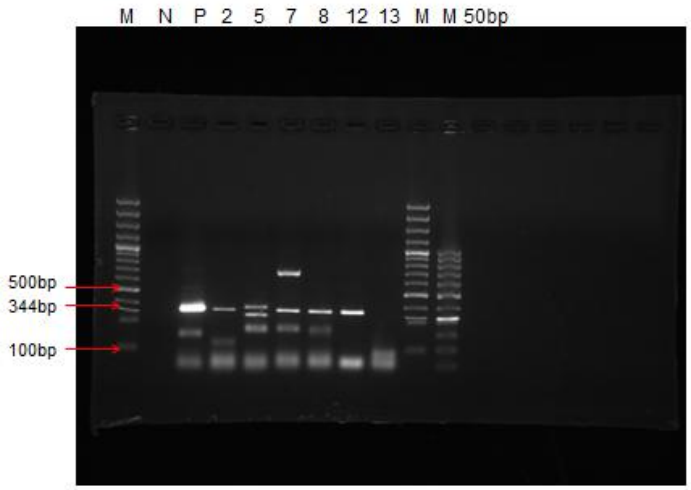


Figure 3.1: PCR of positive flea samples on agarose gel

Legend: M=Marker; N=Neg; P=Pos; 2,5,7,8,12 &13 samples

3.1.3 Blood Collection by Cardiac Puncture from the rat or mouse

Three possible approaches:

1. Hold the mouse or rat by the scruff of skin above the shoulders using the thumb and forefinger and the other fingers hold the rear legs so that its head is up and its rear legs are down.

Figure 3.2: Cardiac blood collection from the rat

2. Lay animal on back and push syringe vertically through sternum;
3. Lay animal on side and insert the needle perpendicular to the chest wall.

Use a 1 ml syringe and a 21 or 22 gauge needle. Insert the needle 5 mm from the center of the thorax towards the animal's chin, 5-10 mm deep, holding the syringe 25-30^{o} away from the chest (Fig. 3.5)

If the blood doesn't appear immediately, withdraw 0.5 cc of air to create a vacuum in the syringe. Withdraw the needle without removing it from under the skin and try a slightly different angle or direction. When blood appears in the syringe, hold it still and gently pull back on the plunger to obtain the maximum amount of blood available. Pulling back on the plunger too much will cause the heart to collapse. If blood stops flowing, rotate the needle or pull it out slightly.

CHAPTER FOUR

4.0 PAPER III: Factors that precipitated human plague in Zambia from 1914 to 2014 - An overview for a century (100 years)

Stanley S. Nyirenda[1,4], Bernard M. Hang′ombe[2], Buketi S. Kilonzo[3]

1- Central Veterinary Research Institute, Ministry of Fisheries and Livestock Lusaka, Zambia

2- Department of Clinical Microbiology, School of Veterinary Medicine, University of Zambia

3- Pest Management Centre, Sokoine University of Agriculture, Morogoro, Tanzania

4- Department of Microbiology and Parasitology, Sokoine University of Agriculture, Morogoro, Tanzania.

Corresponding author: stanleynyirenda@yahoo.co.uk

(Received 14 March 2016; Accepted 5 July 2016)

Published in the Journal of Zoonotic diseases; (2016); 1(1):1-14

Citation: Nyirenda, SS., Hang'ombe, BM. andKilonzo, BS. (2016). Factors that precipitated human plague in Zambia from 1914 to 2014-An overview for a century (100 years).*Journal of Zoonotic diseases,*1(1):1-14.

CHAPTER FIVE

5.0 PAPER IV: Identification of risk factors associated with transmission of Plague disease in Eastern Zambia

Stanley S. Nyirenda[1,2], *Bernard M. Hang'ombe*[3], *Jackson Mwanza*[1], *Robert Machang'u*[2] *and Bukheti S. Kilonzo*[4]

[1]*Central Veterinary Research Institute, Balmoral,P.O. BOX 33980, Lusaka, Zambia*

[2]*Department of Microbiology, Parasitology and Immunology, Sokoine University of Agriculture P.O. BOX 3019, Morogoro, Tanzania*

[3]*Department of Clinical Microbiology, The University of Zambia,P.O. BOX 32379, Lusaka, Zambia*

[4]*Pest Management Centre, Sokoine University of Agriculture,P.O. BOX 3010, Morogoro, Tanzania*

*Corresponding author:

Nyirenda Stanley S., Central Veterinary Research Institute, Balmoral, P.O. BOX 33980, Lusaka, Zambia. Email:stanleynyirenda@yahoo.co.uk, +260977801323

Publication status: Paper published in**American Journal of Tropical Medicine and Hygiene. 2017;97(3):826-830**

Citation: Nyirenda, S.S., Hang′ombe, B.M., Machang′u, R., Mwanza, J. and Kilonzo, B.S. (2017). Identification of Risk Factors Associated with Transmission of Plague Disease in Eastern Zambia. *Am.J. Trop. Med. Hyg.* 97(3):826-830.

CHAPTER SIX

6.0 PAPER V: Molecular epidemiological investigations of plague in Eastern Province of Zambia

Stanley S. Nyirenda*[1,2], Bernard M. Hang′ombe[3], Edgar Simulundu[4], Evans Mulenga[3], Ladslav Moonga[3], Robert S. Machang′u[2], Gerald Misinzo[2] and Bukheti S. Kilonzo[5]

[1]Central Veterinary Research Institute, Balmoral, Lusaka, Zambia

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

[3]Department of Paraclinical studies, School of Veterinary Medicine, The University of Zambia, Lusaka, Zambia

[4]Department of Disease Control, School of Veterinary Medicine, The University of Zambia, Lusaka, Zambia

[5]Pest Management Centre, Sokoine University of Agriculture, Morogoro, Tanzania

*Corresponding author: Stanley S. Nyirenda

Email: stanleynyirenda@yahoo.co.uk

6.1 Abstract

Background: Plague is a flea-borne zoonotic and invasive disease caused by a gram negative coccobacillus bacterium called *Yersinia pestis*. Plague has caused three devastating pandemics globally namely: the Justinian, Black Death and Oriental plague. The disease in the Eastern Province of Zambia has been reported in Nyimba and Sinda Districts in the past 15 years. The aim of this study was to investigate the molecular epidemiology of plague in the two affected districts. Polymerase Chain Reaction (PCR), targeting Plasminogen activator gene (*pla* gene) of *Y. pestis*, was performed on suspected human bubo aspirates (n=7), rodents (n=216), shrews (n=27) and fleas (n=1494). Of these, one positive sample from each source or host was subjected to sequencing followed by phylogenetic analysis.

Results: Theplasminogen activator gene (*pla* gene) of *Y. pestis* was detected in 42.8% bubo aspirates, 6.9% rodents, 3.7% shrew and 0.8% fleas. The fleas were from pigs (n=4), goats (n=5) and rodents (n=3). The sequencing and phylogenetic analysis suggested that the *pla* gene of *Y. pestis* in Nyimba and Sinda was similar and the isolates demonstrated a high degree of similarity with Antiqua strainsfrom the Republic of Congo and Kenya.

Conclusion: It can be concluded that *pla* gene of *Y. pestis*was present in various hosts in the two districts and the strains circulating in each district were similar and resembles those in the Republic of Congo and Kenya.

Keywords: *Yersinia pestis*, Plague, Phylogenetic analysis, Zambia

6.2 Introduction

Plague is a flea-borne zoonotic disease caused by *Yersinia pestis*, a gram-negative coccobacillus, non-motile and non-spore-forming bacterium. The genus *Yersinia* is

amember of the family *Enterobacteriaceae*, which consists of 11 species, of which *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*are human pathogens. *Yersinia pestis*is mainly transmitted by flea vectors, particularly *Xenopsylla* spp, but can also be transmitted mechanically by other flea species and hematophagous arthropods such as ticks and lice during epizootic periods[1,2]. The bacteria infect mammalsand rodents, which are considered to be the natural reservoirs once they survive the initial infection. The bacterium is believed to have originated from Central Asia, Transbajkalian and Mongolian steppes in the former Soviet Union (FSU) and spread out globally[3,4]. *Yersinia pestis* has caused one of the most devastating historical pandemics of the world. It has caused three plague pandemics that affected many people, who succumbed to the infection[5]. Though these pandemics originated from the same place, Justinian plague strain wereconstrained to be the direct ancestor of those associated with the second and third pandemics, also known as the Black Death and the Oriental plague, respectively. The Black Death pandemic, was the result of a separate emergence of *Y. pestis* from rodents into the human population and it gave birth to subsequent *Y. pestis* infections, including those that were responsible for the Oriental plague. The Black death spread in Europe and Africa, and probably back to China and the causal agent re-emerged as a new strain causing the outbreaks of plague that followed[6]. The third plague pandemic, probably started in the Yunnan province of China[7]. This episode spread to Hong Kong and later established fresh rodent foci in Asia, Africa, and North America,giving rise to an extant strain of the bacterium[8].

In Central and Southern Africa, there were possibly two routes in which the spread of the disease could have transpired. The first route of strain 1.ANT group in the second pandemic came through Egypt down to East and Central Africa, including the Uganda and Republic of Congo (Congo- Brazzaville). The second route of 1.ORI group could be in the

third pandemic and spread through Madagascar and Southern Africa during the 19th century. It has been documented by Ziwa *et al*. (2013), that plague was introduced to Eastern, Central and Southern Africa from the Middle East or the Far East by medieval traders, including slave and ivory caravans or via pilgrims to and from Egypt and Saudi Arabia[9].

Zambia, being a landlocked country, could probably be the intersection of the two routes of transmission, and it is most likely that the two different strains of the bacteria, 1.ANT and 1.ORI groups are present in this country (Fig 1). Zambia experienced plague outbreak in three zones,namely: Eastern, Southern and North western[10][11][12]. In the Eastern part of the country, four districts have reported Plague outbreaks since 1917. These are Chama, Lundazi, Sinda and Nyimba[11]. However, in the past 15 years, the disease has occurred in the latter two districts, with the latest outbreak occurring in the Nyimba district in March 2015[13]. No efforts have been made so far to identify the biotype or biovars of the bacterium in the area. The objective of this study was,therefore, to investigate the molecular epidemiology of plagueamong different potential reservoir hosts and their flea vectors in Sinda and Nyimba districts.

