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A pilot study of *Leptospira* in rodents in North-Eastern Kenya

By

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Declaration

I, Martin Wainaina Kimari, hereby declare that this dissertation is my own work and that I have not plagiarized work from other sources. I confirm that I have cited all the sources, including books, journals, conference proceedings, theses and websites from which I obtained information for completing this work. The work in this dissertation has not been submitted to any other university for the award of any degree.

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Abstract

Leptospirosis is a neglected zoonotic disease that disproportionately affects poor populations in the world. Prevalence data in human populations in pastoral communities has been shown to be high. The disease is therefore contributing to an unknown toll on livestock productivity as well as human health in these areas. Rodent populations in irrigated areas of Kenya have also seen a rise and this could lead to an increase transmission of rodent-borne diseases. This pilot study therefore aimed at demonstrating the presence of the bacteria in rodent carriers in Tana River and Garissa counties of Kenya, areas that are characterized by irrigation and pastoral activities respectively.

Kidney and blood samples from 67 rodents previously collected from these areas (mainly mice and multimammate rats) were analyzed using PCR. Prevalence of leptospires in rodent carriers was found to be 41.8% (28/67). Prevalence in the towns was: 16% (4/25) in Bura; 42% (8/19) in Hola; 82% (9/11) in Ijara and 58% (7/12) in Sangailu. Prevalence was found to be influenced with the area of sampling, with rodents from the pastoral areas being more likely to have the bacteria than those from the irrigated areas (Odds Ratio = 6.095). Prevalence showed no association with the species and age of rodents. Sequencing data revealed the species in circulation among rodents is *Leptospira interrogans*.

This pilot study is one of the few to demonstrate the bacteria in rodent carriers in North-Eastern Kenya, which illustrates the underplayed public health importance of the disease in this part of Kenya. The high rodent prevalence of these bacteria poses risk of transmission of the disease in animal and human populations. These results demonstrate the need for policy makers to consider disease emergence and transmission in these marginalized parts of Kenya. More epidemiological knowledge of the disease like circulating serotypes and

role of animal hosts in the area will greatly aid in forming public health policy aimed at controlling the disease.

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Abbreviations

AMREF	African Medical and Research Foundation
APHIAplusNAL	AIDS, Population, and Health Integrated Assistance, Northern Arid Lands
ASAL	Arid and Semi-Arid Land
AU-IBAR	African Union-Interafrican Bureau for Animal Resources
BLAST	Basic Local Alignment Search Tool
bp	Basepair
C	celcius
Cat. No.	Catalogue Number
CFT	Complement-Fixation Test
CI	Confidence Interval
CIE	Counter-immunoelectrophoresis
CSF	Cerebrospinal Fluid
DALY	Disability-Adjusted Life Years
DDDAC	Dynamic Drivers of Diseases in Africa Consortium
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMJH	Ellinghausen-McCullough-Johnson-Harris
ESRC	Economic and Social Research Council
g	gram
GIS	Geographic Information System
HIV	Human Immunodeficiency Virus
HRH	Human Resources for Health

HRP	Horse-radish Peroxidase
IFAT	Indirect Fluorescent Antibody Test
Ig	Immunoglobulin
IHA	Indirect Haemagglutination Test
ILRI	International Livestock Research Institute
LA	Latex Agglutination Test
MAT	Microscopic Agglutination Test
MEGA	Molecular Evolutionary Genetics Analysis
ml	milliliter
mm	millimeter
n	number
NCBI	National Center for Biotechnology Information
SAT	Slide Agglutination Test
sq km	square kilometers
NFW	nucleus-free water
ng/µl	nanogram per microliter
NMK	National Museums of Kenya
NZD	Neglected Zoonotic Disease
OD	optical density
OIE	World Organization for Animal Health
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses

PSAT	Patoc-slide agglutination test
rcf	Relative Centrifugal Force
rpm	revolutions per minute
rRNA	ribosomal Ribonucleic Acid
RVF	Rift Valley Fever
SEL	Sensitized erythrocyte lysis test
TAE	Tris Acetate EDTA
µl	microliter
µM	micromolar
UV	ultraviolet
WHO	World Health Organization

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1. Introduction

Leptospirosis is a disease that affects animals and human beings. The aetiological agent for this disease is all pathogenic bacteria of the genus *Leptospira*; family *Leptospiraceae* and order Spirochaetales. *Leptospira* bacteria are largely divided into pathogenic and non-pathogenic strains based on the old serological classification (Hovind-Hougen, 1979), with an extra intermediate (opportunistic) group being included in the recent genotypic classification (Levett, 2015). The bacteria can infect all mammals.

Following infection, the bacteria inhabit the renal tubules and urogenital tract where they multiply and are ultimately shed in the urine of the host animal. Human infection can be brought about by direct or indirect contact with urine from an infected animal though there are other routes of infection (Bharti *et al.*, 2003).

