KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI, GHANA

COLLEGE OF SCIENCE

DEPARTMENT OF THEORETICAL AND APPLIED BIOLOGY

RODENT-BORNE PATHOGENS IN *MASTOMYS* SPECIES AND HUMANS IN GHANA

A THESIS PRESENTED TO THE DEPARTMENT OF THEORETICAL AND APPLIED BIOLOGY, KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

BY

SHIRLEY CAMERON ODOOM

(20068317)

JUNE, 2014

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DECLARATION

This dissertation is the outcome of research work undertaken by Shirley Cameron Odoom at the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon and Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, under the supervision of Professor K. Obiri-Danso, Professor D.A. Boakye and Professor W.K. Ampofo. This thesis either in whole or in part has not been presented elsewhere for another degree.

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DEDICATION

To Commander Karl C. Kronmann and my dear husband, Mr. Kwame Nimo-Paintsil.

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LIST OF ABBREVIATIONS

VHF-Viral Haemorrhagic Fever

LASV-Lassa virus

IFA-Indirect Immunoflourescence assay

ELISA-Enzyme-Linked Immunosorbent assay

RT-PCR-Reverse Transcription Polymerase Chain Reaction

AST-Aspartate transaminase

TCID- Tissue culture infectious doses

PFU-Plague forming units

GPC-Glycoprotein precursor protein

NP-Nucleoprotein

Z-Zinc-binding protein

LPS-Lipopolysaccharide

IgM-Immunoglobulin M

IgG-Immunoglobulin G

BLAST-Basic Local Alignment Search Tool

CDC-Center for Disease Control

NMIMR-Noguchi Memorial Institute for Medical Research

US NAMRU-3-United States Naval Medical Research Unit Number Three

EID-Emerging Infectious Diseases

PUU-Puumala Serotype

HTN/DOBV-Hantaan/Dobrava Serotype

LEPTO-Leptospirosis

ABSTRACT

Small mammals such as mice and rats are abundant in many regions throughout Sub-Saharan Africa, and some of these have been described as reservoirs for many human pathogens including Lassa fever. Lassa fever is a viral haemorrhagic fever unique to West Africa. Lassa virus, the aetiologic agent of Lassa fever is zoonotic, with *Mastomys* species as the reservoir. Outbreaks with high fatality rates have been reported in Nigeria and Sierra Leone. It is presumed to exist in West Africa, but there has been little research in most of these countries, despite reports of disease in travellers to Burkina Faso, Cote d'Ivoire, Ghana and Mali. In addition to Lassa virus, some of these small mammals are known to habour other agents such as Hantaviruses, *Borrelia crocidurae*, *Leptospira* and *Leishmania* species. The remarkable expansion of human population has led to increasing contact with these rodents, thereby disrupting their habitats and increasing opportunities for disease transmission. The magnitude of the potential for human disease involving rodent-borne agents in Ghana is largely unknown.

A recent risk map was therefore used to select study sites in Ghana in an attempt to help define the burden of some of these pathogens. Small mammals were collected, identified by species and screened for the presence of pathogens during the rainy seasons of 2010 and 2011 from ten communities in Ghana together with 657 human sera from healthy adults living in the same communities (7 predicted high risk and 3 low risk). Sherman collapsible traps were set on three consecutive nights in houses (indoors) and surrounding farmlands (outdoors) totaling 9,269 night traps.

In all, 764 small mammals were captured constituting ten genera of seventeen species: *Praomys* (n=333), *Mastomys* (n=231), *Mus* (*Nannomys)* (n=133), *Gerbilliscus* (n=6), *Crocidura* (n=21), *Rattus* (n=5), *Lophuromys* (n=3), *Lemniscomys* (n=2), *Taterillus* (n=29), and *Uranomys* (n=1). Of the total captured, 66% were trapped indoors with *Praomys daltoni* (312/504), the predominant species, followed by *Mastomys natalensis* (180/504). The pygmy mice, *Mus* (*Nannomys)* (130/260) were abundant in the outdoor captures which constituted 50%. There were more *Mastomys* occurrence in Upper West (n=36), Upper East (n=32) and Northern (n=75) regions than in the southern sector.

Whole blood of two rodents (0.3%) *Mus* (*Nannomys*) spp. was positive for Arenavirus presence by conventional polymerase chain reaction assay (PCR). All rodent lung tissues were negative for Hantaviruses (Dobrava and Puumala serotypes). Using an in-house enzyme-linked immunosorbent assay (ELISA), human serum showed evidence of Arenavirus antibodies in 34 samples (5.2%). Puumala and Dobrava serotype antibodies were also detected with commercial kit, constituting 11% and 12% respectively. Twenty one percent (21%) of the human serum showed evidence of *Leptospira* antibodies using SERION ELISA classic test kit. Human exposure to the zoonotic infections studied was observed to cut across all age groups. Seropositivity was highest for anti-LASV at site 7-Eastern region (29%), anti-hantavirus (Dobrava serotype) at site 10-Brong Ahafo region (26%), and anti-*Leptospira* at site 8-Northern region (19%). Fifty six individuals (21% of positives) had been exposed to more than one of the rodent-borne infections

tested, whereas 208 (78%) had been exposed to only one type of infection. Putative rodent reservoirs of the different pathogens found in the human sera were captured in most of the study sites but human exposure could not be linked to their presence in the rodents.

This study suggests that 40% of residents in rural farming communities in Ghana have been exposed to at least one rodent-borne disease (Arenavirus, Hantavirus, or *Leptospira*).

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Some species of mice have been shown to be reservoir hosts for a number of pathogens that cause human diseases. Many of these diseases have specifically been associated with *Mastomys* mice which are abundant in Sub-Saharan Africa (Fiedler 1988). Some of these diseases of wild animals, the zoonotic diseases, can be transmitted to humans when they come into contact with contaminated rodent excreta, tissues, or blood. Over 200 zoonotic diseases have been described, for which rodents serve as hosts for the etiologic agent (World Health Organisation, WHO, 2014). The rodent-borne haemorrhagic fevers represent one such group. The potential for human disease involving rodent-borne agents in Ghana is unknown. Knowledge of the extent of these rodent-borne diseases in Ghana is essential in making public health decisions to prevent outbreaks.

Lassa fever, one of the viral haemorrhagic fevers, is a disease of West Africa. It occurs when humans become infected by Lassa virus, an arenavirus carried by rodents. Most human infections are thought to be mild, but outbreaks of haemorrhagic fevers with considerable mortality also occur (CDC Lassa fever Fact Sheet, 2004). Lassa fever is one of several infections associated with rodents, which may affect the health of citizens of Ghana and other West African countries. In 2011, there were unconfirmed reports of deaths in Ghanaian citizens living in a rural area due to Lassa fever, but there have been no systematic studies to determine whether the virus is present in Ghanaian rodents (Dzotsi *et al*., 2012). If the virus is present in rodents in Ghana, then there could be a risk for outbreaks and unrecognized disease in rural areas. In most rural farming communities, rodent activities in houses normally increase during the dry and at the beginning of the rainy season as a result of a habitat change driven by the seasonally changing availability of food stuffs for the rodents. When the harvest is completed, the crops (groundnuts, maize, cassava, and rice) are stored

sequentially inside houses where farmers and their families live (Saltzmann 1978). Simultaneously, the fields are used for pasture or are burnt prior to being cultivated at the beginning of the next rainy season (Saltzmann 1978, personal observation). These rural farming practices may attract the rodents from the proximal cultivations into the houses. There is therefore a need to study what diseases may be affecting the health of the people as a result of the influx of the rodents.

Lassa fever was first described in 1969 in a town called Lassa, in Borno State, Nigeria located in the Yedseram river valley at the south end of Lake Chad (Frame *et al.*, 1970). Clinical cases of this disease had been known in Sierra Leone for over a decade earlier but had not been connected with this viral pathogen (Buckley and Casals, 1970; Frame *et al.*, 1970). The ancestors of Lassa virus may have spread through West Africa between 300 and 800 years ago, but were not recognized until 1969 (Ehichioya *et al*., 2011). Since 1969, there have been reported outbreaks of Lassa virus infection in various parts of Nigeria including Jos, Onitsha, Zonkwua, Abo Mbaise, Epkoma and Lafia (Carey *et al.*, 1972; Bowen *et al.*, 1975; Monath, 1975; Fisher-Hoch *et al.*, 1995; Menakayi, 2000). The virus is endemic in West African countries, with estimated 300,000500,000 cases annually and approximately 5,000 deaths (Ogbu *et al.*, 2007). Outbreaks of the disease have been reported from Sierra Leone, Liberia Nigeria, and human cases have also been reported from Guinea, Mali, Burkina Faso, and the Democratic Republic of Congo.

The reported animal host for Lassa fever virus is the Natal Multimammate Mouse (*Mastomys natalensis*), a rodent indigenous to most of Sub-Saharan Africa (Werner and Dietrich, 2004). No other animal host for this virus has since been described.

In some areas, human infection could occur from the consumption of the rodents themselves, but the most common mode of human infection is probably from ingestion of food (such as grain) or

inhalation of dust contaminated with rodent faeces or urine (Werner and Dietrich, 2004). Additionally, the blood of infected rodents contains high virus titers and possibly be a source of transmission during peridomestic rodent hunting in rural West Africa (ter Meulen *et al.,* 1996).

Outbreaks of Lassa fever can take place anytime of the year (McCormick, 1987). The virus cannot be spread through casual skin-to-skin contact without exchange of body fluids (McCormick, 1987). Person-to-person transmission of the disease can also occur through contaminated medical equipment, such as reused needles or when a person comes into contact with the virus in blood, tissue, or excretions of an infected individual (Fisher-Hoch *et al.,* 1995). Lassa fever occurs in all age groups and in both men and women. Persons at greatest risk are those residing in rural areas where *M. natalensis* is usually found, presumably in areas with significant close contact between humans and *Mastomys* rodents, such as subsistence farming communities where rodents have access to food stored in farmer's houses. Health care workers are at risk if proper barrier nursing and infection control are not maintained (WHO Newsletter, 2005).

Approximately 15-20% of patients hospitalized for the disease die from the illness; however, about 80% of human infections with the virus are mild or asymptomatic, and overall 1% of infections result in death (CDC Lassa fever Fact Sheet, 2004). Antibody prevalence to Lassa virus was described as 7% in Guinea, 15-20% in Sierra Leone and Liberia, and over 20% in Nigeria (Bloch, 1978; McCormick *et al.,* 1987; Bausch *et al.,* 2001). In a very limited study in Ghana, a seroprevalence of 3.8% has been reported (Emmerich *et al*., 2008).

Lassa fever was associated with high mortality in fetuses and pregnant women (Price *et al.,* 1988). For foetuses in early pregnancy, the death rate was 92%; that for the third trimester was 75%, and 100% in the neonatal period for full-term babies. The mortality rate for gravid women was 7% in the first two trimesters, 30% in the last trimester and 50% for pregnant women who

delivered within one month. The high mortality was related to the presence of high concentrations of the virus in both foetal tissue and in the placenta. It was suspected that maternal T-cells could not attack virus in the placenta due to lack of the expression of class I or class II Major Histocompatibility Class (MHC) antigens. On the contrary, the general mortality rate for non-pregnant women is 13% (Price *et al.*, 1988; Harper, 2004).

Previous observations indicate possible transmission of the disease from an endemic area to a non-endemic one. As a result of an incubation period of up to 21 days, the virus may be imported into parts of the world where the disease is not endemic as the infected person is asymptomatic or shows early unspecific signs of Lassa fever (McCormick, 1987). About 25 cases of imported Lassa fever have been reported worldwide (Woodruff *et al.*, 1973; Zweighaft *et al.*, 1977; Cooper *et al.*, 1982; Hirabayashi *et al.*, 1988; Mahdy *et al.*, 1989; Holmes *et al.*, 1990; Johnson and Monath, 1990). Four cases of the disease were imported into Europe in the year 2000 (Gunther *et al.*, 2000; WHO Records, 2000) and another case occurred in 2003 (CDC Report, 2003). At least two of these patients died from the disease (Schmitz *et al.*, 2002) showing that even state-of-the-art intensive care in Europe cannot necessarily prevent a fatal outcome.

In 2007, 960 serum samples from human subjects across West Africa were analysed to determine antibody response to Lassa virus (LASV) using a standard indirect immunofluorescence assay (IFA) and a highly sensitive reverse ELISA. These samples had been collected during previous population-based investigations for other tropical diseases. The samples from Ghana had been collected during studies on the presence of anti-malaria antibodies in the areas of Tamale (n=280), Agogo (n=100), and Kumasi (n=100). Three different LASV strains were used as antigens namely; Josiah strain from Sierra Leone, AV strain from Ivory Coast and CSF strain from Nigeria. In 88 of the 960 subjects (Guinea, Liberia, Ivory Coast, Ghana, Benin and Nigeria) anti-Lassa antibodies

were detected with both assays. The antibody prevalence according to geographic region was: 12% in Macenta and 20% in Gueckedou (both in Guinea); 20% in Ivory Coast; 3.8% in Ghana; 9.9% in Benin; and 10.3% in Nigeria (Emmerich *et al*., 2008). This study also noted that strain specific antibodies were important, and seroprevalence in Ghana may be underestimated if an unknown strain circulating in Ghana did not closely match the strains used for testing.

Increasing inter-border traffic and international travels, necessitates the need for better information about Lassa virus and the disease it causes. On the other hand, the discovery that genetic distance among Lassa virus strains shows a relationship with geographic distance suggests that the breeding populations of *Mastomys* species, the rodent hosts of the virus, have exhibited little regional movement since Lassa fever was first recognized in the 1960s (Bowen *et al.,* 2000). However, the geographically restricted occurrence of the disease is not well understood as its rodent host is prevalent in many regions of sub-Saharan Africa (Granjon *et al.,* 1997). Research is therefore urgently needed as there are undefined increasing viral haemorrhagic fevers and febrile illnesses of unknown etiology, to determine if Lassa virus is present in *Mastomys* species populations in any of the countries separating Nigeria from Guinea;

Liberia and Sierra Leone including Cote D'Ivoire, Ghana, Togo, Benin and Burkina Faso.

Hantavirus is another agent known to cause haemorrhagic fever with renal syndrome (HFRS) in Europe and Asia which can lead to hantavirus cardio-pulmonary syndrome (HCPS) in America but other types of the virus have not been associated with human disease (Martinez *et al*., 2005). The virus is transmitted to humans via rodent bites and aerosol derived from rodent excreta but human-to-human transmission has been reported with the Andes virus in South America (Martinez *et al.,* 2005).

Since 2006, indigenous hantaviruses have been reported from Africa. The Sangassou strain was found in an African wood mouse (*Hylomyscus simus*) in Guinea (Klempa *et al.,* 2006). Discovery of newer African hantaviruses, Tanganya strain and recently Azagny strain, were even more surprising because they were found in shrews (Klempa *et al.*, 2007, Kang *et al.,* 2011). The detection of hantaviruses in small mammals other than rodents, such as shrews and moles (Kang *et al.,* 2011), raises questions regarding actual hantavirus host range.

In addition to viral haemorrhagic fevers, rodents are known or suspected of harboring other human pathogens. In particular, *Borrelia crocidurae*, the causative agent of Tick Borne Relapsing Fever in West Africa, may be important in Ghana. *B. crocidurae* is transmitted by the tick, *Ornithodoros sonrai*, an ectoparasite of reservoir rodents such as *Mastomys* species. A study found that about 10% of febrile patients in neighbouring country Togo, were PCR positive for *B. crocidurae* (Nordstrand *et al.,* 2007). *Mastomys* and related rodent species in West Africa have also been described as reservoirs for *Leptospira* and *Leishmania* species (Gratz, 1997).

Human leptospirosis (*Leptospira* spp. infection) is a worldwide public health problem that is of greatest concern for humid tropical and subtropical regions. The magnitude of the problem in these areas is larger not only because of the climatic and environmental conditions the bacteria face outside their hosts but also because of the frequency of contacts between people and sources of infection. Leptospirosis affects all mammals and can spread between domestic pets, livestock, wild animals and humans. In Africa, it is thought that commensal rodents are the most important source of transmission to humans (Dalu and Feresu, 1997; Machangu *et al.*, 1997; WHO Leptospirosis Manual, 2003). Leptospirosis infection in humans presents with symptoms that are similar to that of other better known parasitic, viral and bacterial infections such as malaria, Rift valley fever and

brucellosis. Hence, it may be frequently misdiagnosed and its impact on African communities is largely undocumented. As very little data has been documented on its occurrence in Ghana, we sought to assess the existence of the exposure to the bacteria in the human population in this study.

Leishmaniasis has not been frequently reported from Ghana, and an outbreak in the Ho District of the Volta Region, was investigated by the Naval Medical Research Unit Number 3 (NAMRU3) and the Noguchi Memorial Institute for Medical Research (NMIMR) in 2005. While the reservoir for this outbreak was never determined, rodents have been identified as a reservoir for this parasite in other areas of Africa, notably in Senegal, Nigeria and Kenya (Dedet *et al.,* 1981; Githure *et al.,* 1984; 1986; Ikeh *et al.,* 1995).

1.2 Rationale

Rodents such as the multimammate rats are abundant in many regions throughout Sub-Saharan Africa, and have been described as reservoirs for many human pathogens (Hugh-Jones *et al.,* 1995). Lassa fever is a viral hemorrhagic fever unique to West Africa, for which *Mastomys* rodents are the reservoir. Lassa fever outbreaks with high fatality rates have occurred in Nigeria and Sierra Leone, but it is also thought to be a minor illness in most of those infected in endemic areas (McCormick *et al.,* 1987). Rodents harboring the virus are asymptomatic and can shed the virus for months. Lassa fever has been reported in travellers to West Africa, and has been associated with significant nosocomial spread (Macher and Wolfe, 2006). Unfortunately, very little is known about the prevalence of the virus in most countries of West Africa. It is presumed to exist in all West African countries, but there has been little research in most of these countries, despite the cases reported in travelers to Benin, Cote d'Ivoire, Ghana and Mali (Saltzmann, 1978; Gunther *et al.,* 2000; Akoua-Koffi *et al.,* 2006; Atkin *et al.,* 2009). Laboratory confirmed cases of Lassa fever were reported in the Ashanti Region of Ghana by the Ministry of Health (Dzotsi *et al.*, 2012).

Experts in the field have pointed to the need for studies to examine the prevalence in rodents (Ogbu *et al.,* 2007). These studies are important to understanding the potential for outbreaks and to guide public health interventions.

A risk map of Lassa fever in West Africa used models derived from environmental data (such as rainfall, temperature, vegetation) and disease data from humans and rodents where available (Fichet-Calvet & Rogers 2009). The authors of this publication concluded that 10% of the area of Ghana was a Lassa fever risk area, with areas of risk, concentrated in bands across the northern and southern ends of the country.

There have been reports of novel hantavirus genome sequences in Africa, Sangassou virus (SANV) in the African wood mouse (*Hylomyscus simus*) and Tanganya virus (TGNV) in the Therese's shrew (*Crocidura theresae*) both discovered in Guinea (Klempa *et al.,* 2006; 2007). A seroepidemiological study in 2010, in the forest region of the same country clearly showed that local hantaviruses infect humans (seroprevalence of 1.2% in the general population). Other hantaviruses, Azagny virus harbored by the West African pygmy shrew (*Crocidura obscurior*) and Magboi virus detected in the slit-faced bat (*Nycteris hispida*) have been found in the neighboring countries Cote d'Ivoire (Kang *et al*., 2011) and Sierra Leone (Weiss *et al*., 2012), respectively. These findings further confirm that hantavirus infections in West Africa need to be considered.

The precise incidence and distribution of TBRF in West Africa are unknown (Rodhain, 1976; Trape, 1991; Trape *et al*., 1996). Reports of the disease are few and the distribution of the vector seems typically limited to the Sahel and Saharan regions (Trape *et al*., 1996); however, findings of investigations done in Senegal indicate that TBRF is, after malaria, the most common cause of outpatient visits to a rural dispensary near Dakar. Levels of TBRF infection in small communities infested with *O. moubata* (*B. duttonii*) in east Africa (Tanzania, Zaire) and *O. sonrai* (*B.*

Crocidurae) in West Africa (Senegal, Mauritania, Guinea) can be high with up to 24.4% of febrile patients in local medical facilities being infected and as such TBRF should be considered an important public health issue (Dupont *et al*., 1997; Vial *et al*., 2006).

Leptospirosis disease is a global condition found across the world. Rates of leptospirosis are highest in tropical and sub-tropical locations, particularly in rural areas. However, the condition is becoming increasingly widespread in urban areas that have poor sanitation. Up to 80% of individuals in tropical areas are estimated to have positive seroconversion rates, indicating either past or present infection (Levett and Haake, 2009).

Leishmaniasis is also endemic in West Africa (Sheik-Mohamed and Velema, 1999). Although the disease has a long history in West Africa, it appears to be one of the less recognized or underreported parasitic infections in this region (Desjeux *et al*., 1981). The first known cases of cutaneous leishmaniasis (CL) in the Volta Region of Ghana were recognized in 2002 (Kweku M., personal communication), based on the histological examination of biopsy samples. Since then, there have been sporadic, unconfirmed outbreaks in different communities all over the Ho District. Leishmaniasis is not known to be transmitted from humans to humans; the unpredictable nature of the outbreaks is an indication that the parasite may be harbored by certain animal populations and is picked up by one or several vector species which then incidentally infect humans.

1.2.1 Aim of Study

The aim of this project was to look for evidence of rodent-borne pathogens, particularly Lassa virus in Ghana.

1.2.2 Specific objectives

The study sought to:

1. Collect and identify rodents in and around ten selected sites in Ghana (one site in Upper West region; three sites in the Northern region; one site in Upper East region; three sites in Brong Ahafo region; one in Ashanti region and one in Eastern region);
2. Screen rodents for the presence of Lassa virus and other human pathogens in rodent samples using real-time Polymerase Chain Reaction (RT-PCR);
3. Determine human seroprevalence rates for antibodies to Lassa virus, and other rodentborne infections, in rural areas.

CHAPTER TWO

LITERATURE REVIEW

2.1.1 Lassa fever-the disease

Lassa fever is an acute viral zoonotic illness caused by an arenavirus (family *Arenaviridae*) responsible for a severe haemorrhagic fever characterized by muscle aches, sore throat, nausea, vomiting and chest and abdominal pain (Centers for Disease Control and Prevention, CDC, Fact Sheet 2004). The family *Arenaviridae* comprises 23 virus species (Charrel and de Lamballerie, 2003). It is serologically, phylogenetically, and geographically divided into two major complexes, the Old World complex (Africa, Europe, and Asia) and the New World complex (North and South America). The Old World complex consists of the prototype arenavirus lymphocytic choriomeningitis virus (LCMV), Lassa virus, Mopeia virus, Mobala virus and Ippy virus. The New World complex is larger and includes Tacaribe virus, Pichinde virus, Junin virus, Machupo virus, Sabia virus, and Guanarito virus. Virus species differ in antigenicity, geographical occurrence, natural host species and their potential to cause disease after transmission to humans.

The majority of arenaviruses are not associated with human disease. However, Lassa, Junin, Machupo, Guanarito and Sabia viruses can cause viral haemorrhagic fever (VHF) upon transmission to humans (Peters, 2002; Charrel and de Lamballerie, 2003).

Lassa virus exhibits constant, asymptomatic infection, with profuse urinary virus excretion in *Mastomys natalensis*, the reservoir host. The blood of infected rodents contains high virus titers which may be a source of transmission during peridomestic rodent hunting in rural West Africa (ter Meulen *et al*., 1998). Transmission between humans has been reported as a result of exposure to blood, sexual contact and breast feeding (McCormick *et al*., 1987).

The incubation period of Lassa fever in infected humans ranges from 1 to 24 days (Mertens *et al*., 1973) and 7 to 18 days (McCormick *et al*., 1987). The disease is associated with a wide spectrum of clinical manifestations. It is estimated that 80% of human infections are asymptomatic or have a mild self limited febrile illness (CDC Lassa fever Fact Sheet, 2004). Other times, the disease progresses to severe and often fatal hemorrhagic fever. Patients presenting with Lassa fever, may initially have signs and symptoms similar to malaria, influenza or viral gastroenteritis, making it clinically difficult to distinguish the disease from other febrile illnesses seen in West African hospitals (Monath *et al*., 1974; Bausch *et al*., 2001). Pharyngitis which is often exudative and characterized by elevated liver enzymes, particularly aspartate transaminase (AST), and proteinuria are frequent signs (White, 1972). Some clinical complications include pleural and pericardial effusions, facial edema, neurological manifestations and bleeding from mucosal surfaces (Knobloch *et al*., 1980; McCormick *et al*., 1987). Patients often go into shock in the terminal stage, although mucosal bleeding itself is usually not of a magnitude to produce shock (McCormick *et al*., 1986; Fleischer *et al*., 2000).

Most patients are usually viremic by day 3 of infection (Johnson *et al*., 1987; Demby *et al*., 1994). Persistent viremia is a poor prognostic sign, and nearly all patients with fatal Lassa fever remain viremic until death, with terminal serum viral loads ranging from 10^3 to 10^8 50%-tissue culture infectious doses ($TCID_{50}$)/ml (Johnson *et al*., 1987). Death usually occurs after a mean period of 12 days after onset of illness. In patients surviving Lassa virus infection, duration of illness is about 2 weeks. The peak of viremia is normally around days 4 to 9, and virus is being cleared from the circulation about 3 weeks after onset of symptoms (Mertens *et al*., 1973; Trappier *et al*., 1993). Although the virus may no longer be found in the blood during convalescence, pericarditis can occur, especially in males (Harper, 2004). Sensorineural deafness and ataxia are neurological

complications of the convalescence phase that are assumed to be caused by the immune process (Solbrig and McCormick, 1991; Cummins *et al*., 1990). Temporary or permanent deafness in one or both ears occurs in 29% of Lassa fever patients (Liao *et al*., 1992).

The overall case fatality rate for patients admitted to hospitals in endemic areas is about 15%. In young infants, the specific clinical picture that is observed is the "swollen baby syndrome" which is characterized by widespread edema, abdominal distension, and bleeding usually with a case fatality rate of about 80% (Monson *et al*., 1987). In pregnant women, Lassa fever is associated with infection of the fetus (Walker *et al*., 1982) and loss of the fetus or newborn in nearly 90% of cases (Monson *et al*., 1987; Price *et al*., 1988). The risk of death is also higher for mothers in the third trimester; however, evacuation of the uterus significantly improves the mother's chance of survival (Price *et al*., 1988).

2.1.2 Distribution of the disease

Lassa fever is widespread in West Africa, affecting two million persons per annum with 5,000 to 10,000 fatalities annually (McCormick, 1999). Since its initial discovery, outbreaks of Lassa fever have occurred repeatedly in Sierra Leone: Panguma, Kenema, 1971–1983, 1997, Liberia: Zorzor, 1972; Phebe 1972, 1977, 1982; Ganta 1977, 1982 and Nigeria: Jos,1970, 1993; Onitsha, 1974; Zonkwa, 1975; Vom, 1975–1977, Imo, 1989; Lafia, 1993; and Irrua, 2004 (Carey *et al*., 1972; Bowen *et al*., 1975; Frame *et al*., 1984; Fisher-Hoch *et al*., 1995; Bajani *et al*., 1997; McCormick and Fisher-Hoch, 2002; Omilabu *et al*., 2005). In Guinea, some acute but isolated cases were recorded in hospitals (Bausch *et al*., 2001) and a single rural outbreak was recorded on the Sierra Leone border with Guinea in 1982–83 (Boiro *et al*., 1987). Between these two areas of West Africa, namely in Côte d'Ivoire, Ghana, Togo and Benin, no major outbreak has ever been recorded, though isolated cases suggest the presence of Lassa virus (Saltzmann, 1978; Gunther

and Lenz, 2004; Akoua-Koffi *et al*., 2006). In 2009, the first case from Mali was reported in a traveler living in southern Mali; Ghana reported its first cases in late 2011. Isolated cases have also been reported in Côte d'Ivoire and Burkina Faso and there is serologic evidence of Lassa virus infection in Togo and Benin (CDC Fact Sheet, 2015). Lassa fever therefore appears to have 2 geographically separate endemic areas: the Mano River region (Guinea, Sierra Leone, and Liberia) in the West, and Nigeria in the East (Figure 2.1 below).

Evidence of Lassa virus infection in some African countries has been demonstrated either by sporadic human cases, or by rodent or human seroprevalence studies. It should be noted however that data are often sparse and under-diagnosis in some of these countries is likely (British Health Protection Agency, 2009).

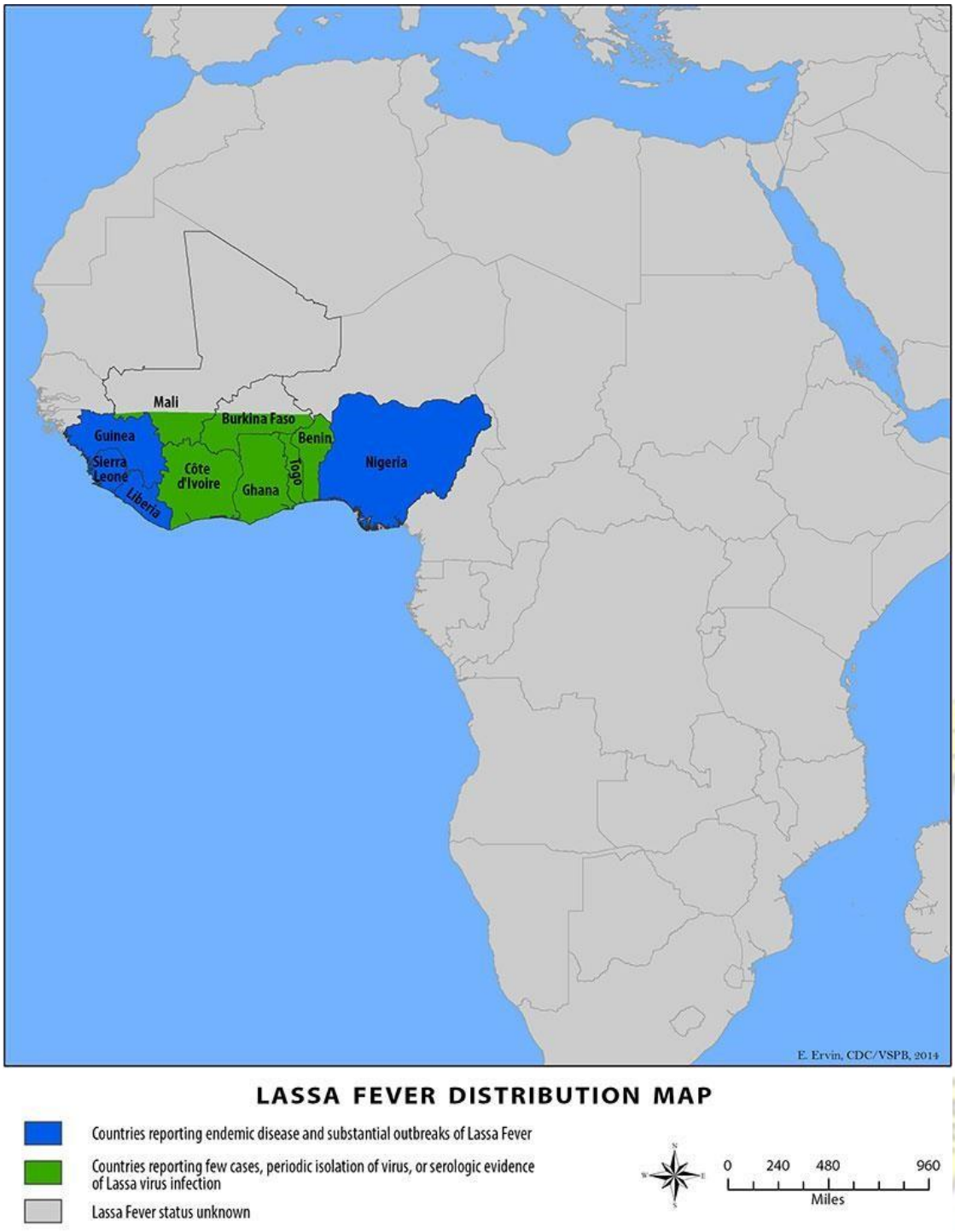

Figure 2.1: Distribution of Lassa fever in Africa (Source: Centers for Disease Control and Prevention, CDC, 2014)

2.1.3 Imported Lassa fever

The virus may be imported into other regions of the world due to an incubation period of up to 3 weeks, while the infected person is asymptomatic or shows early non-specific signs of Lassa fever. Because the environmental conditions, including the presence of the *Mastomys* reservoir, are suitable throughout West Africa, it is presumed that the virus is present all over the region. This presumption is strengthened by reports of disease in travelers to West African countries who have been diagnosed with Lassa fever on return (Macher & Wolfe, 2006). About 25 cases of imported Lassa fever have been reported worldwide (Woodruff *et al*., 1973; Zweighaft *et al*., 1977; Cooper *et al*., 1982; Hirabayashi *et al*., 1988; Mahdy *et al*., 1989; Johnson and Monath, 1990; Holmes *et al*., 1990). Five cases of Lassa fever were imported into Europe between 2000 and 2003 (WHO Records, 2000; CDC Report, 2003) with a casereported in a visitor to Mali in the United Kingdom (Atkin *et al*., 2009). The acquisition of a viral hemorrhagic fever in a traveler to sub-Saharan Africa usually receives great attention, in part because of the possibility of secondary infections in contact persons. In imported cases, the diagnosis is often delayed because the early unspecific symptoms are misinterpreted as malaria or bacterial infection, and because most laboratories do not have diagnostic capacity. In one case the diagnosis was even more difficult since the patient did not travel through established endemic regions and Lassa fever was not initially suspected (Gunther *et al*., 2000). In another case, Lassa fever was not suspected due to the atypical course of the disease (Gunther *et al*., 2001).

2.1.4 Properties of Lassa virus

Lassa virus is an enveloped, single-stranded, bisegmented RNA virus belonging to the Arenaviridae family. It does not have a conventional negative-strand coding arrangement and the isolates of the virus differ in their genetic, serologic, and pathogenic characteristics (Bowen *et al*.,

1997; Jahrling *et al*., 1985). Lassa virus is spherical in shape and measures between 70 and 150 nm in diameter (Figure 2.2a). Its envelope has a smooth surface with T-shaped spikes built with glycoprotein measuring 7 to 10 nm. The envelope encloses the genome with a helical nucleocapsid which measures between 400 and 1300 nm in length (Auperin *et al*., 1986; Auperin and McCormick, 1989).

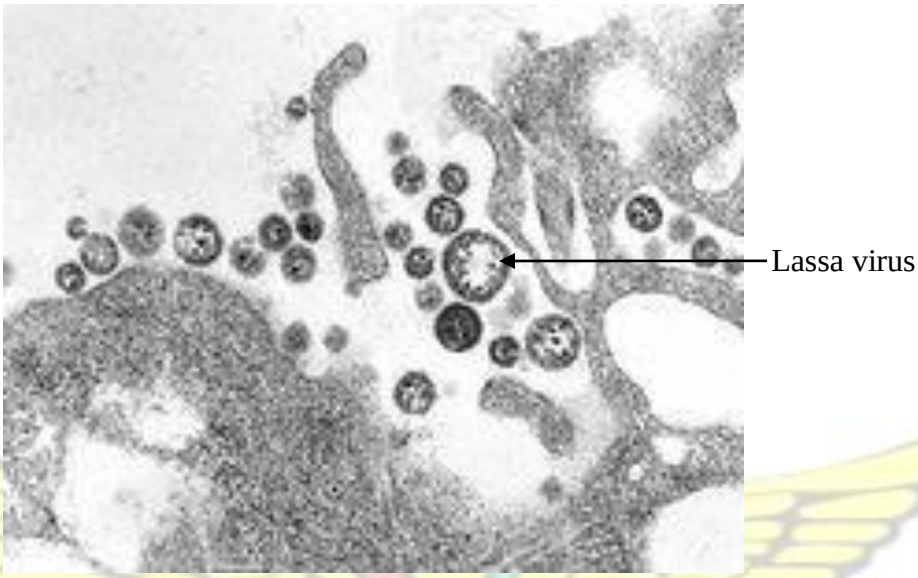

Figure 2.2a: Lassa virus electron micrograph (Image courtesy: C.S. Goldsmith and M. Bowen, Center for Disease Control and Prevention, CDC, Atlanta, USA)

Lassa virus can be inactivated in ultraviolet, gamma irradiation, heat from 56–100ºC and pH range between 5.5 and 8.5. Chemical agents like 0.5% sodium hypochlorite, 0.5% phenol and 10% formalin are good inactivants against the virus (McCormick, 1987). The arenavirus genome consists of a small (s) and a large (l) RNA fragment, sizes 3.4 and 7 kb, respectively and the sRNA encodes the viral glycoprotein precursor protein (GP) and the nucleoprotein (NP), while the lRNA encodes the viral polymerase (L) and a small, zinc-binding (Z) protein (Gunther *et al*., 2000). The sequencing of Lassa virus sRNA has enabled the identification and molecular characterization of four Lassa virus strains namely: strain Josiah, originating from Sierra Leone (Auperin and

McCormick, 1989), the strain Nigeria30 and strain LP (Demby *et al*., 1994; ter Meulen *et al*., 1998), both from Nigeria and the strain AV imported into Germany by a traveler who had visited Ghana, Côte D'Ivoire, and Burkina Faso (Gunther *et al*., 2000). Thus, three of the four lineages are found in Nigeria and the other lineage, (strain Josiah) is in Guinea, Liberia and Sierra Leone (Bowen *et al*., 2000). Sequencing of sRNA of Lassa virus indicated a considerable genetic variation among the strains of the virus, however, phylogenetically, strain AV appears to be the most closely related to strain Josiah from Sierra Leone (Gunther *et al*., 2000).