There are three biotypes (biovars) of *Y. pestis* recognised by their distinct biochemical characteristics in their ability to reduce nitrates (NO_3^-) to nitrites (NO_2^-) and ferment glycerol. Biotype Antiqua is positive for both of the biochemical characteristics, Orientalis biovar forms nitrites from nitrates but does not ferment glycerol because of a 93 bp deletion in the*glycerol 3-phosphate dehydrogenase (glpD)*gene, while Medievalis biovar ferments glycerol, but does not form nitrites from nitrates due to a G to T nucleotide mutation that results in a stop codon in the*napA*gene [14][15].

Based on the whole genome sequences to discover Single Nucleotide Polymorphisms (SNP), a global analysis demonstrated that the three *Y. pestis* strains could be separated into several populations with distinctive geographic patterns, including 0.ANT (Antiqua; Asia), 0.PE (Pestoides in group of Antiqua; Angola and Asia), 1.ORI (Orientalis; North and South America, Madagascar, Southeast Asia), 2.MED (Mediaevalis; Asia), 1.ANT (Antiqua; East and Central Africa), and 2.ANT (Antiqua*;* Asia), 3.ANT (Antiqua; China and Mongolia) and 4.ANT (Antiqua; Mongolia) [6][4].

Strains of the three biotypes demonstrate no difference in their virulence or pathogenesis in animals and humans[16]. Regardless of the biotypes or biovars, the bacterium possesses three plasmids which contribute to its virulence namely; Calcium Dependant (pCD1), Murine Toxin/Fraction1(MT1/pFra1) and Pesticin, Coagulase and Plasminogen activator(pPCP1). Plasmid PCP1 has 9.5 kb and is made up of fivedifferent genes namely:i) Pesticin (1,07 4bp *pst*), ii) Pesticin immunity- a transcriptional regulator gene (426 bp *pim*), iii) the replication regulation proteins (195 bp rop), iv) IS100 (made up of 1,02 kb transposase and 782 bp ATP-binding protein) and v) Plasminogen activator gene (939 bp *pla*gene)[17][18]. The *pla* gene, only found in *Y. pestis,* is 1.4 kb and has 936 bp open reading frame (ORF). It is responsible for cleaving fibrin deposits that trap the microorganisms. consequently, helps to facilitate dissemination of the bacteria in the host. This gene is targeted for molecular diagnosis and epidemiology of the disease in this study as described elsewhere[19][20][21].

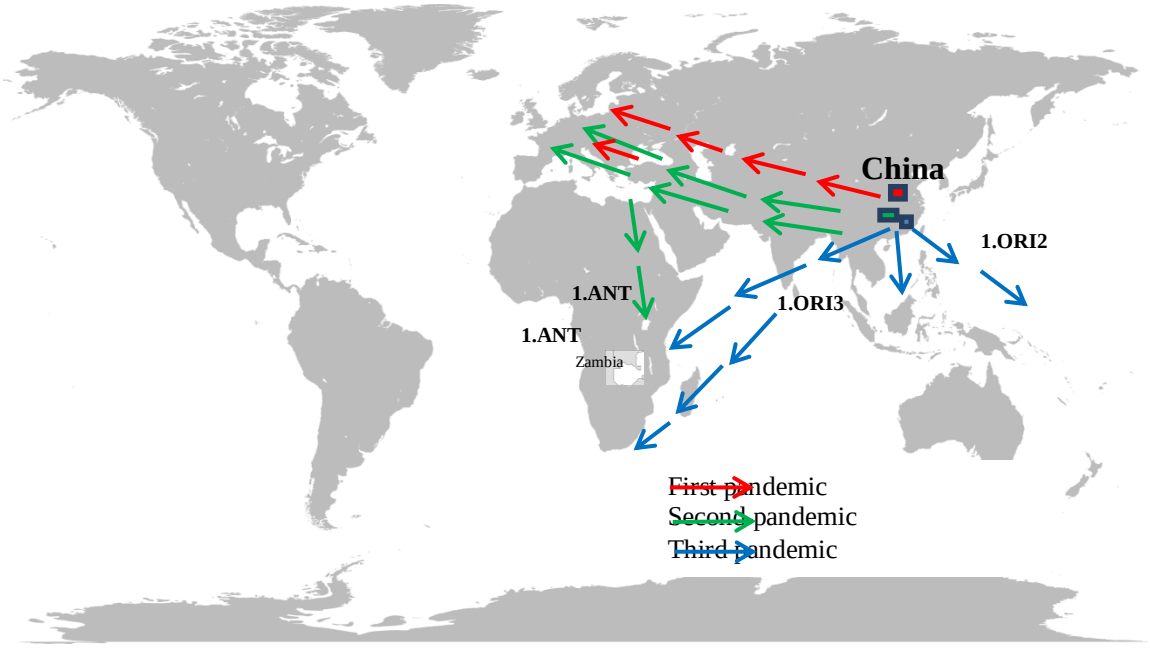


Figure 6.1: Geographical spread of *Yersinia pestis* from the suspected source to Africa

6.3 Materials and methods

6.3.1 Study areas

This study was carried out in the Eastern part of Zambia in the two districts of Sinda and Nyimba, both of which have a recent history of the plague outbreaks. These areas experience high rainfall between the months of January and March[11]. The study was conducted between March 2015 and August 2016.

6.3.2 Sample collection

6.3.2.1 Domestic animals

The villages were selected randomly and a maximum of 20 animals from each species, including pigs, goats and sheep, were sampled in each village upon obtaining the consent from the livestock keepers. Depending on the species, each animal was assigned a unique number. The first number was picked randomly followed by a systematic technique (Systematic Random Sampling). Each restrained domestic animal was laid on a

whiteplastic sheet and inspected for flea infestation. The animal was brushed with cotton wool soaked in 90% diethyl ether, to anaesthetise the ectoparasites, and then scrubbed with most an appropriate animal brush to remove fleas and other ectoparasites from its fur. Fleas fell onto the white plastic sheetand those which remained attached to the animal skin/fur were gently removed with a pair of fine forceps and put in small vials containing 70% ethanol.Animals below the age of six months and those which came from other villages, in the past six months, were excluded from the study.

6.3.2.2 Rodent and shrew trapping

Each selected village was divided into six zones from which three zones were selected at random. Sherman's live traps (50×65×157 mm) baited with peanut butter mixed with soya flour were set at a distance of 10m apart in the nearby bushes and left overnight. Wire cage traps (145×100×230 mm) (Hoga-lab, Kyoto, Japan), baited with *Stolothrissa tanganicae (Kapenta)* fish and tomatoes, were set in selected houses in the zones. Traps were inspected the following morning and captured animals were taken to a nearby mobile laboratory for organ and flea collection. Trapping continued for three consecutive days in the same area as previously described elsewhere[13].

6.3.2.3 Collection of fleas from rodents and shrews

Each captured rodent or shrew was put in a bag containing cotton wool soaked with 90% diethyl ether to anaesthetise the host and the ectoparasites. The small mammals were then transferred to a silver basin and brushed with a hard toothbrush to remove fleas and other ectoparasites. Fleas which fell into the basin and those which remained attached to the animal fur were collected with a pair of fine forceps and placed in small vials containing 70% ethanol.

6.3.2.4 Collection of organs and bubo aspirates

Each rodent or shrew was aseptically dissected and organs (spleen, liver, lung, kidney and heart) were collected, divided into two parts in two separate vials and stored at -20^{o}C until required for use. Clinically suspected human plague cases were examined and about 0.5 ml of bubo aspirate was collected and inoculated into 5 mlof Brain Heart Infusion (BHI) medium(Oxoid, Hampshire, England).

6.3.2.5 Flea Identification

Fleas were pooled (1 to 5) according to species and location, and from each pool, one to two fleas were removed, processed, and identified using main key features such as pronotal combs, genal combs, and the shape of head and reproductive organs (*spermathecae* in females and penis plates in males) as described by Kilonzo, 1999[22].

6.4 DNA extraction, PCR and sequencing

DNA extraction and PCR processing, from the rodent organs, human bubo aspirates and fleas, were performed as previously described[23][24] and five positive samples were selected for the purpose of sequencing to determine their biovarsor biotypes (Table 1).

DNA amplification was done using forward primers *Yp2 pla1* (5' ATC TTA CTT TCC GTG AGA AG3') and reverse primer Yp2 pla2 (5' CTT GGA TGT TGA GCT TCC TA 3'), which amplifies 479 bp region corresponding to nucleotides 971 to 990 and 1431 to 1450 of the *pla* locus sequence, respectively[18][25].PCR products were purified from agarose gel using a Wizard_SV Gel and Clean-Up System (Promega) in accordance with the manufacturer’s protocol. Purified PCR products weresubjected to cycle sequencing using a BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems). Products from the cycle sequencing reaction were purified by ethanol/EDTA/sodium acetate precipitation and separated on a 3130 Genetic Analyzer

(Applied Biosystems). Nucleotide sequences were assembled and edited using GENETYX ATGC software, version 4.0.10 (GENETYX Co., Tokyo, Japan), which was compared with other *pla* genesof *Y. pestis*elsewhere using basic local alignment search tool (BLAST) in MEGA 6 software as described previously[26].

6.5 Phylogenetic data analysis

Molecular evolutionary analyses were conducted using Molecular Evolutionary Genetics Analysis version (MEGA) version 6.0[27]. Phylogenetic analysis of the derived sequences was performed with these *Y. pestispla* gene reference sequences after an alignment was made via ClustalW. In the constructed phylogenetic tree, the sequences obtained were compared with other reference sequences of *Y. pestispla* gene obtained from the GenBank, homology search of *pla* gene of *Yersinia pestis* using BLAST technique[28]. The topological reliability of the trees was inferred by the bootstrap method with 1000 replicates.