Leptospirosis is a systemic zoonotic disease. It mainly affects humans and domestic animals such as cattle, swine and dogs (Adler & de la Peña Moctezuma, 2010). The disease in humans presents mainly as either a biphasic illness or the fulminant form of the disease. The biphasic illness is characterized by an undifferentiated febrile syndrome and pyuria while the fulminant form, termed Weil's disease, is characterized by renal and hepatic insufficiencies as well as other hematological manifestations such as thrombocytopenia (Bharti *et al.*, 2003).

The most important carriers of the *Leptospira* bacteria are rodents, which pass the disease to domestic and wild animals directly or indirectly through urine contamination of shared water sources/bodies. Knowledge gaps have curtailed efforts to give current figures of the burden of leptospirosis in many parts of the world. Estimates however place it to have a

global burden 70% more than that of cholera (Torgerson, Hagan, Costa, *et al.*, 2015), making it an important bacterial zoonosis to be considered in global health. The disease also decreases livestock productivity and causes considerable reproductive wastage. Few studies exist on the impact that animal leptospirosis has on productivity of animals of economic importance and low-cost interventions needed to reduce transmission of the infection between animal hosts and human populations (Schneider, Jancloes, Buss, *et al.*, 2013).

2. Leptospirosis in the world

2.1 Global Outlook

Leptospirosis is regarded as having the widest geographical reach of all of the zoonotic diseases (Evangelista & Coburn, 2010). Recent modelling studies have revealed that the disease is a leading contributor of human mortalities and morbidities in the world *(Costa et al.*, 2015) with most of these cases found to be in the most resource-scarce regions. The disease is thought to contribute 2.90 million Disability-Adjusted Life Years (DALYs) globally per annum (Torgerson, Hagan, Costa, *et al.*, 2015). Leptospirosis-HIV co-infections have also been recorded in many parts of the world with the severity ranging from mild and resolving illness to fulminant disease, causing high mortalities in some of these patients (Kuppalli, Chandrasekaran, Rio, *et al.*, 2011; Biggs, Galloway, Bui, *et al.*, 2013; Jones & Kim, 2001; Ganoza, Segura, Swancutt, *et al.*, 2005; Pai, 2013). The interaction of leptospirosis with key infectious diseases in global health needs further investigation as it may contribute significant disease burden in many immunosuppressed patients in the world.

Environmental drivers interplaying with changes in climate, and certain anthropogenic factors, have been demonstrated to lead to incidences of leptospirosis in many parts of the world. For instance, higher than normal rainfall and extreme weather events are natural disasters that can cause a rise in rodent populations, as well as those of other animal reservoirs. This is due to the scattering of debris and garbage, interference of sewerage systems as well as the increased growth of vegetation leading to increased food availability. Flood waters that drive reservoir animals out of their habitats can lead them to move to

human populations and increase human-animal contact. This can ephemerally lead to increased transmission of the disease (Lau *et al.*, 2010).

Conversely, a decrease in rainfall can also increase the human-to-animal contact by reducing the water available on the surface as well as forcing rodents into human habitations in order to forage for food (Cook *et al.*, 2008). The interaction of these factors is therefore complex and dynamic in nature.

2.2 Africa

Leptospirosis poses a disease burden in Africa that is not fully elucidated due to the lack of adequate data (Costa *et al.*, 2015). A lack of adequate diagnosis and surveillance has hindered investigations into the extent that leptospirosis impacts upon human health and livestock productivity.

Leptospirosis is regarded as a neglected zoonotic disease (NZD) (WHO, 2011), and therefore a disease that disproportionately affects the impoverished and marginalized populations due to its characteristic association with poverty. With the development of rapid diagnostic tests for malaria, more and more febrile illnesses in sub-Saharan Africa are being recognized as non-malarial (de Vries *et al.*, 2014). Some of these previously mis-diagnosed infections may be as a result of leptospirosis, the symptoms of which can be similar to those of malaria.

A recent systematic review (Allan *et al.*, 2015) compared the knowledge about human acute leptospirosis as well as the cases of the infection in animal hosts in Africa. A summary of the data gathered from present literature using preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines is summarized in Table 1 below. This review only sought to highlight current cases and therefore excluded studies that; didn't include original research data, didn't focus on naturally-occurring human or animal leptospirosis, used serological diagnostic methods, used experimental animal inoculations, didn't met the case definitions and those that reported of returning travelers.

Table 1: Results of systematic literature review of peer reviewed and grey literature in Africa between 1930 and 2014, with the numbers representing the articles identified based on the inclusion criteria used. This has been adapted from Allan *et al.*, (2015).