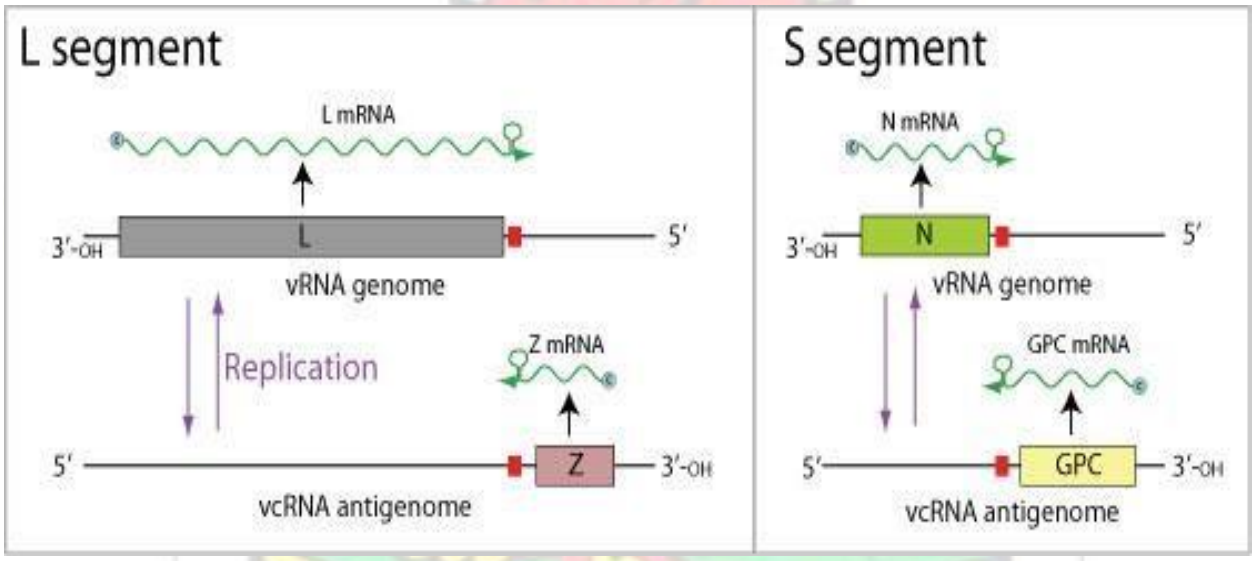

Figure 2.2b: Lassa virus genome (Source: Swiss Institute of Bioinformatics, Lausanne, Switzerland, 1999)

2.1.4.1 Lassa virus replication

The first step in viral replication is adsorption on cell surface receptors that are found to be widely distributed and highly conserved molecules (Southern, 1996). The glycoprotein of the spikes is

responsible for the interactions with cell surface receptors (Burns and Buchmeier, 1993). The second step is the penetration of the virus, then deproteinisation, and finally liberation of RNA genome into the infected host cytoplasm where both replication and transcription take place. During the process, the cell nucleus provides capped cellular mRNA for priming transcription, and the nuclear membrane provides structural support (Southern, 1996). It has been observed that the 5'end of the S derived subgenomic mRNAs extend beyond the end of the genomic RNA template and the length of such an extension varies between 1 and 7 nucleotides and terminates at 5' cap structure (Auperin *et al*., 1984). The initiation of replication and transcription starts from the terminus of the template. As the RNA polymerase rails on the template to add new nucleotides that will form polynucleotide of the new strand, the first two slip back on the template to create non-templated nucleus, a process peculiar to arenaviruses (McCormick, 1987). After biosynthesis of macro-molecules, the virions are assembled through a process not yet understood. Matured virions are released through budding from the plasma membrane of acutely infected cells (Ogbu *et al*., 2007).

2.1.5 Transmission and pathogenesis of Lassa virus

Rodents in the *Mastomys natalensis* species complex host Lassa virus. Infected rodents remain carriers throughout their life without exhibiting clinical symptoms but excrete the virus through urine, saliva, respiratory secretion and exposed blood vessels through micro or macro trauma (Keenlyside *et al*., 1983). Humans most likely become infected through contact with infected rodent excreta, urine, tissues, or blood (Monath *et al*., 1974; McCormick, 1987). Transmission to man is through the faecal-oral route, or via the respiratory tract by inhalation of contaminated air containing the virus. Infected persons represent a serious contamination threat to the environment. The virus can be isolated in the blood, faeces, urine, throat swab, vomit, semen and saliva of

infected persons and secretion of the virus from infected individuals can continue for 30 days or more (Fisher-Hoch *et al*., 1995; CDC, 2004).

On acquisition of the virus, Lassa fever infection is initiated in the human victim. Lassa fever is a generalized infection with dissemination of the virus to multiple organs via the blood stream, lymph vessels, respiratory tract, and/or digestive tract (Gunther and Lenz, 2004). The blood vessels are always the most affected and the virus multiplies in cells of the reticuloendothelial system. The capillary permeability is increased, and along with disseminated intravascular coagulation, leads to haemorrhagic syndrome (Frame *et al*., 1970). Haemorrhage may be present in the intestine, liver, myocardium, lungs and the brain and when infected these organs are often inflamed and enlarged, and the tissues are infiltrated and necrotic (Fleischer *et al*., 2000; Gunther *et al*., 2001). Other observable pathological changes include black vomit with traces of blood, watery diarrhoea that gives rise to dehydration and reduction in the volume of blood in circulation and low blood pressure, depressed lymphocyte counts and platelet function as well as moderate thrombocytopenia (Fisher-Hoch *et al*., 1988).

2.1.6 Prevention and control of Lassa fever

Prevention of primary transmission of Lassa virus from its host to humans can be achieved by avoiding contact with *Mastomys* rodents, especially in the geographic regions where outbreaks occur (WHO Newsletter, 2005). Storing food in rodent-proof containers and keeping the home clean helps to deject rodents from entering homes. Use of these rodents as a food source should not be encouraged. Trapping in and around homes can help reduce rodent populations. However, the wide distribution of *Mastomys* in Africa makes complete control of this rodent reservoir impractical (Monath *et al*., 1974; Keenlyside *et al*., 1983; Granjon *et al*., 1997).

Lassa fever is a highly virulent and contagious viral infection. Therefore, when caring for infected patients, further transmission of the disease through person-to-person contact or nosocomial routes can be avoided by taking preventive precautions against contact with patient secretions by establishing strict barrier nursing (McCormick, 1987; Fisher-Hoch *et al*., 1995). Such precautions include wearing protective clothing, using infection control measures, such as complete equipment sterilization and isolating infected patients from contact with unprotected persons until the disease has run its course.

Body fluids, excreta and other materials that might have been contaminated should be handled carefully and disposed properly preferably by burning. All instruments used on the patient, if not disposable must be subjected to autoclaving immediately. Complete care should be taken when collecting pathological materials for laboratory investigations. In addition to these practices, the correct procedure for transporting materials suspected to contain highly virulent virus or microorganisms must be observed (McCormick, 1987; McCormick *et al*., 1987). Absolute precautionary measures must be taken while carrying out bacteriological and biochemical investigations in the blood and urine samples of suspected cases and such manipulations must be done in biosafety chambers. All those who had contact directly with suspected Lassa haemorrhagic fever patients have to be traced, monitored and specimens should be collected for laboratory diagnosis. Those who test positive have to be isolated and treated as soon as possible with ribavirin (WHO Newsletter, 2005).

Health education strategies for preventing infections in people living in endemic areas must be instituted and should focus on rodent control and minimizing contact with rodent excreta. Furthermore, emphasis should be placed on measures to control virus transmission from cases that

include routine use of standard precautions, isolation of suspected cases and surveillance of contacts (WHO Records, 2005).

2.1.6.1 Vaccination

Although the prevention of human contact with the *Mastomys* rodents is an essential factor in the control of Lassa fever, widespread prevention of such contact is currently not feasible in the endemic regions of West Africa. Therefore, provision of a vaccine for community and hospital use is an imperative public health need as vaccination is the most viable control measure (FisherHoch and McCormick, 2001). Currently, there is no vaccine against Lassa virus available for use in humans (Fisher-Hoch and McCormick, 2001). Several experimental vaccines have been developed and were successfully tested in guinea pigs, monkeys, and mice. A previous report indicates that a vaccinia virus expressing the Lassa virus glycoprotein protected four non-human primates against lethal challenge with Lassa virus (Fisher-Hoch *et al*., 1989). This was followed by the finding that single administration of a vaccine expressing the full-length Lassa virus glycoprotein affords protection against Lassa fever in primates, with or without expression of the nucleoprotein (Fisher-Hoch and McCormick, 2000).

The challenge, however, is to overcome the scientific and political obstacles in producing a human use vaccine candidate. It is well established that the G-protein gives protection but its duration is unknown and if the N-protein is also included there may be a better duration of protection. However, it is unclear whether the N-protein as a vaccine may possibly enhance the infection (Fisher-Hoch and McCormick, 2004). Adequate funding and applications of new vaccine technologies give hope that there may soon be a vaccine in clinical trials. However, the difficulty of conducting clinical trials in endemic areas of the West African sub-region coupled with lack of political stability remain serious problems.

2.1.7 Treatment of Lassa fever

All persons suspected of Lassa fever infection should be admitted to isolation facilities and their body fluids and excreta properly disposed of. Ribavirin, an antiviral drug primarily used in the treatment of hepatitis C, is effective in the treatment of Lassa fever, but only if administered early in the course of illness (Jahrling *et al*., 1980). Ribavirin is a prodrug which appears to interfere with viral replication by inhibiting RNA-dependent nucleic acid synthesis (Crotty *et al*., 2002). The drug is relatively inexpensive, but the cost of the drug is still very high for many of those in poverty-stricken West African states. In a study of Lassa fever in Sierra Leone (McCormick *et al*., 1986), it was observed that patients with a high risk of death who were treated for 10 days with intravenous ribavirin, begun within the first six days after the onset of fever, had a case-fatality rate of 5% (1 of 20) ($p = 0.0002$ by Fisher's exact test), while patients whose treatment began seven or more days after the onset of fever had a case fatality rate of 26% (11 of 43) ($p = 0.01$). The study confirmed the efficacy of ribavirin in the treatment of Lassa fever as well as for post-exposure prophylaxis.

Anti-Lassa fever plasma may be used as adjunctive therapy in very ill patients. Supportive treatment, including correction of fluid and electrolyte imbalances, is imperative. For infected pregnant women, particularly during the third trimester, uterine evacuation appears to reduce maternal mortality (Price *et al*., 1988).

2.1

.8 Diagnosis of Lassa fever infection

The signs and symptoms of Lassa fever is often difficult to distinguish from common diseases in the tropics such as severe malaria, typhoid fever, yellow fever and other viral haemorrhagic fevers. Definitive diagnosis requires specific testing that is available only in highly specialised laboratories (WHO Records, 2005). As the symptoms of the disease are so varied and nonspecific, clinical diagnosis is normally difficult especially early in the course of infection. Hence, in order to make accurate diagnosis of Lassa fever, clinical manifestation, epidemiological data, and result of laboratory findings should be taken into consideration (Ogbu *et al*., 2007).

Lassa fever is diagnosed by detection of Lassa antigen or RNA, anti-Lassa virus antibodies, or virus isolation techniques (WHO Newsletter, 2005). In the laboratory, the virus can be isolated using laboratory animals such as guinea pigs, albino mice, Vero cell or African green monkeys. Albino mice inoculated intracerebrally die between 3 and 5 days. Lassa virus causes conspicuous cytopathic effect on confluent monolayer of Vero cell culture within 96 hours. The antigens to be used for viral isolation can be obtained from the patient's blood, urine, pleural fluid, throat swab and in case of death, pathological materials from liver, kidney, spleen and heart (McCormick, 1987; Cummins, 1990; Gunther and Lenz, 2004). The virus can be seen under electron microscope using specimens obtained from infected persons. Although virus isolation remains the most sensitive, it is still uniquely a research tool and requires use of high containment Biosafety Level Four (BSL-4) laboratory facilities.

.8.1 Culture and isolation of virus

The classical method to detect Lassa virus is inoculation of Vero cells with serum, cerebrospinal fluid (CSF), throat washing, pleural fluid or urine of the patient (Buckley and Casals, 1970;

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Henderson *et al*., 1972; Jahrling *et al*., 1985). It is imperative that specimen for laboratory analysis be collected as soon as possible from the patient suspected of having the infection. Lassa virus is infectious by aerosol and the human and rodent specimens should be processed with appropriate precautions in BSL-4 laboratories (Cummins 1990; WHO Records, 2005). The specific diagnosis is readily made by the isolation and identification of the virus. This is usually done by the inoculation of blood from the patient into Vero cell cultures. Growth in cell culture and immune detection are hardly affected by the variability of the virus. Virus isolation also facilitates a detailed geno- and phenotypic characterization of the isolate. The major disadvantages are the length of time (days to weeks) required to isolate a virus as well as the need for BSL-4 facilities.

Virus antigen can be detected by enzyme-linked immunosorbent assays (ELISA) using Lassa virus-specific antibodies (Niklasson *et al*., 1984; Bausch *et al*., 2000). These tests are easy to conduct and rapid, and can be performed with inactivated specimens, which is advantageous in the field if sophisticated equipment is not available. The minimal concentration of infectious virus required for detection by ELISA ranges from 10^2 to 10^5 PFU/ml depending on the type of material tested. However, for unknown reasons the antigen ELISA becomes increasingly insensitive concomitant with the appearance of specific antibodies (Niklasson *et al*., 1984). Consequently, antigen ELISA is clinically less sensitive than virus isolation (36%), although the high concentration of virus early in the course of Lassa fever often allows antigen detection in serum (Jahrling *et al*., 1985; Bausch *et al*., 2000; Bausch *et al*., 2001).

.8.2 Serological techniques

The indirect fluorescent-antibody (IFA) test has traditionally been employed in the laboratory diagnosis of acute Lassa virus infection (Wulff *et al*., 1978; McCormick *et al*., 1987). The

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interpretation of IFA results is complicated by the presence of antibody during both acute and convalescent stages of infection. The appearance of antibody early in the course of Lassa infection may be useful in identifying patients with poor prognosis. However, due to lack of specificity in populations in non-endemic areas (VanderWaals *et al*., 1986), the technique has been largely replaced by ELISA for Lassa virus antigen and Lassa virus-specific immunoglobulin M (IgM) and G (IgG) antibodies (Jahrling *et al*., 1985; Ivanov *et al*., 1986; Bausch *et al*., 2001).

Usually, IgM and IgG antibodies are detectable in nearly half of the patients during the first days of illness, with about 15% only IgM-positive. Therefore, serological testing is not suitable for early diagnosis of Lassa fever. Furthermore, patients with fatal Lassa fever show lower antibody titers or may not develop antibodies at all (Johnson *et al*., 1987; Schmitz *et al*., 2002). The fraction of seropositive patients increases further during the course of disease and is close to 100% by day 18, when viremia is already decreasing (Johnson *et al*., 1987). Therefore, serological assays are the methods of choice for diagnosis of Lassa fever in the convalescence phase. Specimens can be inactivated by heat (Mitchell and McCormick, 1984) to facilitate testing under standard laboratory conditions.

The most common test for detection of IgM and IgG antibodies to Lassa virus is indirect immunofluorescence using virus-infected cells. An IgG seroconversion with a >4-fold increase in the IgG titer or detection of IgM together with an IgG titer ≥256 was considered evidence of acute infection (Johnson *et al*., 1987). However, the immunofluorescence test probably is not

completely specific for Lassa virus because of the extensive crossreactivity among African arenaviruses (Wulff *et al*., 1978; Meunier *et al*., 1985; Swanepoel *et al*., 1985).

Enzyme-linked immunosorbent assay (ELISA) or immuno blot tests using recombinant protein (NP, GPC, and Z protein) as antigen have also been developed and used for seroprevalence studies or for diagnosis of acute infection (Hummel *et al*., 1992; Lukashevich *et al*., 1993; ter Meulen *et al*., 1998; Gunther *et al*., 2001). The great advantage of these assays is that their preparation does not require BSL-4 laboratories. On the other hand, the high background in African sera of antibodies against components of bacterial or insect cell expression systems complicates the use of recombinant proteins for serological diagnostics in endemic regions. Immunoglobulin G and IgM ELISA havebeen developed using gamma irradiated virus (Elliot *et al*., 1982) from infected cells as an antigen (Niklasson *et al*., 1984; Bausch *et al*., 2000). The clinical sensitivity of these assays is comparable to indirect immunofluorescence.

2.1.8.3 Detection and quantification of Lassa virus RNA by reverse-transcription polymerase chain reaction (RT-PCR) assay

The RT-PCR assay is the method of choice for rapid and early diagnosis of Lassa fever (Drosten *et al*., 2002). Since Lassa virus is an RNA virus, its RNA must be reverse transcribed into complementary DNA (cDNA) prior to PCR. A one-step RT-PCR system is also available which is based on an optimized mixture of a retroviral RT and a *Taq* polymerase that is heat-activated only following the reverse transcription step. Using this system, high analytical sensitivity of the Lassa virus PCR is achieved without the need of nested steps or Southern hybridization (Drosten *et al*., 2002). Detection is also faster and the risk of contamination is reduced. Lassa virus PCR products can be detected in real time using intercalating dyes, like SybrGreen (Drosten *et al*.,

2002). This technique allows rapid measurement of virus RNA concentration in serum or other body fluids (Gunther *et al*., 2001; Schmitz *et al*., 2002). The RNA concentration is important as a prognostic parameter, in therapy monitoring, and in the risk assessment of virus transmission to contact persons.

Various diagnostic Lassa virus PCRs target the S RNA segment encoding GPC and NP (Trappier *et al*., 1993; Demby *et al*., 1994; ter Meulen *et al*., 1998). However, sequence information on the L RNA segment of arenaviruses is also available (Vieth *et al*., 2004). The L gene, encoding the viral RNA polymerase, may be particularly suited as a PCR target because RNA polymerases share conserved amino acid motifs even between different virus families (Poch *et al*., 1990). The L gene has been found to contain highly conserved regions that have been used to develop a Lassa virus-specific PCR assay (Vieth *et al*., 2007). As a result of the high degree of conservation at these sites, this assay is also able to detect other Old World arenavirus species such as LCMV, Mopeia virus, and Ippy virus, and may therefore be more robust with regard to virus variability than assays that solely detect Lassa virus.

2.2.1 Hantavirus infection and disease

Two severe diseases are associated with hantavirus infection in humans: haemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). Haemorrhagic fever

with renal syndrome, endemic in Europe is caused by the Old World hantaviruses Dobrava (DOBV), Puumala (PUU), and Tula (TULV) viruses whereas Hantaan virus (HNTV) and Seoul virus (SEOV) are the most prominent HFRS pathogens in Asia (Brummer-Korvenkontio *et al.*, 1982; Lee and van der Groen, 1989; Klempa *et al.*, 2006). The New World hantaviruses Andes (ANDV) and Sin Nombre (SNV) cause HCPS in the Americas (Duchin *et al.*, 1994; Mertz *et al.*, 1997; Peters and Khan 2002). Infection with PUU and TULV varies from moderate disease to subclinical courses whereas infection with DOBV, SEOV, HNTV, ANDV and SNV tends to affect various organs and can lead to more severe and even fatal disease (Peters *et al.*, 1999; Schutt *et al.*, 2004).

While rodents have long been known to serve as reservoirs of hantaviruses, recent studies indicate a far richer genetic diversity among hantaviruses harbored by shrews and moles of multiple species (Yadav *et al.*, 2007; Song *et al.*, 2007; Arai *et al.*, 2008; Kang *et al.*, 2009). Until recently, there have been reports of novel hantavirus genome sequences in Africa,

Sangassou virus (SANV) in the African wood mouse (*Hylomyscus simus*) and Tanganya virus (TGNV) in the Therese's shrew (*Crocidura theresae*) both discovered in Guinea (Klempa *et al.*, 2006; 2007). These findings stimulated a seroepidemiological study in 2010, in the forest region of Guinea which clearly showed that local hantaviruses infect humans (seroprevalence of 1.2% in the general population). Moreover, a 4.4% seroprevalence of hantavirus-specific antibodies in patients with fever of unknown origin and detection of SANGV specific IgM and IgG antibodies in one patient, indicate that, hantaviruses may constitute a medical problem in Guinea (Klempa *et al*., 2010). Additional hantaviruses, Azagny virus harbored by the West African pygmy shrew (*Crocidura obscurior*) and Magboi virus detected in the slit-faced bat (*Nycteris hispida*) have been found in the neighboring countries Cote d'Ivoire (Kang *et al*., 2011) and Sierra Leone (Weiss *et*

al., 2012), respectively, further confirming that hantavirus infections in West Africa need to be considered.

2.2.2 Geographic distribution of Hantavirus

Hantavirus is a well recognized human pathogen in most world continents; with indigenous African representatives being identified recently. Hantaan (HTN) virus causes severe HFRS in Asia (Lee *et al*., 1978). Seoul (SEO) virus, reported to cause moderately severe HFRS, is linked to urban rats and therefore is potentially found all over the world (Lee *et al*., 1982). Dobrava (DOB) virus, which was isolated on the Balkan Peninsula, has been found to be an important cause of the more severe type of HFRS in this region (Avsic-Zupanc *et al* 1992). Puumala (PUU) virus, the cause of a milder form of the disease (nephropathia epidemica [NE]), is found in Scandinavia, western Russia, the Balkan region, and several central European countries (Lee and van der Groen, 1989).

Although HFRS or HCPS are not recognized diseases in Africa, the pathogenic potential of SANGV, TGNV, and probably other, yet undiscovered African hantaviruses, should not be underestimated. In Africa, where medical health care conditions are relatively limited, hantavirus-associated disease may be confused with other severe diseases or may be unrecognized.

2.2.3 Classification and properties of Hantavirus

Hantaviruses belong to the *Bunyaviridae* family. They differ from other bunyaviruses in one important ecological aspect in that; they are not transmitted by arthropod vectors. Transmission to humans is mainly through inhalation of aerosolized infected rodent excreta or bites (Plyusnin *et al.,* 1996). In their natural hosts, hantaviruses produce chronic infections with no apparent harm. They are strictly associated with one (or few closely related) small mammal species as their natural reservoir hosts that is reflected also in their phylogeny.

Hantaviruses are enveloped viruses with a genome that consists of three single-stranded, negative sense RNA segments designated S (small), M (medium), and L (large) (Plyusnin *et al*., 1996). The S RNA encodes the nucleocapsid (N) protein. The M RNA encodes a polyprotein that is co-translationally cleaved to yield the envelope glycoproteins Gn (formerly G1) and Gc (formerly G2) (Jonsson *et al.*, 2010).

The L RNA encodes the L protein, which functions as the viral transcriptase/replicase. Within virions, the genomic RNAs of hantaviruses are thought to complex with the N protein to form helical nucleocapsids.

The virions are about 120 to 160 nanometres (nm) in diameter. The lipid bilayer of the viral envelope is about 5 nm thick and is embedded with viral surface proteins to which sugar residues are attached. These glycoproteins tend to associate with each other and have both an interior tail and an exterior domain that extends to about 5 nm beyond the envelope surface. Inside the envelope are the nucleocapsids which are composed of many copies of the nucleocapsid protein N, that interact with the three segments of the viral genome to form helical structures. The virally encoded RNA polymerase is also found in the interior (Plyusnin *et al*., 1996).

2.2.4 Pathogenesis of Hantavirus

The pathogenesis of Hantavirus infections is unclear due to lack of animal models (rats and mice do not seem to acquire severe disease) (Safronetz *et al*., 2011). While the primary site of viral replication in the body is not known, in HFRS, the main effect is on the blood vessels while in HCPS, most symptoms are associated with the lungs. In HFRS, there is increased vascular permeability and decreased blood pressure due to endothelial dysfunction and the most dramatic damage is seen in the kidneys, whereas in HCPS, the lungs, spleen, and gall bladder are most affected. Early symptoms of HCPS tend to present similarly to influenza (muscle aches, fever and

fatigue) and usually show up around 2 to 3 weeks after exposure. Later stages of the disease (about 4 to 10 days after symptoms start) will include difficulty breathing, shortness of breath and coughing (Canadian Lung Association, 2009). Depending in part on which hantavirus is responsible for the illness, HFRS can appear as a mild, moderate, or severe disease. Approximately 200,000 cases of HFRS involving hospitalization are reported each year throughout the world, with more than half in Asia. Death rates range from less than 0.1% for HFRS caused by Puumala (PUU) virus to approximately 5% to 10% for HFRS caused by HTN
virus (Vapalahti *et al.*, 2003; Maes *et al.*, 2004).

Hantavirus cardiopulmonary syndrome (HCPS) is an often fatal disease caused by hantavirus infection. The symptoms are very similar to those of HFRS and include tachycardia and tachypnea. Additionally, patients will develop difficulty breathing, coughing and shortness of breath (Canadian Lung Association, 2009). Such conditions can lead to a cardiopulmonary phase, where cardiovascular shock can occur, and hospitalization of the patient is required.

2.2.5 Prevention and treatment of Hantavirus

As Hantavirus can be transmitted by rodent saliva, excreta, and bites, control of rats and mice in areas frequented by humans is important for disease prevention. General prevention can be accomplished by destroying rodent nests, sealing any cracks and holes in homes where mice or rats could use for entry and use natural predators such as cats in the home. There is no effective antiviral therapy for HPS, although the drug ribavirin has shown a treatment effect in reducing HFRS mortality (Huggins *et al.*, 1991). However, the earlier hantavirus infection is treated, the better the chances are for recovery. Patients with suspected hantavirus infection are usually admitted to hospital and given oxygen to help them breathe (Maes *et al.*, 2004).

2.2.6 Diagnosis of Hantavirus

The most practical approach for the laboratory diagnosis of hantavirus infection in humans is the detection of Immunoglobulin M (IgM) antibodies in acute serum samples using an enzymelinked immunosorbent (ELISA) IgM capture assay. The ELISA tests to detect IgG antibodies may also be used to confirm diagnosis, two serum samples taken two to three weeks apart are required to demonstrate rising titers of IgG antibodies. Less commonly used serological tests, such as immunofluorescent assay and particle agglutination, can also be applied to hantaviral diagnosis (Niklasson *et al*., 1999). In fatal cases, fresh frozen tissue, fixed tissue, and blood can be used to confirm the diagnosis by reverse transcriptase-polymerase chain reaction (RT-PCR), immunohistochemistry, or ELISA methods, respectively. Collection of blood clots from initial samples of all suspect cases is also recommended for subsequent RT-PCR on selected seropositive individuals. The RT-PCR is a molecular diagnostic technique targeting specific regions of the virus genome and is available only at certain research laboratories. For routine diagnosis, this molecular technique is not recommended, but is valuable in defining the virus genotype, detecting known viruses, and performing certain epidemiological studies (Aitichou *et al*., 2005).

Some Old World hantaviruses have occasionally been isolated from patient serum or whole blood drawn within three to nine days of onset of illness. However, propagation of hantaviruses is difficult and this is not a recommended diagnostic procedure (Lee 1999). The lungs are massively edematous at postmortem, but microscopic studies find little necrosis. There are scant to moderate hyaline membranes, intact pneumocytes, and scarce neutrophils (Nolte *et al*., 1995).

However, there is interstitial infiltration by T lymphocytes and activated macrophages (Zaki *et al*., 1995).

These findings differ from those of typical adult respiratory distress syndrome and much pneumonia. Hantaviral antigens are detected primarily in endothelial cells, and those in the lung are heavily involved. Lesser amounts of antigen are found in scattered endothelial cells throughout

the body, as well as occasional involvement of macrophages, myocytes, and many other cell types. In light of the rapid progression of HCPS, effective clinical management depends heavily on careful fluid management, hemodynamic monitoring, and ventilatory support (Death-Valley, 2005).

2.3.1 Tick-Borne Relapsing Fever-the disease

In West Africa, tick-borne relapsing fever (TBRF) is caused by the spirochaete *Borrelia crocidurae* (Leger, 1917; Goubau, 1984; Trape, 1991). The pathogen is transmitted by *Ornithodoros species* ticks (formerly *Alectorobius species*) (Mathis *et al*., 1934; Horak *et al*., 2003), which are ectoparasites that live on rodents and other insectivores (Godeluck *et al*., 1994; Trape *et al*., 1996). Infection of African TBRF in humans is presumably related to their closeness to tick-infested burrows and huts. People are generally infected during their sleep, when the host burrows open into their bedrooms (Mathis *et al*., 1934; Trape *et al*., 1996). *B. crocidurae* causes an acute febrile illness in people. If left untreated, patients have relapsing remitting fever over several months, and severe meningoencephalitic complications can ensue (Goubau, 1984; Charmot, 1986).

Spirochetes of the genus *Borrelia* are known to cause two major types of human disease, Lyme disease, which occurs primarily in temperate regions, and relapsing fever (RF), which occurs in

both temperate and tropical regions. Borreliosis is related to climatic and other environmental parameters required for the vectors and reservoir hosts (Trape *et al*., 1996; Helmy, 2000). *Borrelia*-related disease is endemic in tropical and subtropical regions; *B. hermsii* and *B. turicatae* cause tick-borne RF (TBRF) in North America. In Europe, TBRF is uncommon; *B. hispanica* is the causative agent in Spain, Portugal, Greece, and Cyprus (Goubau and Munyangeyo, 1983; Anda *et al*., 1996).

The precise incidence and distribution of TBRF in West Africa are unknown (Rodhain, 1976; Trape, 1991; Trape *et al*., 1996). Reports of the disease are few and the distribution of the vector seems typically limited to the Sahel and Saharan regions (Trape *et al*., 1996); however, findings of investigations done in Senegal indicate that TBRF is, after malaria, the most common cause of outpatient visits to a rural dispensary near Dakar. Levels of TBRF infection in small communities infested with *O. moubata* (*B. duttonii*) in east Africa (Tanzania, Zaire) and *O. sonrai* (*B. Crocidurae*) in West Africa (Senegal, Mauritania, Guinea) can be high with up to 24.4% of febrile patients in local medical facilities being infected and as such TBRF should be considered an important public health issue (Dupont *et al*., 1997; Vial *et al*., 2006). High prevalence rates were found in rodents from several regions of Senegal and studies of clinic outpatients suggested that borreliosis is a major cause of morbidity in many rural areas of this country (Trape *et al*., 1991). The frequent occurrence of relapsing fever in Senegal was first suspected in the mid-1930s (Advier *et al*., 1934). However, since diagnosis is difficult because of the frequent low density of *B. crocidurae* in patients' blood (Trape *et al*., 1991), case reports were rare and the disease has remained poorly studied. Furthermore, as a result of a drought in sub-Saharan countries (since 1970)**,** the tick has now colonised the Sudan savanna (Trape, 1991; Trape *et al*., 1996).

The primary clinical manifestations of RF are recurrent high fever interrupted by afebrile periods, hepatomegaly, splenomegaly, and anemia. These signs are similar to those of malaria. The fever peaks are associated with high spirochetemias, and antigenic variation leads to new antigenic variants of a major surface protein and the recurrence of high numbers of borreliae in the blood (Meier, 1985; Barbour, 1990; Shamaei-Toussi *et al*., 1999). When patients have high fever, spirochetes may achieve sufficiently high cell densities in the blood to be observed directly by microscopy when wet mounts of blood or Giemsa-stained blood smears are examined. Between peaks, the bacteria are too scarce to be visualized in the blood. Treatment with various antimicrobial drugs is effective (WHO, 1997; Cadavid and Barbour, 1998); however, borreliae may rapidly invade the brain, and infection of the central nervous system may persist if not treated or if treated with antimicrobial drugs that do not readily penetrate the bloodbrain barrier (Cadavid and Barbour,1998).

Research on RF in Africa has been limited, and little is known regarding the presence and geographic distribution of the spirochetes and tick vectors. Studies in Senegal indicate that RF is widely distributed and prevalent in this country; investigators speculate that RF may cause illness in rural areas throughout much of West Africa (Trape *et al*., 1991).

2.3.2 Tick-borne relapsing fever distribution

Relapsing fever-associated *Borrelia* species have been named on the basis of the cospeciation concept, taking into account the geographic endemicity of the vectors. Some of these organisms have a worldwide distribution: *Borrelia recurrentis* is transmitted by human body lice (Felsenfeld, 1965), and *B. anserine* is restricted to birds and *Argas* ticks. Other species are geographically restricted. The specificity of the association between *Borrelia* species and vectors has been questioned, as *B. duttonii*, *B. crocidurae*, and *B. hispanica*, which are naturally transmitted in

nature by *Ornithodoros moubata*, *O. sonrai*, and *O. erraticus* respectively, could be experimentally adapted to lice (Felsenfeld, 1965).

Tick-borne relapsing fever (TBRF) occurs in Africa, Spain, Saudi Arabia, Asia, and certain areas in Western United States and Canada. In 1969, the number of cases in South America was 278, with one death. In 1976, 15 cases were reported in the United States. Sporadic cases occur in the western United States, namely, Canada (British Columbia), Mexico, Guatemala, Panama, Columbia, Venezuela, Ecuador and Argentina. Although endemic relapsing fever is usually sporadic, at times group outbreaks occur (Oliver *et al*., 1996). In 1973, there was an outbreak with 62 cases among tourists at Grand Canyon National Park in Arizona (USA) who were lodged in rustic wooden cabins infested by rodents and their ticks. Another outbreak occurred under similar circumstances in California with 6 cases among 11 tourists (Harwood and James, 1979). At least six TBRF species are known to occur in Europe or close to its boundaries. The greatest endemic risk in Europe lies in the Iberian Peninsula, particularly in the Mediterranean part, and in Asia Minor. Reports of imported TBRF cases have come from the UK, Belgium and France. The disease caused by *Borrelia* spp. are rarely reported in travelers from disease-endemic countries, most likely because most infections are benign, and no diagnosis is made (Rebaudet and Parola, 2006).

2.3.3 Tick-borne relapsing fever biology

The relapsing fever *Borrelia spp* are gram negative helical bacteria normally 0.2 to 0.5 microns in width and 5 to 20 microns in length. They are visible with darkfield or phase contrast but not standard light microscopy without special stains (Barbour *et al*., 2005) and have the cork-screw shape typical of all spirochetes (Figure 2.3). These bacteriahave a unique process of DNA rearrangement in their linear DNA which frequently generates a variable major protein that allows

the organism to evade the human immune system and therefore causes recurrent patterns of fever and other symptoms (Barbour, 1990).

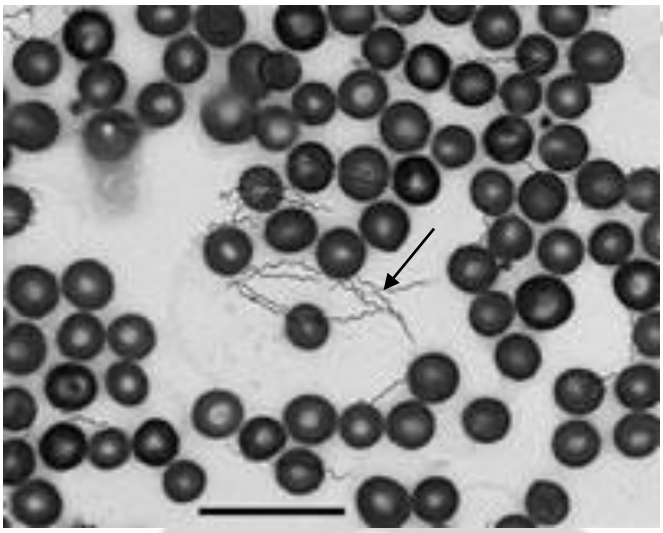

Figure 2.3: Peripheral blood smear showing Spirochaetes (arrow) and blood cells (Emerging Infectious Disease, 2003)

Borrelia is transmitted to humans through the bite of infected soft ticks (genus *Ornithodoros*). The larvae of most *Ornithodoros* species take brief blood meals lasting between 1 to 2 hours, usually at night. Between meals the larvae live in the nesting materials in their host burrows. Individual ticks will take many such blood meals during each stage of their life cycles, including the development of eggs by adult females. The bites of soft ticks are usually painless and the persons who are bitten while asleep are usually unaware that they were bitten (Marquardt 2005; Anderson and Magnarelli 2008; CDC: National Center for Emerging and Zoonotic Infectious Diseases, 2012).

In the tick, *Borrelia* can be found in all the tissues including salivary glands and ovaries of certain subspecies of ticks (Schwan and Piesman, 2002). Infected *Ornithodoros* ticks can transmit

relapsing fever spirochetes to humans through their saliva while feeding. When a tick feeds on an infected person, spirochaetes are ingested with the blood. These multiply in their gut and enter the haemocoel, where they increase to enormous proportions (Barbour, 2005). The spirochaetes pierce all organs of the tick's body including the salivary gland, the coxal (excretory) organ and the reproductive system, leading to transovarial infection. People are infected both by the bite of the tick and from coxal fluid, but not from the faeces. In *O. moubata* adults (Figure 2.4), the coxal fluid is the source of spirochaetes, but in the nymphs and other species of *Onithodoros*, it is the salivary glands (Barbour, 2005). Since the infection is also in the ovaries of certain ticks, such as *O. hermsi*, they can transmit their infections over many generations from female ticks to their offspring. Soft ticks can live up to 10 years. In certain parts of Russia the same tick has been found to live almost 20 years (CDC, 2012).

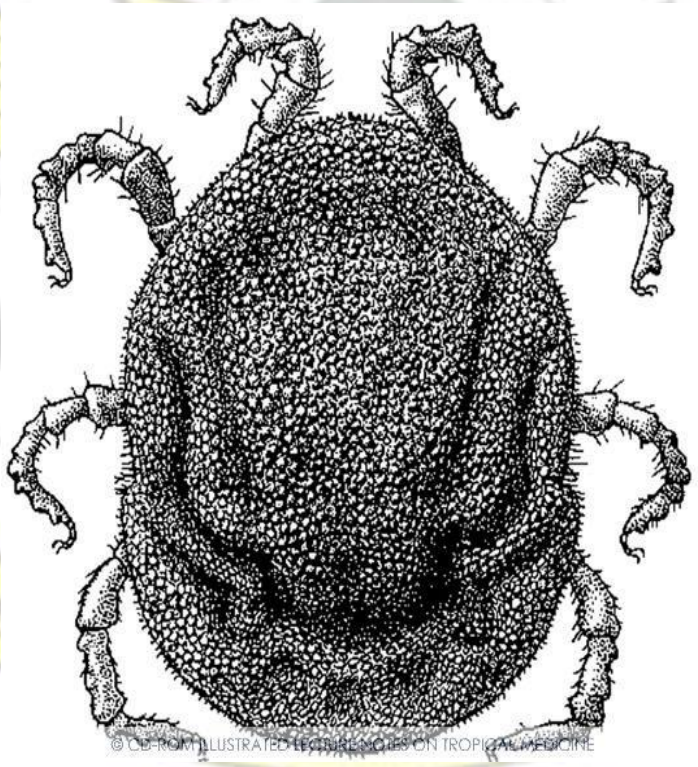

Figure 2.4: Soft tick, Ornithodoros moubata (Source: Institute of Tropical Medicine, Antwerp, Belgium, 2003)

Ticks rest in cracks and crevices of poorly built houses, emerging at night to feed on sleeping occupants. Their eggs are coated with a waxy protective layer allowing them to remain viable for several months. These are laid in walls, floors and furniture and will normally hatch in 1 to 4 weeks if conditions are suitable. Soft ticks once established are very persistent occupants (Webber, 2005).