6.6 Results

6.6.1 Molecular detection of *pla* gene of *Y. pestis*

A total of 216 rodents, 27 shrews, 245 pigs, 232 goats and 31 sheep were sampled for fleas, where a total of 22 fleas from rodents, 1456 fleas from pigs and 16 from goats, were collected. There were no fleas collected from shrews and sheep. Organs were collected from the rodents (n=216) and the shrews (n=27). Seven human bubo aspirates were also collected. Altogether, *Echidnophaga larina (*Jordan & Rothschild) (n=1064), *Xenopsylla cheopis* (Rothschild) (n=22),*Echidnophaga gallinacea* (Westwood) (n=389) and*Ctenocephaides canis* (Curtis) (n=19) were collected from the two districts.

The*pla* gene was detected in 6.9% rodents, 3.7% shrews, 42.9% human bubo aspirates and 0.94% fleas (Tables 6.1 & 6.2). The results also show that *Mastomys natalensis* had the highest number of positive *Y. pestispla* gene (Table 6.3).

Table 6.1: Flea vector species and their PCR results

District	**Host/source of fleas**	**Host sampled (n)**	**Fleas collected**	**Species of fleas collected**	**SFI**	**No. of PCR positive**
Nyimba	Rodents	120	15	*Xenopsylla cheopis*	0.12	3
	Shrews	17	0	-	-	-
	Pigs	2	3	*Ctenocephalides canis*	1.5	0
	Pigs	9	7	*Echidnophaga gallinacea*	0.8	4
	Goats	83	16	*Ctenocephalides canis*	0.19	5
	House	10	1	*Ctenocephalides canis*	0.1	0
Sinda	Pigs	121	382	*Echidnophaga gallinacea*	3.2	0
	Pigs	124	1064	*Echidnophaga larina*	8.6	0
	Goats	232	0	-	-	-
	Sheep	31	0	-	-	-
	Rodents	96	7	*Xenopsylla cheopis*	0.07	0
	Shrews	10	0	-	-	-
	Total	**855**	**1495**			**12**

Table 6.2: PCR results from rodents, shrews and humans

District	Animal spp	No. of animals sampled	PCR positive	Positive (%)
Nyimba	Rodents	120	6	5.0
	Shrews	17	1	5.9
	Human	7	3	42.8
Sinda	Rodents	96	9	9.4
	Shrews	10	0	0
	Total	**250**	**19**	

Table 6.3: PCR results of tissues from rodents (per specie) and shrews

Rodent spp	Sinda district		Nyimba district	
	No. sampled	No. positive (PCR)	No. caught	No. positive (PCR)
Mastomys natalensis	52	5	68	5
*Gerbillurus*spp	22	3	0	0
Rattus rattus	19	0	34	1
Crocidura spp	10	0	19	1
Saccostomus spp (Pouched mouse)	3	1	31	0
Steatomys parvus (Fat mouse)	0	0	5	0
Total	**106**	**9 (9.4%)**	**137**	**7 (5.1%)**

6.6.2 Sequencing and phylogenetic analysis

From the 31 positive amplicons for *pla* gene of *Y. pestis*, five amplicons one from each species were selected for sequencing; *Homo sapiens*(Human), *E. gallinacea*,*X. cheopis*,*Mastomysnatalensis*and *Gerbillinus* spp (Table 6.4). A phylogenetic tree was constructed with the known strains of*Y. pestis pla* gene,which included: Antiqua CP009903.1 (1.ANT), Nairobi CP010294.1 (1.ANT), Nepal 516 CP000307.1 (2.ANT), CO92 L109969.1 (1.ORI), KIM F053945.1 (2.MED), Angola CP009936.1 (0.PE3),

Eldorado CP009782.1 (1.ORI2), Microtus 9001 AE017046.1 (2.MED) and *Yersinia pestis* D182038 CP001592.1 (2.ANT) strains while sequences of the samples are in appendix 1.Strains from Nyimba demonstrated a high degree of similarity with *Y. pestis*of Antiqua (1.ANT) (Accession No. CP009903.1), isolated from a human in the Republic of Congo. In contrast, the Sinda strain showed that it was (98%-99%) related to the Nairobi strain (Accession No. CP010294.1), isolated from wild rodent in Nairobi, Kenya (Fig 6.2).

Table 6.4: Isolation frequency and host distribution of *Y. pestis* isolates in this study

Host		**Species**	**No. of *Y. pestis* DNA extracts**	**District**
Host	Rodent	*Mastomys natalensis*	5	Nyimba
		Mastomys natalensis	5	Sinda
		*Gerbillurus*spp	3	Sinda
		Rattus rattus	1	Nyimba
		*Crocidura*spp	1	Nyimba
		Saccostomus spp	1	Sinda
	Human	*Homo sapiens*	3	Nyimba
Vector	Fleas from Goat	*Ctenocephalides canis*	5	Nyimba
	Fleas from Pigs	*Echidnophaga gallinacea*	4	Nyimba
	Fleas from Rodents	*Xenopsylla cheopis*	3	Nyimba
		Total	**31**	

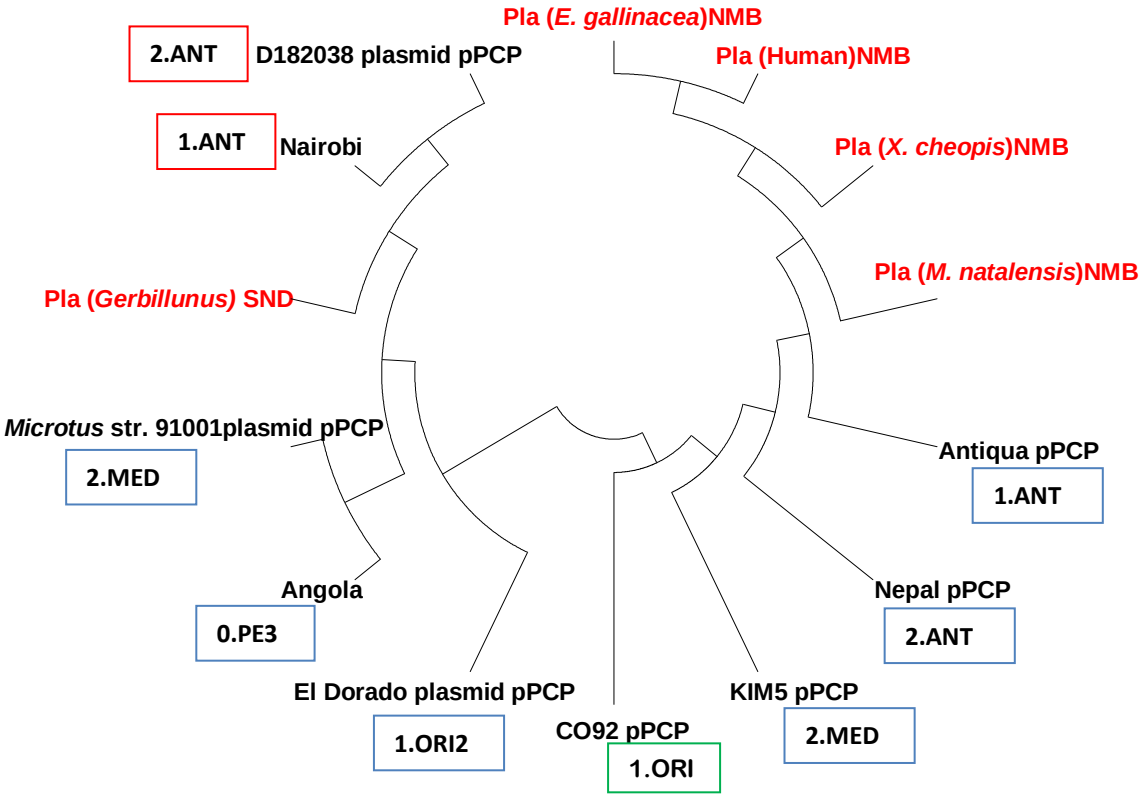


Figure 6.2: **Dendrogram exhibiting the similarity *Y. pestis* strains from Sinda and Nyimba districts using Neighbour-Joining clustering method. ANT=Antiqua, MED=Medievalis, ORI=Orientalis, PE= Pestoide**

6.7 Discussion

The presence of *pla*gene of *Y. pestis* in rodents, fleas, a shrew and human samples was an indication that the bacterium was present and was the cause of the disease outbreak in Nyimba, where sampling was done just after an outbreak of plague. The results showed that during the outbreak different species of flea vectors have the potential to acquire and mechanically transmit the bacterium to other animals as previously described [29]. In contrast, in Sinda district, the flea vectors were negative for *pla* gene, suggesting that during the quiescent period, fleas did not harbour the bacterium, although the latter was still isolated from the rodents, which are the natural reservoirs of *Y. pestis*. It is, therefore,

established that the bacterium was in circulation among the reservoir hosts and the disease could break out anytime if environmental conditions for both the hosts and flea vectors, become favourable.

From our findings, it is demonstrated the isolates from Nyimba districts were closely related to those of Antiqua (1.ANT) from the Republic of Congo, isolated from human samples. The Nyimba isolates may have originated from China and migrated during the trade voyages as the steamships sailed from the infected regions to non-infected areas of the world[30]. This is consistent with the findings by Morelli *et al*. 2010[4], that the isolates in the East and Central Africa involve the 1.ANT group, which they estimated was between 628 and 6,914 years and predated the trade voyages in China[8][31]. Intercontinental trade carried large consignments of cereals which provided a suitable environment for rodents harbouring flea-vector, *Rattusrattus*and *Xenopsylla cheopis* respectively, which are primary natural reservoirs and vectors of the *Y. pestis*[30].