Region	Country	Both animal carrier and human illness data	Animal carrier data only	Human data only
Eastern Africa	Comoros	2	-	-
	Kenya	9	-	-
	Madagascar	6	-	-
	Mayotte	3	-	-
	Réunion	5	-	-
	Tanzania	9	-	-
	Zimbabwe	-	8	-
	Mozambique	-	-	1
	Seychelles	-	-	2
Southern Africa	South Africa	14	-	-
	Botswana	-	2	-
Middle Africa	Cameroon	-	1	-
	Democratic Republic of the Congo (DRC)	-	-	3
	Gabon	-	-	3
Western Africa	Ghana	4	-	-
	Benin	-	1	-
	Guinea	-	1	-
	Nigeria	-	4	-
	Mali	-	-	1
	Senegal	-	-	3
Northern Africa	Egypt	10	-	-
	Tunisia	-	4	-
	Algeria	-	-	1
	Morocco	-	-	3

The other African countries not included didn't have data for this infection based on the inclusion criterion used. Data from cohort and surveillance studies in the same time period

however reveal extensive scientific evidence that the disease is an important contributor of febrile illnesses in hospitals across the continent (Allan, Biggs, Halliday, *et al.*, 2015). The study also attested to many cases of *Leptospira* infection in domestic and wild animals through the continent, highlighting the need for studies that integrate animal and human infections, a one-health approach, to better understand the overall epidemiology of this disease in different settings across the continent. This is especially important for a continent that has a high number of poor people that depend on livestock (Grace *et al.*, 2012).

2.3 Eastern Africa

2.3.1 Tanzania

Leptospirosis has been widely reported in Tanzania, which has wider epidemiological data than most African countries (Table 1). Confirmed cases of the disease have been reported at 17.5% (70/400) (Machang'u, 2006) and 8.4% (70/870) (Biggs, Bui, Galloway, *et al.*, 2011) among hospital patients in different parts of the country. Febrile patients in Kilosa district of Tanzania were recently revealed to have confirmed leptospirosis cases as well (13% [26/200]) (Chipwaza *et al.*, 2015). The provisional diagnosis of some of these patients suffering from leptospirosis was malaria and other common febrile illnesses. This serves to show the under-recognized nature of the disease among febrile patients in the country. Co-infections of leptospirosis and other febrile illnesses have also been shown in these patients. This finding shows how differential diagnosis is difficult without the use of diagnostic tests (Chipwaza, Mhamphi, Ngatunga, *et al.*, 2015).

Leptospiral antibodies in humans, rodents and various domestic and wild animals in the Katavi-Rukwa ecosystem of Tanzania have been shown to be widely prevalent and that common serogroups circulate among these hosts (Assenga *et al.*, 2015).

Leptospira have also been demonstrated directly through molecular techniques and indirectly by use of serological techniques in other species in the country such as: rodents with 11 % (3/27) prevalence (Mgode, Mhamphi, Katakweba, *et al.*, 2005); pigs with 4.42% (17/385) prevalence (Kessy, Machang'u & Swai, 2010); bats at 19.4% (7/36) prevalence (Mgode, Mbugi, Mhamphi, *et al.*, 2014); fish at 54.2% (26/48) prevalence (Mgode, Mhamphi, Katakweba, *et al.*, 2014); rodents and shrews at 10% (50/500) prevalence (Machang'u, 2006); cattle at 30.3% (198/654) (Schoonman & Swai, 2010); as well as dogs and cats (Mgode, Machang'u, Mhamphi, *et al.*, 2015). All these studies therefore point to the varied number of animal hosts that commonly interact with humans and that can act as transmitters of pathogenic leptospires.

2.3.2 Uganda

Though Uganda has little published data on this disease in both human and livestock populations, efforts still exist to demonstrate the disease in the country.

A pilot study conducted in Southwestern Uganda that demonstrated an overall seroprevalence of 42.39% (39/92) in African buffalo and 29.35% (27/92) in cattle in Southwestern Uganda (Atherstone *et al.*, 2014). Other studies have been done in dogs and buffaloes around national parks in Uganda. A seroprevalence of 26.7% (28/105) among dogs (Millán, Chirife, Kalema-Zikusoka, *et al.*, 2013) and 0% (0/42) seroprevalence among the buffaloes sampled (Kalema-Zikusoka, Bengis, Michel, *et al.*, 2005) was demonstrated

in these different studies. These observations show that peri-domestic animals can be a potential source of the infection in human populations in the country, a theory that can only be corroborated by further investigations with a one-health approach so as to demonstrate the role of different animal hosts in the epidemiology of the disease.

The presence of suitable reservoir hosts, suitable environment for survival of the bacteria and the fact that surrounding countries have reported the disease make for a compelling argument that the disease is present in the country (Atherstone, Picozzi & Kalema-Zikusoka, 2014), despite there being no reported human and animal cases (AU-IBAR, 2016).