2.3.3.1 Pathogenesis of Tick-borne relapsing fever

Tick borne relapsing fever has an average incubation period of 7 days after the tick bite but vary from 4 to 18 days. The disease is characterized by initial pyrexia that lasts 3 to 4 days which begins and disappears suddenly. The fever which may reach 41ºC is accompanied by chills, profuse sweating, vertigo, cephalalgia, myalgia and vomiting. At times, erythemas, petechiae and jaundice of varying degrees of severity may be observed. After several days without fever, the attacks of fever recur many times lasting longer than in the first episode. The primary characteristic of the disease is the syndrome of periodic fevers. There are generally 3 to 7 relapses of fever, with intervals of 4 to 7 days (Barbour, 1990). Periodic recurrences are attributed to antigenic changes or mutations in the borreliae, against which the patient has no immunity. Borreliae in the first attack, are antigenically different from those isolated in relapses and there is no protective immunity among these serotypes (Barbour, 1990). Long-term complications such as iritis (inflammation of the iris of the eye), depression, and heart failure occasionally occur.

Tick-borne relapsing fever (TBRF) contacted during pregnancy can cause spontaneous abortion, premature birth, and neonatal death (Melkert and Stel, 1991). The maternal-fetal transmission of *Borrelia* is believed to occur either transplacentally (Steenbarger, 1982) or while traversing the birth canal. In one study, perinatal infection with TBRF was shown to lead to lower birth weights, younger gestational age, and higher perinatal mortality (Jongen and van Roosmalen *et al.*, 1997). In general, pregnant women have higher spirochete loads and more severe symptoms than

nonpregnant women. Higher spirochete loads have not, however, been found to correlate with fetal outcome (CDC: National Center for Emerging and Zoonotic Infectious Diseases, 2012).

Skin manifestations do occur in some victims. Rash occurs in up to 50% of patients, depending on the particular species of *Borrelia* involved (McGinley-Smith and Tsao, 2003). A variety of skin lesions have been associated with TBRF; the most common of these are described as macules (flat discolourations), 1 to 2 cm in size, itchy, with irregular borders, or circular, sharply demarcated, blanching, 18 to 24 mm rose-coloured macules, resembling erythema multiforme. Papules (small lumps), petechiae (small red or purple spots due to bleeding into the skin), purpura (bleeding into the skin, includes petechiae and bruises), and facial flushing have been described. Petechiae may also occur on mucous membranes. The rash usually appears towards the end of the first febrile episode or during the symptom-free interval. The rash usually lasts 24 to 48 hours and may be present over the entire body or in localised areas. Lesions can occur on the face, trunk or extremities (McGinley-Smith and Tsao, 2003).

2.3.4 Prevention and control of Tick-borne relapsing fever

To prevent infection, humans should avoid sleeping in rodent infested buildings. Tick bites should be limited by the use of insect repellent containing *N*, *N*-Diethyl-*meta*-toluamide (DEET) (on skin or clothing) or permethrin (applied to clothing or equipment). Rodent-proof buildings are required in endemic areas. Rodent nesting material should be identified and removed from walls, ceilings and floors. In combination with removing the rodent material, fumigate the building with preparations containing pyrethrins and permethrins. Ticks can be controlled with insecticides such as malathion, permethrin or propoxur, sprayed around houses. In carrying out this, special attention needs to be paid to any cracks and crevices where ticks may hide. More than one treatment is often needed to effectively rid the building of soft-ticks. In addition, insecticides can be mixed with the floor or wall plaster during construction or repair work.

Infants and adults can be protected from house-invading ticks by sleeping under mosquito net (Webber, 2005).

2.3.5 Treatment of Tick-borne relapsing fever

Treatment of TBRF is with a single dose of 300,000 units of procaine penicillin immediately, followed the next day by tetracycline 500 mg four times daily for 10 days. This regimen provides adequate treatment; while at the same time minimizes reactions (Webber, 2005). A common and potentially serious complication of relapsing fever treatment is the Jarisch-Herxheimer reaction, caused by the massive release of cytokines (primarily TNF-alpha, IL-6 and IL-8) during the spirochete die-off. The reaction usually begins 2 to 4 hours after antibiotic administration and is similar to the crisis stage of the fever cycle. Typical presentations are elevated fever, increased respiration and heart rate, excessive sweating, chills, and sudden changes in blood pressure. Fatalities from the J-H reaction can occur. Research suggests that administration of anti-TNFalpha antibodies can ameliorate the severity of the J-H reaction, but aspirin, acetaminophen and corticosteroids are ineffective (Cadavid and Barbour, 1998).

Another common regimen is 100 mg of doxycycline every 12 hours or 500 mg of erythromycin every 6 hours, for one week. Intravenous penicillin is recommended in cases of suspected or proven central nervous system involvement. The mortality rate for untreated TBRF is between 5 to 10%. Treated properly, the death rate is reduced to around 1%, but TBRF patients often report residual symptoms even after treatment. These symptoms are usually associated with delayed diagnosis and initiation of treatment (Cadavid and Barbour, 1998).

2.3.6 Diagnosis of Tick-borne relapsing fever

Diagnosis of TBRF depends on observation of borreliae in the blood, bone marrow, cerebrospinal fluid or urine (CDC: National Center for Emerging and Zoonotic Infectious Diseases, 2012). The

spirochaetes can be visualized in fresh specimens by dark-field microscopy or in stained smears by light microscopy at a magnification of 400X to 1000X. *Borreliae* are the only spirochaetes in the blood that are stainable with aniline dyes (Giemsa or Wright's). Thin and thick smears are diagnostic in 70% cases, and repeated smear increase this yield (Parola and Raoult, 2001). The *Borrelia* index (number of spirochaetes per white blood cells in a thick smear) provides a clue to the severity of illness; indices greater than two are associated with more severe complications. Organisms are rarely visualized in the blood between relapses and become increasingly difficult to find during subsequent relapses. Serologic tests are unreliable because of antigenic variations. Furthermore, serologic testing is not useful for making an immediate diagnosis, as it requires an acute sample to be taken within 7 days of symptom onset and a convalescent sample taken at least 21 days after symptoms start (Schwan *et al*., 1999).

Quantitative buffy coat is 100 times more sensitive than thick films *in vitro* and may be useful in diagnosis of relapsing fever. However, this requires special equipment (van Dam *et al*., 1999). The organism can be grown in culture using fortified Kelly's growth medium (Kelly, 1971; Stoenner *et al*., 1982). In this technique, a couple of drops of blood are added to the medium, which is incubated at 30-37°C and kept for 2 to 6 weeks. Dark-field microscopy is used to periodically check for spirochetes.

In early 19th century research, blood from patients was inoculated into mice and the spirochetes amplified in murine blood (Moursund, 1942). Polymerase chain reaction (PCR) amplification has been developed for identification of most *Borrelia* species (Fukunaga *et al*., 1996). A real-time PCR assay for *B. recurrentis* was described in 2003, and it does not cross-react with other *Borrelia* species (Jiang *et al*., 2003).

Polymerase chain reaction methods that are available for detection of *Borrelia* DNA in various specimens include standard PCR, nested PCR, competitive PCR and real-time PCR (Wang *et al*., 2010). With these methods, direct detection of *Borrelia* species infection in tick vectors, host reservoirs as well as clinical specimens can be carried out. However, the efficiency of a PCR assay is determined by various factors. Among these, the selection of an appropriate gene target and primer set for PCR amplification are the most important. In general, a PCR primer set yielding an amplicon of 100 to 300 bp is recommended, as it has high amplification efficiency under standard PCR conditions and can attenuate the effects of DNA fragmentation during sample processing (Wang *et al*., 2010). Although PCR assays targeting numerous *Borrelia* genes have been employed extensively in research settings, only a few of these genes have been widely utilized for PCR-based detection of *Borrelia* species. These include the chromosomally encoded genes *rrs*, *flaB*, *recA* and *p66*, and the plasmid-encoded gene *ospA* (Wang *et al*., 2010).

2.4.1 Leptospirosis-the disease

Leptospirosis is a bacterial disease that affects humans and animals. It is caused by spirochaete bacteria of the genus *Leptospira* which includes a small number of pathogenic and saprophytic species (CDC, 2005). *Leptospira* was first observed in 1907 in kidney tissue slices of a leptospirosis victim who was described as having died of "yellow fever" (Stimson, 1907). The genus *Leptospira* is divided into 20 species based on DNA hybridization studies (Brenner *et al*., 1999; Bharti *et al*., 2003). Members of *Leptospira* are also grouped into serovars according to their

antigenic relatedness. There are currently over 200 recognized serovars. A few serovars are found in more than one species of *Leptospira*.

Leptospirosis in animals is often subclinical. The disease is maintained in nature by chronic renal infection of carrier animals, which excrete the organism in their urine, contaminating the environment. Leptospires may persist for long periods in the renal tubules of animals by establishing a symbiotic relationship with no evidence of disease or pathological changes in the kidney (Inada *et al*., 1916). As a result, animals that serve as reservoirs of host-adapted serovars can shed high concentrations of the organism in their urine without showing clinical evidence of disease. This leptospiruria in animals often occurs for months after the initial infection. Rodents are implicated most often in human cases.

Human infection occurs either by direct contact with infected urine or tissues, or more commonly by indirect exposure to the organisms in damp soil or water. The infection in man is contracted through skin abrasions and the mucosa of the nose, mouth and eyes. Exposure through water contaminated by urine from infected animals is the most common route of infection (Inada *et al*., 1916). Most human infections are most likely asymptomatic; the spectrum of illness is extremely wide, ranging from undifferentiated febrile illness to severe multisystem disease with high mortality rates. Most cases occur in the warm season and in rural areas because leptospires can persist in water for many months. They survive best in freshwater, damp alkaline soil, vegetation, and mud with temperatures higher than 22°C (Inada *et al*., 1916). The extreme variation in clinical presentation is partly responsible for the significant degree of underdiagnosis. Human-to-human transmission is rare. Leptospiruria in humans is more transient, rarely lasting more than 60 days. Humans and nonadapted animals are incidental hosts. With rare exceptions, man represents a dead

end in the chain of infection because person-to-person spread of the disease is rare (Feigin and Anderson, 1975).

2.4.2 Leptospirosis distribution

The disease is a global condition found across the world. Specific serovars vary with locality. Rates of leptospirosis are highest in tropical and sub-tropical locations, particularly in rural areas. However, the condition is becoming increasingly widespread in urban areas that have poor sanitation. Up to 80% of individuals in tropical areas are estimated to have positive seroconversion rates, indicating either past or present infection. Although leptospirosis is generally associated with tropical countries and heavy rainfall, most cases actually occur in temperate climates, possibly because of under reporting in some countries. High-risk areas include the Caribbean islands, Central and South America, Southeast Asia, and the Pacific islands (Levett and Haake, 2009).

It is estimated that globally 10 million people catch leptospirosis every year (WHO factsheet, 2010). It is hard to estimate how many people die from leptospirosis because many cases occur in parts of the developing world where causes of death are not routinely reported. However, some experts have estimated that the fatality rate from leptospirosis could range from 5 to 25% (WHO Factsheet, 2010). Deaths from leptospirosis are higher in countries where access to good quality healthcare is limited.

2.4.3 Morphology of Leptospires

Leptospira, (Figure 2.5), are spiral-shaped bacteria that are 6 to 20 μm long and 0.1 μm in diameter with a wavelength of about 0.5 μm (Levett, 2001). One or both ends of the spirochete are usually hooked. *Leptospira* have a gram-negative-like cell envelope consisting of a cytoplasmic and outer membrane. However, the peptidoglycan layer is associated with the cytoplasmic rather than the outer membrane, an arrangement that is unique to spirochetes. Motility is conferred by the rotation

of two axial flagella underlying the membrane sheath, which are inserted at the opposite ends of the cell and overlap in the central region (Mathias *et al*., 2008).

The outer membrane contains a variety of lipoproteins and transmembrane outer membrane proteins (Cullen *et al*., 2002). The protein composition of the outer membrane differs when comparing *Leptospira* growing in artificial medium with *Leptospira* present in an infected animal (Palaniappan *et al*., 2002; Nally *et al*., 2007). Several leptospiral outer membrane proteins have been shown to attach to the host extracellular matrix and to factor H. These proteins may be important for adhesion of *Leptospira* to host tissues and in resisting complement, respectively (Choy *et al*., 2007).

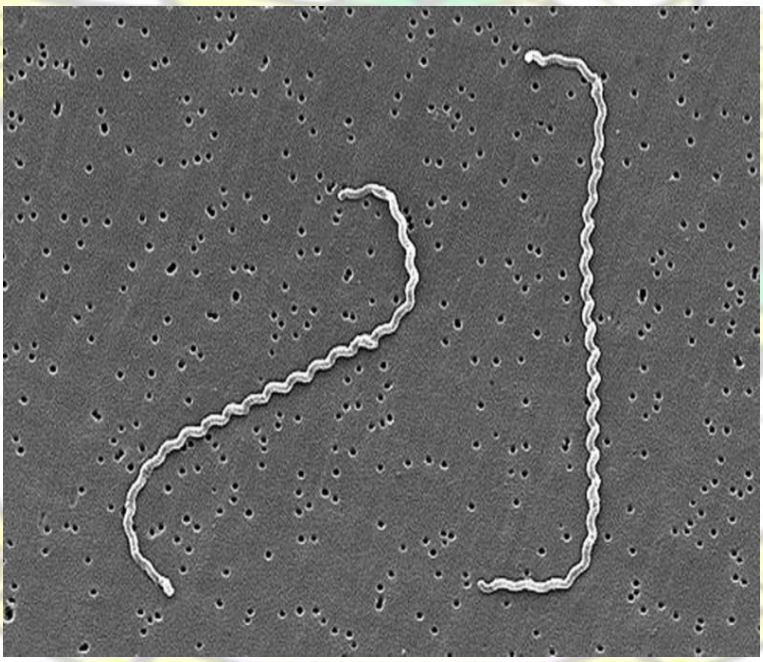

Figure 2.5: Electron micrograph of Leptospira interrogans. (Courtesy Rob Weyant, Centers for Disease Control and Prevention, 1982)

The outer membrane of *Leptospira*, like those of most other gram-negative bacteria, contains lipopolysaccharide (LPS). Leptospiral LPS has low endotoxin activity (Levett, 2001). An unusual feature of leptospiral LPS is that it activates host cells via TLR2 rather than TLR4 (Werts *et al*., 2001). The unique structure of the lipid A portion of the LPS molecule may account for this observation (Que-Gewirth *et al*., 2004). Finally, the LPS O antigen content of *L. interrogans* differs in an acutely infected versus a chronically infected animal (Nally *et al*., 2005). The role of O antigen changes in the establishment or maintenance of acute or chronic infection, if any, is unknown. Differences in the highly immunogenic LPS structure account for the numerous serovars of *Leptospira* (Levett, 2001). Consequently, immunity is serovar specific; current leptospiral vaccines, which consist of one or several serovars of *Leptospira* endemic in the population to be immunized, protect only against the serovars contained in the vaccine preparation.

Leptospira, both pathogenic and saprophytic, can occupy diverse environments, habitats and life cycles; these bacteria are found throughout the world, except in Antarctica (Madigan and Martinko, 2005). High humidity and neutral (6.9-7.4) pH are essential for their survival in the environment, with stagnant water reservoirs being the natural habitat for the bacteria. *Leptospira* are typically cultivated at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Ellinghausen and McCullough 1965), but the formula was modified by replacing rabbit serum with polysorbate 80-albumin to enhance growth of fastidious strains (Johnson and Harris, 1967). Growth of pathogenic *Leptospira* in an artificial nutrient environment such as EMJH becomes noticeable in 4 to 7 days; growth of saprophytic strains occurs within 2 to 3 days. The minimal growth temperature of pathogenic species is 13 to 15°C. Because the minimal growth temperature of the saprophytes is 5 to 10 °C, the ability of *Leptospira* to grow at 13°C can be used to distinguish saprophytic from pathogenic *Leptospira* species (Isenberg, 1992). The optimal pH for growth of *Leptospira* is 7.2 to 7.6.

Leptospira are aerobes whose major carbon and energy source during *in vitro* growth is longchain fatty acids, which are metabolized by beta-oxidation (Johnson and Gary, 1963; Henneberry and Cox, 1970). Fatty acids are provided in EMJH in the form of Tween 20 (Johnson and Harris, 1967). Fatty acid molecules are bound by albumin in EMJH and are released slowly into the medium to prevent its toxic accumulation. Like most bacteria, *Leptospira* require iron for growth (Faine, 1959). *L. interrogans* and *L. biflexa* have the ability to acquire iron in different forms (Louvel *et al*., 2006). A TonB-dependent receptor required for utilization of the ferrous form of the iron has been identified in *L. biflexa*, and an ortholog of the receptor is encoded in the genome of *L. interrogans*. *L. interrogans* can also obtain iron from heme, which is bound to most of the iron in the human body. The HbpA hemin-binding protein, which may be involved in the uptake of hemin, has been identified on the surface of *L. interrogans* (Asuthkar *et al*., 2007) Although other pathogenic species of *Leptospira* and *L. biflexa* lack HbpA, yet another heminbinding protein, LipL41, may account for their ability to use hemin as a source of iron (Asuthkar *et al*., 2007). Although they do not secrete siderophores, *L. biflexa* and *L. interrogans* may be capable of obtaining iron from siderophores secreted by other microorganisms (Louvel *et al*., 2006).

2.4.3.1 Pathogenesis of Leptospirosis

Symptoms of the disease begin from 2 to 25 days after initial direct exposure to the urine or tissue of an infected animal (Levett, 2001). This can even occur via contaminated soil or water. Veterinarians, pet shop owners, sewage workers, and farm employees are at particularly high risk. People participating in outdoor sporting activities like canoeing, rafting, hiking, and camping can also come into contact with contaminated water or soil. The illness typically progresses through two phases: The first phase of nonspecific flu-like symptoms includes headaches, muscle aches, eye pain with bright lights, followed by chills and fever. Watering and redness of the eyes occurs and symptoms seem to improve by the fifth to ninth day. The second phase begins after a few days

of feeling well. The initial symptoms recur with fever and aching with stiffness of the neck. Some patients develop serious inflammation of the nerves to the eyes, brain, spinal column (meningitis), or other nerves. Right upper area abdominal pain may occur. Less common symptoms relate to disease of the liver, lungs, kidneys, and heart (WHO Factsheet, 2010).

After leptospires gain entry via intact skin or mucosa, they multiply in blood and tissue. The resulting leptospiremia can spread to any part of the body but particularly affects the liver and kidney (Palaniappan *et al*., 2007). In the kidney, leptospires migrate to the interstitium, renal tubules, and tubular lumen, causing interstitial nephritis and tubular necrosis. When renal failure develops, it is usually due to tubular damage, but hypovolemia from dehydration and from altered capillary permeability can also contribute to renal failure. Liver involvement is seen as centrilobular necrosis with proliferation of Kupffer cells. Jaundice may occur as a result of hepatocellular dysfunction. Leptospires may also invade skeletal muscle, causing edema, vacuolization of myofibrils, and focal necrosis. Muscular microcirculation is impaired and capillary permeability is increased, with resultant fluid leakage and circulatory hypovolemia (Faine *et al*., 1999).

In severe disease, a disseminated vasculitic syndrome may result from damage to the capillary endothelium. Leptospires may invade the aqueous humor of the eye, where they may persist for many months, occasionally leading to chronic or recurrent uveitis (Pappachan *et al*., 2007). Despite the possibility of severe complications, the disease is most often self-limited and nonfatal. Over time, a systemic immune response may eliminate the organism from the body but may also lead to a symptomatic inflammatory reaction that can produce secondary end-organ injury (Palaniappan *et al*., 2007).

2.4.4 Control and prevention of Leptospirosis

Prevention of leptospirosis can be achieved by avoidance of high-risk exposures, adoption of protective measures, immunization and use of chemoprophylaxis, in varying combinations depending on the environmental circumstances and the degree of human activity.

High-risk exposures include immersion in fresh water, such as swimming, and contact with animals and their body fluids (Faine *et al*., 1999). Removal of leptospires from the environment is impractical, but reducing direct contact with potentially infected animals and indirect contact with urine-contaminated soil and water remains the most effective preventive strategy available. Consistent application of rodent control measures is important in limiting the extent of contamination. In tropical environments, walking barefoot is a common risk-factor (Douglin *et al*., 1997).

Immunization of animals with killed vaccines is widely practiced, but is short-lived and animals require periodic boosters (Wang *et al*., 2007). These vaccines however, do not prevent infection and renal colonization as a result; they tend to have little effect on the maintenance and transmission of the disease within the animal population in which they are applied.

Human immunization is not widely practiced. Vaccines are used in Europe and Asia. Travelers, who visit endemic regions or will be engaged in activities that increase likelihood of exposure, can take 200 mg of doxycyline per week by mouth starting before and during the time period of potential exposure. This regimen is recommended for those with short-term exposure and is not for repeated or long-term exposure (Guidugli *et al*., 2000).

2.4.5 Treatment of Leptospirosis

Antibiotics have been used to treat leptospirosis since penicillin first became available. Antibiotic therapy consistently prevents or reduces the duration of leptospiruria. A few randomized or

placebo-controlled trials have also been performed but yielded conflicting results (McClain *et al*., 1984; Edwards *et al*., 1988; Costa *et al*., 2003). Severe disease is usually treated with intravenous penicillin and mild disease with oral doxycycline. Once daily ceftriaxone has been shown to be as effective as penicillin (Panaphut *et al*., 2003).

Antibiotic therapy should be initiated as soon as disease is suspected. Supportive therapy is essential for hospitalized patients. Jarisch-Herxheimer reactions were reported in patients treated with penicillin (Vaughan *et al*., 1994; Emmanouilides *et al*., 1994). Patients receiving penicillin should be monitored because of the increased morbidity and mortality of such reactions.

2.4.6 Diagnosis of leptospirosis infection

2.4.6.1 Direct detection methods

Visualization of leptospires in blood or urine by darkfield microscopy can be used. However, artefacts are commonly mistaken for leptospires, and the method has both low sensitivity (40.2%) and specificity (61.5%) (Vijayachari *et al*., 2001). A range of staining methods has been applied to direct detection, including immunofluorescence staining, immunoperoxidase staining and silver staining. These methods are not widely used because of the lack of commercially available reagents and their relatively low sensitivity.

A monoclonal antibody-based dot-enzyme-linked immunosorbent assay (ELISA) for detection of leptospiral antigen in urine assay was developed (Saengjaruk *et al*., 2002). This assay has not been evaluated widely and is not available commercially.

Several polymerase chain reaction (PCR) assays were established for the detection of leptospires (Levett, 2001). The main advantage of PCR is the prospect of confirming the diagnosis during the early acute stage of the illness, before the appearance of immunoglobulin M (IgM) antibodies, when treatment is likely to have the greatest benefit. In fulminating cases, in which death occurs

before seroconversion, PCR may be of great value (Brown *et al*., 1995). Leptospiral DNA has been amplified from serum, urine, aqueous humor and a number of tissues obtained at autopsy. For early diagnosis, serum is the optimal specimen.

Histological diagnosis has traditionally relied on silver impregnation staining (Stimson, 1907), but immunohistochemical staining offers greater sensitivity and specificity (Alves *et al*., 1987; Guarner *et al*., 2001).

2.4.6.2 Isolation and identification

Leptospires can be isolated from blood, CSF, and peritoneal dialysate fluids during the first 10 days of illness before antibiotic therapy are initiated. Urine can be cultured after the first week of illness. Specimens are collected into sterile containers without preservatives and processed within a short time of collection. Best results are obtained when the delay is less than an hour, as leptospires do not survive well in acidic environments (Levett, 2003).

Cultures are performed in albumin-polysorbate media such as EMJH or PLM-5. Primary cultures are performed in semi-solid medium, to which 5-fluorouracil is usually added as a selective agent. Cultures are incubated at 30°C for several weeks, as initial growth may be very slow.

Isolated leptospires are identified to serovar level either by traditional serologic methods or by molecular methods such as pulse field gel electrophoresis (Levett, 2003).

2.4.6.3 Indirect detection methods

Most leptospirosis cases are diagnosed by serology. The reference standard assay is the microscopic agglutination test (MAT). In this assay, live antigens representing different serogroups of leptospires are reacted with serum samples and then examined by darkfield microscopy for agglutination (Levett, 2003). However, this is a complex test to maintain, perform, and interpret.

A serologically confirmed case of leptospirosis is defined by a fourfold rise in MAT titer to one or more serovars between acute phase and convalescent serum specimens run in parallel. A titer of at least 1:800 in the presence of compatible symptoms is strong evidence of recent or current infection (Levett, 2003).

The interpretation of MAT is complicated by cross-reaction between different sreogroups, especially in acute-phase samples (Levett, 2001). Cross-reactivity in acute samples is attributable to IgM antibodies, which may persist for several years (Cumberland *et al.*, 2001). The MAT is a serogroup-specific assay, and cannot be used to interpret the identity of the infecting serovar (Levett, 2003). However, knowledge of the presumptive serogroup may be of epidemiological value in determining potential exposures to animal reservoirs.

2.5.1 Leishmaniasis-the disease

Leishmaniasis is a disease caused by protozoan parasites that belong to the genus *Leishmania* and is transmitted by the bite of certain species of sand fly (subfamily Phlebotominae). Although the majority of the literature mentions only one genus transmitting *Leishmania* to humans (*Lutzomyia*-Old World) in the Americas, a 2003 study by Galati suggested a new classification for the New World sand flies, elevating several subgenera to the genus level. Elsewhere in the New World, the genus *Phlebotomus* is considered the vector of leishmaniasis (Myler and Fasel, 2008). Most forms of the disease are transmissible only from animals (zoonosis), but some can be spread between humans. Human infection is caused by about 21 of 30 species that infect mammals (Lainson and Shaw, 1987; Herwaldt, 1999). These include the *L. donovani* complex with three species (*L. donovani, L. infantum,* and *L. chagasi*); the *L. mexicana* complex with four main species (*L. mexicana, L. amazonensis,* and *L. venezuelensis*); *L. tropica; L. major; L. aethiopica*; and the subgenus *Viannia* with four main species (*L. (V.) braziliensis, L. (V.) guyanensis, L. (V.) panamensis*, and *L. (V.) peruviana*). The different species are morphologically indistinguishable, but they can be differentiated by isoenzyme analysis, DNA sequence analysis, or monoclonal antibodies (Lainson and Shaw, 1987; Herwaldt, 1999).

The disease is zoonotic, mainly affecting small mammals (rodents and canids) and is transmitted to man through the bite of an infected female sandfly seeking a blood meal. Leishmaniasis can be spread through blood transfusions or contaminated needles (Otero *et al*., 2000). *Leishmania* parasites can also cross to the placenta (Meinecke *et al*., 1999). The clinical forms of the disease are varied and unpredictable ranging from relatively mild but debilitating cutaneous leishmaniasis (CL) which result in disfiguring scars, to the more destructive visceral leishmaniasis (VL), 90% cases of which are fatal if untreated. Other forms of the disease include diffuse cutaneous leishmaniasis (DCL); which produces widespread skin lesions resembling leprosy and is

particularly difficult to treat. Mucocutaneous leishmaniasis (ML) the other form of infection commences with skin ulcers which spread causing tissue damage particularly to nose and mouth (Berman, 1995; Singh and Sivakumar, 2003).

Leishmaniasis-related disabilities impose a great social burden, as infections can occur on a large scale with large numbers of cases that are personally and socially disruptive (Herwaldt, 1999). Cutaneous leishmaniasis is believed to have been prevalent in a number of West African countries for several decades and although it has been under-reported and underestimated in its extent and severity, there were reports of its incidence as far back as 1911 in Niger. Other cases have also been reported in Mali, Burkina Faso, Senegal, Nigeria, Guinea Conakry and Cameroon (Boakye *et al.*, 2005). In the mid to late 1990s, incidences of epidemic proportion were reported in Ouagadougou, the capital of Burkina-Faso which is on the northern border of Ghana. In a retrospective study of disease incidence in Ouagadougou, 1,845 cases were identified from 1996 to1998 (Traore *et al*., 2001).

The first known cases of cutaneous leishmaniasis (CL) in the Volta Region of Ghana were recognized in 2002 (Kweku M., personal communication), based on the histological examination of fifteen biopsy samples at the Korle-Bu Teaching Hospital, Accra, Ghana. These were obtained from individuals living in six different communities in the Ho District. Leishman-Donovan bodies were identified in ten out of the fifteen samples, leading to the first suspicion of cutaneous leishmaniasis as the cause of an epidemic first identified in 1999 at Nyive, a village on the Ghana-Togo border. A follow up on the presumptive diagnosis in three schools where some of the cases were discovered, revealed 12.2% to 32.3% of pupils having similar lesions (Kweku M., personal communication). In the 2004 annual report of the Ghana Health Service (GHS), a total of 2,348 cases before 2003 and 6,185 cases between 2003 to October 2004 were described in the section on

CL surveillance and disease situation in the Ho District. Since 2004, there have been sporadic, unconfirmed outbreaks in different communities all over the Ho District. Leishmaniasis is not known to be transmitted from humans to humans; the unpredictable nature of the outbreaks is an indication that the parasite may be harbored by certain animal populations and is picked up by one or several vector species which then incidentally infect humans.

2.5.2 Geographic distribution of Leishmaniasis

It was estimated that 350 million people in 88 countries on five continents (Africa, Asia, Europe, North America and South America) are at risk around the world with 1.5 million new cases of CL each year (CDC, 2000). It is believed that worldwide 12 million people are affected by leishmaniasis; this figure includes cases with overt disease and those with no apparent symptoms. Of the 1.5 to 2 million new cases of leishmaniasis estimated to occur annually, only 600 000 are officially declared. Five hundred thousand new cases of VL occur annually, of which 90% are in five countries: Bangladesh, Brazil, India, Nepal and Sudan. Ninety percent (90%) of all cases of MCL occur in Bolivia, Brazil and Peru whereas 90% of all cases of CL occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia and Syria, with 1 to 1.5 million new cases reported annually worldwide. In Iran, CL is endemic in south, east north and some part of the center of Iran. In Africa, leishmaniasis is endemic to countries mostly in the North, Central, East and the Horn of Africa. The disease is also endemic in West Africa (Sheik-Mohamed and Velema, 1999). Although the disease has a long history in West Africa, it appears to be one of the less recognized or under-reported parasitic infections in this region (Desjeux *et al*., 1981). Since the first published work indicating the presence of leishmaniasis in Niger in 1911, other cases of leishmaniasis have been reported in West Africa mostly from Mali, Nigeria and Senegal as well as Cameroon. Other countries in the sub-region that also reported cases in the past included, Haute Volta (present day

Burkina Faso), Mauritania, Gambia and Guinea. Based on this information, cutaneous leishmaniasis is proposed to be endemic in a belt running from

Mauritania, Gambia and Senegal in the west to Nigeria and Cameroon in the east (Dyce-Shar, 1924).

The geographical distribution of leishmaniasis is limited by the distribution of the sand fly, its susceptibility to cold climates, its tendency to take blood from humans or animals only and its capacity to support the internal development of specific species of *Leishmania* (Abdulla and Riad, 2002).

2.5.3 Morphology of *Leishmania* and vector

Leishmania parasites are found in two morphologic forms during their life cycle. In humans and other mammalian hosts, they exist within macrophages as round to oval nonflagellated amastigotes and in the arthropod vectors (sand flies) the parasites exist as elongated flagellated promastigotes (Figure 2.6). About 30 species of phlebotomine sand flies can become infected when taking a blood meal from a reservoir host. Sand flies are small mosquito-like insects 1.5 to 4 mm in length and their small size allows them to pass through ordinary mesh screens and mosquito netting (Figure 2.7). They are noiseless when they fly so their presence is not readily noticeable. Sand flies are usually most active during twilight, evenings and at night but less active during the hottest time of the day (Sawalha *et al*., 2003).

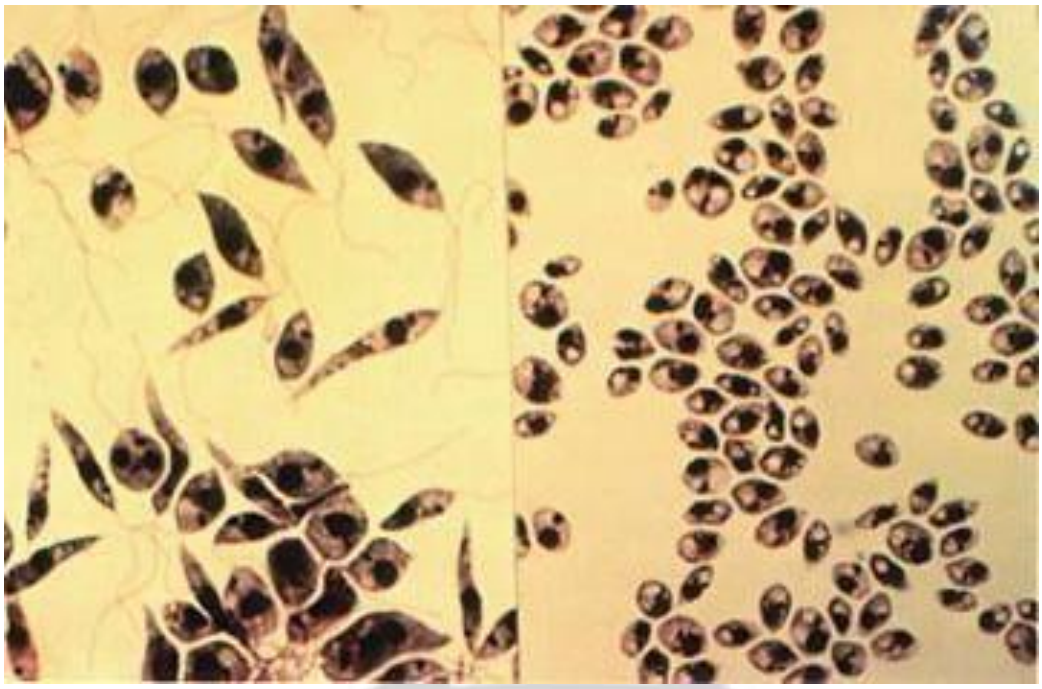

Figure 2.6: Leishmania promastigotes (left) characterized by a flagellum and a kinetoplast, anterior to the nucleus and amastigotes (right). (Source: Brian E. Keas, 1999)

Figure 2.7: The sand fly vector (Source: Jose Ribeiro, National Institute of Allergy and Infectious Diseases, NIAID, 1999)

2.5.4 Pathogenesis of Leishmaniasis

The sand flies inject the infective stage, metacyclic promastigotes, during blood meals (Burchmore and Barrett, 2001). Metacyclic promastigotes that reach the puncture wound are phagocytized by macrophages and transform into amastigotes (Dacie *et al*., 2006). Amastigotes multiply in infected cells and affect different tissues, depending in part on which *Leishmania* species is involved (Soto and Toledo, 2007). These differing tissue specificities cause the differing clinical manifestations of the various forms of leishmaniasis. Sand flies become infected during blood meals on an infected host when they ingest macrophages infected with amastigotes (Arevalo *et al*., 2007; Sunder *et al*., 2007). In the sand fly's midgut, amastigotes released from the macrophages transform into promastigote forms (Burchmore and Barrett, 2001). The flagellated promastigotes divide repeatedly and block the gut resulting in the movement of the organisms forward into the pharynx and buccal cavity. The promastigotes then become attached to the epithelial lining of the sand fly using flagella through specific lectin-like receptors on the sandfly epithelium attaching to lipophosphoglycans secreted by the parasite (Chaves *et al*., 2003). The flagella are also used to penetrate the epithelial cells to allow leakage of cell sap, an important nutrient source for the promastigotes.

For the infection process, promastigotes release chitinolytic enzymes, which are inhibited by haemoglobin. When the sandfly is between blood meals and feeding on plant juices these enzymes become activated. It has been suggested that the enzymes damage the fly's cardiac valve, which normally prevents back flow of the blood meal. When the fly takes a blood meal, the damaged valve allows flow in both directions thus permitting some promastigotes to escape back into the host's skin (Burchmore and Barrett, 2001). The secretion of chitinases by the promastigotes trapped together with the blood meal, within the peritrophic membrane of the sand fly; allow them

to escape through the same membrane to continue their development. If release of this chitinase is inhibited, the *Leishmania* is excreted with the residual blood meal (CDC Fact Sheet, 2003).