The Sinda isolate was closely related to the Nairobi strain [32] (Fig 6.2). This entails that the strain may have originally spread from China to East Africa through the Nile route of Arab traders downsouth to Central Africa during the second pandemic [4,26]. Both strains may have migrated forth south probably along the Great Rift Valley during the seasonal migration of rodents along Rukwa valley down to Luangwa valley[33]. The plague outbreak in Nyimba occurred in the Luangwa valley, whereas Sinda is a few kilometres from the Valley but closer to Mozambique. As previously described by Davies, 1953[34], there was a similarity between the Luangwa Valley rodent species and those in Southern Tanganyika (current Tanzania) and Nyasaland (current Malawi) near the border with Northern Rhodesia (current Zambia). Our findings are consistent with those described by Davis *et al*. 1960[33], that the strain isolated from Mukomba in Lundazi district in eastern Zambia, were biochemically glycerol positive and reduced nitrates to nitrites indicating

that it was Antiqua strain of *Y. pestis*isolated[15]. This bacterium may have been transported along the valley from Northern to Southern Luangwa by either rodents or by human migration[35][30].

6.8 Conclusion

It is concluded that *Y. pestis* was present in the study area and the isolates from Nyimba district were similar despite coming from different hosts or sources. The Nyimba isolates demonstrated a high degree of similarity with Antiqua (1.ANT) strain from the Republic of Congo, while the Sinda strain was similar to Nairobi (1.ANT) strain in Kenya.

6.9 Disclosures

Abbreviations

PCR: Polymerase Chain reaction; MEGA: Molecular Evolutionary Genetic Analysis, BLAST: Basic Local Alignment Search Tool

6.9.1 Funding

This workwas supported by a PhD grant from the Intra-ACP Academic mobility scheme Africa (Mwalimu Nyerere) and the “Caribbean and Pacific under the commission of the European Union” and the project “Enhancing community of practice in one Health for Infectious diseases through post-graduate training” Grant No. 2012-3166/001, of the Sokoine University of Agriculture (SUA), Morogoro, Tanzania.

6.9.2 Acknowledgements

The authors would like to thank the Director, Department of Livestock in the Ministry of Fisheries and Livestock for supporting this work, members of staff of the Nyanje RCZ Mission Hospital, Chinambi Health Center, District Veterinary Offices and Community

health personnel in Nyimba and Sinda districts for organizing the villagers and the sampling team. We would like to thank the villagers for their co-operation during the study.

Authors' contributions

NSS designed and conducted the research and wrote the manuscript; ME, NSS, ML sample collection and analysis; HMB and SE laboratory protocols and data analyses; MG, KBS and SE read and edited the manuscript; MRS and HMBproofread the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for Publication

Not Applicable.

6.9.3 Ethics approval and consent to participate

Ethical approval to conduct this study was sought from the Institutional Ad hoc committee of the Sokoine University of Agriculture, Tanzania, the Biomedical Research Ethics Committee (BREC),Zambia. (Assurance No. is FWA00000338). Consent to participate was granted by persons whose animals and houses were sampled.

6.10 References

1 Kiefer D, Dalantai G, Damdindorj T, Riehm JM, Tomaso H, Zöller L, Dashdavaa O, Pfister K, and Scholz HC. Phenotypical Characterization of Mongolian Yersinia pestis Strains.*Vector Borne Zoonotic Dis.* 2012;12,(3):183–8.

2 Ratovonjato S, Rajerison J, Rahelinirina M and Boyer R. *Yersinia pestis* in *Pulex irritans* fleas during plague outbreak, Madagascar. *Emerg. Infect. Dis.* 2014; 20:1414–1415.

3 Li Y, Cui Y, Hauck Y, Platonov ME, Dai E, Song Y and Guo Z. Genotyping and Phylogenetic Analysis of Yersinia pestis by MLVA : Insights into the Worldwide Expansion of Central Asia Plague Foci. *PLos one.* 2009;4 (6).

4 Morelli PG, Song Y, Mazzoni CJ, Eppinger M, Roumagnac R, Wagner DM, Feldkamp M, Kusecek B, Vogler AJ, Li Y, Cui Y, Thomson NR, Jombart T, Leblois EC, Lichtner P Rahalison L, Petersen JM, Balloux F, Keim P, Wirth T, Ravel J, Yang R and Achtman M. Phylogenetic diversity and historical patterns of pandemic spread of Yersinia pestis. *Nat Genet.* 2010; 42(12):1140–1143.

5 Harbeck M, Seifert L, Hänsch S, Wagner DM, Birdsell D, Parise KL, Wiechmann I, Grupe G, Thomas A, Keim P, Zöller L, Bramanti B, Riehm JM, and Scholz HC. Yersinia pestis DNA from skeletal remains from the 6th century AD reveals insights into Justinianic Plague. *PLoS Pathog.* 2013; 9(5):e1003349.

6 Cui Y, Yu C, Yan Y, Li D, Li Y, Jombart T, Weinert LA. Historical variations in mutation rate in an epidemic pathogen , *Yersinia pestis*. *Proc Natl Acad Sci*. 2013;110(2):577–82. C

7 Pollitzer R. Plague Studies-A summary of history and a survey of the present distribution of the disease. *Bull. World Health Organ.*1951;4(4): 475–533.

8 Wagner DM, Klunk J, Harbeck M, Devault A, Waglechner N, Sahl JW, Enk J,. Birdsell DN, Kuch M, Lumibao C, Poinar D, Pearson T, Fourment M, Golding B, Riehm JM, Earn DJD, Dewitte S, Rouillard J, Grupe G, Wiechmann I,. Bliska JB, Keim PS,. Scholz HC, Holmes EC, Poinar H.*Yersinia pestis* and the Plague of Justinian 541 – 543 AD: a genomic analysis. *Lancet Infect Dis*. 2014;(13):1–9.

9 Ziwa MH, Matee MI, Hang'Ombe BM, Lyamuya EF, Kilonzo BS. Plague in Tanzania: An overview. *Tanzan J Health Res.* 2013;15(4):1–9.

10 Neerinckx S, Bertherat E and Leirs H. Human plague occurrences in Africa: an overview from 1877 to 2008. *Trans. R. Soc. Trop. Med. Hyg.* 2010;104:97-103

11 Nyirenda SS., Hang'ombe BM. and Kilonzo B. Factors that precipitated human plague in Zambia from 1914 to 2014-An overview for a century (100 years). *J Zoonotic Dis.* 2016;1(1):1–14.

12 McClean KL, An outbreak of plague in northwestern province, Zambia. *Clin. Infect. Dis. 1995;* 21(3): 650–2.

13 Nyirenda, SS, Hang'ombe BM, Kilonzo BS, Kabeta MN, Mundia, C, Sinkala Y. Molecular, serological and epidemiological observations after a suspected outbreak of plague in Nyimba, Eastern Zambia. *Trop Doct*. 2016;1(1):1–6.

14 Haensch S, Bianucci R, Signoli M, Rajerison M, Schultz M, Kacki S, Vermunt M, Weston DA, Hurst D, and Achtman M. Distinct Clones of *Yersinia pestis* Caused the Black Death.*PLoS Pathogens.*2010; 6(10):1–8.

15 Perry RD and Fetherston JD. *Yersinia pestis*--etiologic agent of plague. *Clin. Microbiol. Rev.* 1997;10(1):35–66.

16 Chain PSG, Hu P, Malfatti S, Radnedge L, Larimer F, Vergez LM, Worsham P, Chu MC, and Andersen GL. Complete genome sequence of *Yersinia pestis* strains Antiqua and Nepal516: evidence of gene reduction in an emerging pathogen. *J. Bacteriol 2006;*188(12): 4453–63.

17 Rajanna C, Revazishvili T, Rashid MH, Chubinidze S, Bakanidze L, Tsanava S, Imnadze, P, Bishop-Lilly KA, Sozhamannan S, Gibbons SH, MorrisGJJ, Sulakvelidze A. Characterization of pPCP1 plasmids in *Yersinia pestis* strains isolated from the former Soviet Union. *Int J Microbiol.* 2010;1–9.

18. Sodeinde O, Goguen J. Nucleotide sequence of the plasminogen activator gene of *Yersinia pestis:* relationship to ompT of *Escherichia coli* and gene E of *Salmonella typhimurium*. *Infect Immun*. 1989;57(5):1517–23.

19. Giles TA, Greenwood AD, Tsangaras K, Giles TC, Barrow PA, Hannant D, Yon L. Detection of a *Yersinia pestis* gene homologue in rodent samples. *Peer J.* 2016.e2216

20 Malek MA, Hammani A, Beneldjouzi A, Bitam I. Enzootic plague foci, Algeria. *New Microbes New Infect.* 2015;4:13–6.

21. Bos KI, Herbig A, Sahl J, Waglechner N, Fourment M, Forrest SA, Klunk J, Schuenemann JV, Poinar D, Kuch M, Golding GB, Dutour O, Keim P, Wagner DM, Holmes EC, Krause J, Poina HN, Broca P, Etudes H. Eighteenth century *Yersinia pestis* genomes reveal the long-term persistence of an historical plague focus. *eLife* 2016; 1–11

22 Kilonzo BS. Basic procedures for collecting, processing and identification of common fleas. Morogoro, Tanzania; 1999.

23 Hang'ombe BM, Nakamura I, Samui KL, Kaile D, Mweene AS, Kilonzo BS, Sawa H, Sugimoto C, Wren BW.Evidence of *Yersinia pestis* DNA from fleas in an endemic plague area of Zambia. *BMC Res Notes*. 2012;5(1):72.

24. Engelthaler DM, Gage KL, Montenieri J, Chu M, Carter LG. PCR detection of *Yersinia pestis* in fleas: comparison with mouse inoculation. *J Clin Microbiol*. 1999;37(6):1980–4.

25. Tsukano H, Itoh KI, Suzuki S and Watanabe H. Detection and identification of Yersinia pestis by polymerase chain reaction (PCR) using multiplex primers. *Microbiology and Immunology* 1996;40(10):773-775.

26 Ndashe K, Simulundu E, Hang BM, Ogawa H, Takada A, Mweene AS. Molecular characterization of infectious bursal disease viruses detected in vaccinated commercial broiler flocks in Lusaka, Zambia. *Arch Virol.* 2016;161(3):513–9.

27 Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA 6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution.* 2013;30(12):2725-9.

28 Bethesda (MD): National Library of Medicine (US) NC for BI. National Center for Biotechnology Information NCBI). USA; 1988. Available from: https://www.ncbi.nlm.nih.gov/

29 Andrianaivoarimanana V, Katharina K, Elissa N, Duplantie JM, Carniel E, Rajerison M, Jambou R. Understanding the Persistence of Plague Foci in Madagascar. *PLoS Negl. Trop.Dis.* 2013;7(11):2382.

30 Dennis E, Gage DT, Gratz KL, Poland N and Tikhomirov JD. Plague manual: Epidemiology, distribution, surveillance and control, Geneva, 1999.

31 Liang Y, Hou X, Wang Y, Cui Z, Zhang Z, Zhu X, Xia L, Shen X, Cai H, Wang J, Xu D. Genome rearrangements of completely sequenced strains of *Yersinia pestis*. *J. Clin. Microbiol.* 2010;48(5):1619-23.

32 Ayyadurai S, Flaudrops C, Raoult D, Drancourt M. Rapid identification and typing of *Yersinia pestis* and other *Yersinia* species by matrix-assisted laser mass spectrometry. *BMC Microbiol.* 2010;10(1):285.

33. Davis DH, Fisher BW, Goldring F. The Luangwa Valley plague outbreaks and their significance in relation to Savannah plague in Central Africa. *Bull World Health Organ.* 1960;23:405–8.

34. Davis DH. Plague in Africa from 1935 to 1949; a survey of wild rodents in African territories. *Bull World Health Organ*. 1953;9:665–700.

35 Wang H, Cui Y, Wang Z, Wang X, Guo Z, Yan Y, Li C, Cui B, Xiao X, Yang Y, Qi Z, Wang G, Wei B Yu S, He D,. Chen H, Chen G, Song Y, Yang R. A dog-associated primary pneumonic plague in Qinghai Province, China. *Clin. Infect. Dis.* 2011;52(2):185–90

CHAPTER SEVEN

7.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

7.1 General Discussion

The detection of IgG antibodies against *Fra1* antigen of *Y. pestis* in pigs, goats and sheep in this region of Zambia can suggest that these animals were previously or currently exposed to the bacterium. The possible exposure may be either by bites from infected fleas or by consuming carcasses infected with the bacterium as the animals are not confined but rather are allowed to roam freely within and around the villages and only come back to their shelters in the evenings (Goel *et al*., 2014). The results indicate that the animals may act as potential sentinel hostsfor plague in the area(Nelson *et al.*, 1985).

The demonstration of *Y. pestis* DNA plasminogen activator gene and IgG antibodies against Fra1 antigen in the rodents and shrews indicates that these animals were infected with the bacterium either through common flea bites or rarely from soil contamination, as the bacterium can persist in the soil for substantial periods of time(Ayyadurai *et al.*, 2010; Boegler*et al.*, 2012). The fact that *Mastomys*spp. had the highest infection rate as compared with other rodents is consistent with observations reported by Kilonzo *et al*., (2005) that *M. natalensis* was the major natural reservoir host of plague in Tanzania′s Lushoto district, playing an important role in maintaining the disease in the study area(Kilonzo *et al*., 2005).

The absence of*Y. pestispla* gene in all the collected fleas from rodents and pigs in Sinda district suggested that during quiescent periods, the flea ectoparasites do not harbour the bacterium. It has been previously reported that highly susceptible rodents with active *Y. pestis* infections are rare during interepizootic periods and if flea numbers are very low at

such times, it is also unlikely that sufficient numbers of fleas will become infected to maintain transmission at substantial rates. This can also be supported by the low population density of *X. cheopis,* the natural vector of plague, as measured by SFI (0.067) and a PII (2.8%). These findings in the recent study are consistent with the previous studies(Hinnebusch *et al*., 1998; Haule *et al*., 2013; Wimsatt and Bigginsb, 2009).The findings of the present study further suggest that fleas should be tested for *Y. pestis* DNA during the active phase of plague outbreaks for confirmation of infection and during interepidemic periods to confirm disease quiescence or detect infection activity. Ratovonjato *et al.* (2014) suggested that most species of fleas can at least briefly carry the plague bacterium during outbreaks and some are capable of transmitting the pathogens to other mammals at varying rates, depending on the flea specie involved (Ratovonjato *et al*., 2014).

The demonstration of *Y. pestis* DNA *pla* gene in five pools containing fleas from different animals in Nyimba district suggests that these arthropods were infected with the plague bacterium from an infected animal**.** As the rodents from where the fleas came were PCR negative, the insects must have fed on infected animals prior to infecting their current host animals. The findings that positively infected fleas were from different species, is an indication that any flea can acquire *Y. pestis* from infected animals and serve as the potential vector, host or carrier.These results are consistent with findings from Madagascar(Ratovonjato *et al.*, 2014).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.

The findings of this study suggest that socio-cultural behavior, climate and contact with the rodents largely contributed to the spread of plague disease in Zambia as observed in most

villages that experienced an outbreak of the disease. The type of behaviour included polygamy in which the husband move from one house to another in the same or different villages and in so doing he could move with infected fleas. Furthermore, witchcraft reportedly contributed to the spread of the disease in these areas as most people believed that the disease was caused by magic, or evil spirits hence most patients were reluctant to visit medical facilities for appropriate and prompt treatment(Ngulube *et al.*, 2006; Worsfold, 1955).

The findings in this study further suggest that heavy rains were important positive contributing factor to outbreak and spread of plague in Zambia, indeed, most outbreaks usually occurred between October and March; which are suitable for breeding of flea vectors as such most epizootic and human disease are favoured by sufficient populations of fleas and susceptible rodents(Perry and Fetherston, 1997). Likewise, heavy rainfall floods rodent burrows and consequently compel occupants together with their flea ecto-parasites to migrate to human habitations. Ben Ari *et al*. (2011), observed that abundance of rodent fleas was affected by suitable temperature, rainfall and relative humidity and that warm, moist weather was most suitable and associated with probable high flea index, as such weather has a direct effect on the development, survival, behaviour and reproduction of the insects(Ben Ari*et al.*, 2011).

The results revealed that most people in the studied communities commonly eat rodents, which are the most preferred hosts for flea vectors and potentially plague carriers. The rodent provides a ready source of protein in their diets, especially when other protein sources are scarce or expensive. Communities use various methods for catching, handling and preparing the animals. This culinary behavior is, therefore, associated with plague transmission in eastern Zambia. These rodent handling practices inevitably facilitate fleas to infest humans and other susceptible animals in the vicinity and spread plague if they are

carrying *Y. pestis*. The study also revealed that a good number of younger males were mostly involved in the rodent hunting, transportation and preparing. The younger males prepare the delicious meal on their own at a place traditionallycalled *Sangweni or Mphara*, a place where males gather, eat together and discuss how best they can live in the society. That could be the reason as to why most affected people with plague were younger males than adult males in most plague disease outbreaks (Davis *et al.*, 1960). The behavioral deeds are closely consistent with observation elsewhere including Kenya where herdsmen contracted plague after carrying goat kids on their shoulders (Ministry of Health, 1978). Wang *et al.*(2011) reported a case of a person infected with pneumonic plague after carrying a dead dog on his shoulders for burying it at the distance, and in Russia, where a ten-year-old boy got infected with *Y. pestis* after skinning a marmot (Will, 2016).

From our findings, it evidently sounds that all isolates from Nyimba districts were closely related to those of Antiqua (1.ANT) from Republic of Congo, isolated from human samples. This illustrated that Nyimba isolates may have originated from China and may have migrated during the trade voyages as the steamships migrated to non-infected areas of the world (Dennis *et al*., 1999). This is consistent with the findings elaborated by Morelli *et al*., (2010), who stated that the isolates in the East and Central Africa involve the 1.ANT group, which they estimated was between 628 and 6,914 years and slightly predated the trade voyages in China (Wagner *et al.*, 2014; Liang *et al.*, 2010). These intercontinental trade carried a large consignment of cereals which provided a suitable environment for the rodents and their flea-vector such as *Rattusrattus*and *Xenopsylla cheopis* respectively, which are suitable natural reservoirs and vectors of the *Y. pestis*(Dennis *et al*., 1999).

The Sinda strain or isolate was closely related to the Nairobi strain (Ayyadurai *et al.*, 2010). This entails that the strain might have originally spread from China to East Africa

through the Nile route by the Arab traders and migrated South to Central Africa during the second pandemic (Cui *et al.,* 2013). Both strains may have migrated to south probably along the Great Rift Valley during the seasonal migration of rodents and fleas along Rukwa valley down to Luangwa Valley (Davis *et al*., 1960) as the outbreak in Nyimba occurred in the Luangwa valley whereas Sinda is a few kilometres from the Valley but closer to Mozambique. As previously described by Davis(1953), there was a similarity of the Luangwa Valley rodent species with those in Southern Tanganyika (Tanzania) and Nyasaland (Malawi) near the border with Northern Rhodesia (Zambia). These findings are consistent with those described by Davis *et al.* (1960), who stated that the strain isolated from Mukomba in Lundazi district in eastern Zambia, were biochemically glycerol positive and reduced nitrates to nitrites. This shows that it was Antiqua strain of *Y. pestis*isolated. The bacterium might have been transported along the valley from Northern to Southern Luangwa by either rodent and their fleas, or by human migration (Wang *et al.*, 2011; Dennis *et al*., 1999).

7.2 Conclusion

Based on the results from the present study, the following conclusions are drawn.

1. Detection of specific anti-*Yersiniapestis* antibodies to Fra1 antigen in pigs, goats, rodents and sheep, and the *Y. pestis* DNA *pla* gene in rodents and fleas gives the vivid picture that the pathogen of plague is still in circulation in the study area. Hence, if environmental factors become favourable, plague can break out. The results have also revealed that the domestic animals may be considered as the sentinel of the plague in the endemic area.
2. The circulating *Y. pestis* isolate from the Nyimba and Sinda districts were similar despite coming from different hosts and was closely related to 1.ANT from both Republic of Congo and Nairobi respectively (Wagner *et al*., 2014; Liang *et al*., 2010).