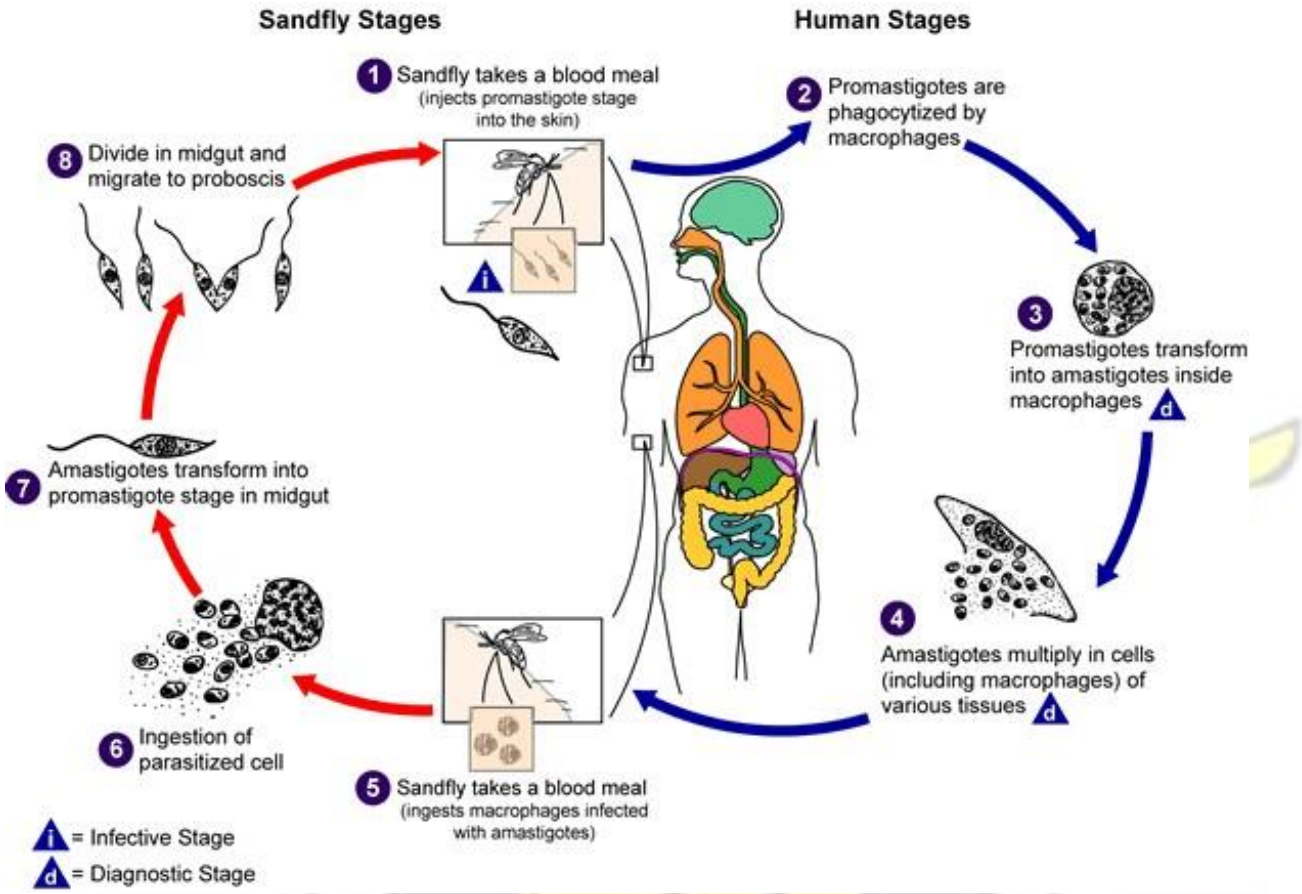

Figure 2.8: Life cycle of the *Leishmania* parasite (Source: CDC Parasites and Health, 2000)

In cutaneous leishmaniasis, the infection site is usually localized to the site in which the sand fly bite occurs. The amastigotes multiply in the reticuloendothelial system of the skin. The incubation period spans from one day up to several months. A symptom of the developing infection is the appearance on the surface of the skin a small red papule, the result of the development of a thin crust on the skin at the bite site, which hides the developing ulcer underneath. However, several

sites of infection, which are close together, may coalesce to form a large sore on the skin. If kept clean the sores will heal spontaneously within 2 months to 1 year.

These sores are often the sites of secondary infection and can result in permanent disfiguration. Some secondary infections include Yaws caused by spirochaetes and a disease condition known as Myiasis where ulcers become infected with fly maggots (Markle and Makhoul, 2004). Once an infection has been cleared the host is immune to reinfection.

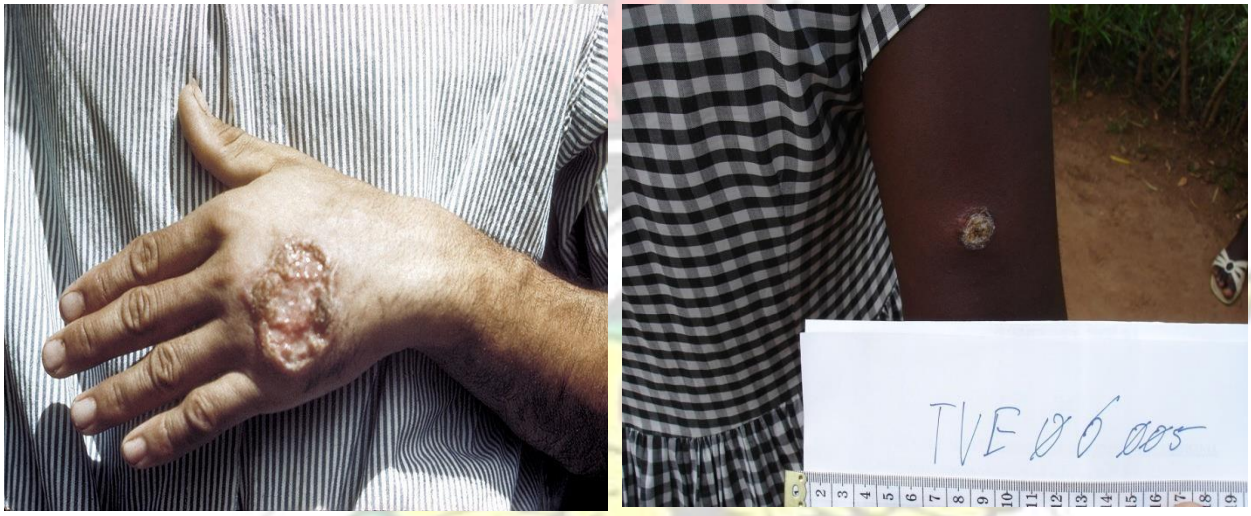

Figure 2.9: Cutaneous leishmanisis ulcer: on hand of Central American adult (Left) (Source: National Institute of Allergy and Infectious Diseases, NIAID) and on upperarm of a Ghanaian student (Right) (Source: Naiki Puplampu, picture taken at Taviefe, Ho District, 2006)

2.5.5 Control and prevention of Leishmaniasis

Animals like dogs, rats; gerbils, other small mammals and rodents serve as reservoirs of the disease. One important way of prevention is to suppress both vector and reservoir (Lane, 1993). This can be achieved through adopting personal protective measures, particularly at night by minimizing the amount of exposed skin. Application of insect repellent like N,

Ndiethylmetatoluamide (DEET) and treatment of clothes and bed nets with Permethrin can also be used in endemic areas.

2.5.6 Treatment of Leishmaniasis

The most commonly used, cheapest and most effective chemotherapies for *Leishmania* infections are antimony-containing compounds such as Meglumine or Amphotericin B7 (Medical Letter, 2000). However, these drugs cause severe allergies to most users. They are also not safe for pregnant women and those breast-feeding. Sodium stibogluconate (Hepburn, 2003) may be used to minimize or avoid the allergies associated with the use of antimony compounds. This drug is however less effective and produces drug-resistant *Leishmania*. Paromomycin, an antimony topical ointment may be used to treat cutaneous-*Leishmania* infections. Fluconazole is believed to decrease healing time. Pentoxifylline has been used with N-methylglucamine antimoniate to successfully treat CL that is unresponsive to antimonial drugs (Báfica *et al*., 2003). Drugs such as Pentostam and Glucantime are often used although Aminosidine has been found to be more effective for CL and better tolerated (CDC Fact Sheet, 2003). However, either prolonged use or inefficient drug therapy has resulted in drug resistance. More than 60% of the clinical cases are resistant to the first line drug, antimony (Sundar and Chatterjee, 2006). There is concern about the possibility of development of resistance to the new oral drug miltefosine in the near future as there are already reports of relapse among miltefosine treated cases (Pandey *et al*., 2009).

Vaccination would be the method of choice and a number of vaccine trials were carried out both in South America and the Middle East with mixed success. Vaccine candidates consisted of whole killed promastigotes administered together with BCG (as an adjuvant) against CL (CDC Fact Sheet, 2003). Attempts to develop vaccines for parasitic agents such as heat killed, subunit, or DNA vaccines have not resulted in a successful vaccine candidate that could be applicable to humans (Kedzierski *et al*., 2006; Palatnik-de-Sousa, 2008). However, past experience has revealed

that individuals who recover from *Leishmania* infection develop a long lasting protection from future infections suggesting that a successful vaccine candidate needs to cause a controlled infection that evokes a protective immunity. Experience from other pathogens has suggested that a live-attenuated parasite vaccine could fulfill such requirements (Kedzierski, 2010).

2.5.7 Diagnosis of *Leishmania* parasites

Traditionally, investigations relating to *Leishmania* diagnosis and research use the identification of amastigotes by histology or direct microscopy, and the growth of promastigotes in culture (Vega-Lopez, 2003; Costa *et al*., 2003). However, isolation or demonstration of parasites is poor due to low parasite load and high rate of culture contamination (Singh and Sivakumar, 2003). Several recombinant proteins have been developed to improve detection of the parasites. In this regard, a recombinant protein, kinesin (39 kDa) referred to as rK39 was identified as the most promising molecule (Rosati *et al*., 2003). This protein serving as antigen has been shown to carry immunodominant epitopes that increases antigenic sensitivity. The rK39 antigen has been put into various formats including that of an enzyme-linked immunosorbent assay (ELISA) for the specific diagnosis of VL and has been found to be valuable in monitoring drug therapy and detecting relapse of the disease (Kumar *et al*., 2001). Molecular techniques targeting various genes of the *Leishmania* parasite have also been developed (Faber *et al*., 2003). This is to obviate the inconclusiveness of the conventional reported methods of smear, culture, and histopathology of a skin biopsy specimen for detecting the presence of the *Leishmania* parasite. Compared to the conventional methods, the molecular technique involving polymerase chain reaction (PCR) is reported to be the most sensitive single test for detecting *Leishmania* parasites (Faber *et al*., 2003). The technique offers considerable advantages in the collection and transport of specimens and DNA extraction procedures that are more efficient in laboratory and field-based protocols (Vega-

Lopez, 2003). It also allows for fast identification of *Leishmania* at species and subspecies level (Vega-Lopez, 2003; Singh and Sivakumar, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Criteria for Study Site Selection

Using environmental data such as rainfall, temperature, vegetation and altitude obtained from a variety of sources; both satellites and ground-based meteorological stations were recently used to create Lassa fever 'risk maps' (Fichet-Calvet and Rodgers, 2009). According to these risk maps, the LF risk area covers approximately 80% of the area of each of Sierra Leone and Liberia, 50% of Guinea, 40% of Nigeria, 30% of each of Côte d'Ivoire, Togo and Benin and 10% of Ghana. The analyses showed quite clearly that Lassa fever requires a particular combination of high (but not the highest) rainfall, and with a particular form of variability and seasonal timing, whereas its rodent reservoir occurs over regions experiencing a much wider range of rainfall conditions.

Temperature appeared to be less important in determining LASV distribution, although there are large differences between different areas; for example the annual mean and maxima in high risk areas were 27°C and 32°C respectively, whereas in low risk areas the mean temperature was approximately 38°C. Such high temperatures are known to increase LASV decay (Stephenson *et al*., 1984).

These results suggest that the survival of the virus outside of the vertebrate host might be a key to determining its distribution, and that this survival depends upon moisture or rainfall conditions above more or less all other environmental variables. In the case of Lassa, the virus appears to survive better in humid conditions, during the rainy season. Rodents more often tend to be contaminated during their frequent movements at this season, for mating or dispersing into the surrounding fields (Fichet-Calvet *et al*., 2008).

A risk map of Ghana was extracted from the predicted risk maps published by Fichet and Rogers (2009), with red areas indicating high predicted risk and green areas indicating low predicted risk based on the listed factors. Using this map, ten sites were selected for further study; 7 high risk (high probability for Lassa virus presence and rodent vector) and 3 low risk (low probability for Lassa virus and rodent vector presence) (Figure 3.1). For each site, detailed maps were created from the Centre for Remote Sensing and Geographic Information Services (CERSGIS), University of Ghana, Legon, to select specific candidate villages for study. Candidate communities were identified using these detailed maps and the Ghana Population and Housing Census statistics of the year 2000 (as the 2010 statistics data had not been completed), targeted villages with a population size between 500 and 2000 people, and located at least 20 km from any urban center and 5 km distant from any major road. These selection criteria were used because large or highly trafficked communities could have the indigenous rodent populations outnumbered by introduced species such as *Rattus sp*., rather than the targeted *Mastomys* species (Demby *et al*., 2001). Communities that were surveyed cut across several ecological zones ranging from moist semi-deciduous forest (south); guinea savannah (north) to the transitional zone (middle belt).

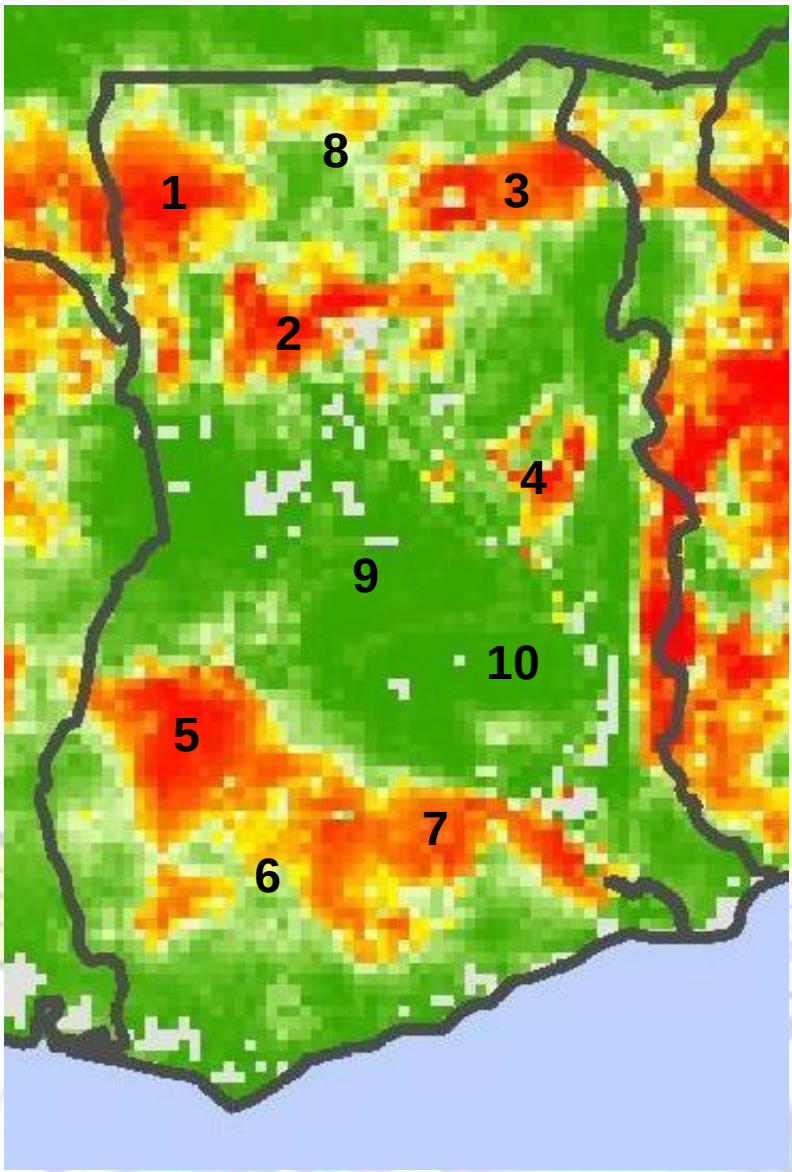

Figure 3.1: Map of Ghana showing location of study sites in the high and low endemic Lassa areas (Source: Fichet-Calvet and Rodgers, 2009). Red areas indicate high predicted risk and green areas indicate low predicted risk.

Site numbers 1, 2, 3, 4, and 8 fall within the Guinea Savannah ecozone, whereas sites 9, 10 and 5, 6, 7 fall within the Transitional zone and Moist Semi-Deciduous forest respectively.

Table 3.1: Study sites selected for study.

Site Number	Region	Community	Latitude	Longitude
1	Upper West	Sankana	10º 12’ N	2º 36’ W
2	Northern	Bowena	9º 33’ N	1º 39’ W
3	Northern	Nagbo	10º 25’ N	0º 20’ W
4	Northern	Wald	8º 12’ N	0º 18’ W
5	Brong Ahafo	Atronie	7º 10’ N	2º 23’ W
6	Ashanti	Adinkra	6º 7’ N	1º 48’ W
7	Eastern	Pankese	6º 31’ N	0º 50’ W
8	Upper East	Nakong-Atinia	10º 48’ N	1º 26’ W
9	Brong Ahafo	Pumpumtifi	8º 01’ N	1º 46’ W
10	Brong Ahafo	Kofe Djan	7º 47’ N	0º 30’ W

After analyses of road maps and census statistics of the sites in Table 3.1, a 20 km zone was created around all the sites. Two to three candidate villages at sites 5, 6, 7, 9 and 10 that met these criteria were visited. However, this was not done at sites 1, 2, 3, 4 and 8 because of the very sparsely populated nature of settlements in these areas. At each village, the chiefs and elders were briefed on the protocol, population size was reassessed and village interest in participating in the study was assessed. In line with traditional cultural practice, a bottle of schnapp or kola nuts was presented to the chief or community leader of each site during the initial meeting. The Director-General of the Ghana Health Service (GHS) approved the study before hand and GHS Regional and District Officers were visited for each site to seek their support and assistance.

Scientific and Ethical review and approval of the study was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR) Science and Technical Committee (STC), the Naval Medical Research Unit No. 3 (NAMRU-3) Science Review Board (SRB), the NMIMR Institutional Review Board (IRB), the NAMRU-3 IRB, and the NAMRU-3 Institutional Animal Care and Use Committee (IACUC). The study numbers for NMIMR and NAMRU-3 IRBs are 013/09-10 and 1008-(DOD #NAMRU3.2010.0008) respectively. Rodent trapping was authorized by the Forestry Commission of Ghana (Permission number FCWD/GH-01).

3.1.1 Community selection for each site

The villages described below were chosen carefully considering all three factors for the selection criteria: population between 500 and 2000, distance greater than 20 km from any urban center or 5km from a paved road, and willingness to participate.

An observation in some of the villages in the northern sector was that houses in the villages were very sparsely distributed because inhabitants farm on the land that surrounds their home. As a result of this, most farmers do not walk long distances to the village outskirts to farm. These farmlands were very dry during the study visits because crops had been harvested and the inhabitants were waiting for the rains to start so they could cultivate new crops.

Secondly, vegetation in this part of the country was found to be mostly grassland, particularly savanna with clusters of drought-resistant trees such as baobabs, acacias and shea.

3.1.2a Sample size determination for rodents

For Lassa virus, the primary interest was whether or not the pathogen was present in rodents in predicted high risk areas. In a similar study in Guinea, the prevalence of PCR positive samples from high prevalence areas was 13.2% and 0 from low prevalence areas. Therefore an expected prevalence of 10% or greater was adopted in high risk areas, with an acceptance of values greater

than 1% to confirm the presence of Lassa virus. Sample size determinations were therefore calculated with the assumption that the number of *Mastomys* rodents in each village was 1,000.

Using Epi Info version 6 approved by the Centers for Disease Control and Prevention, the sample size required was determined to be 41 rodents with a 95% confidence level. Sample size calculations were made with the following assumptions: (1) The population size was 1000 *Mastomys* rodents; (2) The anticipated prevalence rate was 10%; (3) A 9% range in the expected prevalence rate was acceptable; (4) The sample size was calculated using a 95% confidence level.

3.1.2b Sample size determination for humans

For human seroprevalence to Lassa virus, prior studies have found seroprevalence rates of 25 to 55% in high prevalence areas, and 0 to 11% in low risk areas (Kernéis *et al*., 2009). Because Lassa fever has rarely been reported from Ghana, it was anticipated that the human seroprevalence would be closer to 0, and values greater than 11% suggestive of local transmission of disease. It was estimated the villages studied would have populations of 1000 to 1500. Again, using Epi Info version 6, a required sample size of 41 individuals was calculated with a 95% confidence level. Sample size calculations were made with the following assumptions: (1) The population size was 1250 people; (2) The anticipated prevalence rate was 25%; (3) A 13% range in the expected prevalence rate was acceptable; (4) The sample size was calculated using a 95% confidence level.

While this study was designed primarily for determining whether Lassa virus was present in Ghana, a sample size calculation was made for other serologic tests. For these tests, prevelance rates of 20% and population size of 1500 was assumed. Using Epi Info version 6, a required sample size of 59 was calculated with a 95% confidence level. Sample size calculations were made with the following assumptions: (1) The population size was 1500; (2) The anticipated prevalence rate was

20%; (3) A 10% range in the expected prevalence ratewas acceptable; (4) The sample size was calculated using a 95% confidence level.

Therefore, the target set was toattempt to trap 42 to 100 *Mastomys* rodents per village, and obtain blood for serology from 42 to 70 adult volunteers.

3.1.3 Sample site descriptions

Sampling Site 1 (Natorduori): Using the Ghana Population and Housing Census (2000), a village named Natorduori was selected as it satisfied the population and location criteria. This village is located in the Nadowli district in the Upper West region. A map was obtained from CERSGIS, University of Ghana with the coordinates in table 3.1.

Natorduori located at N 10°15.315' W 002°37.536' was about 12 km from the paved road. A meeting with the chief and some inhabitants including the community Chairman was arranged for a briefing on the protocol. The people expressed interest in participating in the study. The population as quoted in the 2000 Ghana Population and Housing Census was 494, but appeared to be more, about 619 in total (a previous year census conducted by the community health nurse). The village had no electricity. Housing was very sparsely distributed with dry vegetation in between the houses (Plate A). Cows, guinea fowls, goats, pigs and chickens were a common sight in the village. Most of the inhabitants in the village practised subsistence farming, with the preferred crops being mainly groundnuts, millet, beans, rice, bambara beans (*Vignea subterranea*), tiger nuts and yam. The main source of water in the community was a borehole. There was no clinic in the community and inhabitants had to walk 6 km to assess healthcare in the neighbouring village (Nator).

Farmers normally leave their homes at about 5.00-6.30 am to their farms and return around 11.00 am to rest and went back late afternoon.

Study team visited the GHS Regional Directorate in Wa for a briefing on the research protocol.

Plate A: Dry vegetation on farmlands (Source: Shirley Nimo-Paintsil picture taken at Natorduori, Nadowli District, March 16, 2011)

Plate B: Farmlands located in between houses in Natorduori village, Nadowli District (March 16, 2011). In the picture are 3 study team members and an inhabitant of the community (as tour guide)

Sampling Site 2 (Bowena): The village is located at N 09°32.734' W 001°37.995' in the West Gonja district of Northern Region near Mole National Park. Bowena was assessed and deemed eligible for the study.

Bowena is about 53 km from the Mole National Park with savanna woodland vegetation. Only 12 km of this total distance was paved and one has to drive through the forest on pathways to get to the community. The total population was reported as 846 (Population and Housing Census, 2000). The village had no electricity. Most of the men had returned from their farms at the time of our visit (about 5 pm). Housing was sparsely distributed with farmlands on the outskirts of the community. Cows, guinea fowls, goats and chickens were seen roaming in the community. Most of the farmers were subsistence farmers and grew mainly groundnuts, yam, maize and cassava.

Farm produce were stored in shelters built on farmers compounds. Water source was mainly from boreholes. A brief meeting on the research protocol was held with the Regional GHS Director.

Sampling Site 3 (Teanoba): A village named Teanoba located at N 10°23.685' W 000°21.940' in the Northern region, near Nalerigu, was identified as suitable for the study.

Teanoba is 29 km on the Gambaga paved road in the East Mamprusi District. The study team met with the chief and elders and briefed them on the research protocol. The chief and elders positively confirmed their willingness to participate in the study. The population in the village was described as 475 (Population and Housing Census, 2000), but the elders indicated that the population was about 1000. There were solar street lights in the community with houses being sparsely distributed. Cows, guinea fowls, donkeys, pigs, chickens, sheep and goats were a common sight. The main crops cultivated were millet, groundnuts, maize and okro (okra). Harvested crops were stored in storage structures on their compound.

The majority of the adult population were farmers and water source for the community was from boreholes.

Sampling Site 4 (Jirandogo): After an assessment, Jirandogo located in the Kpandai district of the Northern region, was found suitable as a study site.

Jirandogo, located at N 08°20.827' W 000°20.750' was about 40 km off the Salaga paved road. The team met with the chief and some elders for a briefing on the research protocol. They indicated their interest to participate. The village population according to the community health nurse was 1239 people. There was electricity in the community with the houses quite close to each other. Cows, guinea fowls, pigs, chickens, sheep and goats were a common sight. The main crops

cultivated were yam, maize, millet, and groundnuts. Harvested crops were stored in storage structures on their compound. The vegetation was mainly shrubs and grassland.

The majority of the adult population were farmers and the community relies on the River Daka which is about 2 miles away for water.

Sampling Site 5 (Gyedu, Ntotoroso and Amomaso): Using the Ghana Population and Housing Census (2000) document, three villages that satisified the population and location criteria were identified: Gyedu, Ntotoroso and Amomaso. These communities were in the Berekum district of the Brong Ahafo region. According to the map, all three communities were supposed to be along feeder roads but the first two were along a paved/major road and seemed rather big in size.

Amomaso, located at N 07° 07.355' W 002° 19.648' was about 9 km from the paved road. A meeting with the chief and some inhabitants was arranged. The people expressed their interest in participating in the study. The population was 805 (Population and Housing Census, 2000), but was described by local inhabitants asnearly 1400. The village had electricity. Most men were at home during our visit because most of their farms, located on the outskirts of the community, were flooded. Housing was sparsely distributed with vegetation in between houses. Goats and chickens were a common sight in the community. The farmers in the village were mainly subsistent farmers, and cultivated mainly cassava, maize, rice, pepper, tomatoes, cocoyam, yam, plantain, palm nut and cocoa.

Sampling Site 6 (Ankaakur, Kronko No. 1 and Hia): Three possible villages (Ankaakur, Kronko No. 1 and Hia) were identified that satisfied the population and location criteria. These villages were located in the Amansie East district of the Ashanti region. During the assessment visit, it was discovered that inhabitants of Kronko No. 1 had relocated to join Hia because their village had

earlier been flooded forcing them to move. Hia therefore seemed very densely populated with many houses and no vegetation in between houses.

Ankaakur, located at N 06° 10.559' W 001° 47.557' was about 16 km from the paved road and a meeting with the chief was arranged. After briefing him and some elders on the research protocol, he expressed the willingness of the community to participate in the study. The population was listed as 635 (Population and Housing Census, 2000), but was found to be about 800 people. The village had electricity. Housing was sparsely distributed with vegetation in between houses. Goats and chickens were common. The village community were mostly subsistence farmers, cultuivating mainly cassava, maize, cocoyam, yam, garden eggs, tomatoes, plantain and cocoa. Shelters had been built on their compounds to store food stuffs/farm produce.

Plate C: Sparse distribution of houses with vegetation in between (Source: Shirley NimoPaintsil, picture taken at Ankaakur, Amansie East District, June 20, 2010)

Plate D: Vegetation surrounding houses (Source: Shirley Nimo-Paintsil, picture taken at Ankaakur, Amansie East District, June 20, 2010)

Sampling Site 7 (Ehiawenwu and Apradan): The villages of Ehiawenwu and Apradan located in the Eastern region near Pankese were identified as candidate villages and visited to investigate their suitability. Village representatives stated their community's willingness to participate. Apradan had electricity and the population was about 2000 while Ehiawenwu located at N 06° 26.970' W 000° 51.113' had no electricity and the population as reported by the assemblyman was 1150. On the basis of size, Ehiawenwu was chosen as a more suitable study village. The terrain was hilly with tropical rain forest ecology. The main crops were cassava, maize, plantain, cocoa and palm nut.

Plate E: Tropical rain forest vegetation surrounding Ehiawenwu ((Source: Shirley NimoPaintsil, picture taken at Ehiawenwu, East Akim District, May 19, 2010)

Sampling Site 8 (Doninga Naveransa): Doninga Naveransa, located in the Upper East region near Sandema was visited to investigate its suitability as a study site.

Doninga Naveransa is 20 km from the paved road of Sandema in the Builsa district. The village chief and some elders met with the team, and after the briefing on the research protocol, they expressed interest to participate. The population was 535 (population and Housing Census, 2000), but the village community nurse said the number was about 800 based on a recent census 2012 count. There was no electricity with houses being very sparsely distributed. Farmlands were between houses. Cows, guinea fowls, donkeys, pigs, chickens, sheep and goats were a common sight. The main crops cultivated were millet, groundnuts, rice and yam. Harvested crops were stored in structures near their homes.

The majority of the adult population were farmers and water source for the community was from boreholes.

Plate F: Sparse distribution of houses (Source: Shirley Nimo-Paintsil, picture taken at Doninga Naveransa, Builsa District, March 17, 2011)

Plate G: Farmlands surrounding houses (Source: Shirley Nimo-Paintsil, picture taken at Doninga Naveransa, Builsa District, March 17, 2011)

Sampling Site 9 (Atakurom and Mangoase): Two villages, Atakurom and Mangoase that satistfied the selection criteria were visited. The majority of the inhabitants of Atakurom had immigrated to a neighbouring village as a result of flooding that occurred earlier.

Mangoase located at N 07° 58.167' W 001° 39.850' within the Techiman municipality was about 7 km from the paved road. A meeting with some elders was arranged since the chief was out on his farm. The elders indicated their interest in participating and promised to inform the chief. Population was about 1602 in total. The village had no electricity. Housing was sparsely distributed with vegetation in between them. Goats, sheep and chickens were a common sight. The inhabitants were mostly subsistence farmers, with main crops being cassava, beans, groundnuts, maize and yam. Farm produce were normally stored in shelters on their farms and on their compounds.

Sampling Site 10 (Dwan, Krobo and Monkwo): Three possible villages (Dwan, Krobo and Monkwo) all within the Sene district of Brong Ahafo region were identified. During our investigation, we discovered that the first two villages were rather small with few scattered houses contrary to the 2000 census. Monkwo on the otherhand seemed more eligible.

Monkwo located at N 07° 40.455' W 000° 38.002' was about 8.7 km from the paved road of Kwame Danso. A meeting with some elders was arranged since the chief was on his farm. The elders indicated that they would be interested in participating and would inform the chief accordingly. Population was about 806 in total. The village had no electricity. Most men were in the field farming at the time of our visit. Houses were sparsely built with vegetation in between the houses. Goats, sheep and chickens were in abundance. The village comprised mostly of

subsistence farmers, who grew mainly rice, cassava, beans, millet, groundnuts, maize and yam. Some inhabitants were also fishermen on the Volta Lake. They usually laid their nets late afternoon and harvested in the morning. Farm produce were stored in shelters on their farms whilst food stuff for individuals and their families were stored in shelters on their compounds.

3.2 Field procedures

A durbar was held the day before field activities at each sampling site (village). All proposed activities, including trapping of rodents and collection of human serum were explained to the inhabitants. Each community was informed that the team would spend several days with them collecting rodents and human serum. The traps used to trap the rodents were shown to the village community and further informed that the traps would be setup at night in the houses and fields of those who had earlier agreed to participate. Children were also asked not to tamper with the traps, to avoid the introduction of errors in the study. In the mornings, the team members wore N-100 masks that had been fit tested, gowns, and gloves for personal protection and collected traps with rodents to a necropsy station. All local inhabitants were reminded to not touch traps and to wait for the team to collect them in the morning. At each village, the chief assigned two volunteers to assist with selection and preparation of the necropsy station, accompanied team members to houses and fields, helped with management of supplies, and ensured traps were not taken overnight. A central location was also designated for phlebotomy where each day volunteers from the village came to provide blood samples after informed consent was obtained. A question and answer session followed and all questions were answered.

After this traps were set for the first night as described below in 3.2.2.

3.2.1 Blood Sampling from Human Volunteers

Human subject enrolment: Inhabitants in villages selected for rodent trapping provided human blood samples from healthy adults. Informed Consent was obtained from volunteers (≥ 18 years

of age), using forms approved by the IRBs. In addition, an approved questionnaire was administered to gather demographic data and some risk factors (see appendix A). Since most village residents were illiterate, community nurses assigned by the GHS, served as witness and translator for most volunteers. The nurse also helped administer questionnaires. A phlebotomist, experienced in similar studies, and trained in the ethics of research, conducted informed consent in English, Twi and or other local dialects. If dialects were unknown to the phlebotomist, the community nurse, village linquist (okyeame) or other volunteers helped to translate. Blood was collected in a sterile disposable needle and syringe or directly into the collection tube via butterfly needles. Prior to collecting blood, the top of the serum sample vial was disinfected thoroughly with an alcohol pad and allowed to air dry. The following procedure was used to collect blood in the field:

1. A tourniquet was applied to the participant's arm just above the antecubital fossa and a vein selected for venipuncture.
2. The area over the vein was thoroughly cleaned with alcohol swab.
3. Needle was introduced into the skin and five milliliters of blood specimen was drawn in a tiger top tube (serum separator included) for serological tests.
4. Specimens were labeled and kept in a cool box with ice packs (4ºC) until centrifugation was done.

Blood specimens were centrifuged at 3000 rpm for 10 minutes to obtain serum, using a centrifuge with power provided by a generator brought by the team. Each serum sample was aliquoted into two labeled cryovials and kept in a liquid nitrogen tank.

3.2.2 Rodent trapping and processing

Rodents were captured by traps (Sherman LFA live trap, H.B. Sherman Traps, Inc., Tallahassee, FL) set along marked lines in fields and houses for three consecutive nights. Sampling was carried

out from August to November 2010 and from April to August 2011. The traps were baited daily with a mixture of pounded peanuts, dried smoked fish and corn flour. Traps were set prior to sunset and left overnight. Outdoor trap-lines were set in farm fields that cultivated maize, millet, peanuts, yams, cassava, rice, cocoyam, palm fruits and also in fallow lands and pastures, with each trap-line having 20 traps set approximately 5 meters apart. The beginning and end of each trap line was marked with red and white tape. Indoor trap-lines were set in houses near a straight line through the village, houses where occupants were willing to participate. One to four traps were set in each family dwelling selected, with no more than 2 per room. Most of the houses were made of mud with thatched roof. Sixty to eighty traps were placed in houses

(depending on the number of participating homes) and 100 to 160 traps in the farm fields.

Records were made of the location of traps relative to housing, date of collection, and the geographic location. Coordinates at the beginning and end of each trap line were recorded by a Global Positioning System (GPS).

Traps were checked in the mornings for rodents and a trap tally form was used to record the number of traps with rodents, the number of traps with snails or other non rodents, the number closed but empty, the number of traps missing, and the total number of traps set.

All field personnel participating in the handling of rodents or traps wore protective clothing, including disposable surgeon's gowns, wellington boots, latex gloves, safety goggles, and Pathogen level 3 (P3) masks (all personnel were fit tested for masks prior to field work). Temporary processing sites for rodents were established in secluded areas in each village, away from any human activity or livestock.

Trapped rodents were handled according to standard procedures for Biosaftey Level 3 (BSL-3) work in the field (Mills *et al.*, 1995). Traps containing captured rodents were collected in open plastic trash bags and conveyed to the processing site. When an unintended mammal or reptile was

captured, the animal was released. Likewise when a captured rodent tried to escape, it was not re-trapped or re-captured as this would have been too stressful on the rodent and also expose personnel to bites and scratches.

Captured rodents were euthanized directly in the traps by placing a cotton ball soaked with isofluorane into the trap and sealing the trap in a plastic bag. After they became motionless and unresponsive, rodents were described morphologically, weighed, and measured (length of head and body, tail, hind foot, and ear) for preliminary identification according to the keys published for West Africa (Duplantier and Granjon, 1993; Rosevear, 1969). The number of nipples were counted for females. Rodents were then disinfected with 70% ethanol and dissected using sterilized forceps and scissors. Cardiac puncture was performed on each rodent. Three drops of the blood obtained by this procedure were placed on a filter paper and the remaining blood aliquoted into a labeled cryovial. If pregnant, the number of fetuses were weighed and counted. Spleen, kidneys, eyes, liver and ectoparasites were collected in separate labeled cryovials and stored in liquid nitrogen (blood, liver, spleen and kidney).

Samples in liquid nitrogen were transferred to a -70ºC freezer upon arrival at the laboratory at Noguchi Memorial Institute for Medical Research (NMIMR), Legon. Organs such as kidney in 70% ethanol and eyes in 10% formaldehyde were stored at 4ºC. Carcasses were stored in formalin for further morphological identification. At the end of each day's necropsy, every member of the team, including personal protective wear was disinfected thoroughly with incidine or 70% alcohol and all disposables burnt.

		Indoor (Houses)						
Month	**Village/Site**	**No. traps**	**Trapping success (%)**	**TN**	**No.**	**Trapping**	**TN**	**Total TN/village**

Traps success (%)

Outdoor (Farmlands)

Table 3.2: Overall Distribution of traps set by Village/Site and Habitat

Aug-10	Ehiawenwu/7	232	25.0	236	465	6.5	680	916
Nov-10	Ankaakur/6	180	28.9	224	675	2.8	760	984
Apr-11	Amomaso/5	177	20.3	260	606	2.0	940	1200
Apr-11	Bowena/2	167	18.0	300	415	2.4	800	1100
May-11	Teanoba/3	172	25.6	200	551	2.5	600	800
May-11	Jirandogo/4	85	54.1	120	494	10.1	600	720
Jun/Jul 11	Doninga/8	178	15.2	314	528	3.4	600	914
Jun/Jul 11	Natoduori/1	170	17.6	210	523	6.1	599	809
Jul/Aug 11	Menkwo/10	120	19.2	186	571	6.8	800	986
Jul/Aug 11	Mangoase/9	173	20.8	240	413	7.0	600	840
Total TN/habitat		**1654**	**23.1**	**2290**	**5241**	**4.8**	**6979**	**9269**

Trapping success for each site (indoor and outdoor) was estimated. Trapping success was defined by the number of rodents caught, divided by the number of trapping nights {TS = ∑number of rodents trapped/∑trapping nights}. Trapping nights (TN) was the product of number of traps set per night and the number of nights traps set. Traps which were missing or found closed for any reason other than rodent capture, were not counted in trapping nights. In calculating trapping success, only traps set for three consecutive nights were included (Table 3.2). Traps set on additional nights, and or trap-lines set for less than three consecutive nights were not included in trapping success, though the rodents captured were still processed and tested in the same manner. Three consecutive nights were used to provide a standard number to allow comparisons, since trapping success decreased on each consecutive night in a particular location, in addition to nightly variations due to weather conditions.

3.3 Laboratory procedures

3.3.1 Serological testing

All human serum specimens collected were screened for the presence or absence of immunoglobulin G (IgG) antibodies against Lassa virus using an in-house assay from United States Army Medical Research Institute of Infectious Disease (USAMRIID). Furthermore, IgG antibodies to Hantavirus (Dobrava and Puumala serotypes) and *Leptospira* were also assessed using Progen and Gentaur kits respectively, following manufacturer's instructions.