3. Plague in Zambia usually occurs during hot and rainy season between October and March, which suitable environment for the breeding of fleas (Ben Ari *et al*., 2011) and that disease outbreaks are generally associated with increased rodent and flea populations (Nyirenda *et al*., 2016). This is consistent with the observation of Boisier *et al*. (1997) in Madagascar, where human plague occurred during the same period of time (Boisier *et al.*, 1997). Other factors which facilitated the disease outbreaks and spread included socio-cultural aspects such as beliefs in witchcraft, the lifestyle of residents such as polygamy, overcrowding in a single-roomed house and man-rodent contacts during rodent hunting and eating.
4. The pattern of plague in Zambia demonstrates the typical re-emerging nature of the disease and its ability to break out after many years of quiescence if favourable conditions prevail as observed in the Luangwa valley, where the first and second outbreaks occurred in 1917 and 1956, respectively, and in Chitokoloki in Zambezi plain, where the first, second and third outbreaks occurred in 1937, 1954 and 1994 respectively (Worsfold, 1955; Davies *et al.,* 1960). In Namwala, the first outbreak occurred in 1997 and the second occurred in 2007 (Neerinckx*et al.,* 2012; Hang'ombe *et al.,* 2012) while in Nyanje in Sinda, the first and second outbreaks occurred in 2001 and 2007 respectively (Ngulube *et al.,* 2006).
5. Improving surveillance service in established potential foci in Zambia is recommended in order to get a clear picture on the endemicity of the disease in the country. Data acquired from diagnosis and surveillance may serve as a basis for studying the epidemiology and ecology of the disease, which will facilitate early detection and subsequent appropriate control measures to reduce morbidity and mortality rates of the disease (Kilonzo *et al.,* 1997).
6. Most people come into contact with flea-ectoparasites on rodent carcasses following hunting, transportation and preparation of the small mammals for food. This exposes

them to the risk of being bitten by infective fleas and may acquire the disease (Eisen *et al*., 2014). The findings may also explain partially why there are still some suspected cases of plague in the study area.

7. Institution of education campaigns on the importance of preventing or reducing contact with fleas and rodent carcasses to minimize the transmission of the plague pathogen to humans and other warm-blooded animals.
8. The results of this study will serve as an important reference for government policy makers to support prevention and management of plague in the case of outbreaks in the communities. Further studies are needed to be carried out to design a diagnostic kit based on the local strains of the bacterium in order to facilitate early detection of the disease in the endemic foci.

7.3 Recommendation

The results of this study have shown that *Y. pestis* is circulating in the study area. It is recommended, therefore, that:-

1. Education campaigns should be instituted to sensitise the community through workshops, seminars, drama groups, artistic drawings and the use of reading materials on the dangers of the bacterium, translated into the local languages.
2. Indoor spraying or dusting to eliminate or reduce flea ectoparasites should be re-enforced in the community. Herdsmen and people working in the bush should apply insect repellent to prevent the fleas from jumping on them in plague-endemic areas. This should be given as a way of reducing the bite from these insects.
3. Plague Rapid Diagnostic Test (PRDT) kit should be introduced in the area to facilitate quick screening of the disease. This would help the clinicians to quickly diagnose and resume appropriate treatment. This should include also all plague endemic areas in the country.

4. It is recommended that plague surveillance should be conducted across the country using the sentinel animals such as rodents, dogs, cats, goats and sheep to identify the anti-plague antibodies.

7.4 References

Ayyadurai, S., Flaudrops, C., Raoult, D. and Drancourt, M. (2010). Rapid identification and typing of *Yersinia pestis* and other *Yersinia* species by matrix-assisted laser mass spectrometry. *BMC Microbiology* 10(1):285-291.

Ben-Ari, T., Simon, N., Gage, K. L., Kreppel, K., Laudisoit, A., Leirs, H. and Stenseth, N. C. (2011). Plague and Climate: Scales Matter. *PLoS Pathogens* 7(9):1-6.

Boegler, A.K, Christine, G.B., John, M.A., Katherine, M., Jennifer, H. L., Jeannine, P. M., Kenneth, G. L. and and Eisen, R. J. (2012). Evaluation of the infectiousness to mice of soil contaminated with *Yersinia pestis*-infected blood. *Vector Borne and Zoonotic Diseases* 12(11):948–952.

Boisier, P., Rasolomaharo, M., Ranaivoson, G., Rasoamanana, B., Rakoto, L., Andrianirina, Z., Andriamahefazafy, B. and Chanteau, S. (1997). Urban epidemic of bubonic plague in Majunga, Madagascar: epidemiological aspects. *Tropical Medicine and International Health* 2(5):422–427.

Cui, Y., Yu, C., Yan, Y., Li, D., Li, Y., Jombart, T. and Weinert, L. A. (2013). Historical variations in mutation rate in an epidemic pathogen, *Yersinia pestis.Proceedings of the National Acad Sciences* 110(2):577–582.

Davis, D. H. (1953). Plague in Africa from 1935 to 1949; a survey of wild rodents in African territories. *Bulletin of the World Health Organization* 9:665–700.

Davis, D. H., Fisher, B. W. and Goldring, F. (1960). The Luangwa Valley plague outbreaks and their significance in relation to Savannah plague in Central Africa. *Bulletin of the World Health Organization* 23:405–408.

Dennis, D.T, Gage, K.L, Gratz, N., Poland, J.D. and Tikhomirov, E. (1999). *Plague manual: Epidemiology, distribution, surveillance and control*. Geneva, 100pp.

Eisen, R. J., MacMillan, K., Atiku, L. A., Mpanga, J. T., Zielinski-Gutierrez, E., Graham, C. B., and Gage, K. L. (2014). Identification of risk factors for plague in the West Nile region of Uganda. *The American Journal of Tropical Medicine and Hygiene* 90(6):1047-1058.

Goel, S, Kaur, H, Gupta, AK, Chauhan, U and Singh, A. (2014). Socio-epidemiological determinants of 2002 plague outbreak in Himachal Pradesh, India: a qualitative study. *BMC public health* 14(1):325-331.

Hang'ombe, B.M., Nakamura, I., Samui, K.L., Kaile, D., Mweene, A.S., Kilonzo, B.S., Hirofumi, S., Chihiro, S. and Brendan, W.W. (2012). Evidence of*Yersinia pestis* DNA from fleas in an endemic plague area of Zambia. *BMC Research Notes*5(1):72-81.

Haule, M., Lyamuya, E. E., Hangrsquo, B. M., Kilonzo, B. S. and Matee, M. I. (2013). Investigation of fleas as vectors in the transmission of plague during a quiescent period in North-Eastern. Tanzania. *Journal of Entomology and Nematology*5(7):88-93.

Hinnebusch, B.J., Gage, K.L. and Schwan, T. (1998). Estimation of vector infectivity rates for plague by means of a standard curve-based competitive polymerase chain reaction method to quantify *Yersinia pestis* in fleas. *American Journal of Tropical Medicine and*

Hygiene 58:562–569.

Kilonzo, B.S., Mvena, Z.S.K., Machang'u, R.S. and Mbise, T.J. (1997). Preliminary observations on factors responsible for long persistence and continued outbreaks of plague in Lushoto district, Tanzania. *Acta Tropical* 68:215–227.

Kilonzo, B.S., Mhina, J., Sabuni, C. and Mgode, G. (2005). The role of rodents and small carnivores in plague endemicity in Tanzania. *Belgium Journal of Zoology* 135:119–125.

Liang, Y., Hou, X., Wang, Y., Cui, Z., Zhang, Z., Zhu, X., Xia, L., Shen, X., Cai, H., Wang, J., Xu, D., Zhang, E., Zhang, H., Wei, J., He, J., Song, Z., Yu, X., Yu, D. and Hai, R. (2010). Genome Rearrangements of Completely Sequenced Strains of *Yersinia pestis*. *Journal of Clinical Microbiology* 48(5):1619–1623.

Ministry of Health, (1978). Plague in Kenya. Health information Bulletin, Communicable disease control, Nairobi, Kenya. 28pp.

Morelli, G., Song, Y., Mazzoni, C.J., Eppinger, M., Roumagnac, P., Wagner, D.M., Feldkamp, M., Kusecek, B., Vogler, A.J., Li, Y., Cui, Y., Thomson, N.R., Jombart, T., Leblois, R., Lichtner, P., Rahalison, L., Petersen, J.M., Balloux, F., Keim, P., Wirth, T., Ravel, J., Yang, R., Carniel, E., Achtman, M. (2010). *Yersinia pestis* genome sequencing identifies patterns of global phylogenetic diversity. *Nature Genetics* 42(12):1140–1145.

Neerinckx, S., Bertherat, E. and Leirs, H. (2010). Human plague occurrences in Africa: an overview from 1877 to 2008. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 104(2):97–103.

Nelson, J., Decker, R., Barnes, A., Nelson, B., Quan, T., Gillogly, A. and Phillips, G. (1985).

Plague surveillance using wild boar and wild carnivore sentinels. *Journal of Environmental Health* 47(6):306–309.

Ngulube, T. J., Mwanza, K., Njobvu, C. A. and Muula, A. S. (2006). Knowledge , attitudes and public health response towards plague in Petauke. *Tropical Doctor* 36:223–225.

Nyirenda, S.S., Hang'ombe, B.M. and Kilonzo, B.S. (2016). Factors that precipitated human plague in Zambia from 1914 to 2014-An overview for a century (100 years). *Journal of Zoonotic Disease* 1(1):1–14.

Perry, R. D. and Fetherston, J. D. (1997). *Yersinia pestis*--etiologic agent of plague. *Clinical Microbiology Reviews* 10(1):35–66.

Ratovonjato, J., Rajerison, M., Rahelinirina, R. and Boyer, S. (2014). *Yersinia pestis* in Pulex irritans fleas during plague outbreak, Madagascar. *Emerging Infectious Diseases* 20:1414–1415.