3.3.1.1 Test for Anti-Lassa Immunoglobin G (IgG) by ELISA

High-affinity 96 well plates (Costar, USA) were coated with immobilized LASV antigen isolated from cells infected with LASV (Applied Diagnostics Department, USAMRIID, USA). Rows A, B, E and F of each plate was coated with LASV positive cell slurry and negative cell slurry in rows C, D, G and H at 1:1000 dilution and incubated overnight at 4°C. Blocking buffer was prepared by adding 5 g of milk powder to 100 mL of Phosphate Buffered Saline (PBS). A BioTek EL x 50 plate washer (BioTek Instruments Inc., Vermont,USA) was primed with wash buffer (PBS + 1% Tween 20 at 1:1000 dilution). Coated plates were then washed with 3 x 250 µL program. Human serum, LASV positive and negative sera controls were diluted at 1:100 in blocking buffer to a volume of 450 µL each. Samples including controls were loaded in quadruplicates (positive control=column 1, rows A-D; negative control=column 2, rows A-D; unknown sample 1=column 3, rows A-D) (as shown in figure 3.2) and incubated in a humid chamber for 1 hour at 37°C.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag
B	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag
C	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag

D	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag
E	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag
F	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag	+ Ag
G	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag
H	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag	- Ag

Figure 3.2: An ELISA plate map showing how samples were loaded

A 1:2000 dilution of horse radish peroxidase (HRP)-conjugated secondary antibody in blocking buffer was prepared and a 100 µL of the resulting solution aliquoted into each well after washing plates and incubated in a humid chamber for 1 hour at 37°C. After 1 hour, plates were removed from the incubator and washed. One-step ABTS HRP substrate solution was allowed to attain room temperature and 100 µL added to each well and incubated for 30 minutes at 37ºC. Positive wells developed green colour within the first few minutes of addition. Plates were read with an EL x 808 reader (BioTek Instrumnets, Vermont, USA) using the Gen5 Reader Control protocol (BioTek Instruments, Inc., USA). An optical density (OD) value greater than 0.2 over background [as determined by subtraction of the average of both OD values of the LASV (+) and (-) slurry of the same sample] served to indicate presence of anti-Lassa IgG.

3.3.1.2 Test for Hantavirus Dobrava/Hantaan and Puumala IgG by ELISA

Commercial enzyme immunoassays (PROGEN BioTechnik GmbH, Heidelberg, Germany) for the detection of IgG antibodies against Puumala and Hantaan/Dobrava serotypes of hantavirus were used.

Patient sera were pretreated with sample buffer (Phosphate Buffered Saline pH 7.4 with detergent and 0.01% thimerosal) of 20X concentration in a 1:100 dilution using microtitre tubes. One hundred microliters of undiluted negative, positive and reference controls as well as diluted patient sera were pipetted into their respective ready-to-use microtest wells. Microtest wells had been coated by the manufacturer with recombinant nucleocapsid protein of either Puumala or Hantaan virus. The strips were covered with adhesive foil and incubated at 37°C for 45 minutes. The microassay strips were emptied and washed four times using 200 µL wash buffer per well for each wash. Excess buffer was removed from the wells by tapping the strips onto absorbent paper. Each well was filled with 100 µL anti-IgG peroxidase conjugate, covered with adhesive foil and incubated at 37°C for 45 minutes. Strips were emptied after incubation and wash procedure repeated (4 x 200 µL per well). One hundred microliters of substrate solution, tetramethylbenzidine (TMB) was pipette into each well and incubated at room temperature for 10 minutes. Stop solution, 0.5 M sulfuric acid, was added to each well at a volume of 100 µL per well. Optical density (OD) for each well was read within 20 minutes at 450 nm with reference wavelength of 650 nm.

Quality control was performed for each batch of test runs by validation of the ELISA kit controls using the following ratios and absorbent values:

Postive control IgG/Reference control IgG > 1.8

Negative control IgG/Reference control < 0.5

For calculation and interpretation of results, the ratio of the absorbence of the patient sample and the reference control was determined by:

Patient sample/Reference control = Q

Q values greater than 1.5 indicated detection of specific IgG antibodies against Puumala or Hantaan/Dobrava depending on type of kit used.

3.3.1.3 Test for *Leptospira* IgG by ELISA

A quantitative Serion ELISA classic IgG test kit (Serion GmbH, Würzburg, Germany) was used for the detection of genus-specific human antibodies against *Leptospira* in serum.

Patient samples were diluted in sample diluent at 1:100 using mirotitre tubes. One hundred microliters of diluted patient samples and ready-to-use control/standard sera and negative control were pipetted into their respective microtest wells. One well was spared for the substrate blank (diluent only). Incubation was done at 37°C for 60 minutes in a moist chamber. Plates were washed with 4 x 300 µL program after incubation and 100 µL of anti-human-IgG conjugate was pipetted into each well and incubated at 37°C for 30 minutes. After incubation, plates were washed and 100 µL of substrate (Para-nitrophenylphosphate) added to each well following incubation for 30 minutes at 37°C. The reaction was stopped by aliquoting 100 µL of stop solution (1.2 N Sodium hydroxide) into each well. The optical density (OD) for each well was read within 60 minutes at 405 nm against substrate blank with reference wavelength between 620 nm and 690 nm.

The OD value for the substrate blank was subtracted from all OD values prior to calculations. Interassay variations were compensated by multiplication of the current measured value obtained with a patient's sample with the correction factor, F. The factor 'F'= given reference value/mean of standard sera. Each patient's OD value was then multiplied by 'F'. Values greater than 9U/ml indicated presence of detectable IgG antibodies.

3.3.2 Molecular Identification of Rodent Species

3.3.2.1 Molecular Identification of *Mastomys* Species using Cytochrome b (Cytb) gene analysis (Lecompte *et al*., 2002)

Karyotypes (diploid number, 2N and autosomal fundamental number, aFN) remain the most reliable characters to date for unambiguous species assignation (Green *et al*., 1980; Duplantier *et al*., 1990a; Granjon *et al*., 1997; Lavrenchenko *et al*., 1998).

Deoxyribonucleic acid (DNA) was extracted from 70% ethanol-preserved kidney samples of 231 *Mastomys* species using DNEasy Blood and Tissue kit (Qiagen, Hilden, Germany) following manufacturer's instructions, with a final elution of 200 µl of AE buffer. Double-stranded PCR amplifications of the entire cytb gene were performed in 50 µl reaction volumes.

Each specific reaction included 1 µl each of forward and reverse primers (10 mM), 1 µl of a deoxynucleoside-triphosphate mixture (10 mM), 5 µl of reaction buffer (10X) (Invitrogen), 1.5 µl of $MgCl_2$ (50 mM), 0.2 µl of Platinum *Taq* DNA polymerase (Invitrogen) and 1 µl of template. The following thermal cycling parameters were used: 3 minutes at 94°C, 40 cycles (30 s at 94°C, 30 s at 55°C, 60 s at 72°C), with a final extension of 10 minutes. Each suspected *Mastomys* specimen was tested with *M. natalensis* pairs of specific primers, and those that tested negative were run on *M. erythroleucus* primer pair in separate PCR reactions.

Cytochrome *b* species-specific primers for *Mastomys coucha, M. erythroleucus, M. huberti* and *M. natalensis* (Lecompte *et al*., 2002) are listed below:

M.coucha F-279: 5' TTG-TTC-CTT-CAC-GTA-GGA-CGG 3'

R-635: 5' TTA-GGC-CTG-TTG-GGT-TAT-TGG-AT 3'

M. erythroleucus F-49: 5' CAT-TCA-TTG-ACC-TAC-CTG-CT 3'

R-505: 5' AGA-ATC-CCC-CTC-AAA-TTC-AC 3'

M. huberti F-174: 5' TAC-TAT-AAC-AGC-ATT-TTC-ATC-G 3'

R-702: 5’ AGT-ATA-GTA-TGG-GTG-GAA-TGG-GAT-TTT-G 3’

M. natalensis F-607: 5’ CGG-GCT-CTA-ATA-ACC-CAA-CG 3’

R-813: 5’ TTC-TGG-TTT-GAT-ATG-GGG-AGG-T 3’

When the run was complete, 2.5% Agarose gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was prepared in 100 ml 1 X Tris-borate-EDTA (TBE buffer) (Thermo Fisher Scientific Inc., Pennsylvania, USA). Five microliters of each PCR product was mixed with 1µl loading dye, as well as the positive and negative controls, together with 100 base pair ladder from Fermantas (Thermo Fisher Scientific, Rockford, Illinois, USA). The gel was run at 80 volts for 30 minutes. After the run, gel was observed using Bio-Rad Gel Doc System. The positive control amplification product had a molecular weight of 200 bp (See Appendix C).

3.3.2.2 Molecular Identification of *Nannomys* Species using Cytochrome b (Cytb) gene analysis (Lecompte *et al*., 2005)

Chromosomal diversity within the *Nannomys* species is extensive and many studies have shown the karyotype to be a useful taxonomic marker (Lecompte *et al*., 2005). Deoxyribonucleic acid (DNA) was extracted from 70% ethanol-preserved kidney samples of 133 *Nannomys* species using DNEasy Blood and Tissue kit (Qiagen, Hilden, Germany) following manufacturer’s instructions, with a final elution of 200 µl of AE buffer. Deoxyribonucleic acid PCR amplifications were performed in 50 µl reaction volumes.

Each specific reaction included 1 µl each of forward and reverse primers (10 mM), 1 µl of a deoxynucleoside-triphosphate mixture (10 mM), 5 µl of reaction buffer (10X) (Invitrogen), 1.5 µl of $MgCl_2$ (50 mM) , 0.5 µl of Platinum *Taq* DNA polymerase (Invitrogen) and 1 µl of template. The following thermal cycling parameters were used: 3 minutes at 94°C, 40 cycles (30s at 94°C, 30s at 55°C, 60s at 72°C), with a final extension of 10 minutes.

Cytochrome *b* species-specific primers for *Mus* (*Nannomys*) species (Lecompte *et al*., 2005):

L7: 5'-ACC AAT GAC ATG AAA AAT CAT CGT T-3'

H15915: 5'-TCT CCA TTT CTG GTT TAC AAG AC-3'

At the end of the run, DNA fragments in resulting PCR products were purified using NucleoSpin Gel and PCR Clean-up kit following manufacturer's instructions (MACHERY-NAGEL GmbH & Co., Germany). In the purification process, contaminations were removed by simple washing steps with ethanolic wash buffer (Buffer NT3) and DNA was eluted under low salt conditions with slightly alkaline elution buffer (5 mM Tris/HCL, pH 8.5). Product was then sequenced using Big Dye Terminator vs 3.1 (Life Technologies, Applied Biosysytems) in a total volume of 15 µl which constituted 2 µl Big Dye® Terminator Mix vs 3.1 (Applied Biosysems, Texas, USA), 2 µl 5X Big Dye Terminator Buffer, 2 µl Primer (L7) 0.6 µM, 4 µl sterile distilled water and 5 µl template (purified PCR product). The reaction mix was run in an Applied BioSystems GeneAmp PCR System 2700 with the following cycling conditions 94ºC 2 minutes/ (94ºC 30s; 50ºC 15s; 60ºC 4 minutes) X 25 cycles/4ºC hold. The DNA sequences obtained were purified using AgenCourt CleanSeq® Dye Terminator Removal Kit (Agencourt Bioscience Corporation, Massachusetts, USA) following the manufacturer's instructions. After the purification process, sequences were aligned to concensus sequence, assembled and analysed by comparing with sequences available at BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BioEdit sequence alignment editor software to determine species.

3.3.3 Ribonucleic Acid (RNA) extraction of rodent blood (Qiagen RNeasy Mini Kit: Qiagen GmbH, Hilden, Germany)

For whole blood samples, 200 µL was transferred from cryovial into clean microtube and centrifuged at 5000 rpm for 5 minutes. Supernatant was carefully transferred into a new tube

without disturbing the blood clot at the bottom. Five hundred and sixty microliters of buffer RLT (lysis buffer) was added to 140 µL of the supernatant, vortexed for 1 minute and centrifuged briefly. The lysate was pipetted directly into a QIAshredder spin column (Qiagen, Hilden, Germany) placed in a 2 ml collection tube and spun at full speed for 2 minutes.

For dried blood spots on filter paper, 560 µL buffer RLT was added to a microtube with blood spots that had been punched out of the filter paper with disc sizes of 40 µm. This was incubated at 37°C for 30 minutes, vortexing every several minutes. After incubation, sample was vortexed for 1 minute and briefly centrifuged. The lysate was transferred into a QIAshredder spin column placed in a 2 ml collection tube and spun at full speed for 2 minutes.

To the lysate obtained from both whole blood and dried blood spots, 560 µL of absolute ethanol was added and mixed well by pipetting. Seven hundred microliters of the resulting solution, including any precipitate, was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 1 minute at 10,000 rpm. The flow-through was discarded and the collection tube reused. Three hundred and fifty microliters buffer RW1 (guanidine thiocyanate) was added to the RNeasy spin column to remove contaminants, centrifuged for 1 minute at 10,000 rpm to wash the spin column membrane and the flow-through discarded. A DNase I incubation mix (10 µL DNase I stock solution + 70 µL reconstituted buffer RDD) (Qiagen, Hilden, Germany) was prepared and 80 µL added directly to the RNeasy column membrane and placed on a bench top (20-30°C) for 15 minutes. After DNase digestion, 350 µL buffer RW1was added to the RNeasy spin column and centrifuged for 1 minute at 10,000 rpm to further remove contaminants. Flow-through was discarded and 500 µL buffer RPE (concentrated buffer for washing membrane-bound RNA) added to the spin column. It was then centrifuged for 1 minute at 10,000 rpm to wash the spin column membrane. The wash was repeated after which RNeasy spin column was placed in a new 2 ml collection tube and centrifuged at full speed for 1 minute to eliminate any carryover of

buffer RPE. To elute RNA, the spin column was placed in a 1.5 ml collection tube and 30 µL RNase-free water was directly added to the spin column membrane.

The extracted RNA was stored at -70°C.

3.3.4 Molecular Analysis of Rodents for pathogens

3.3.4.1 Detection and quantification of Arenavirus RNA by reverse-transcription polymerase chain reaction (RT-PCR) (Demby *et al*., 1994)

In this assay, the primer set used targets the S gene encoding the glycoprotein precursor protein (GPC) and the nucleoprotein (NP), useful for the screening of large numbers of samples for the presence of Arenaviruses. The whole blood samples of rodents were tested for the presence of arenavirus GPC gene RNA by using Power SYBR Green RNA-to-C 1-Step PCR Kit (Applied Biosystems, Foster City, CA, USA).

Enzyme, primers, and RNA standards were allowed to thaw. Once thawed, they were vortexed briefly to mix and placed on ice.

Preparation of RNA standards:

While reagents were on ice, 1 uL of stock LASV JOS 17178 (Applied Diagnostics Department, USAMRIID, USA) was diluted into 9 uL RNase free water to become solution A (1X10^4 pfu). Solution A was serially diluted 5 times by taking 1 uL of solution A into 9 ul of RNase free water to become solution B, vortexed and centrifuged briefly. Six serial dilutions were obtained by this procedure (Solutions A-F) to generate a standard curve. Samples were kept on ice while the Master Mix was prepared.

Preparation of Master Mix:

In one tube, the RT Enzyme Mix (125x), the RT-PCR Mix (2x) (Qiagen, Hilden, Germany), both forward and reverse primers, and the Nuclease free water were mixed according to the setup below (Table 3.3a). The mixture was vortexed briefly to mix and centrifuged.

Table 3.3a: Master mix setup for arenavirus RNA detection in rodent samples

Reaction Component	Volume (ul)/Reaction	Volume (ul)/ 96-well plate
RT Enzyme Mix (125x)	0.16	16
RT-PCR Mix (2x)	10	1000
10uM Forward Primer (200nM final)	0.4	40
10uM Reverse Primer (200nM final)	0.4	40
RNA Template	2	200
Nuclease-free Water	7.04	704
Total volume	20	2000

The primer set below, adopted from Demby *et al* (1994) was used for each RT-PCR reaction. 5'-ATATAATGATGACTGTTGTTCTTTGTGCA-3' - Josiah RV (80F2)

5'-ACCGGGGATCCTAGGCATTT-3' - Josiah FW (36E2)

To each well of the 96-well plate, 18 uL of Master mix was added and 2 ul of each of the standards was aliquot into the respective wells in triplicate. Two microliters nuclease free water was added to the non-template controls, also known as the negative control. Two microliters of extracted rodent sample was then added in duplicate (as shown in figure 3.3). The plate was run on ABI 7300 thermocycler (Applied Biosystems, Singapore) under the cycling conditions below (Table 3.3b) with a dissociation step at the end of the run.

Table 3.3b: Master mix setup for arenavirus RNA detection in rodent samples

Step	Temp (°C)	Duration	Cycles
RT Step	48	30 min	Hold
Enzyme Activation	95	10 min	Hold
Denature	95	15 sec	45
Anneal/Extend	60	1 min	

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.E+04	2.E+04	2.E+04	Blank	Sample 1	Sample 1	9	9	17	17	25	25
B	2.E+03	2.E+03	2.E+03	Blank	2	2	10	10	18	18	26	26
C	2.E+02	2.E+02	2.E+02	Blank	3	3	11	11	19	19	27	27
D	2.E+01	2.E+01	2.E+01	Blank	4	4	12	12	20	20	28	28
E	2.E+00	2.E+00	2.E+00	Blank	5	5	13	13	21	21	29	29
F	2.E-01	2.E-01	2.E-01	Blank	6	6	14	14	22	22	30	30
G	NTC	NTC	NTC	Blank	7	7	15	15	23	23	31	31
H	Blank	Blank	Blank	Blank	8	8	16	16	24	24	32	32

Figure 3.3: A PCR (MicroAmp Optical 96-well) plate map showing how samples were loaded

3.3.4.2 Detection of LASV and related Old World arenavirus targeting the L gene (Vieth *et al*., 2007)

Rodent samples that tested positive with the Demby *et al* (1994) primer set were repeated with another set of primers which targets the L RNA (L and Z gene) segment of arenaviruses (Vieth *et al*., 2007). This L-gene PCR assay could be applicable: 1) as a complementary diagnostic test for LASV and LCMV, 2) to identify unknown Old World Arenaviruses and 3) for screening potential reservoir host for unknown Old World Arenaviruses.

This second RT-PCR was performed using SuperScript II One-Step RT-PCR with Platinum *Taq* system (Invitrogen) (Invitrogen Corporation, California, USA). The final PCR assay (20 µl) contained 3 µl of RNA, 1X reaction mix (buffer containing 0.2 mM dNTP and 1.2 mM MgSO4), 0.625 mM additional MgSO4, 0.4 µl of SuperScript/Platinum *Taq* mix, 0.3 µM primer LVL3359A-plus, 0.3 µM primer LVL3359D-plus, 0.3 µM primer LVL3359G-plus, 0.6 µM primer LVL3754A-minus and 0.6 µM primer LVL3754D-minus.

The primer sequences were as follows:

LVL3359D-plus 5'-AGAATCAGTGAAAGGGAAAGCAAYTC-3' Forward

LVL3359G-plus 5'-AGAATTAGTGAAAGGGAGAGTAAYTC-3' Forward

LVL3754A-minus 5'-CACATCATTGGTCCCCATTTACTATGRTC-3' Reverse LVL3754D-minus 5'-CACATCATTGGTCCCCATTTACTGTGRTC-3' Reverse

The reactions were done in a 9700 thermocycler (Applied Biosystems, Singapore) with the following temperature profile: reverse transcription at 50°C for 30 minutes; initial denaturation at 95°C for 2 minutes; 45 cycles at 95°C for 20 s, 55°C for 1 minute and 72°C for 1 minute; and a final extension at 72°C for 10 minutes. Five microlitres of the amplicons were analyzed on 2% agarose gels (FMC BioProducts, Rockland, ME) by electrophoresis at 100 volts in 1 X TAE buffer (0.04 M Tris acetate, 1 mM EDTA [pH 8.0]) and visualized by UV light (High Performance Ultraviolet Transilluminator, California, USA) after staining with ethidium bromide (0.3 mg/ml) (Life Technologies, Applied Biosystems). A PCR result was considered positive when a 400 bp band was observed.

The PCR products of the positive samples were purified using Qiaquick PCR purification kit

(Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified material was then sequenced using Big Dye Terminator vs 3.1 in a total volume of 10 µl which constituted 2µl Big Dye® Terminator Mix vs 3.1, 2 µl 5X Big Dye Terminator Buffer, 1.6 µl Primer (S/A) 2 µM, 2.4 µl sterile distilled water and 2 µl template (purified PCR product). The reaction mix was processed in an Applied BioSystems GeneAmp PCR System 2700 following the cycling conditions 94°C 2 minutes/ (94°C 30s; 50°C 15s; 60°C 4 minutes) X 25 cycles/4°C hold. The DNA sequences obtained were purified using AgenCourt CleanSeq® Dye Terminator Removal Kit (Massachusetts, USA) following manufacturer's instructions. The sequences were then aligned to concensus sequence and assembled using BioEdit software.

To further classify the arenaviruses identified, the fragments of glycoprotein (GP) and nucleoprotein (NP) genes were sequenced on both sides using primers OWS1+, OWS1000-, OWS2165A+, OWS2165B+, OWS2840A-, OWS2840B-, OWS2770+, OWS3400A- and OWS3400- (Ehichovia *et al*., 2011). The fragments were assembled and aligned to concensus sequence in MacVector software (MacVector, Inc. Cary, NC, USA), then analyzed by phylogeny using PhyML software (Guindon *et al*., 2010).

3.3.4.3: Detection of Hantaviruses with HAN-L-F1 and HAN-L-R1 primers using One-step PCR

Hantavirus RNA was detected by reverse transcription PCR performed with pan-hanta viral large (L) segment specific primers (Klempa *et al.*, 2006).

The PCR was carried out using the Qiagen One-Step PCR kit (Qiagen, Hilden, Germany). The final PCR assay (25 µl) contained 5 µl of RNA extracted from lung tissue, 1X reaction buffer, 1X Q-Solution, 0.4 mM dNTP-mix, 1 µl of Enzyme-mix, 0.6 µM primers HAN-L-F1 and HAN-L-F2 (Klempa *et al*., 2006).

Primer sequences were as follows:

HAN-L-F1 5’ – ATG TAY GTB AGT GCW GAT GC – 3’

HAN-L-F2 5’ – AAC CAD TCW GTY CCR TCA TC – 3’

The reactions were run on a 9700 thermocycler (Applied Biosystems, Singapore) with the temperature profile: reverse transcription at 50°C for 30 minutes; initial denaturation at 95°C for 15 minutes; 45 cycles at 95°C for 30 s, 52°C for 1 minute and 72°C for 1 minute.

When the run was complete, 3% Agarose gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was prepared in 100 ml 1 X Tris Acetate-EDTA (TAE buffer) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After complete dissolution of the gel, it was poured slowly in a casting tray using the appropriate comb. The amplification product was loaded in a 3% agarose gel (prepared in 100 ml 1 X TAE buffer) with ethidium bromide (Life Technologies, Applied Biosystems) as well as the positive and negative controls together with 100 base pair ladder from New England Biolabs (Massachchusetts, USA). The gel was run at 100 volts for 30 minutes. The gel was viewed with Bio-Rad Gel Doc System. The positive control showed an amplification product of 450 base pair using the HAN-L-F1 and HAN-L-F2 primers.

3.3.4.4 Deoxyribonucleic acid purification from rodent kidney (QIAamp DNA Mini Kit) (Qiagen, Hilden, Germany)

Approximately 10 mg of kidney was cut up into small pieces and placed in a 1.5 ml microcentrifuge tube. One hundred and eighty microliters of lysis Buffer ATL was added after which 20 µl Proteinase K was added and mixed by vortexing. Incubation of resulting mixture was done at 56ºC in a water bath. The mixture was vortexed occasionally until complete lysis was achieved. The tube was centrifuged briefly to remove drops from the inside of the lid. Two hundred microliters Buffer AL was added to the sample and pulse-vortexed for 15 seconds. Sample was incubated at 70ºC for 10 minutes and briefly centrifuged. Two hundred microliters of absolute ethanol was added to the sample and mixed by pulse-vortex for 15 seconds. Sample was centrifuged and the

mixture including the precipitate was carefully applied to the QIAamp Mini spin column in a 2 ml collection tube. The cap was closed and centrifuged at 8000 rpm for

1 minute. The collection tube was replaced 500 µl Buffer AW1 was added to the spin column. The column was centrifuged at 8000 rpm for 1 minute. The spin column was placed in a clean collection tube and 500 µl of Buffer AW2 was added to the spin column. The column was centrifuged at full speed (14,000 rpm) for 3 minutes. The collection tube was replaced with a new one and centrifuged at full speed for 1 minute to avoid carryover of Buffer AW2. The

QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl elution Buffer AE added. The spin column was incubated at room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute. Eluted DNA was stored at -20ºC.

3.3.4.5 Identification of pathogenic *Leptospira* by PCR using LEPAT 1 and LEPAT 2 primers set (Murgia *et al*., 1997)

Screening of total DNA extracted from kidney for the presence of pathogenic *Leptospira* in the rodent samples was performed by amplifying a 330 bp partial fragment of the ribosomal 16S gene (rrs) by a polymerase chain reaction (PCR) with primers Lepat 1 and Lepat 2 as described by Murgia *et al*., (1997) (Lepat 1: 5'-GAG-TCT-GGG-ATA-ACT-TT-3' Lepat 2: 5'-TCA-CATCG(CT)-TGC-TTA-TTT-T-3'). The DNA of known pathogenic *Leptospira* species (serovar Pomona, serogroup Pomona) was used as control.

The Master mix was prepared per reaction according to table 3.3c below:

Table 3.3c: Master mix setup for detection of *Leptospira* DNA in rodent sample

Reaction Component	Volume (ul)/Reaction

Water	14.375
10X Buffer	2.5
25mM $MgCl_2$	1.5
dNTPs	0.5
Primer forward (Lepat 1)	0.5
Primer reverse (Lepat 2)	0.5
Taq polymerase (1U)	0.125
DNA template	5
Total volume	**25**

To each well of the PCR reaction plate/strip 20 µl of the Master mix was added. Five microliters of DNA template, positive control and nuclease-free water (negative control) was then aliquoted into their respective wells. The reaction was run in a GeneAmp system 9700 (Applied Biosystems, Singapore) according to the cycling conditions below (Table 3.3d).

Table 3.3d: Thermal cycling conditions for detection of *Leptospira* DNA in rodent sample

Temperature (ºC)	**Duration**	**Cycles**
93	3 mins	
93	1 min	35
48	1 min	
72	1 min	
72	10 mins	

When the run was complete, 3% Agarose gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was prepared in 100 ml 1 X Tris Acetate-EDTA (TAE buffer) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). After complete dissolution of the gel, it was poured slowly in a casting tray using the appropriate comb. The amplification product was loaded in the gel as well

as the positive and negative controls together with 100 bp ladder from Promega (Wisconsin, USA). The gel was run at 100 volts for 30 minutes. After the run, gel was stained in Ethidium Bromide (Life Technologies, Applied Biosystems) for 20 minutes then de-stained in distilled water for another 20 minutes. The gel was viewed using Bio-Rad Gel Doc System. The positive control showed an amplification product of 330 bp using the Lepat 1 and Lepat 2 primers (See Appendix C).

3.3.4.6 Nested Polymerase Chain Reaction (PCR) for the *fla*B gene targeting *Borrelia* species (Assous *et al*., 2006)

3.3.4.6.1 Screening for the presence of *Borrelia* species

Preliminary screening of total DNA extracted from kidney for the presence of *Borrelia* in the samples was performed by amplifying a 750 bp partial fragment of the flagellin gene (*fla*B) by a polymerase chain reaction (PCR) with primers BOR1 and BOR2 as described by Assous *et al*., (2006) (BOR1: 5'-TAA TAC GTC AGC CAT AAA TGC-3' BOR2: 5'-GCT CTT TGATCA GTTATC ATT C-3'). *Borrelia burgdorferi* DNA was used as the positive control (VIRCELL, Granada, Spain) and a negative control was included in each run. Amplified DNA was purified with the Qiaquick PCR purification kit (Qiagen, Hilden Germany) as described earlier and used for sequencing. The amplified fragments were sequenced directly with primers used for the amplification reaction. Both strands of each fragment were sequenced. Sequences were analyzed by comparing with sequences available at BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using BioEdit sequence alignment editor software to determine species with the BioEdit software.

3.3.4.6.2 Amplification and sequencing of the flagellin (*fla*B) gene

Determination of the flagellin type of different isolates was performed by sequencing a *fla*B fragment (750 bp) amplified using the genus-specific set of primers BOR1 and BOR2. For PCR amplification 1U BIOTAQ™ DNA polymerase (Bioline, GmbH Germany) was used in a reaction

mixture containing 1.6 mM $MgCl_2$. Reaction conditions were as follows: initial denaturation step of 95°C for 1 minute, followed by 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s with a final extension step of 72°C for 5 minutes.

Five microlitres of the amplicons were analyzed on 2% agarose gels (FMC BioProducts, Rockland, ME) by electrophoresis at 100 volts in 1 X TAE buffer (0.04 M Tris acetate, 1 mM EDTA [pH 8.0]) and visualized by UV light (High Performance Ultraviolet Transilluminator, California, USA) after staining with ethidium bromide (0.3 mg/ml) (Life Technologies, Applied Biosystems).

3.3.4.7 Identification of *Leishmania* species with Internal Transcribed Spacer 1 (ITS1) assay

Internal transcibed spacer 1 (ITS1) PCR was amplified from DNA using the 5-biotinylated primers LITSR (5'-CTG GAT CAT TTT CCG ATG-3') and L5.8S (5'-TGA TAC CAC TTA TCG CAC TT-3') (El Tai *et al*., 2001) as described in the reaction mix below (Table 3.3e):

Table 3.3e: Master mix setup for detection of *Leishmania* DNA in rodent sample

Reaction component	**Volume (µl)/reaction**	**Working concentration**
H_2O	14.35	
dNTPs(10mM)	0.5	0.2mM
$MgCl_2$ (25mM)	1.5	1.5mM
10X Buffer	2.5	1X
Primer LITSR (30uM)	0.5	0.5uM
Primer L5.8S(30uM)	0.45	0.5uM
AmpliTaq	0.2	2U
Template	5	
Total volume	**25**	

Leishmanial DNA (20 ng) isolated from *L. major* reference strains (Cairo, Eygpt) was used as a positive control. Reaction buffers without leishmanial DNA were also included as negative controls in each PCR analysis to ensure reliability, validity and to check for possible

contaminations of the amplification reactions. All PCRs were carried out in a 25-µl volume using the following cycling conditions: initial denaturation at 95°C for 2 minutes then 34 cycles of denaturation at 95°C for 20 s, primer annealing at 53°C for 30 s, DNA extension at 72°C for 1 minute, and further 6 minutes extension after the last cycle. Ten microlitres of the ITS1 amplicons were analyzed on 1.5% agarose gels (FMC BioProducts, Rockland, ME) by electrophoresis at 100 volts in 1 X TAE buffer (0.04 M Tris acetate, 1 mM EDTA [pH 8.0]) and visualized by UV light (High Performance Ultraviolet Transilluminator, California, USA) after staining with ethidium bromide (0.3 mg/ml) (Life Technologies, Applied Biosystems). A PCR result was considered positive when a 300 to 350 bp (ITS1) band was observed. The product size of the ITS1 PCR differs with the *Leishmania* species (Schonian *et al*., 2003).

3.3.4.7.1 RFLP analysis of the ITS1 PCR amplicon (Schonian *et al*., 2003)

The digestion of ITS1 amplicon with restriction enzyme HaeIII, distinguishes all medically relevant *Leishmania* species via restriction fragment length polymerase assay (RFLP).

The PCR products (10 µl) were digested with BsuRI (MBI Fermentas), a HaeIII prototype, according to the manufacturer's instructions, and the restriction fragments were analyzed by gel electrophoresis at 120 volts in 1 × TrisAcetate-EDTA buffer in 2.5% agarose gels (FMC BioProducts, Rockland, ME). The fragments were visualized by UV light (High Performance Ultraviolet Transilluminator, California, USA) and the sizes of the restriction products determined.

3.4 Sequencing of amplification product

3.4.1 Purification of PCR products using Qiaquick PCR purification kit (Qiagen)

Five volumes of Buffer PB was added to one volume of PCR sample and mixed by vortexing. This was then applied to the spin column to bind DNA and centrifuged at full speed for 30-60 seconds. The flow through was discarded and the column placed in the same collection tube. Seven hundred and fifty microliters of Buffer PE (reconstituted with absolute ethanol) was added to the column

and centrifuged at full speed for 30-60 seconds. To avoid carryover of any buffer, the flow through was discarded and the column was centrifuged at full speed for 1 minute. The spin column was then placed in a clean 1.5 ml microcentrifuge tube and 40 µl Buffer EB (10 mM Tris-HCL, pH 8.5) was added to the center of the spin column membrane. This was allowed to stand for 1 minute and then centrifuged at full speed for 1 minute.

3.4.1.1 Cycle sequencing using Big Dye Terminator vs 3.1 (Applied Biosystems, Texas, USA)

The Master mix was prepared following the reaction mix below:

Table 3.3f: Master mix setup for sequencing of amplification products

Reaction Component	Unit volume (µl)
Big Dye® Terminator Mix vs 3.1	2.0
5X Big Dye Terminator Buffer	2.0
Primer (S/A) 2µM	1.6
Sterile Distilled Water	2.4
Template (purified PCR product)	2.0
Total volume	**10.0**

Each well of the PCR reaction plate/strip was loaded with the master mix and template according to the plate/strip map and run in the Applied BioSystems GeneAmp PCR System 2700 following the cycling conditions 94ºC 2 minutes/ (94ºC 30s; 50ºC 15s; 60ºC 4 minutes) X 25 cycles/4 º C hold.

3.4.1.2 Purification of sequenced DNA using AgenCourt CleanSeq® Dye Terminator Removal Kit (Massachusetts, USA)

Six to ten microliters of magnetic particles was added to the sample (10 µl of sequenced DNA). Forty two (42) µl of 85% ethanol was added and allowed to sit on the magnetic field for 3 minutes. Whilst the tube was still in the magnetic field, the liquid was aliquoted from the tube. Again, 100

µl of 85% ethanol was added and left to sit on the magnetic field for another 3 minutes. With the tube still sitting in the magnetic field, all the liquid was pipetted from the tube, until tube was completely dry. Tubes on the magnetic field were kept for 8-10 minutes (Drying of ethanol). Fifty microliters of distilled (Nuclease-free water) was then added to the tube. The tube was removed from the magnetic field onto a plastic rack for 5 minutes. The rack was tapped on the bench to allow the beads to fall off the wall. The tube was placed back onto the magnetic field for 1-2 minutes. With the tubes still on the magnetic field, 40 µl of the clear liquid (DNA) was pipette into the 96-well plate to be used in the Genetic Analyzer. Plate was assembled and loaded onto the analyzer. The sequences obtained after the run were aligned to consensus sequence and assembled using BioEdit software.

CHAPTER FOUR

RESULTS

4.1 Rodent Capture and Identification

Out of the 9,269 night traps that were set up, 764 small mammals were captured. These mammals were found to belong to ten genera: *Praomys* (n=333), *Mastomys* (n=231), *Mus (Nannomys)* (n=133), *Gerbilliscus* (n=6), *Crocidura* (n=21), *Rattus* (n=5), *Lophuromys* (n=3), *Lemniscomys* (n=2), *Taterillus* (n=29), and *Uranomys* (n=1) as presented in table 4.1 below.

Table 4.1: Genus/Species distribution of small mammals captured

Genus/Species	Site # 1	Site # 2	Site # 3	Site # 4	Site # 5	Site # 6	Site # 7	Site # 8	Site # 9	Site # 10	Total
Praomys daltoni	2	5(1)	57(55)	53(51)	57(56)	60(60)	57(55)	4	5(5)	31(29)	331(312)
Praomys tullbergi	0	0	0	0	0	0	2	0	0	0	2
Mus (Nannomys)baoulei	0	0	0	2	0	0	0	0	0	3	5
Mus (Nannomys)mattheyi	12(1)	4	7	21	0	0	0	8(1)	13	24	89(2)
Mus (Nannomys)minutoides	0	0	0	0	0	16	7	0	5	1	29
Mus (Nannomys)musculoides	1(1)	0	0	0	0	0	0	4	0	0	5(1)
Mus (Nannomys)setulosus	0	0	0	0	1	0	4	0	0	0	5
Mastomys natalensis	33(30)	59(54)	1(1)	14(11)	10(4)	0	3	32(32)	40(35)	17(13)	209(180)
Mastomys erythroleucus	3	0	0	0	0	1	6	0	2	10	22
Crocidura sp.	1(1)	0	1	1	0	3	7(1)	5(1)	3(2)	0	21(5)
Rattus rattus	0	0	0	0	0	1(1)	3(2)	0	1(1)	0	5(4)
Lophuromys sikapusi	0	0	0	0	0	0	3	0	0	0	3
Lemniscomys straitus	0	0	0	0	0	0	2	0	0	0	2
Gerbilliscus kempi	0	1	0	4	0	0	0	0	0	0	5
Gerbilliscus gambianus	1	0	0	0	0	0	0	0	0	0	1
Taterillus gracilis	12	0	2	5	0	0	0	1	9	0	29
Uranomys ruddi	0	0	0	0	0	0	0	0	0	1	1
Total/village	**65(33)**	**69(55)**	**68(56)**	**100(62)**	**68(60)**	**81(61)**	**94(58)**	**54(34)**	**78(43)**	**87(42)**	**764(504)**

Numbers in brackets represent Indoor capture. Site names and coordinates: 1 = Natorduori (10°15.49'N 02°37.56'W), 2 = Bowena (09°32.82'N 01°37.95'W), 3 = Teanoba (10°23.70'N 00°21.96'W), 4 = Jirandogo (08°20.83'N 00°20.75'W), 5 = Amomaso (07°07.36'N 02°19.66'W), 6 = Ankaakur (06°10.56'N 01°47.56'W), 7 = Ehiawenwu (06°26.97' N 00°51.11' W), 8 = Doninga (10°37.16'N 01°25.31'W), 9 = Mangoase (07°58.11'N 01°39.79'W), 10 = Monkwo (07°40.45'N 00°37.98'W).