Wagner, D. M., Klunk, J., Harbeck, M., Devault, A., Waglechner, N., Sahl, J. W., Enk, J., Birdsell, D. N., Kuch, M., Lumibao, C., Poinar, D., Pearson, T., Fourment, M., Golding, B., Riehm, J. M., Earn, D. J. D., Dewitte, S., Rouillard, J., Grupe, G., Wiechmann, I., Bliska, J. B., Keim, P. S., Scholz, H. C., Holmes, E. C. and Poinar, H. (2014). *Yersinia pestis* and the Plague of Justinian 541 – 543 AD : a genomic analysis. *Lancet Infectious Diseases* 14(4):319-326.

Wang, H., Cui, Y., Wang, Z., Wang, X., Guo, Z., Yan, Y., Li, C., Cui, B., Xiao, X., Yang, Y.,

Qi, Z., Wang, G., Wei, B., Yu, S., He, D., Chen, H., Chen, G., Song, Y. and Yang, R. (2011). A dog-associated primary pneumonic plague in Qinghai Province, China. *Clinical Infectious Diseases* 52(2):185–190.

Will, S. (2016). Fears of a mass Bubonic plague outbreak in Russia:Thousands given emergency vaccinations after boy contracts the disease. *The Daily Mail* 14 July, p.2.

Wimsatt, J. and Bigginsb, D.E. (2009). A review of plague persistence with special emphasis on fleas. *Journal for Vector Borne Disease* 46:85–99.

Worsfold, J. T. (1955). An outbreak of plague at Chitokoloki, Balovale. *Central African Journal of Medicine* 1(3):116–117.

APPENDICES

Appendix 1: Sequences of *pla* gene of the samples from different hosts

Nmae	Host	*Y. pestis* gene targeted	Nucleotide	Country	Accession number	Reference
Sinda	Rodent-*Gerbillurus*spp	Pla	5'TAATTTGACCTTCTCCATGCCCTGAAAGACGTGGAGAATGTCAAGGCAAAACAACAAAATGAGC GCCCCGTCATTATGGTGAAAAAGGTGATTTTTCACACATCTGAAGGTTGGTCAAACACCAACCTCC TTTCCACAGACATCCTCCCCGCTAGGGGAGGATGAAAAGAGAGATATGATCTGTATTTTTCAGAA GCGATATTGCAGACCCGCCGTCACAGTATAATTTTTATTGGAAATACCGGCAGCATCTCCGCCAAT AGAGACAGAATCTCCACTATTCTTATCAATGGTCTGAGTACCTCCTTTGCCCTCATCATATTTACTG TATGTAAATTCCGCAAAGACTTTGGCATTAGGTGTGACATAATATCCAGCGTTAATTACGGTACCA TAATAACGTGAGCCGGATGTCTTCTCACGGAAAGTAAGA3'	Zambia	This study	This study
Nyimba	Rodent – *Mastomysnatalensis*	Pla	5′CCGTGAGAAGACATCCGGCTCACGTTATTATGGTACCGTAATTAACGCTGGATATTATGTCACAC CTAATGCCAAAGTCTTTGCGGAATTTACATACAGTAAATATGATGAGGGCAAAGGAGGTACTCAG ACCATTGATAAGAATAGTGGAGATTCTGTCTCTATTGGCGGAGATGCTGCCGGTATTTCCAATAAA AATTATACTGTGACGGCGGGTCTGCAATATCGCTTCTGAAAAATACAGATCATATCTCTCTTTTCA TCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTCAGATGTGT GAAAAATCACCTTTTTCACCATAATGACGGGGCGCTCATTCTGTTGTTTTGCCTTGACATTCTCCAC GTCTTTCAGGGCATGGAGAAGGTCAAATTAGACATGGAACGCTACTCTCCTTCCTGTAGGAAGC3′	Zambia	This study	This study
Nyimba	Flea-*Xenopsyllacheopis*	Pla	5′CCGTGAGAAGACATCCGGCTCACGTTATTATGGTACCGTAATTAACGCTGGATATTATGTCACAC CTAATGCCAAAGTCTTTGCGGAATTTACATACAGTAAATATGATGAGGGCAAAGGAGGTACTCAG ACCATTGATAAGAATAGTGGAGATTCTGTCTCTATTGGCGGAGATGCTGCCGGTATTTCCAATAAA AATTATACTGTGACGGCGGGTCTGCAATATCGCTTCTGAAAAATACAGATCATATCTCTCTTTTCA TCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTCAGATGTGT GAAAAATCACCTTTTTCACCATAATGACGGGGCGCTCATTCTGTTGTTTTGCCTTGACATTCTCCAC GTCTTTCAGGGCATGGAGAAGGTCAAATTAGACATGGAACGCTACTCTCCTTCCTGTAGGAAGC3′	Zambia	This study	This study
Nyimba	Flea-*Echidnophaga. gallinacea*	Pla	5′CCGTGAGAAGACATCCGGCTCACGTTATTATGGTACCGTAATTAACGCTGGATATTATGTCACAC CTAATGCCAAAGTCTTTGCGGAATTTACATACAGTAAATATGATGAGGGCAAAGGAGGTACTCAG ACCATTGATAAGAATAGTGGAGATTCTGTCTCTATTGGCGGAGATGCTGCCGGTATTTCCAATAAA AATTATACTGTGACGGCGGGTCTGCAATATCGCTTCTGAAAAATACAGATCATATCTCTCTTTTCA TCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTCAGATGTGT GAAAAATCACCTTTTTCACCATAATGACGGGGCGCTCATTCTGTTGTTTTGCCTTGACATTCTCCAC GTCTTTCAGGGCATGGAGAAGGTCAAATTAGACATGGAACGCTACTCTCCTTCCTGTAGGAAGC3′	Zambia	This study	This study
Nyimba	Human *(Homo sapiens)*	Pla	5′CCGTGAGAAGACATCCGGCTCACGTTATTATGGTACCGTAATTAACGCTGGATATTATGTCACAC CTAATGCCAAAGTCTTTGCGGAATTTACATACAGTAAATATGATGAGGGCAAAGGAGGTACTCAG ACCATTGATAAGAATAGTGGAGATTCTGTCTCTATTGGCGGAGATGCTGCCGGTATTTCCAATAAA AATTATACTGTGACGGCGGGTCTGCAATATCGCTTCTGAAAAATACAGATCATATCTCTCTTTTCA TCCTCCCCTAGCGGGGAGGATGTCTGTGGAAAGGAGGTTGGTGTTTGACCAACCTTCAGATGTGT GAAAAATCACCTTTTTCACCATAATGACGGGGCGCTCATTCTGTTGTTTTGCCTTGACATTCTCCAC GTCTTTCAGGGCATGGAGAAGGTCAAATTAGACATGGAACGCTACTCTCCTTCCTGTAGGAAGC3	Zambia	This study	This study

Appendix 2: Questionnaire used in the survey

PLAGUE SURVEILLANCE QUESTIONAIRE

INTRA-ACP PLAGUE SURVEILLANCE IN CONJUNCTION WITH THE UNIVERSITY OF ZAMBIA AND SOKOINE UNIVERSITY OF AGRICULTURE IN TANZANIA

This questionnaire is meant to collect data or information on the activities related to rodents, domestic pigs, small ruminants and wild carnivores and plague in Sinda district in Eastern Zambia. The information provided will be useful to health personnel and other relevant authorities to understand the status of Plague endemicity in the area and consequently take appropriate measures to prevent further outbreaks of the disease. Plague disease had broken in 2001 and 2007 in Sinda district in Chief Nyanje, where a number of cases were recorded. This study is meant to investigate the disease in the potential reservoirs and the risk factors of human beings in acquiring the disease.

All information provided here shall be treated as **HIGHLY CONFIDENTIAL**

Please answer ALL the questions appropriately

Sex of respondent ☐ Age of respondent ☐

*Highest Education level ☐ **Employment ☐

*Grade 12, University degree, Diploma etc. ** Farmer, Teacher, Agriculturist etc.

Name of the village *(where the respondent is coming from)*: ……

Name of the Chiefdom: ……

Co-ordinates of the homestead: ……

1. Which types of rodent/s are mostly consumed in the area? (List them)

a		b	
c		d	
e		f	

2. Which is/are mostly hunted or caught rodent/s in the area?

a.		b.	
c.		d.	
e.		f.	

3. How do you catch or hunt these rodents? (List the methods and explain each method)

……

……

4. How many rodents do you normally catch per day or per night?

	Type/s	

5. How do you carry them from the point of catch to the point of processing? Explain the methods.

……

……

6. Have you observed fleas *(Nthata)* or any insects jumping on to you? (If Yes which fleas)……

7. How do you process the rodents prior to consumption? (List and explain the method)

……

……

8. When processing the rodents do you see or find some fleas, ticks, lice and/or mites? (If Yes, list them)

☐ Yes, please list them ☐ No

_____

_____

9. Which fleas are the most abundant in the area?

Dog fleas ☐ Cat fleas ☐

Rodent fleas ☐ Human fleas ☐

Others: ☐ Indicate from which animal _____

10. At which point do you experience fleas jumping from rodents to people or other animals?

During killing ☐ During transportation ☐

During processing ☐ Not at all ☐

11. What time of the year do you experience many fleas?

Rainy season	☐	Winter season	☐
Dry season	☐	All the seasons	☐

12. What type of domestic animals do you keep at your home? If none **GO TO 18**

Cattle	☐	No.	☐	Goat	☐	No.	☐
Dog	☐	No.	☐	Chicken	☐	No.	☐
Sheep	☐	No.	☐	Cat	☐	No.	☐

Others: _____

13. Do some or all the animals which you keep, sleep in the same house as people sleep?

YES ☐ Which animals: _____

_____

NO ☐ **GO TO 18**

14. Do you experience some insects or fleas which are found on animals are also found on the human bodies or human clothes?

YES ☐ NO ☐ **GO TO 18**

15. Do you know any effects or disease, they have on the human body? If yes, please list them

……

……

16. How common are these effects or diseases?

Common	☐	Very common	☐
Rarely	☐	Not at all	☐

17. Have you ever experienced any serious illness, including death of a family member? If yes

a) How many people died? ……

b) How many animals died? …… (Name the animals): ……

18. **FOR OFFICIAL USE ONLY**

Number and type of samples collected

	Blood	Fleas		Blood	Fleas
Sheep			Goat		
Pigs			Dogs		
Cats			Others		

Name of interviewer: ……. Signature: …….