4.1.1 Prevalence of small mammals

The target species for the study, *Mastomys natalensis* and *M. erythroleucus*, represented only 27% (209/764) of captured species. Distribution of *Mastomys* species in the study sites illustrated in table 4.1 were as follows according to frequency of detection: 25.5% (59/231) in site two {Bowena}, 18.2% (42/231) in site nine {Menkwo}, 15.6% (36/231) in site one {Natorduori}, 13.6% (32/231) in site eight {Doninga Nevaransa}, 11.7% (27/231) in site ten {Mangoase}. The remaining sites recorded less than 10%. There were more *Mastomys* in Upper West (n=36), Upper East (n=32) and Northern (n=75) regions than in the southern sector (Table 4.1).

Approximately 66% (504/764) of small mammals were captured indoors with *Praomys daltoni* 62% (312/504) being the predominant species followed by *Mastomys natalensis* 36% (180/504). *Mus (Nannomys) mattheyi, Mus (Nannomys) musculoides, Rattus rattus* and *Crocidura sp* constituted 2% of the indoor captures (Figure 4.1a). The pygmy mice; Mus (*Nannomys)* (133/260) were the most abundant in the outdoor captures constituting 51%. The diversity of rodent species captured outdoors (17 species) was significantly more than indoors (6 species) as shown in figures 4.1a and 4.1b. Species diversity was highest in site 7 (Ehiawenwu) three of which were not captured in the other sites during the trapping nights. The least diversity was observed in Amomaso (site 5) with only three species (Table 4.1).

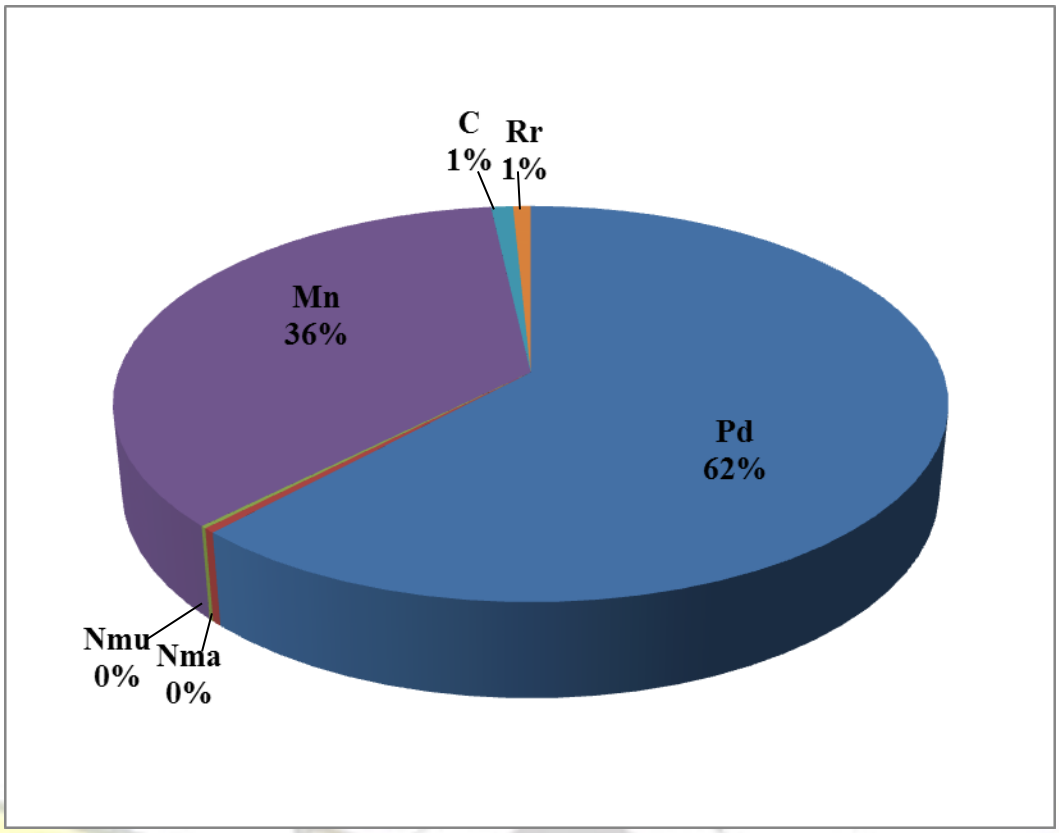

Figure 4.1a: Distribution of rodent species captured indoors

Legend:

Pd-*Praomys daltoni*

Mn-*Mastomys natalensis*

C-*Crocidura sp*

Rr-*Rattus rattus*

Nma-*Nannomys mattheyi*

Nmu-*Nannomys musculoides*

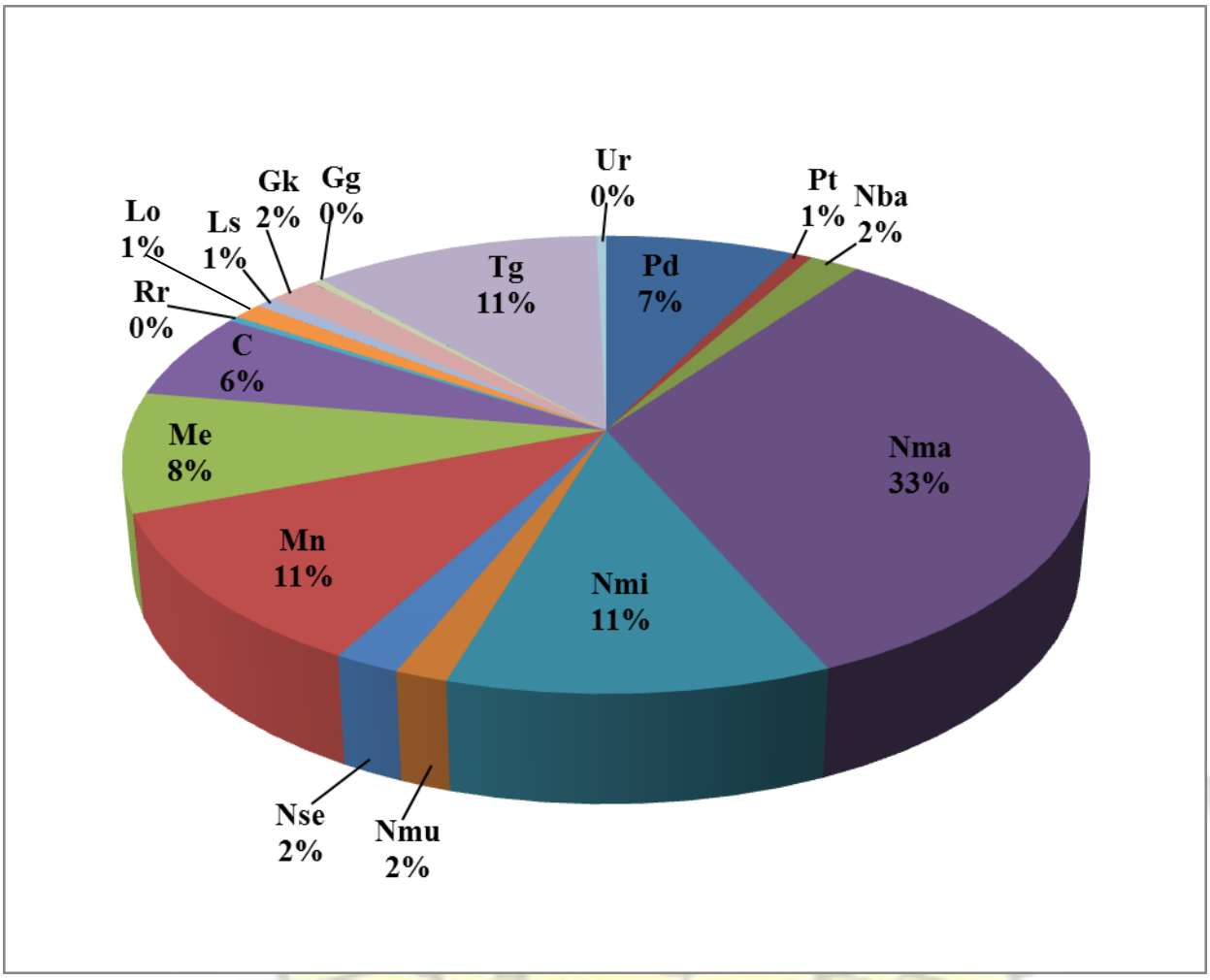

Figure 4.1b: Distribution of rodent species captured outdoors Legend:

Pd-*Praomys daltoni* C-*Crocidura sp.*

Pt-*Praomys tullbergi* Rr-*Rattus rattus*

Nba-*Mus (Nannomys) baoulei* Lo-*Lophuromys sikapusi* Nma-*Mus (Nannomys) mattheyi* Ls-*Lemniscomys straitus*

Nmi-*Mus (Nannomys) minutoides* Gk-*Gerbilliscus kempi*

Nmu-*Mus (Nannomys) musculoides* Gg-*Gerbilliscus gambianus*

Nse-*Mus (Nannomys) setulosus* Tg-*Taterillus gracilis*

Mn-*Mastomys natalensis* Ur-*Uranomys ruddi*

Me-*Mastomys erythroleucus*

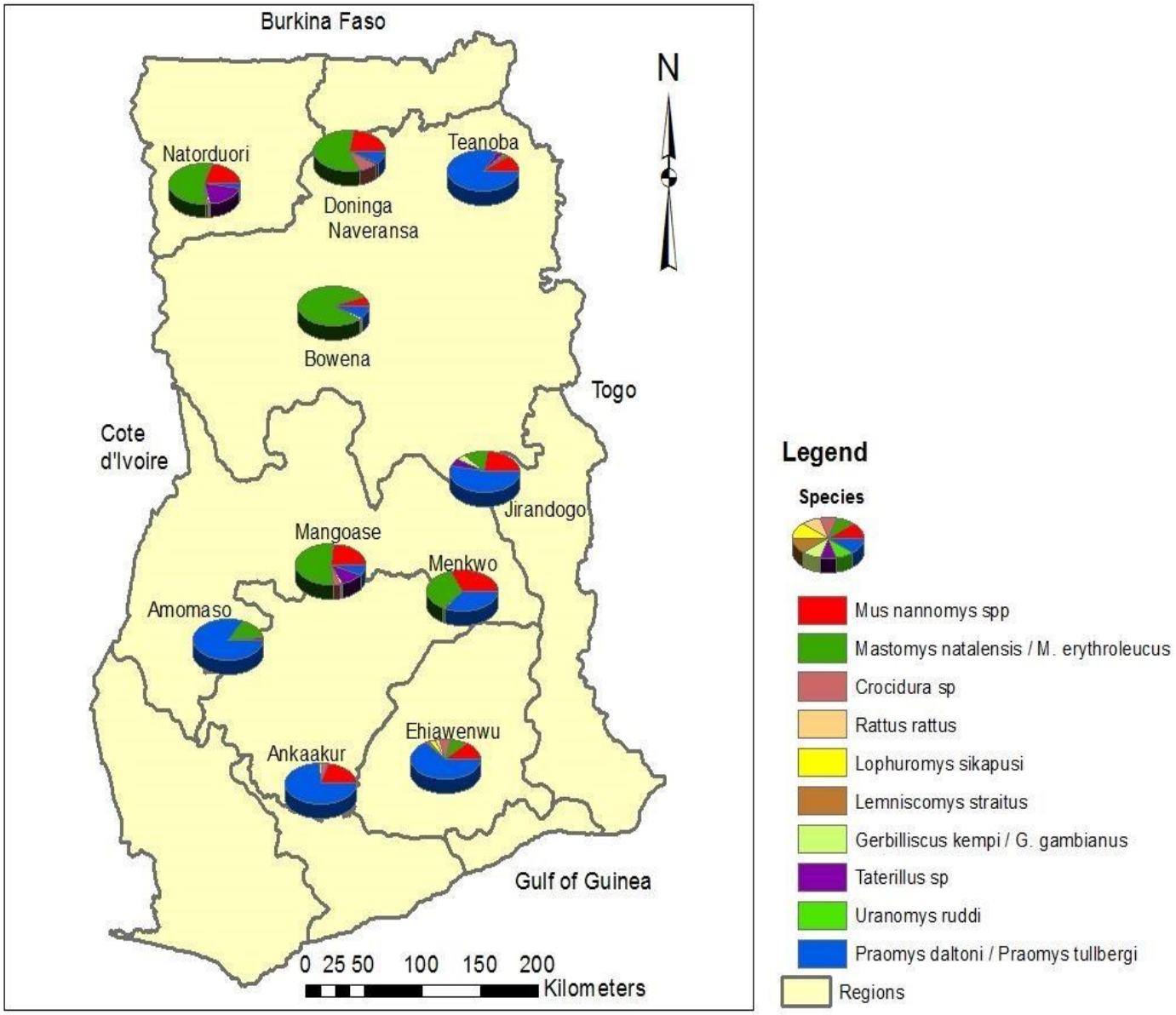

Figure 4.2: Small mammals' distribution at study sites

Trapping success, TS, (number of trapped rodents divided by the number of trapping nights for traps set on three consecutive nights) was 9.2% (635/6895) for all locations; 23% (382/1654) in houses and 4.8% (253/5241) outdoors. The highest TS were observed in Jirandogo (site 4) (Table 4.2).

Table 4.2: Trapping success of rodents at study sites for three consecutive nights of trapping

		Indoor (Houses)			Outdoor (Farmlands)			Total		
Month	**Village/Site**	**No. Rodents**	**TN**	**TS (%)**	**No. Rodents**	**TN**	**TS (%)**	**No. Rodents**	**TN**	**TS (%)**
Jun 2011	Nator Duori/1	30	170	17.6	32	523	6.1	62	693	8.9
Apr 2011	Bowena/2	30	167	18.0	10	415	2.4	40	582	6.9
May 2011	Teanoba/3	44	172	25.6	14	551	2.5	58	723	8.0
May 2011	Jirandogo/4	46	85	54.1	50	494	10.1	96	579	16.6
Apr 2011	Amamaso/5	36	177	20.3	12	606	2.0	48	783	6.1
Nov 2010	Ankaakur/6	52	180	28.9	19	675	2.8	71	855	8.3
Aug 2010	Ehiawenwu/7	58	232	25.0	30	465	6.5	88	697	12.6
Jun 2011	Doninga/8	27	178	15.2	18	528	3.4	45	706	6.4
Jul/Aug 2011	Mangoase/9	36	173	20.8	29	413	7.0	65	586	11.1
Jul/Aug 2011	Monkwo/10	23	120	19.2	39	571	6.8	62	691	9.0
Total		**382**	**1654**	**23.1**	**253**	**5241**	**4.8**	**635**	**6895**	**9.2**

4.2 Screening of rodent samples for Arenaviruses

Two of 764 rodents tested were identified as arenaviruses with real time RT-PCR cycle threshold (C_T) values less than 30 from whole blood and homogenized heart samples (Figure 4.3a). The arenaviruses detected in these two rodents were also present in their spleen, kidney and liver (Figure 4.3b & 4.3c). In both rodent samples, the cycle threshold values for the kidneys were lowest indicating high viral load. The arenaviruses detected in the L gene of each of the tissues amplified at approximately 400 base pairs as shown in figure 4.4. These mice were *Mus (Nannomys) baoulei* and *Mus (Nannomys) mattheyi* identified at Jirandogo (Site 4) and Natorduori (Site 1) respectively. These two viruses were thus named after the villages in which they were discovered, Jirandogo and Natorduori accordingly.

JIRA 076

NATO 023

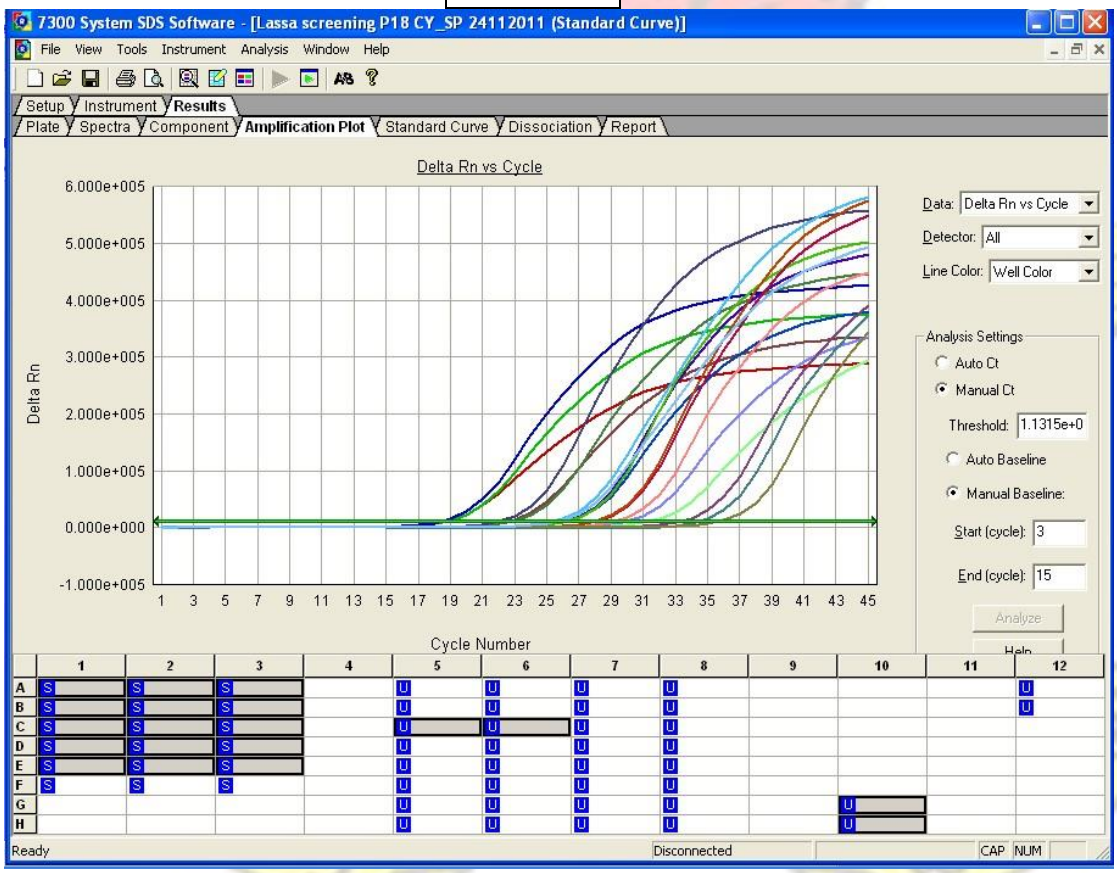

Figure 4.3a: Amplification plot showing the presence of arenavirues by RT-PCR assay in the two rodent samples (JIRA 076-CT=25.5 & NATO 023-CT=28), each in duplicate. The other

amplification curves represent the RNA standards of the positive control, each in triplicate (C_T range: 18 to 35). The assay was done on ABI 7300 thermocycler (Applied Biosystems, Singapore)

Spleen

Kidney

Liver

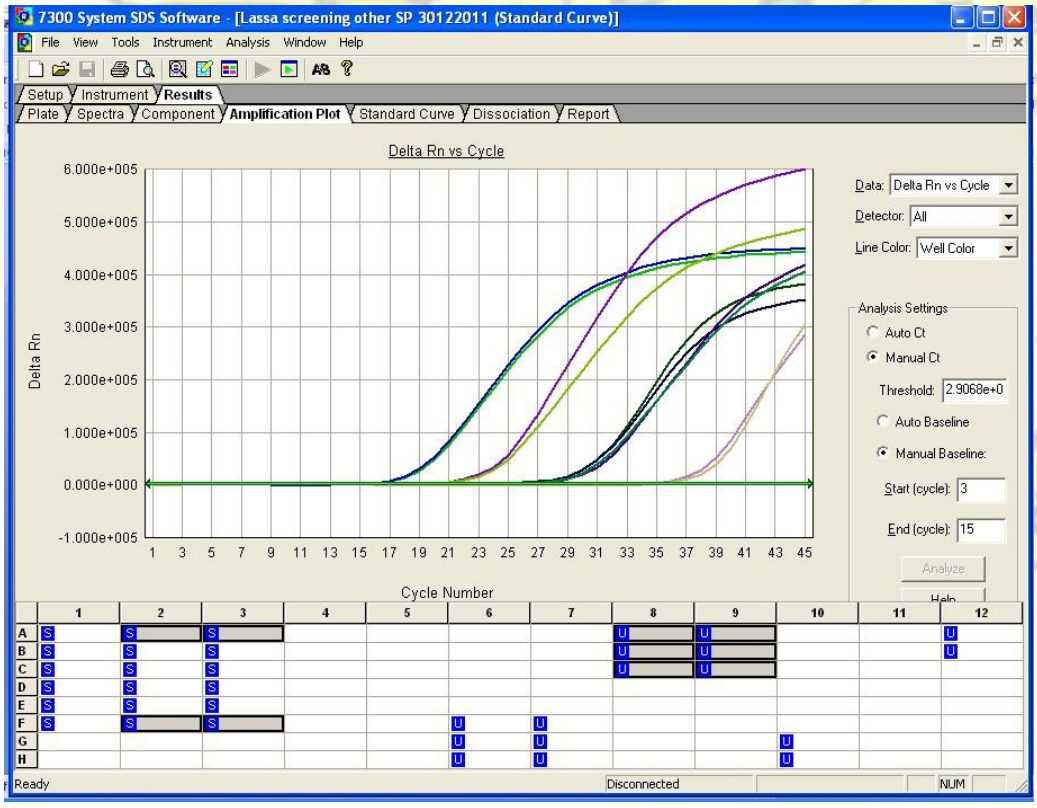

Figure 4.3b: Amplification plot showing presence of arenavirus by RT-PCR assay in spleen (CT=25), liver (CT=27) and kidney (CT=20) of sample JIRA 076, each in duplicate. The first and the last sets of curves represent the RNA standard range (CT range: 16 to 36). The assay was done on ABI 7300 thermocycler (Applied Biosystems, Singapore)

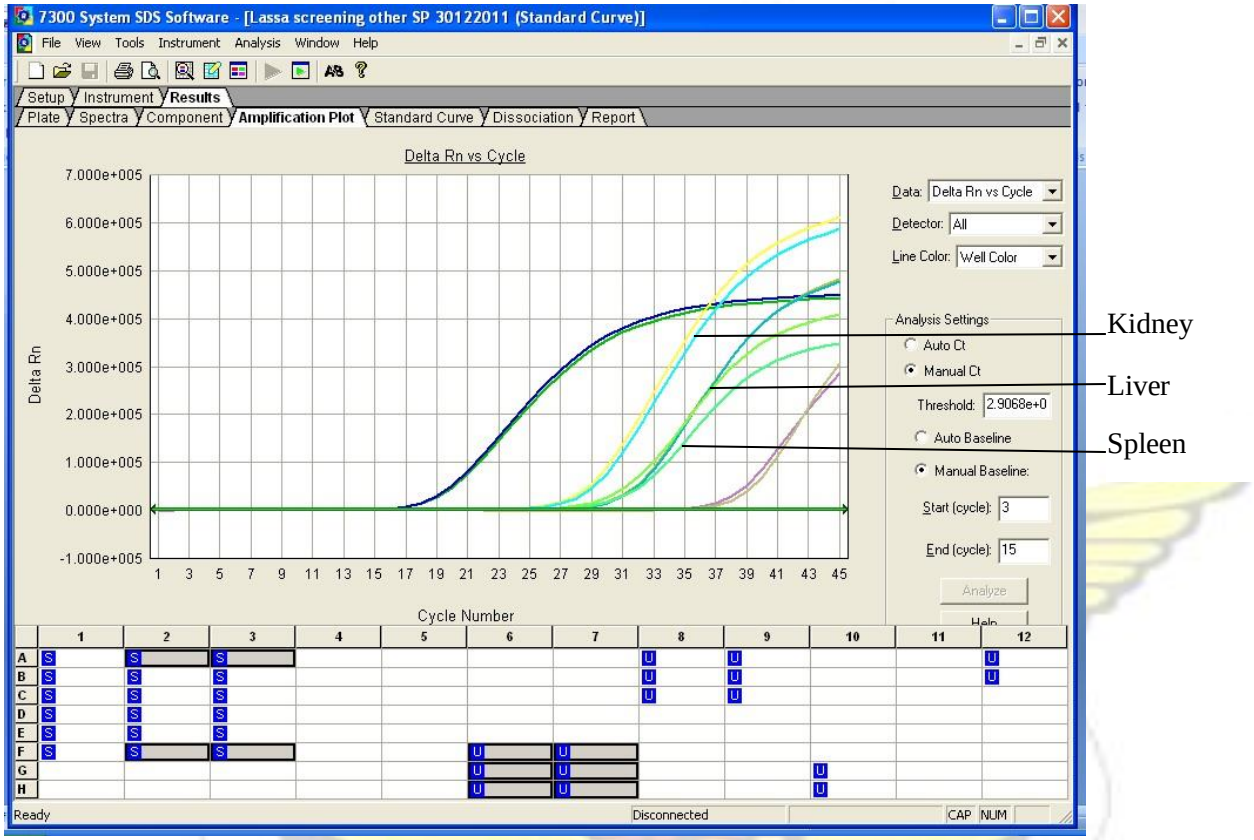

Figure 4.3c: Amplification plot showing presence of arenavirus by RT-PCR assay in spleen (CT=27), liver (CT=26) and kidney (CT=24) of sample NATO 023, each in duplicate. The first and the last sets of curves represent the RNA standard range (CT range: 16 to 36). The assay was done on ABI 7300 thermocycler (Applied Biosystems, Singapore)

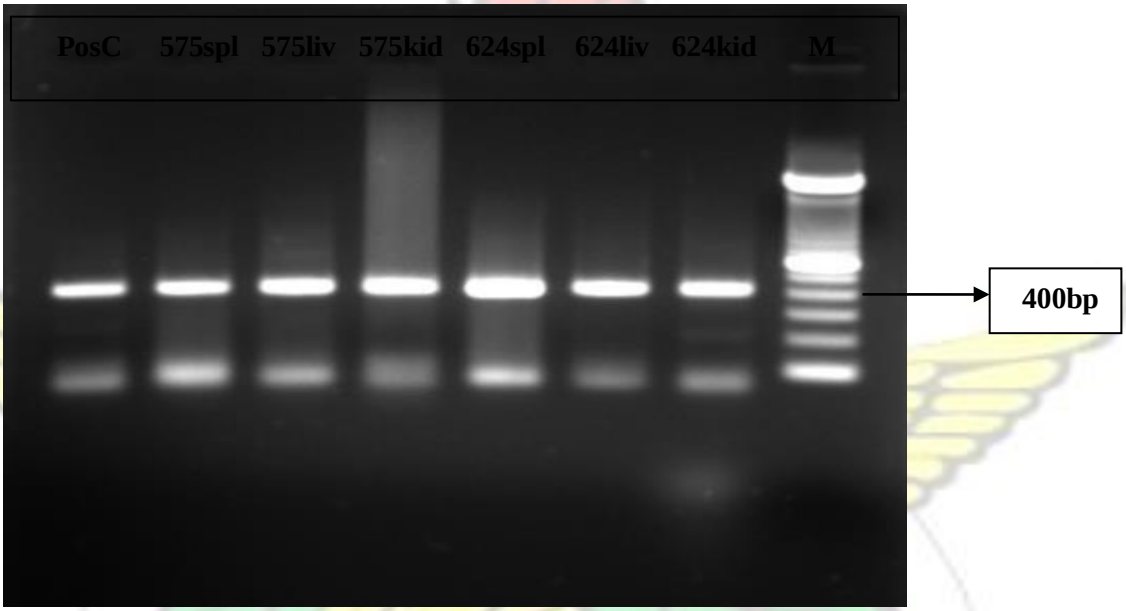

Figure 4.4: Gel electrophoregram for the identification of arenavirus in rodent tissues targeting the L gene. Lane PosC=positive control, Lane 2 to 4=spleen, liver and kidney of sample 1, Lane 5 to 7=spleen, liver and kidney of sample 2, Lane M = 100bp molecular weight marker. All products amplified at approximately 400 bp

A sequence analyses performed on the L genes of these samples showed 78% and 84% maximum identity by BLAST to Lassa virus (Liberia 07-56) and Lymphocytic Choriomeningitis virus

(LCMV) respectively. The viral and murine (cytochrome b) sequences have been deposited in Genbank under the references JX845167-JX845174.

The phylogenetic analysis for GP genes (Figure 4.5a) placed one strain found in *M. baoulei*, named Jirandogo, but this strain also clusters with the Nigerian LASV strain Lili Pinneo (lineage I), with a very low branch support of 20%. For the NP gene tree (Figure 4.5b), the sequence was basal to all Lassa strains with 62% bootstrap support. The L gene tree (Figure 4.5c) showed Jirandogo basal to Sierra Leonean LASV strains (lineage IV) with branch support of 66%. Lineages of Lassa virus clade are indicated by Roman numerals on the right of each tree. At the nucleotide level, identity scores between Jirandogo and other published Lassa strains ranged between 70.9-74.6%, 71.6-74.6% and 76.9-83.8% for GP, NP and L respectively. At the amino acid level, the scores ranged between 79.1-84.2%, 82.0-84.2%, and 93.8-98.2% for GP, NP and L respectively. However, the maximum amino acid difference in NP between Jirandogo and LASV of 18% exceeds the 12% cutoff criteria, and therefore places it outside the LASV clade (Bowen *et al*., 2000).

The other strain found in *M. mattheyi*, named Natorduori, also clustered with LCMV in all three phylogenetic trees (Figures 4.5a, 4.5b & 4.5c). For the NP gene tree (Figure 4.5b), the sequence was basal to all LCMV strains with 100% bootstrap support. The L gene tree (Fgure 4.5c) showed Natorduori basal to Kodoko virus strain which is also closely related to LCMV. At the nucleotide level, identity scores between Natordouri and other published LCMV strains ranged between 82.4-86.6%, 84.2-87.8% and 86.9-91.8% for GP, NP and L respectively. At the amino acid level, the scores ranged between 88.2-93.1%, 92.6-95.9%, and 94.8-99.9% for GP, NP and L respectively. Comparison of the nucleoprotein amino acid sequences indicated that this LCMV-related virus could be designated a novel arenavirus, thus was tentatively named Natorduori strain.

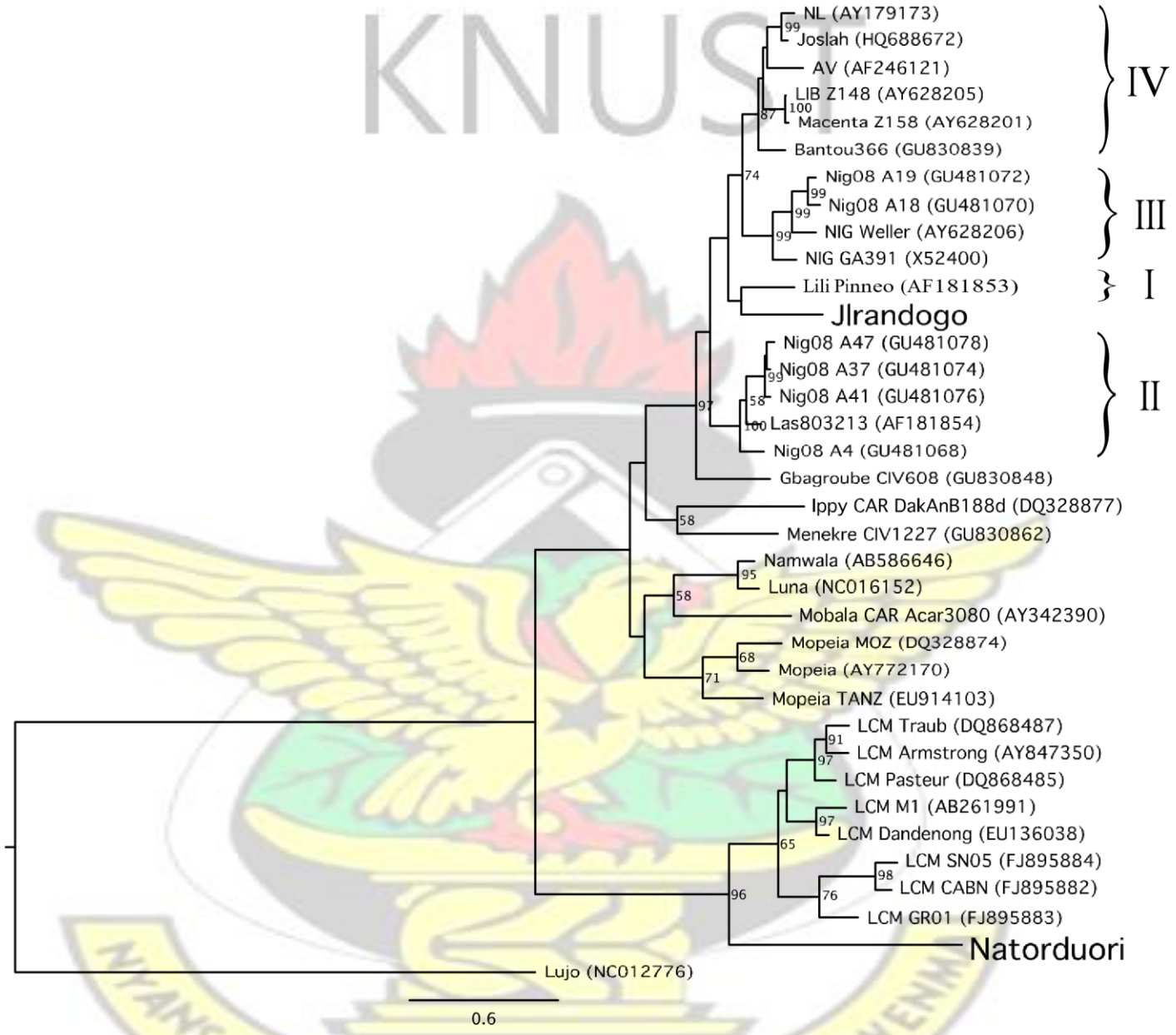

Figure 4.5a: GP gene phylogenetic tree of Jirandogo and Natorduori viruses (partial 1034bp)

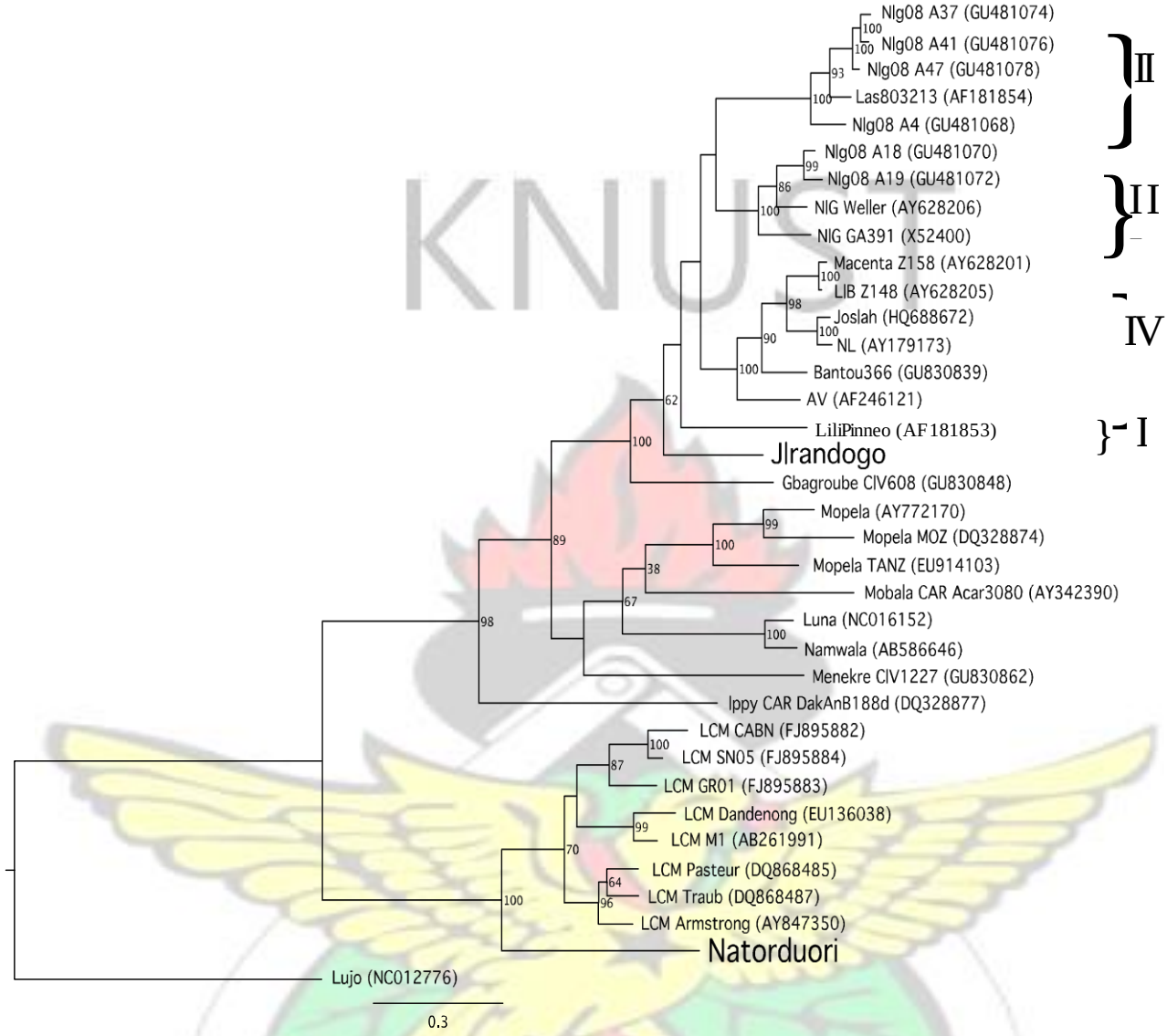

Figure 4.5b: NP gene phylogenetic tree of Jirandogo and Natorduori viruses (partial 1297bp)

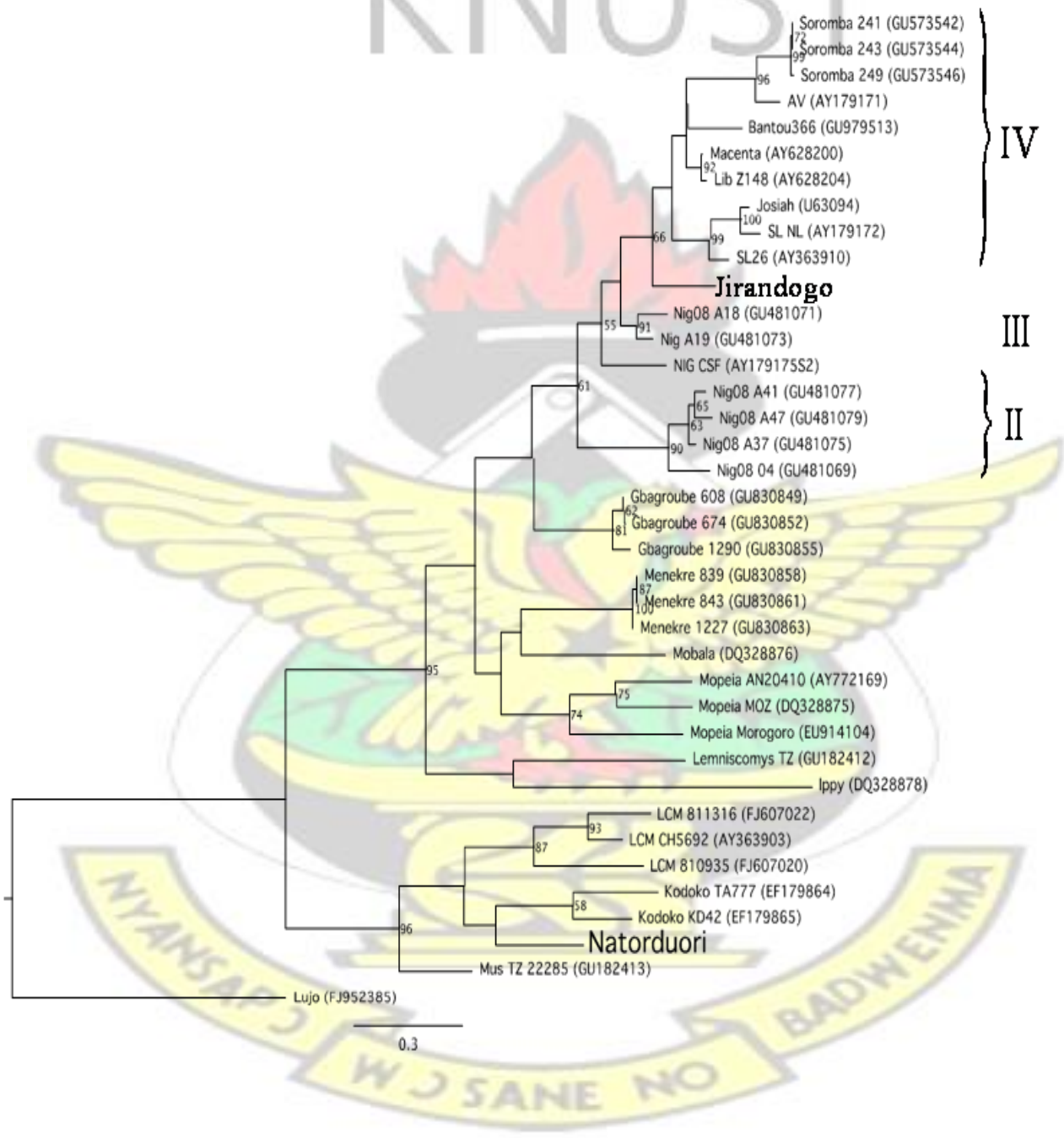

Figure 4.5c: L gene phylogenetic tree of Jirandogo and Natorduori viruses (partial 340 bp)

4.2.1 Screening of tissues for other rodent-borne infections

All lung tissues harvested from formaldehyde-preserved carcasses tested negative for Hantaviruses RNA by PCR. Kidney tissues were negative for *Borrelia, Leptospira* and *Leishmania* DNA. As a result of logistics reasons, all rodent organs were not tested against all the pathogens of interest. In light of this, only appropriate specimen was used (that is, kidney for testing *Borrelia, Leptospira* and *Leishmania* DNA and lung for Hantavirus RNA).

4.3 Demographic data

In all ten study sites, majority of the enrolled subjects were predominantly farmers (486/657) 74%, with the majority from Jirandogo (site 4) and Natorduori (site 1). Of the 264 individuals that had been exposed to one or more rodent-borne infection, 214 (82%) had their houses built with mud and 117 (44.3%) had between 6 to10 persons living in their home. Ninety seven percent (255) of those who had been exposed to one or more rodent-borne pathogen, kept at least one domestic animal with the most common of these being chicken and goats. Examination of records of malaria in the last year among the population that tested positive to at least one rodent-borne pathogen in the last year, showed 46 (17.4%) had had malaria once and 90 (34%) more than once (Table 4.3).

4.3.1 Seroprevalence of pathogens in Human

Six hundred and fifty seven subjects between 18 and 98 years of age, participated in the study (mean ± SD age = 41.4± 18.3 years). Sample sizes for each age group were 152 (18-25 years), 152 (26-35 years), 137 (36-45 years), 72 (46-55 years), 55 (56-65 years) and 86 (>65 years) with 3 unknown ages. The gender ratio (M:F) was 4:5.

Table 4.3: Risk factors of rodent-borne pathogens among enrolled population

Risk factor among enrolled population	Seropositivity for one or more rodent borne infection

Farmer occupation	41% (200/486)
Mud house	41% (214/521)
>6 persons in house	40% (198/496)
Malaria fever one or more times last year	38% (136/360)
Age	
18-25 years	37% (56/152)
26-35 years	31% (47/152)
36-45 years	45% (61/137)
46-55 years	43% (31/72)
56-65 years	44% (24/55)
Above 65 years	50% (43/86)
Sex	
Male	43% (129/297)
Female	37% (132/356)
TOTAL	**40% (264/657)**

4.3.2 Trapping success and human seroprevalence rate

Rodent trapping success (TS) and human seroprevalence for rodent-borne pathogens showed no particular association. Among the ten study sites, Jirandogo (site 4) recorded the highest TS of 16.6% for rodents, but seroprevalence rate in humans was observed to be among the lowest (31.4%). On the otherhand, Doninga (site 8) with one of the lowest TS (6.4%) recorded the highest seroprevalence rate of 60% (Figure 4.6).

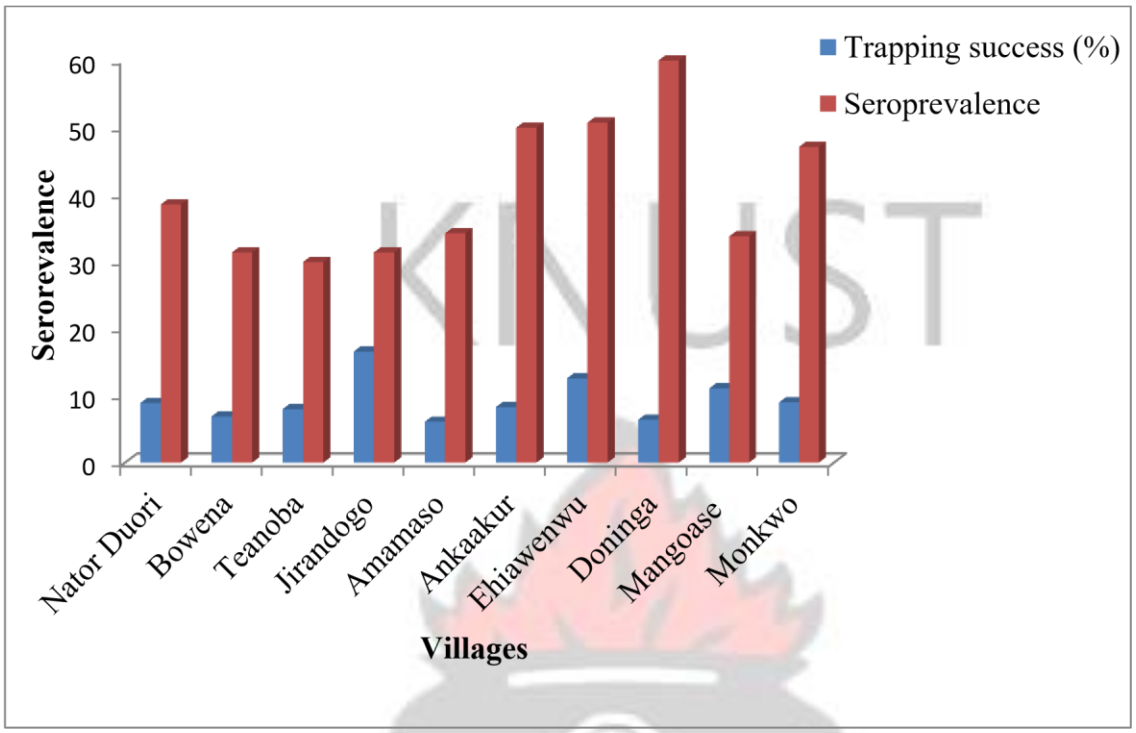

Figure 4.6: Rodent trapping success verses individuals exposed

4.3.3 Immunoglobulin G Analysis in human Serum

4.3.3.1 Lassa Virus Immunoglobulin G (IgG) in humans

A total of 34 LASV IgG positives were detected representing 5.2% seropositivity (Figure 4.7). Generally, human seropositivity appeared to be high in southern Ghana, which was surprising since these sites did not have a high burden of *Mastomys natalensis*.

Seropositivity within the various age groups shows highest occurrence in individuals older than 65 years, 8.1% (7/86) and the lowest in age group 26-35 years recorded 2.0% (3/152).

Overall, Ehiawenwu (site 7) recorded the highest seropositivity 26.5% (9/34) followed by Monkwo (site 9) and Ankaakur with 20.6% (7/34) and 14.7% (5/34) respectively (Figure 4.7). Comparatively, seropositivity within the villages was highest in Ankaakur 15.6% (5/32) and lowest in Doninga Naveransa and Jirandogo (sites 8 and 4 respectively) both recording 2.9% (Figure 4.7).

Recent or past exposure to LASV by presence of IgG was not detected in Bowena and Amomaso (sites 2 and 5) (Figure 4.7). Age group, gender and village analysed by logistic regression showed that village (geographic location) had an effect on an individual's exposure to LASV ($X^2 = 31.685$, $p = 0.000$) whereas the other factors were not significantly associated.

4.3.3.2 Hantavirus Immunoglobulin G in humans

Tests for presence of anti-Dobrava/Hantaan and Puumala IgG antibodies resulted in 12.2% (80/657) and 11.3% (74/657) respectively with Doninga Naveransa/site 8 and Monkwo/site 10 recording the highest numbers. Age group 18-25 years recorded the highest seropositivity for both Dobrava (26%, 21/80) and Puumala (29%, 22/74). There was evidence of antiDobrava/Hantaan and Puumala IgGs in all study sites except for site number 6 where all sera were negative for anti-Dobrava/Hantaan IgG (Figure 4.8). For both Hantavirus serotypes, exposure rates were higher in females compared to the males but the difference was not statistically different.

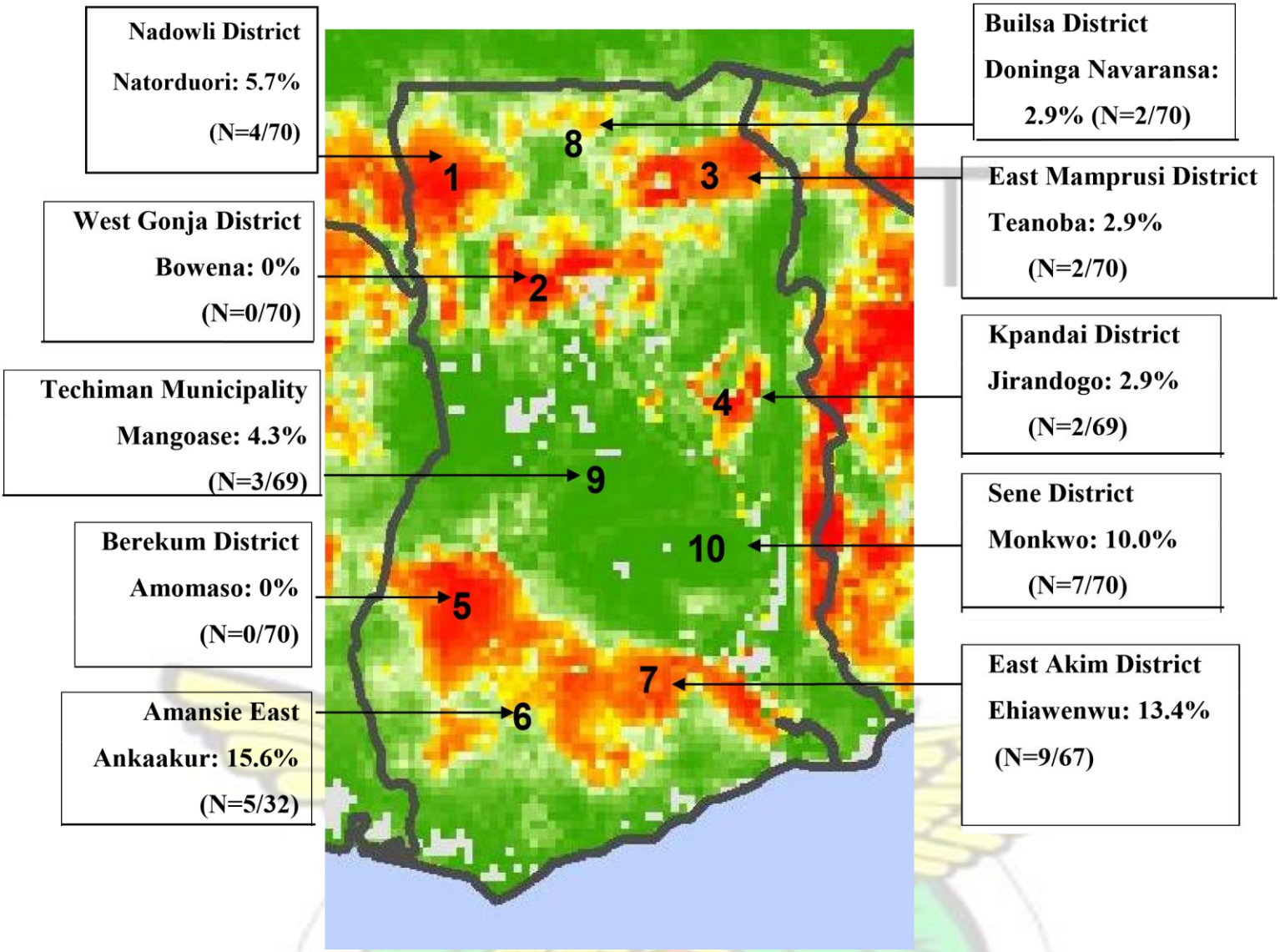

Figure 4.7: Site distribution and prevalence of anti-LASV Immunoglobulin G in humans indicating previous exposure to LASV superimposed on previous risk map for Lassa virus.

4.3.3.3 *Leptospira* Immunoglobulin G in humans

A total of 140 human sera (21%) had anti-*Leptospira* antibodies. Exposure was observed in all ten sites with site 8 recording the highest (19.3%, 27/140) (Figure 4.8). The geographic location of an individual was significantly associated to his or her exposure to anti-*Leptospira* antibodies (X^2 =75.606, p = 0.000). Within the sampled sites, age group 56-65 years recorded the least exposure

rate of 7.9% (11/140). The exposure rate between males and females was not significantly different with the males recording 50% (70/140) and females 48.6% (68/140).

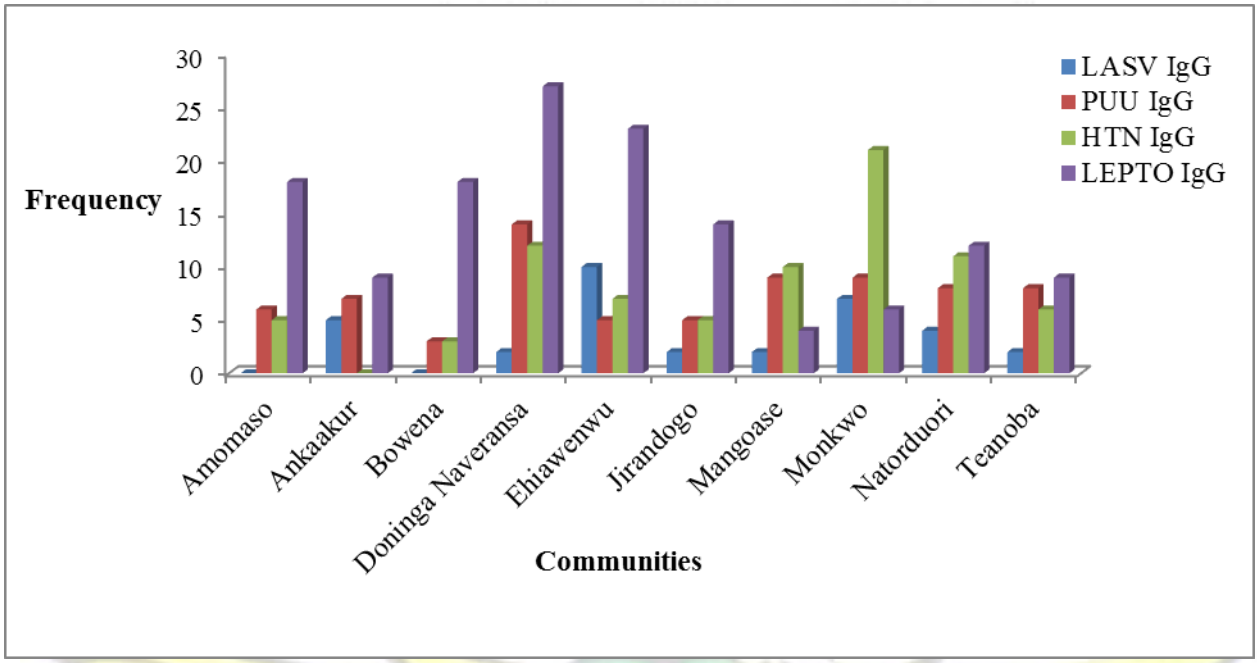

Figure 4.8: Distribution of rodent-borne IgG against LASV, PUU, HTN and LEPTO in study communities

4.3.4 Co-infections of pathogens in humans

Of the total number of seropositive individuals (264), 56 (21%) had been exposed to more than one of the rodent-borne infections tested, whereas 208 (78%) had been exposed to only one type of infection. Common co-infection exposure types observed were Puumala and *Leptospira* (35.7%), Hantaan/Dobrava and *Leptospira* (21.4%) as well as LASV and Puumala (17.9%) with the others recording less than 10% (Figure 4.9).

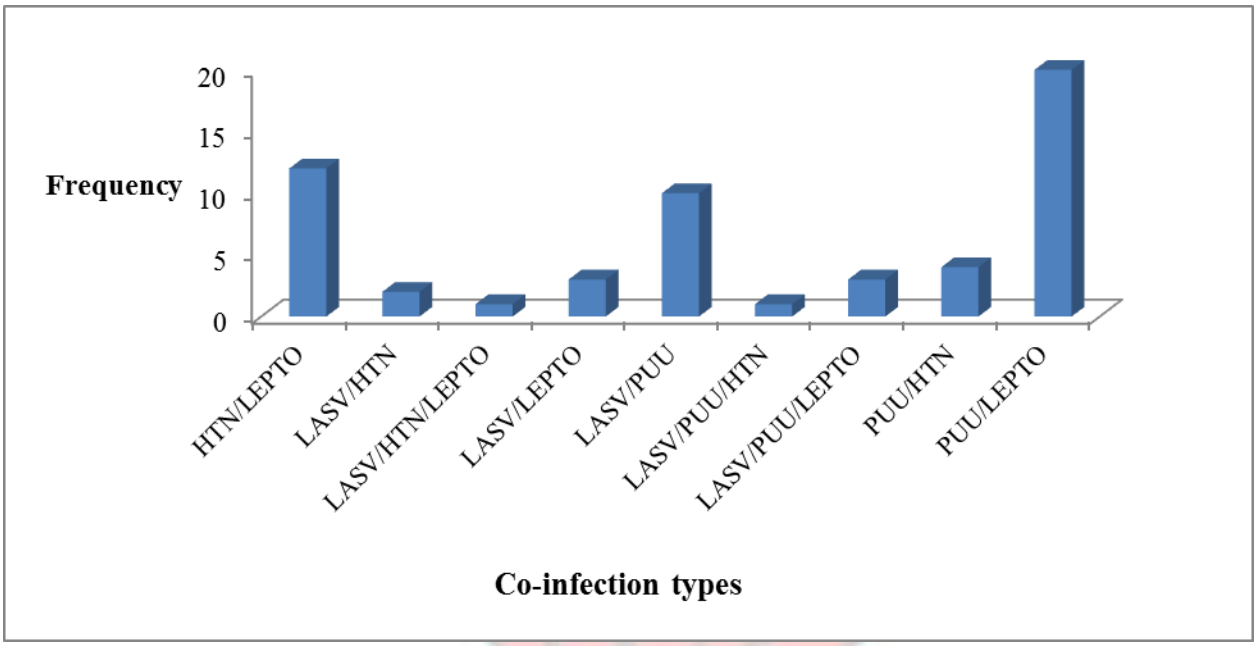

Figure 4.9: Frequency of rodent-borne IgG co-infections

CHAPTER FIVE

DISCUSSION

5.1 Rodent distribution

5.1.1 General distribution of *Mastomys natalensis*

The ecological zones of the human communities surveyed ranged from moist semi-deciduous forest (south); guinea savannah (north) to the transitional zone (middle belt). With Ghana situated between well known Lassa fever endemic regions, one would expect to find the reservoir, *Mastomys natalensis*, in abundance within this area. However, this was not the case in this study. Only 27% (209 of 764 captured rodents) were identified as *M. natalensis*, compared with 54% (601/1123) in Guinea and 80% (82/103) in Mali (Fichet-Calvet *et al.*, 2007; Safronetz *et al.*, 2010). Overall, *Praomys daltoni*, a species not known to harbor Lassa virus (LASV), outnumbered *M. natalensis*, and was the predominant rodent species found indoors in six of the ten study sites contradicting the previous findings that *M. natalensis* is adaptable, opportunistic and frequently in contact with humans (Isaacson 1975).

A study conducted by Fichet-Calvet and Rodgers in 2009, showed that while the reservoir host, *M. natalensis* can and do occur over regions experiencing a much wider range of rainfall conditions, Lassa virus (LASV) required a particular combination of high (but not the highest) rainfall, and with a particular form of variability and seasonal timing. In the same study, vegetation variables in the predictor data sets seemed unimportant as one of the study models independently and quite categorically failed to identify vegetation variables as important in determining LASV distribution. This observation suggest that the survival of the virus outside of the vertebrate host might be a key to determining its distribution, and that their survival depends upon moisture or rainfall conditions above more or less all other environmental variables. In the case of Lassa, the virus appears to survive better in humid conditions, during the rainy season. Rodents will be more often

contaminated during their frequent movements at this season, for mating or dispersing into the surrounding fields (Fichet-Calvet *et al*., 2008). Conversely, viral aerosol stability, seems to be higher when the humidity is lower (Stephenson *et al*., 1984), a condition that obviously occurs more frequently in the dry season.

There are at least four *Mastomys* species described as sibling species with no fully diagnostic external morphological criteria (Granjon *et al*., 1997). These species are widely distributed: *Mastomys coucha* (2N=36, aFN=52-56) in Southern Africa, *Mastomys huberti* (2N=32, aFN=4446) in West Africa, *Mastomys erythroleucus* (2N=38, aFN=50-56) from Senegal to Ethiopia south to Burundi with a relict population in Morocco, and *Mastomys natalensis* (2N=32, aFN=52-54) in nearly all sub-Saharan Africa. Locally, two or three species can be found in sympatry (Duplantier and Granjon, 1988; Lavrenchenko *et al*., 1998). In this study, *M. natalensis* was found together with *M. erythroleucus* in four out of the ten study sites.

The mean trapping success (TS) in this study which was adopted as an indicator of rodent abundance was higher inside houses (indoors) than in the farm fields (outdoors). Indoor species were mainly *M. natalensis* and *Praomys daltoni* constituting 98% (492/504) of total indoor captures. This observation could be attributed to habitat change caused by the seasonal change in availability of food stuff for rodentsas trapping was carried out during the rainy season, when viral prevalence rates in rodents have been shown to be higher (Fichet-Calvet *et al*., 2007). Before the rainy season, crops are harvested and stored inside houses whiles the farm fields are used for pasture or burnt prior to being cultivated at the beginning of the rainy season (Saltzmann 1978).

These ecological factors possibly attract rodents from the fields into houses as the type of farming practices affects the nature of the habitat, shelter and population density of rodents (Makundi *et*

al., 1999). Among the individual study sites, TS was highest in Jirandogo/site 4 with a total of 96 rodents captured in the least number of trapping nights (579) for three consecutive nights. Jirandogo was the only study site where the target of trapping a maximum of 100 rodents per site was exceeded within 3 consecutive nights. This observation was not surprising as there was overcrowding in the community which probably encourage increasing rodent populations.

Hygiene in and around homes were found to be poor in all but one site (Doninga Naveransa) probably accounting for the lowest rodent capture (54 rodents) during the trapping nights. Heaps of firewood, shrubs and rubble were seen in some parts of the human settlements. These are attractive to rodents for shelter and refuge thereby acting as a risk factor for rodent infestation of houses and for transmission of rodent-borne diseases in the house's immediate surroundings alike (Telford 1989).

The type of building material used in constructing the houses varied from sand, clay and cement with straw or aluminium sheets as roofing. The walls of these houses in most of the communities were generally not well done with cracks and rough surfaces that make it easy for rodents to climb. In Doninga Naveransa, even though the houses were made of clay, they were smoothly plastered making it virtually impossible for rodents to climb into the rooms.

It was also observed in Ankaakur/site 6 and Ehiawenwu/site 7 that their graveyards were close to the human dwellings (about 2 meters apart). Even though rodent capture was high in these two communities, (81 and 94 rodents respectively), one could not attribute this to the closeness of these graveyards to the communities as Jirandogo/site 4, Mangoase/site 9 and Monkwo/site 10 which

equally recorded high rodent capture had their graveyards greater than 1 km from their inhabitation.

5.2 Molecular Analysis for Pathogen Detection in Rodents

5.2.1 Arenavirus Detection

A sequence of Lassa virus (LASV) was detected in a pygmy mouse, *Mus (Nannomys) baoulei*. The African pygmy mice, *Nannomys*, an African subgenus of *Mus* sensu lato (Muridae, Murinae), constitute a group of small-sized rodents (<12 g) that are widespread throughout subSaharan Africa (Catzeflis and Denys, 1992). They are a complex of morphologically very similar species (Petter, 1963; 1981; Macholan, 2001), which has led to the description of 5 to 30 species (Marshall, 1981). Based on the phylogenetic analyses, the Jirandogo virus found could belong to a new Lassa strain as the L gene nested within a group containing members of lineages II, III and

IV. However, the maximum amino acid difference in glycoproteins (GP) and nucleoproteins (NP) genes between Jirandogo and other Lassa strains of 20.1 and 16.2% were over the threshold of 12.0%, usually accepted within the Lassa clade (Bowen *et al*., 2000). Lassa virus isolation was previously reported in a pygmy mouse from Nigeria in 1975, but that work was done before the modern era of molecular identification of rodents and viruses, and must be interpreted with caution (Wulff *et al*., 1975). The strain, Natorduori, clearly clustered with LCMV in the phylogenetic trees of the glycoproteins, nucleoproteins and large genes. The new LCMV-related virus belongs to the LCMV clade, but diverges from the typical LCMVs.

Mus species in Africa are sometimes found indoors where exposure to humans is more likely, but most live outdoors in fields, including the two arenavirus positives found in this study. However, it is not certain whether the arenavirus sequences found in these mice are from viruses which are

pathogenic in humans. While arenaviruses, including Lassa, have species specific reservoirs, recent work suggests host switching of arenaviruses may be more common than previously thought (Coulibaly-N'Golo *et al*., 2011). While in this study *Mastomys* species were negative for LASV, their abundant presence in some of the study sites represent potential for virus circulation. Inadvertant introduction of LASV into a community with significant *Mastomys* species could establish a reservoir in Ghana, and subsequent human outbreaks.

It is notable that possible cases of Lassa fever have recently been reported in Ghana by the Ministry of Health from a predicted high risk area near site 5 of this study (Dzotsi *et al*., 2012). These reported cases in humans occurred about 35 km from site 5 (Amomaso), where *Mastomys natalensis* represented 15% of all rodents captured, and showed no evidence of arenavirus infection by PCR testing. The cases in humans were reported on the basis of the results of PCR tests, and further sequencing would be necessary to confirm the finding. Although arenaviruses have species–specific reservoirs, recent work suggests host switching may be more common than previously believed (Coulibaly-N'Golo *et al*., 2011).

The study did not detect any evidence of the presence of Hantavirus, *Leptospira*, *Borrelia* and *Leishmania* pathogens in the rodents captured in the study sites by molecular investigations.

5.3 Seroprevalence in Humans

5.3.1 Antibodies to Arenavirus

The villages surveyed were mainly remote communities; with the major occupation being farming. It was expected that villages with more rodents would have a greater number of their human population being exposed to more rodent-borne pathogens. However, this trend was not observed in the study sites. About 5.2% of the study population, mainly healthy adults, had antibodies to

Lassa virus suggesting LASV or another closely related virus may be infecting humans in rural Ghana. The highest rates were in southern Ghana for unknown reasons. In a study conducted in 2007, 960 serum samples from subjects across West Africa were processed to determine antibody response to Lassa virus (LASV) using a standard indirect immunofluorescence assay (IFA) and a highly sensitive reverse ELISA. The samples that represented Ghana had been collected during studies on the presence of anti-malaria antibodies in the areas of Tamale (n=280), Agogo (n=100), and Kumasi (n=100). The antibody prevalence was 3.8% in Ghana (Emmerich *et al*., 2008) which is lower than that observed in this study.

Past exposure (IgG) to LASV was detected more in the male population 58.8% (20/34) and seropositivity was seen across all age groups with the highest incidence in age group 18-25 years recording 29.4% (10/34). Klein and others in 2010 established that males and females differ in their susceptibility to a diverse array of viral infections. Suceptibility to viral infections as well as their severity often is reduced among females because they typically have a more robust antibody production following viral infection compared to males (Torcia *et al*., 2012). Identification and reaction to viruses differ between males and females during viral infections. This often results in gender differences in cytokine and T cell responses to infections that play a critical role in determining susceptibility to viruses (McClelland & Smith, 2011).

Some studies have also shown that age plays a critical role in determining viral virulence (Grifin *et al*., 1994; Labrada *et al*., 2002). It is commonly considered that maturation of the immune system explains increased resistance to viral infection in older hosts, but this has not been thoroughly confirmed though other differences in cellular differentiation and proliferation may play a role (Labrada *et al*., 2002).

The residences of 29 out of the 34 individuals who had antibodies to LASV were built with mud walls and straw roofs. Most of these walls had gaps between them and the roof allowing rodents and other creeping animals to enter the rooms in search of food. About half the number of individuals who had been exposed to the virus stored harvested food stuff in rooms within the houses.

5.3.2 Antibodies to Hantavirus

For hantavirus exposure, the observed IgG seroprevalence by ELISA of 11.75% is lower compared to that found in the forest of Guinea (12.2%; Klempa *et al*., 2013). However, the IgG seroprevalence observed for this study was higher than those reported earlier from Gabon (8%; Dupont *et al*., 1987) and Central African Republic (4%; Gonzalez *et al*., 1988). The high value observed in this study, could probably be overestimates of antibody prevalence because no confirmatory tests were performed. After confirmatory tests were performed on the serum samples from Klempa and others, the seroprevalence rate was observed to be 1.2% with rather low antibody titres. An array of different serological assays is necessary to avoid false-positive results that might be caused by unspecific reactivity of sera from the African population.

However, it needs to be stated that an array of assays might considerably decrease the sensitivity. Nonetheless, in this very first assessment of Ghana, the priority was to clearly confirm whether human hantavirus antibodies occurred. The focus was therefore, on specificity rather than sensitivity.

Some of the reservoirs of Hantavirus, *Rattus* sp and shrews, captured in this study were mostly outdoors. No evidence of Hantavirus was detected in any of these rodents by molecular assays even though there have been reported cases of indigenous hantaviruses from Africa since 2006.

These include Sangassou virus that was found in an African wood mouse (*Hylomyscus simus*) in Guinea (Klempa *et al.*, 2006), Tanganya virus and Azagny virus, found in shrews and moles respectively (Klempa *et al.*, 2007, Kang *et al.*, 2011). When screening for both Hantavirus serotypes was done, more females 53.9% (83/154) had been exposed to the antibodies compared to the males 45.5% (70/154) but this observation was not statistically significant.

5.3.3 Antibodies to *Leptospira* Species

Leptospirosis has not been widely studied in Africa and the disease mimics many febrile illnesses in endemic areas as its signs and symptoms are nonspecific, making accurate diagnosis on clinical grounds a difficulty (Farr 1995). About 21% (140/657) of the subjects had antibodies to *Leptospira* species via an enzyme immnumoassay that was highly sensitive but not specific. Immunoglobulin G (IgG) concentrations higher than 9 U/ml indicated *Leptospira* infection. It should be noted that not all patients develop a detectable IgG reaction after *Leptospira* infection. About 88% showed a rise in antibody titers after two to three months (Silva *et al*., 1995), whereas some had no IgG response at all. It will however be necessary to carry out a second test to determine the precise nature of the infecting agent in our study population.

It is known that clinical expression can differ according to the species concerned and some of them do not cause clinical symptoms, especially in Africa (Sankale *et al*., 1976). The seroprevalence rate in this survey was however lower than the 33% rate demonstrated by Hogerzeil and others in 1986 within the humid forest areas of Ghana. Other previous studies conducted in West Africa reported 18% among volunteers in Nigeria (Ezeh *et al*., 1991). In East Africa, a study undertaken in Kenya, both on healthy and febrile patients showed that 19% of the cases proved to be reactive against one or more leptospiral antigens (Forrester *et al*., 1969).

5.4 Limitations of the study

There is the possibility that sampling may have been flawed in this study if LASV circulation is seasonal even though in some countries such as Sierra Leone and Guinea, the virus has been detected in *M. natalensis* in both the dry and rainy season (Fraser *et al*., 1974; Keane and Gilles 1977; Bausch *et al*., 2001; Merlin *et al*., 2002). The LASV is more stable in dry compared to humid ambiance and may be transmitted to occasional hosts by aerosols (Stephenson *et al*., 1984; Peters *et al*., 1987).

The antibody prevalence rates observered by ELISA were probably overestimated as confirmatory tests were not performed due to logistic reasons. On the one hand, a series of different serological assays is necessary to avoid false-positive results that might be caused by unspecific reactivity of sera from African people. On the other hand, it needs to be stated that these array of assays might considerably decrease the sensitivity. However, since the priority of this study was to clearly confirm whether human arenavirus, hantavirus and *leptopira* infections occur, the focus was on specificity rather than sensitivity.

5.5 Conclusion

This study has provided data on the level of risk of Lassa fever disease in Ghana which is located in the Lassa fever endemic belt but with virtually no clinical report of Lassa disease. The findings support the conception that Lassa virus is not widely prevalent in Ghana and that the *M. natalensis* reservoir is not as common as in other more highly endemic countries in the West African Region. Although *Mastomys species* were negative for the presence of Lassa virus, their abundant presence in some sites in the Northern Ghana can support virus circulation. Continued vigilance is warranted since Arenaviruses were detected in two pygmy mice from different regions in Ghana suggesting

a potential for human disease. Further genetic characterization of these arenaviruses is crucial to the understanding of their ecology and epidemiology in the region. More information about the frequency of host-switching and the degree of arenavirus circulation among rodents and humans is also needed to better appreciate the implications of this assessment for the risk of Lassa fever outbreaks in Ghana.

The results from this study also provide additional and useful information on the rodent species distribution in the study sites. It describes the preferred habitat by these small mammals within the study areas; indoors (houses) and outdoors (farmlands and surrounding fields).

It is reassuring that, none of the rodents captured were positive for the presence of Hantaviruses, *Leptospira*, *Borrelia* and *Leishmania* species by molecular tests. The absence of these pathogens in the rodent samples adds to the knowledge about their probable infrequent existence in the study sites. In addition, it is likely that the sampling of the rodents did not capture any of the reservoirs that harbored Hantaviruses, *Leptospira, Borrelia* and *Leishmania* species during the sampling period. However, the presence of immunoglobulin G antibodies of Puumala and Dobrava/Hantaan, which are Hantavirus serotypes and *Leptospira* in the human subjects, raises some concern as past exposure is indicated. There is the possibility that these disease-causing pathogens may be circulating in the country but are being misdiagnosed as their symptoms mimic many other febrile infections.

5.5.1 Recommendations

In order to further explore the results observed in this study, it is recommended that further studies with a larger sample size and more detailed analyses be conducted on the small mammal species in Ghana. More information about the frequency of host-switching and the degree of arenavirus and other pathogens circulation among rodents and humans is needed to better understand the implications of the assessment for the risk of Lassa fever and other rodent-borne disease outbreaks

in Ghana. It is however, necessary to undertake a more spatial survey to further enhance our understanding of the distribution of these rodents for disease control purposes.

Until more information regarding small mammals and the pathogens they are likely to harbor is available, it is recommended that rodent control and human behavioral changes be taken seriously in order to ensure effective control of disease infection and outbreaks.

Generally, the primary prevention of rodent-borne diseases can be achieved by avoiding contact with rodents. In improving human behavioral changes, health education strategies should encourage clearing up of bushes, heaps of firewood and rubble around homes as these serve as shelter for rodents. Storing and putting food away in rodent-proof containers and keeping the home clean helps to discourage rodents from entering homes. Holes inside and outside of homes should be sealed to keep rodents out.

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APPENDICES

APPENDIX A: CONSENT FORM

***Title**: Prevalence of Lassa Virus Infections and other Human Pathogens in Mastomys species and Humans in Ghana*

***Principal Investigator**:* Shirley Cameron Odoom
Address: NAMRU-3 Ghana Detachment
Noguchi Memorial Institute for Medical Research
University of Ghana
Legon, Ghana

Introduction

Mice have been shown to have diseases that can affect humans. Close contact with these mice or their urine and feces can sometimes cause diseases in humans. People can have blood tests done, which may show evidence that they have been infected in the past, even if they did not have any symptoms when they were infected and are now completely healthy. In order to be sure that you are informed about being in this research, we are asking you to read (or have read to you) this consent form. You will be asked to sign it (or make your mark) in front of a witness. We will give you a copy of this form. Please ask us to explain anything you may not understand.