Date: ……

Contact persons:-

Nyirenda Stanley

+260977801323

Email- stanleynyirenda@yahoo.co.uk

Appendix 3: Questionnaire used in the survey in *Chi Chewa*

PLAGUE SURVEILLANCE QUESTIONAIRE

INTRA-ACP PLAGUE SURVEILLANCE IN CONJUNCTION WITH THE UNIVERSITY OF ZAMBIA AND SOKOINE UNIVERSITY OF AGRICULTURE IN TANZANIA

Mafunso awa alingalira kufufuza nkhani ya zochitika za tunyama twa mthengo, nkhumba zoweta, mbuzi, mberere ndi matenda ya **Mbambavu (bubonic plague)** mu mzinda wa Sinda kum'mawa kwa Zambia. Mayankho awa azathandiza amene agwira ntchito muvipatala ndi atsogoleri ena kuti amvetsetse za matenda ambambavu ***(plague),*** amene ama pelekedwa ndi tu nthata ku chokera ku makhoswe, kuti ayang'ane bwino katetezgedwe ka matenda awa.Matenda awa, ambambavu, ana buka mmzinda wa Sinda muchaka cha 2001 ndi 2007 mu Mfumu Nyanje. Kafukufuku uyu ***(research)*** uza yang'ana pa nyama zimene zingathe ku sunga matenda awa ndi kupeleka ku anthu ndi zinyama zina zace.

Onse mayankho azankhala ya chisisi (**HIGHLY CONFIDENTIAL)**

Chonde yankhani mafunso onse moyenera

Mkazi kapena Mwamuna ☐ Zaka ☐

*Sukulu | | **Nchito | |

*Grade 12, University degree, Diploma etc ** Farmer, Teacher, Agriculturist etc

Dzina la muzi *(Kumene muchokela)* : ……..…

Dzina la Mfumu: ……

Co-ordinates of the homestead: ……

1. Ndi tunyama bwanji twa mu nthengo tomwe mukonda kudya? (Lembani mndandanda)

a		b	
c		d	
e		f	

2. Ni tunyama bwanji tumene mmagwira koposa?

a.		b.	
c.		d.	
e.		f.	

3. Muma tugwira munjira yotani? (Lembani ndi kufotokoza njirazo)

……

……

4. Kodi mumagwira tu ngati pa tsiku limodzi tu nyama utu?

	Mtundu	

5. Kodi mumanyamula bwanji ngati mwapha nyamazo? Fotokozani.

……

……

……

6. Kodi mumaona tu nthata kapena tuzilombo twina kumukwerani pa nthupi?

……

7. Kodi mumakonza bwanji nyamazo pakukonzekela kudya? (Lembani ndi kufotokoza)

……

……

……

8. Kodi mumaona tu nthata kapena tuzilombo twina monga kababa, nsabwe?

☐ Inde-fotokozoleni ☐ Ayi

_____

_____

9. Kodi ni tunthata bwanji tumaoneka kwambiri?

Twa galu ☐ Twa chona ☐

Twa mbewa ☐ Twa anthu ☐

Twina twace: ☐ Lembani nyama kwamene tu ma choka

10. Kodi ndi nthawi bwanji yamene muona tu nthata ku ku kwelani?

Yakupha	☐	Yopeleka ku nyumba	☐
Yakukonza nyamazo	☐	Kulibe nthawi yili yonse	☐

11. Kodi ndi nthawi bwanji pamene tu nthata tu khala twambiri?

Nthawi ya mvula	☐	Nthawi ya mphepo	☐
Nthawi ya dzuwa/chirimwe	☐	Nthawi zonse	☐

12. Kodi mumasunga zoweta zotani? Ngati mulibe yankhani gawo la 19

Ng'ombe	☐	Zingati	☐	Mbuzi	☐	Zingati	☐
A Galu	☐	Angati	☐	Nkhuku	☐	Zingati	☐
Mberere	☐	Zingati	☐	A Chona	☐	Angati	☐

Zina zache _____

13. Kodi ziweto zimene mwachula zima gona mu nyumba imodzi ndi anthu?

Indee ☐ Zoweta zotani _____

Ai ☐ **Yankhani gawo la 19** _____

14. Kodi mumaona tu zilombotu pa zowetazo?

Indee ☐ Ayi ☐ **Yankhani gawo la 19**

15. Kodi tu zilomboto tumaonekaso pa nthupi kapena zobvala za anthu? …….

16. Kodi mudziwako matenda amene tuzilomboto tu mapeleka kwa anthu? Ngati mwabvomera, zilembeni

……

17. Matenda amene awa (ochulidwa mu 16) amapezeka .....

Kwambiri ☐ Nthawi zambiri ☐

Sapezeka kwambiri ☐ ayi ☐ Sapezeka ☐

18. Kodi matenda awa anaphapo anthu kapena nyama? A) Indee B) Ayi

a) Anthu angati anafa kapena anakhuzidwa? Anafa ☐ Anakhuzidwa ☐

b) Nyama zingati zinafa kapena kukhuzidwa? Zinafa ☐ Zinakhuzidwa ☐

19. **FOR OFFICIAL USE ONLY**

Number and type of samples collected

	Blood	Fleas		Blood	Fleas
Sheep			Goat		
Pigs			Dogs		
Cats			Others		

Name of interviewer: ……. Signature: …….

Date: ……

Contact persons:-

Nyirenda Stanley

+260977801323

Email- stanleynyirenda@yahoo.co.uk

Or

Prof. B.M. Hang'ombe

+260977326288

Email- *mudenda68@yahoo.com*

Appendix 4: Consent to participate in focus group discussion

Consent to participate in Focus group discussion

You have been asked to participate in a focus group sponsored by The University of Zambia and Sokoine University of Agriculture. The purpose of the focus group is to try and understand plague related activities in the area. The information learned in the focus groups will be used to design public health messages intended to understand the association between plague reservoirs, agents and human contact.

You can choose whether or not to participate in the focus group and stop at any time. Although the focus group will be tape recorded, your response will remain anonymous and no names will be mentioned in the report.

There are no right or wrong answers to the focus group questions. We want to hear many different viewpoints and would like to hear from everyone. We hope you can be honest, even when your responses may not be in agreement with the rest of the group. In respect for each other, we ask that only one individual speaks at a time in the group and that responses made by all participants be kept confidential.

I, ……, understand this information and agree to participate fully under the condition stated above

Signed: ……. Date: ……..

Plague focus group participant Demographics		
Date:	**Time:**	**Place:**
What is your specialty/job title?	How long have you been leaving here?	How many plague or related cases have noticed since 2001?
What other sources of income do you have?	Your age (Years) ☐ 10-20 ☐ 21-30 ☐ 31-40 ☐ 41-50 ☐ 51-60 ☐ Over 60	Your gender: ☐ Male ☐ Female

Appendix 5: Focus group discussion welcoming remarks

FOCUS GROUP INTRODUCTION

WELCOME

Thanks for agreeing to be part of the focus group. We appreciate your willingness to participate.

INTRODUCTION

Moderator, assistant moderator

PURPOSE OF FOCUS GROUPS

We have been asked by the SUA and UNZA to conduct the focus groups

The reason we are having these focus groups is to find out activities associated with plague disease

We need your input and want you to share your honest and open thoughts with us.

GROUND RULES

1. **WE WANT YOU TO DO THE TALKING**

   We would like everyone to participate

   I may call on you if I have not heard from you in a while

2. **THERE ARE NO RIGHT OR WRONG ANSWERS**

   Every person's experience and opinions are important

   Speak up whether you agree or disagree

   We want to hear a wide range of opinions

3. **WHAT IS SAID IN THIS ROOM OR PLACE STAYS HERE**

   We want the society to feel comfortable sharing when sensitive issues come up

4. **WE WILL BE TAPE RECORDING THE GROUP**

   We want to capture everything you have to say

   We don’t identify anyone by name in our report. You will remain anonymous

Appendix 6: Plague surveillance focus group discussion (FGD) questionnaire

1a. Which types of rodent/s are mostly consumed in the area? (List them)

a		b	
c		d	
e		f	

1b. Which is/are mostly hunted or caught rodent/s in the area?

a.		b.	
c.		d.	
e.		f.	

2a. How do you catch or hunt these rodents? (List the methods and explain)……

……

……

2b. How many rodents do you normally catch per day or per night?

	Type/s

3. How do you carry them from the point of the catch to the point of processing? Explain

……

……

4. Have you observed fleas *(Thata)* or any insects jumping on to you?……

5. How do you process the rodents prior to consumption? (List and explain the method)

……

……

6. Which fleas are the most abundant in the area? ……

7. At which point do you experience fleas jumping from rodents to people or other animals?

During killing	☐	During transportation	☐
During processing	☐	Not at all	☐

8. What time of the year do you experience many fleas?

Rainy season	☐	Winter season	☐
Dry season	☐	All the seasons	☐

9. What type of domestic animals do you keep at your home?

Cattle	☐	No.	☐	Goat	☐	No.	☐
Dog	☐	No.	☐	Chicken	☐	No.	☐
Sheep	☐	No.	☐	Cat	☐	No.	☐

Others: _____

10. Do some or all the animals which you keep, sleep in the same house as people sleep?

YES ☐ Which animals: _____

_____

NO ☐

11. Do you experience some insects or fleas which are found on animals are also found on the human bodies or human clothes?

YES ☐ NO ☐

12. Do you notice these insects on your clothes? …….

13. Do you know any effects or disease, they have on the human body? If yes, please list them

……

14. How common are these effects or diseases?

Common ☐ Very common ☐

Rarely ☐ Not at all ☐

15. Have you ever experienced any serious illness, including death of a family member?

a) How many people died? ……

b) How many animals died? …… (Name the animals): ……

Name of the Moderator: …… Signature: …… Date: ……

Assistant Moderator: …… Signature: …… Date: ……