Research Purpose/General Information about Research

The Noguchi Memorial Institute for Medical Research is conducting a research to assess whether people living in Ghana have ever been infected with some germs that cause disease. We will test blood samples of people in the study sites for certain diseases, namely Lassa fever, Hantaviruses, Leptospirosis, Borreliosis and Leishmaniasis. Some of these diseases are known to occur in Ghana, but it is not known how common they are. The results of this study will help doctors and public health officials know what diseases are in Ghana and help them decide how to prevent them. You have been randomly selected to be a part of this study. If you agree to be part of this study, we will do the following:

- Ask you some general questions about yourself (e.g. name, age, telephone number) Collect about five millilitres of blood from you.
- If you would like to know the outcome of the test conducted, you will be contacted on telephone.

Possible Risks and Discomforts

This research poses minimal risks or discomforts to you. You may however be inconvenienced by the time you will spend in answering the questions and the little pain you will feel when drawing the blood.

Possible Benefits

You may not directly benefit from this research but the findings may benefit Ghanaians in general in helping to better manage rodent-borne diseases.

Confidentiality

All the information we get from you, the volunteer, will be treated in the strictest confidence. All your responses and information from the analysis of your blood will be handled confidentially and used only for the purposes indicated for this study.

Retention of samples

Some of the blood samples collected will be studied right away. Others will be stored for an indefinite period after the study, but will only be used to analyze the results of this study. No human genetic tests will be performed on your samples. The samples will be stored at Noguchi Memorial Institute for Medical Research, Legon and will be identified only by your study number.

If you provide your permission below, any leftover samples from this study may be used in future research studies to learn more about other mice-related diseasesin collaboration with other institutions but only if approved by the Institutional Review Board at the Noguchi Memorial Institute for Medical Research (NMIMR). You have the right to ask us to dispose of your samples at any time after signing this consent.

Please initial below your decision about permission for future research with your samples. Your decision can be changed at any time by notifying the study doctor or nurse. Your decision regarding your samples will not affect your participation.

_____ YES, you may store my unused samples for an indefinite period of time for future research as described above.

_____ NO, you may not use my samples for other future research. Destroy my unused samples at the end of the study

Compensation
You will receive no monetary compensation for participating in this study. However, you will be given a bar of key soap and a 400 gram can of Milo® (vitamin-fortified chocolate- flavored powder beverage)for your time spent with us.

Leaving the Research
You are free not to take part in this study. If you agree to participate, you can also decide to leave the research at any time. If you do not wish to participate in the study, you will not be penalized in any way.

If You Have a Problem or Have Other Questions
Please call Dr Karl Kronmann on telephone number 024 4333027 or Shirley Cameron Odoom on telephone number 024 4534031 if you have questions about the research.

Your rights as a participant
This research has been reviewed and approved by the Institutional Review Board (IRB) of NMIMR. An IRB is a committee that reviews research studies in order to help protect participants. If you have any questions about your rights as a research participant, you may contact Rev. Dr. Ayete-Nyampong, Chairperson, NMIMR-IRB, mobile 0208152360.

VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research titled "Prevalence of Lassa Virus and Other Human Pathogens in *Mastomys* species and Human Seroprevalence" has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_____

Date Signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_____

Date Signature of Witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____

Date Signature of Person Who Obtained Consent

APPENDIX B: Case Form/Questionnaire

Study Title: "Prevalence of Lassa Virus Infections and other Human Pathogens in Mastomys species and Humans in Ghana"

Date: ___/___/_____ 1.
Case ID No.:

2. _____ _____ _____
Village GPS Coordinates Region

Demographics

3. Name of Participant: _____ **4. Date of Birth: _____/_____/_____** **Age: _____ years**

5.Sex: ☐Female ☐Male

6. Residence Wall Type: ☐ Mud ☐ Block ☐ Other_____ **Roof:** ☐ Aluminum ☐ Straw ☐ Other _____

Do you have: Indoor plumbing? ☐Y ☐N Electricity? ☐Y ☐N

7. Number of persons living in your house: _____

8. Where is food stored? ☐ Separate building ☐ Kitchen ☐ Room in house ☐ Other _____

9. Occupation:☐Business ☐Farmer (Circle all applicable: Rice Maize Livestock other _____) ☐ Other (List)_____

10. Does your family own any of the following?

☐None	☐Swine	☐Pigeons	☐Dogs
☐Sheep	☐Donkeys	☐Ducks	☐Bats
☐Goats	☐Horses	☐Other Birds	☐Grasscutters
☐Cattle	☐Chickens	☐Cats	☐Rabbits

11. Do you drink fresh milk? ☐ Y ☐ N **If yes, what type?** ☐ Cow ☐ Goat

12. Have you been around animals giving birth (live or dead)? ☐ Y ☐ N

13. Have you seen rodents around your house? ☐ Most days ☐ Sometimes ☐ Never

14. Have you been bitten by a tick before? ☐ Y ☐ N

Health

15. How many times have you had malaria or fever this year? _____

16. Have you ever been admitted to the hospital? ☐Y ☐N What was diagnosis? _____

17. Do you have any problems with hearing? ☐Y ☐N

18. Do you sleep under a mosquito net? ☐Y ☐N

Person Completing Form Name: _____ Initials _____

*If lab specimen is collected, complete the following information and send a copy of this form to the lab with the specimen.

19. Date of specimen collection: ____/____/_____

For the Laboratory Use Only

20. Date specimen received by lab: ____/____/_____ **21. Specimen Condition:**☐Adequate ☐Not adequate _____
(Reason)

22. Results: Check (X) below as appropriate:

Disease/ Condition	**Type of antibody**	**Results (+=positive) (-=negative)**	**Titer (if done)**	**Type of test (e.g. ELISA)**
Lassa	IgG	☐+☐ -	_____	
Hantaan/Dobrava	IgG	☐+☐ -	_____	
Puumala	IgG	☐+☐ -	_____	
Leptospirosis	IgG	☐+ ☐ -	_____	

COMMENTS:

APPENDIX C: Forestry Commission of Ghana Permission to Trap Rodents

Receipt No. 045931 of 2-8-2010

FCWD/GH-01

APPLICATION TO STUDY OR CONDUCT RESEARCH WITHIN WILDLIFE PROTECTED AREAS IN GHANA

APPLICANT'S INFORMATION

First name: SHIRLEY CAMERON
Last (family) name: ODOOM
Nationality of passport: GHANAIAN
Passport Number: H0660885
Date of birth: 08/10/1980
Place of birth: ACCRA
Highest degree earned: BACHELOR OF SCIENCE
Specialized field of research: ZOOLOGY
Affiliated institution: NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Institution address: UNIVERSITY OF GHANA
P. O. BOX LG 581 LEGON
Current address:
Email: shirleycameron93@yahoo.com / sodoom@noguchi.mimcom.org
Telephone: 0244 534 031
Fax:

Information on proposed project

Title: PREVALENCE OF LASSA VIRUS AND OTHER HUMAN PATHOGENS IN MASTOMYS SPECIES AND HUMAN SEROPREVALENCE
Proposed Start Date: AUGUST 2010 End Date: DECEMBER 2011

Specimen and /or samples ☐ will / ☒ will not be collected within wildlife protected areas in Ghana during the course of the proposed project

Name and region of proposed research sites in protected areas of Ghana

1. _____
2. _____
3. _____
4. _____
5. _____

To: Executive Director, Wildlife Division, Forestry Commission, P.O. Box MB 239 Accra

If I and my collaborators receive permission to carry out the proposed project, we will comply with any and all laws, regulations, stipulations, and agreements germane to this work, and, in addition, abide by any additional orders and requests made by government officials.

✓I have submitted the following documents with this application for your consideration:

1. **project proposal**
2. **biographical sketches for each of the _____(number of persons) collaborators**
3. **A photocopy of the signature page from the passport**

FORESTRY COMMISSION
WILDLIFE DIVISION
P. O. BOX MB. 239
ACCRA
Approved

Sincerely yours,

Signature 30/07/2010 date

SHIRLEY CAMERON OBOOM
Printed

Project Leader

Official use only
Research project code

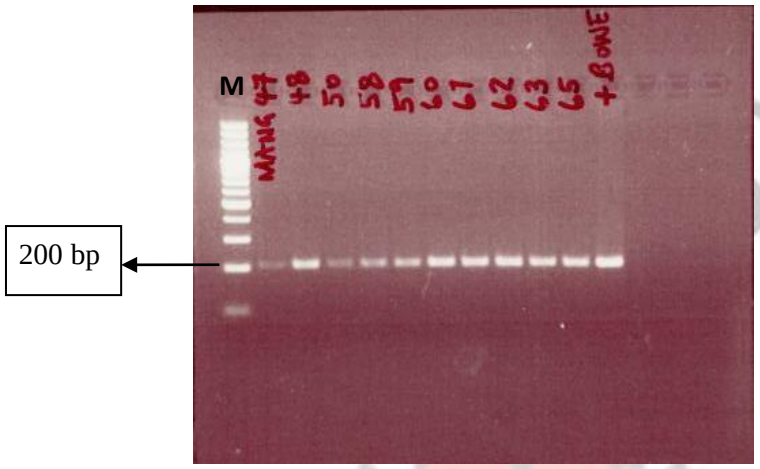
This research/ study is approved under the following conditions:

☐Pay a flat research fee of $ Cdn$ 125.00 or its equivalent in cedis
☐Pay for entrance fee, guide fees and other services that you may require
☐Submit one (1) copy of thesis/report/document to the Executive Director.
☐Provide three (3) copies of any publication emanating from the study.
☐Obtain CITES export permit should there be the need to send specimen outside the country

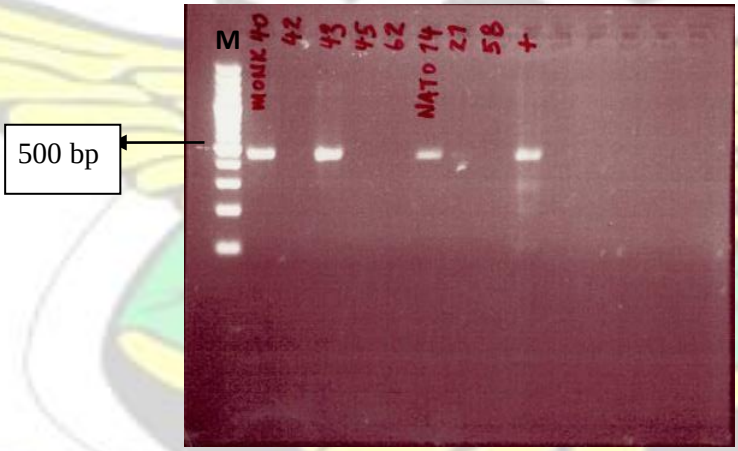
FOR: EXECUTIVE DIRECTOR
(Ag. Asst PR. Manager)
Joseph Yaw Oppong

FORESTRY COMMISSION
WILDLIFE DIVISION
P. O. BOX MB. 239
ACCRA

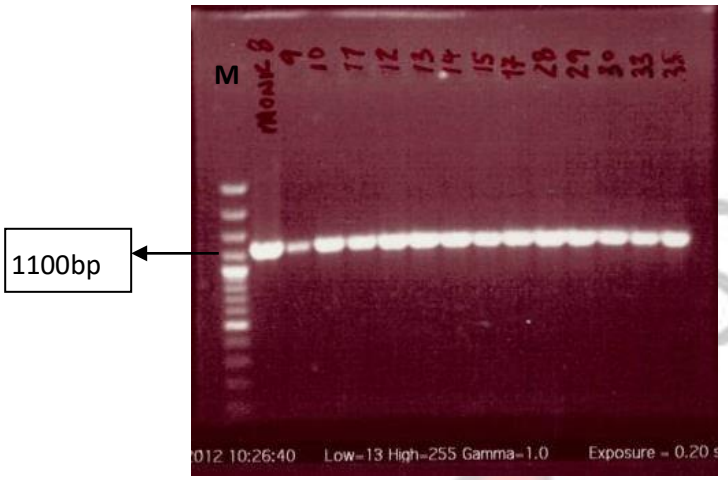
APPENDIX D: Gel electrophoregram pictures



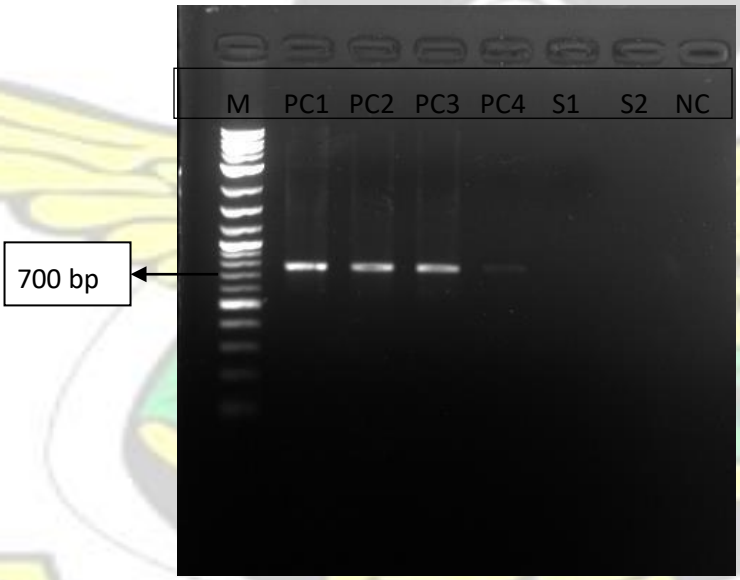
Gel 1: Gel electrophoregram for the identification of *Mastomys natalensis* species. Lane M=100 bp molecular weight marker, Lane 12=positive control, Lane 2 to 11=rodent samples. All products amplified at approximately 200 bp.



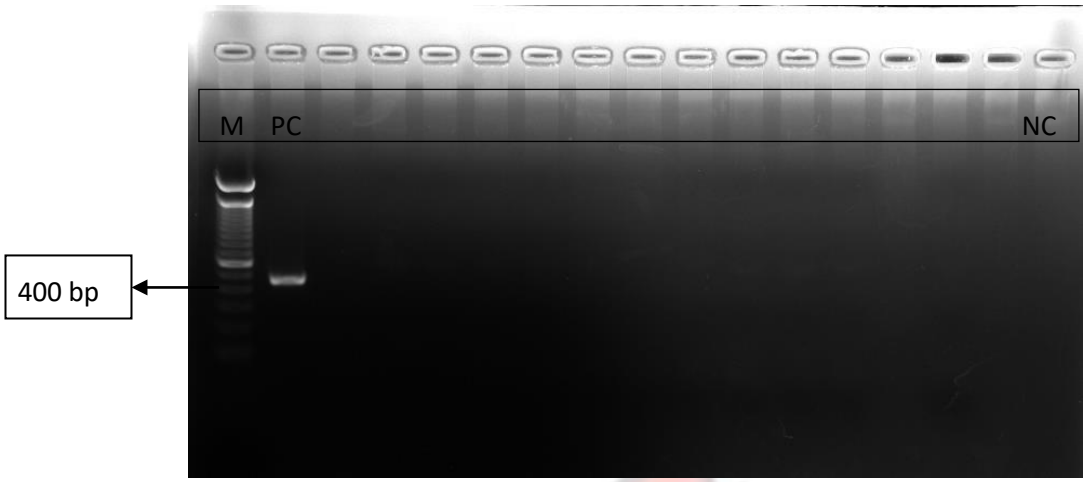
Gel 2: Gel electrophoregram for the identification of *Mastomys erythroleucus* species. Lane M=100 bp molecular weight marker, Lane 10=positive control, Lane 2 to 9=rodent samples. Products amplified at approximately 500 bp.



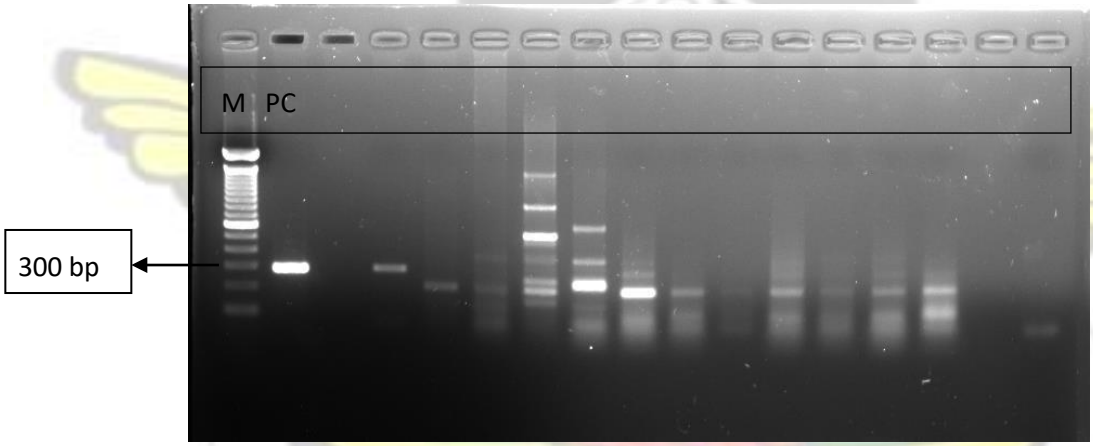
Gel 3: Gel electrophoregram for the identification of *Mus* (*Nannomys*) species. Lane M=100 bp molecular weight marker, Lane 2=positive control, Lane 3 to 15=rodent samples. All products amplified at approximately 1150 bp.



Gel 4: Gel electrophoregram for the identification of *Borrelia* species. Lane M=100 bp molecular weight marker, Lane 2=positive control (undiluted), Lane 3=positive control (1/10 dilution), Lane 4=positive control (1/100 dilution), Lane 5=positive control (1/1000 dilution), Lane 6 & 7=rodent samples, Lane 8=negative control. All products amplified at approximately 750 bp.



Gel 5: Gel electrophoregram for the identification of Hantavirus species. Lane M=100 bp molecular weight marker, Lane 2=positive control, Lane 3 to 16=rodent samples, Lane 17=negative control. All products amplified at approximately 450 bp.



Gel 6: Gel electrophoregram for the identification of *Leptospira* species. Lane M=100 bp molecular weight marker, Lane 2=positive control, Lane 4 to 15=rodent samples, Lane 17=negative control, Lane 3 & 16=blank. All products amplified at approximately 330 bp.

APPENDIX E: Data Analysis

Number of participants per study sites

Village	Frequency	Percent	Cumulative Percent
Amomaso	70	10.7	10.7

Ankaakur	32	4.9	15.5
Bowena	70	10.7	26.2
Doninga Naveransa	70	10.7	36.8
Ehiawenwu	67	10.2	47.0
Jirandogo	70	10.7	57.7
Mangoase	68	10.4	68.0
Monkwo	70	10.7	78.7
Natorduori	70	10.7	89.3
Teanoba	70	10.7	100.0
Total	657	100.0	

Age group range of participants

Age_grp	Frequency	Percent	Cumulative Percent
18-25yrs	152	23.1	23.1
26-35yrs	152	23.1	46.3
36-45yrs	137	20.9	67.1
46-55yrs	72	11.0	78.1
56-65yrs	55	8.4	86.5
Above65yrs	86	13.1	99.5
MissingAge	3	.5	100.0
Total	657	100.0	

Sex of participants

Sex	Frequency	Percent	Cumulative Percent
Female	356	54.2	54.2

Male	297	45.2	99.4
Missing	4	.6	100.0
Total	657	100.0	

LASV IgG in Villages

Village		LASV_IgG		Total
		Neg	Pos	
Amomaso	Count	70	0	70
	% within Village	100.0%	.0%	100.0%
Ankaakur	Count	27	5	32
	% within Village	84.4%	15.6%	100.0%
Bowena	Count	70	0	70
	% within Village	100.0%	.0%	100.0%
Doninga Naveransa	Count	68	2	70
	% within Village	97.1%	2.9%	100.0%
Ehiawenwu	Count	57	10	67
	% within Village	85.1%	14.9%	100.0%
Jirandogo	Count	68	2	70
	% within Village	97.1%	2.9%	100.0%
Mangoase	Count	66	2	68
	% within Village	97.1%	2.9%	100.0%
Monkwo	Count	63	7	70
	% within Village	90.0%	10.0%	100.0%
Natorduori	Count	66	4	70

	% within Village	94.3%	5.7%	100.0%
Teanoba	Count	68	2	70
	% within Village	97.1%	2.9%	100.0%
Total	Count	623	34	657
	% within Village	94.8%	5.2%	100.0%

Chi-Square Tests of LASV IgG in Villages

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	34.095[a]	9	.000
Likelihood Ratio	34.659	9	.000
N of Valid Cases	657		

Puumala (PUU) IgG in Villages

Village		PUU_IgG			
		Equivocal	Neg	Pos	Total
Amomaso	Count	3	61	6	70
	% within Village	4.3%	87.1%	8.6%	100.0%
Ankaakur	Count	0	25	7	32
	% within Village	.0%	78.1%	21.9%	100.0%
Bowena	Count	1	66	3	70
	% within Village	1.4%	94.3%	4.3%	100.0%
Doninga Naveransa	Count	0	56	14	70
	% within Village	.0%	80.0%	20.0%	100.0%
Ehiawenwu	Count	3	59	5	67
	% within Village	4.5%	88.1%	7.5%	100.0%
Jirandogo	Count	0	65	5	70

	% within Village	.0%	92.9%	7.1%	100.0%
Mangoase	Count	3	56	9	68
	% within Village	4.4%	82.4%	13.2%	100.0%
Monkwo	Count	1	60	9	70
	% within Village	1.4%	85.7%	12.9%	100.0%
Natorduori	Count	1	61	8	70
	% within Village	1.4%	87.1%	11.4%	100.0%
Teanoba	Count	0	62	8	70
	% within Village	.0%	88.6%	11.4%	100.0%
Total	Count	12	571	74	657
	% within Village	1.8%	86.9%	11.3%	100.0%

Chi-Square Tests of PUU IgG in Villages

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	27.315[a]	18	.073
Likelihood Ratio	29.296	18	.045
N of Valid Cases	657		

Hantaan (HTN) IgG in Villages

Village		HTN_IgG			
		Equivocal	Neg	Pos	Total
Amomaso	Count	0	65	5	70
	% within Village	.0%	92.9%	7.1%	100.0%
Ankaakur	Count	0	32	0	32

	% within Village	.0%	100.0%	.0%	100.0%
Bowena	Count	0	67	3	70
	% within Village	.0%	95.7%	4.3%	100.0%
Doninga Naveransa	Count	3	55	12	70
	% within Village	4.3%	78.6%	17.1%	100.0%
Ehiawenwu	Count	0	60	7	67
	% within Village	.0%	89.6%	10.4%	100.0%
Jirandogo	Count	0	65	5	70
	% within Village	.0%	92.9%	7.1%	100.0%
Mangoase	Count	1	57	10	68
	% within Village	1.5%	83.8%	14.7%	100.0%
Monkwo	Count	0	49	21	70
	% within Village	.0%	70.0%	30.0%	100.0%
Natorduori	Count	1	58	11	70
	% within Village	1.4%	82.9%	15.7%	100.0%
Teanoba	Count	3	61	6	70
	% within Village	4.3%	87.1%	8.6%	100.0%
Total	Count	8	569	80	657
	% within Village	1.2%	86.6%	12.2%	100.0%

Chi-Square Tests of HTN IgG in Villages

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	52.516[a]	18	.000

Likelihood Ratio	53.041	18	.000
N of Valid Cases	657		

***Leptospira* IgG in Villages**

Village		LEPTO_IgG				Total
		Equivocal	Missing	Neg	Pos	
Amomaso	Count	4	0	48	18	70
	% within Village	5.7%	.0%	68.6%	25.7%	100.0%
Ankaakur	Count	2	1	20	9	32
	% within Village	6.2%	3.1%	62.5%	28.1%	100.0%
Bowena	Count	14	0	38	18	70
	% within Village	20.0%	.0%	54.3%	25.7%	100.0%
Doninga Naveransa	Count	7	0	36	27	70
	% within Village	10.0%	.0%	51.4%	38.6%	100.0%
Ehiawenwu	Count	8	0	36	23	67
	% within Village	11.9%	.0%	53.7%	34.3%	100.0%
Jirandogo	Count	11	0	45	14	70
	% within Village	15.7%	.0%	64.3%	20.0%	100.0%
Mangoase	Count	6	0	58	4	68
	% within Village	8.8%	.0%	85.3%	5.9%	100.0%
Monkwo	Count	13	0	51	6	70
	% within Village	18.6%	.0%	72.9%	8.6%	100.0%
Natorduori	Count	12	0	46	12	70

	% within Village	17.1%	.0%	65.7%	17.1%	100.0%
Teanoba	Count	15	0	46	9	70
	% within Village	21.4%	.0%	65.7%	12.9%	100.0%
Total	Count	92	1	424	140	657
	% within Village	14.0%	.2%	64.5%	21.3%	100.0%

Chi-Square Tests of *Leptospira* IgG in Villages

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	75.606[a]	27	.000
Likelihood Ratio	65.065	27	.000
N of Valid Cases	657		

LASV IgG in Age groups

Age_gp		LASV_IgG		
		Neg	Pos	Total
18-25yrs	Count	142	10	152
	% within Age_gp	93.4%	6.6%	100.0%
26-35yrs	Count	149	3	152
	% within Age_gp	98.0%	2.0%	100.0%
36-45yrs	Count	132	5	137
	% within Age_gp	96.4%	3.6%	100.0%
46-55yrs	Count	67	5	72
	% within Age_gp	93.1%	6.9%	100.0%
56-65yrs	Count	51	4	55

	% within Age_gp	92.7%	7.3%	100.0%
MissingAge	Count	3	0	3
	% within Age_gp	100.0%	.0%	100.0%
above65yrs	Count	79	7	86
	% within Age_gp	91.9%	8.1%	100.0%
Total	Count	623	34	657
	% within Age_gp	94.8%	5.2%	100.0%

Chi-Square Tests of LASV IgG among Age groups

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.091[a]	6	.313
Likelihood Ratio	7.894	6	.246
N of Valid Cases	657		

Puumala IgG in Age groups

Age_gp		PUU_IgG			Total
		Equivocal	Neg	Pos	
18-25yrs	Count	1	129	22	152
	% within Age_gp	.7%	84.9%	14.5%	100.0%
26-35yrs	Count	4	137	11	152
	% within Age_gp	2.6%	90.1%	7.2%	100.0%
36-45yrs	Count	1	118	18	137
	% within Age_gp	.7%	86.1%	13.1%	100.0%
46-55yrs	Count	0	64	8	72
	% within Age_gp	.0%	88.9%	11.1%	100.0%
56-65yrs	Count	1	49	5	55

	% within Age_gp	1.8%	89.1%	9.1%	100.0%
MissingAge	Count	0	3	0	3
	% within Age_gp	.0%	100.0%	.0%	100.0%
above65yrs	Count	5	71	10	86
	% within Age_gp	5.8%	82.6%	11.6%	100.0%
Total	Count	12	571	74	657
	% within Age_gp	1.8%	86.9%	11.3%	100.0%

Chi-Square Tests of PUU IgG among Age groups

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	16.594[a]	12	.166
Likelihood Ratio	16.309	12	.177
N of Valid Cases	657		

HTN IgG in Age groups

		HTN_IgG			
Age_gp		Equivocal	Neg	Pos	Total
18-25yrs	Count	0	131	21	152
	% within Age_gp	.0%	86.2%	13.8%	100.0%
26-35yrs	Count	2	134	16	152
	% within Age_gp	1.3%	88.2%	10.5%	100.0%
36-45yrs	Count	3	121	13	137
	% within Age_gp	2.2%	88.3%	9.5%	100.0%
46-55yrs	Count	0	62	10	72
	% within Age_gp	.0%	86.1%	13.9%	100.0%
56-65yrs	Count	1	45	9	55

	% within Age_gp	1.8%	81.8%	16.4%	100.0%
MissingAge	Count	0	3	0	3
	% within Age_gp	.0%	100.0%	.0%	100.0%
above65yrs	Count	2	73	11	86
	% within Age_gp	2.3%	84.9%	12.8%	100.0%
Total	Count	8	569	80	657
	% within Age_gp	1.2%	86.6%	12.2%	100.0%

Chi-Square Tests of Hantaan IgG among Age groups

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	8.046[a]	12	.782
Likelihood Ratio	10.746	12	.551
N of Valid Cases	657		

***Leptospira* IgG in Age groups**

Age_gp		LEPTO_IgG				
		Equivocal	Missing	Neg	Pos	Total
18-25yrs	Count	16	0	110	26	152
	% within Age_gp	10.5%	.0%	72.4%	17.1%	100.0%
26-35yrs	Count	23	0	100	29	152
	% within Age_gp	15.1%	.0%	65.8%	19.1%	100.0%
36-45yrs	Count	25	1	81	30	137
	% within Age_gp	18.2%	.7%	59.1%	21.9%	100.0%
46-55yrs	Count	8	0	46	18	72
	% within Age_gp	11.1%	.0%	63.9%	25.0%	100.0%

56-65yrs	Count	10	0	34	11	55
	% within Age_gp	18.2%	.0%	61.8%	20.0%	100.0%
MissingAge	Count	0	0	1	2	3
	% within Age_gp	.0%	.0%	33.3%	66.7%	100.0%
above65yrs	Count	10	0	52	24	86
	% within Age_gp	11.6%	.0%	60.5%	27.9%	100.0%
Total	Count	92	1	424	140	657
	% within Age_gp	14.0%	.2%	64.5%	21.3%	100.0%

Chi-Square Tests of *Leptospira* IgG among Age groups

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	18.532[a]	18	.421
Likelihood Ratio	17.187	18	.510
N of Valid Cases	657		

LASV IgG among Gender

Sex		LASV_IgG		
		Neg	Pos	Total
Female	Count	342	14	356
	% within Sex	96.1%	3.9%	100.0%

Male	Count	277	20	297
	% within Sex	93.3%	6.7%	100.0%
Missing	Count	4	0	4
	% within Sex	100.0%	.0%	100.0%
Total	Count	623	34	657
	% within Sex	94.8%	5.2%	100.0%

Chi-Square Tests of LASV IgG among Gender

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	2.809[a]	2	.245
Likelihood Ratio	2.991	2	.224
N of Valid Cases	657		

Puumala IgG among Gender

Sex		PUU_IgG			
		Equivocal	Neg	Pos	Total
Female	Count	8	306	42	356
	% within Sex	2.2%	86.0%	11.8%	100.0%
Male	Count	4	261	32	297
	% within Sex	1.3%	87.9%	10.8%	100.0%
Missing	Count	0	4	0	4
	% within Sex	.0%	100.0%	.0%	100.0%
Total	Count	12	571	74	657
	% within Sex	1.8%	86.9%	11.3%	100.0%

Chi-Square Tests of Puumala IgG among Gender

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	1.544[a]	4	.819
Likelihood Ratio	2.078	4	.721
N of Valid Cases	657		

Hantaan IgG among Gender

Sex		HTN_IgG			
		Equivocal	Neg	Pos	Total
Female	Count	3	312	41	356
	% within Sex	.8%	87.6%	11.5%	100.0%
Male	Count	5	254	38	297
	% within Sex	1.7%	85.5%	12.8%	100.0%
Missing	Count	0	3	1	4
	% within Sex	.0%	75.0%	25.0%	100.0%
Total	Count	8	569	80	657
	% within Sex	1.2%	86.6%	12.2%	100.0%

Chi-Square Tests of Hantaan IgG among Gender

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	1.896[a]	4	.755
Likelihood Ratio	1.815	4	.770
N of Valid Cases	657		

***Leptospira* IgG among Gender**

Sex		LEPTO_IgG				
		Equivocal	Missing	Neg	Pos	Total
Female	Count	46	1	241	68	356
	% within Sex	12.9%	.3%	67.7%	19.1%	100.0%
Male	Count	46	0	181	70	297
	% within Sex	15.5%	.0%	60.9%	23.6%	100.0%
Missing	Count	0	0	2	2	4
	% within Sex	.0%	.0%	50.0%	50.0%	100.0%
Total	Count	92	1	424	140	657
	% within Sex	14.0%	.2%	64.5%	21.3%	100.0%

Chi-Square Tests of *Leptospira* IgG among Gender

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	6.519[a]	6	.368
Likelihood Ratio	7.042	6	.317
N of Valid Cases	657		

LASV IgG Relative to Number of Persons Living in a House

Persons_inhse		LASV_IgG		Total
		Neg	Pos	
1 to 5	Count	146	6	152
	% within Persons_inhse	96.1%	3.9%	100.0%
6 to 10	Count	271	17	288
	% within Persons_inhse	94.1%	5.9%	100.0%
Above10	Count	197	11	208
	% within Persons_inhse	94.7%	5.3%	100.0%
Missing	Count	9	0	9
	% within Persons_inhse	100.0%	.0%	100.0%
Total	Count	623	34	657
	% within Persons_inhse	94.8%	5.2%	100.0%

Chi-Square Tests of LASV IgG Relative to Number of Persons Living in a House

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	1.274[a]	3	.735
Likelihood Ratio	1.766	3	.622

N of Valid Cases	657		

Puumala IgG Relative to Number of Persons Living in a House

Persons_inhse		PUU_IgG			Total
		Equivocal	Neg	Pos	
1 to 5	Count	1	133	18	152
	% within Persons_inhse	.7%	87.5%	11.8%	100.0%
6 to 10	Count	8	245	35	288
	% within Persons_inhse	2.8%	85.1%	12.2%	100.0%
Above10	Count	3	184	21	208
	% within Persons_inhse	1.4%	88.5%	10.1%	100.0%
Missing	Count	0	9	0	9
	% within Persons_inhse	.0%	100.0%	.0%	100.0%
Total	Count	12	571	74	657
	% within Persons_inhse	1.8%	86.9%	11.3%	100.0%

Chi-Square Tests of Puumala IgG Relative to Number of Persons

Living in a House

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	4.762[a]	6	.575
Likelihood Ratio	6.124	6	.409
N of Valid Cases	657		

Hantaan IgG Relative to Number of Persons Living in a House

Persons_inhse		HTN_IgG			Total
		Equivocal	Neg	Pos	
1 to 5	Count	0	132	20	152
	% within Persons_inhse	.0%	86.8%	13.2%	100.0%
6 to 10	Count	4	244	40	288
	% within Persons_inhse	1.4%	84.7%	13.9%	100.0%
Above10	Count	4	184	20	208
	% within Persons_inhse	1.9%	88.5%	9.6%	100.0%
Missing	Count	0	9	0	9
	% within Persons_inhse	.0%	100.0%	.0%	100.0%
Total	Count	8	569	80	657

	% within Persons_inhse	1.2%	86.6%	12.2%	100.0%

Chi-Square Tests of Hantaan IgG Relative to Number of Persons Living in a House

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	6.298[a]	6	.391
Likelihood Ratio	9.269	6	.159
N of Valid Cases	657		

***Leptospira* IgG Relative to Number of Persons Living in a House**

Persons_inhse		LEPTO_IgG				
		Equivocal	Missing	Neg	Pos	Total
1 to 5	Count	15	0	105	32	152
	% within Persons_inhse	9.9%	.0%	69.1%	21.1%	100.0%
6 to 10	Count	39	1	189	59	288
	% within Persons_inhse	13.5%	.3%	65.6%	20.5%	100.0%
Above10	Count	36	0	125	47	208
	% within Persons_inhse	17.3%	.0%	60.1%	22.6%	100.0%

Missing	Count	2	0	5	2	9
	% within Persons_inhse	22.2%	.0%	55.6%	22.2%	100.0%
Total	Count	92	1	424	140	657
	% within Persons_inhse	14.0%	.2%	64.5%	21.3%	100.0%

Chi-Square Tests of *Leptospira* IgG Relative to Number of Persons Living in a House

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	6.785[a]	9	.659
Likelihood Ratio	7.196	9	.617
N of Valid Cases	657		

Rodent-borne disease_exposure Crosstabulation

Village		Rodent-borne_exposure		Total
		No	Yes	
Amomaso	Count	46	24	70
	% within Rodent-borne_exposure	11.7%	9.1%	10.7%
Ankaakur	Count	16	16	32
	% within Rodent-borne_ exposure	4.1%	6.1%	4.9%
Bowena	Count	48	22	70

	% within Rodent-borne_ exposure	12.2%	8.3%	10.7%
Doninga Naveransa	Count	28	42	70
	% within Rodent-borne_ exposure	7.1%	15.9%	10.7%
Ehiawenwu	Count	33	34	67
	% within Rodent-borne_ exposure	8.4%	12.9%	10.2%
Jirandogo	Count	48	22	70
	% within Rodent-borne_ exposure	12.2%	8.3%	10.7%
Mangoase	Count	45	23	68
	% within Rodent-borne_ exposure	11.5%	8.7%	10.4%
Monkwo	Count	37	33	70
	% within Rodent-borne_ exposure	9.4%	12.5%	10.7%
Natorduori	Count	43	27	70
	% within Rodent-borne_ exposure	10.9%	10.2%	10.7%
Teanoba	Count	49	21	70
	% within Rodent-borne_ exposure	12.5%	8.0%	10.7%
Total	Count	393	264	657
	% within Rodent-borne_ exposure	100.0%	100.0%	100.0%

Chi-Square Tests of Rodent-borne disease_exposure

	Value	df	Asymp. Sig. (2sided)
Pearson Chi-Square	26.957[a]	9	.001
Likelihood Ratio	26.843	9	.001
N of Valid Cases	657		

Rodent-borne disease_Coinfection types

Coinfection types	Frequency	Percent	Valid Percent	Cumulative Percent

	12	1.8	1.8	1.8
	2	.3	.3	2.1
HTN/LEPTO	1	.2	.2	2.3
LASV/HTN	3	.5	.5	2.7
LASV/HTN/LEPTO	10	1.5	1.5	4.3
LASV/LEPTO	1	.2	.2	4.4
LASV/PUU	3	.5	.5	4.9
LASV/PUU/HTN	393	59.8	59.8	64.7
LASV/PUU/LEPTO	4	.6	.6	65.3
No infection	20	3.0	3.0	68.3
PUU/HTN	208	31.7	31.7	100.0
PUU/LEPTO	657	100.0	100.0	
Single infection				
Total				