2.3.3 Select Eastern African countries

Little data exists in the other East African countries on leptospirosis. Observational studies in Djibouti have shown seroprevalences as high as 84% (26/31) among camels, horses and cattle (Roqueplo, Davoust, Mulot, *et al.*, 2011).

An early study done in all provinces of Sudan on cattle, goats and rats showed seroprevalences of 15.3% (175/1142), 13.3% (4/30) and 23.3% (13/56) respectively (El Wali, 1980). The presence of the disease in the country was later on demonstrated by Sebek *et al.* (1989) who observed seroprevalences of 54% (n=195) among domestic animals and 9.8% (70/771) in wild animals surveyed in a district in Sudan. Rodent species collected in this study also showed presence of the bacteria.

Human populations in Somalia have also shown presence of leptospiral agglutinins in a riverine and pastoral region of the country. The overall seroprevalence was found to be

50.5% (107/212), with those from the riverine area being 63.5% (68/107) and those from the dry area being 37.1% (39/105). The overall seroprevalence in this study was found to be generally higher than those found in other parts of the world. The close contact of nomads with their animals during cattle-droving, which is common in Somalia, was thought to be a risk factor (Cacciapuoti, Nuti, Pinto, *et al.*, 1982).

Seropositivity has been demonstrated in horses, cows, pigs, goats, sheep, camels and dogs in Ethiopia (Moch, Ebner, Barsoum, *et al.*, 1975). Seroprevalence of human leptospirosis in Ethiopia has also been observed to be 47.46% (28/59) among febrile patients. The country is thought to have favourable conditions for the incidence and transmission of the disease even though having very little documented information about the disease (Yimer, Koopman, Messele, *et al.*, 2004).

2.3.4 Kenya

2.3.4.1 Current outlook on recently published data

Recent studies in urban, rural and arid areas of Kenya exist and serve to paint a picture on what underlying disease burden leptospirosis poses, as well as the diverse geographical zones the disease is found in. Most of these studies currently focus on human populations, with few focused on animal populations.

Doctoral work by Cook (2014) was the first in demonstrating *Leptospira* antibodies among slaughterhouse workers at 13.4% (99/738) prevalence in Busia, Kenya. This study also demonstrated that leptospirosis is an occupational disease among slaughterhouse workers as they had twice the odds of being seropositive for the bacteria than the general population. This shows the need for monitoring of other at-risk populations in the country who are not

necessarily exposed to rodent-carriers like veterinarians, farmers, water-sports athletes, military personnel, among others.

The importance of the disease in rural Kenya is further illustrated by another study in arid and semi-arid lands (ASALs) area of Kenya by Ontiri *et al.* (2014). The unpublished data in this study has cited increase in rodent populations in Tana River county of Kenya due to the increased food available in the farms following increased irrigation activity in the area. Preliminary data from an epidemiological study in the same study area by Bett *et al.* (2015) has also revealed high seroprevalence of *Leptospira* antibodies in the human populations. The seroprevalence was however even higher in the neighboring pastoral population. This demonstrates the need for further investigation of leptospirosis in both of these areas.

The disease has also been demonstrated in Somali pastoralists in remote, arid Northeast Kenya who were positive for Immunoglobulin M (IgM) antibodies with a prevalence of 25% (3/12) (Ari, Guracha, Fadeel, *et al.*, 2011). Antibodies against leptospirosis in Garissa district have also been observed in patients as part of differential diagnosis of rift valley fever (RVF). This was during the largest outbreak of RVF in humans ever recorded in East Africa at the time (Woods, Karpati, Grein, *et al.*, 2002).

The public health risk of exposure to the disease in an urban slum area in Kenya was demonstrated by Halliday *et al.* (2013). They demonstrated 18.3% (41/224) prevalence of the bacteria among rodents collected in the Kibera settlement area in Nairobi, an area with a high number of people with undifferentiated febrile illness.

Over 161 human cases suspected to be leptospirosis and 8 fatalities were reported by the Kenyan Ministry of Health in schools in Western Kenya. These cases are the only reported

outbreak cases to the World Health Organisation (WHO) so far (WHO, 2004). Analysis of global trend of incidences of the disease has shown that Kenya may have high incidences of human leptospirosis, even without studies to estimate the incidence rate in the country (Pappas, Papadimitriou, Siozopoulou, *et al.*, 2008).

The study of leptospirosis and its effects on animal production in the country is not comprehensive, though it could contribute to large reproductive losses in animals. About 27.3% (18/66) of veterinarians perceived the disease to contribute to majority of bovine abortions in the former Nakuru district of Kenya (Okumu, 2014). No cases in animals have been reported to the World Organisation for Animal Health (OIE) (AU-IBAR, 2016).

Disease outbreaks in humans in Kenya and other African and Asiatic countries have been linked strongly to extreme weather events like flooding, which increases rodent populations and offers contaminated flood waters for transmission of the disease (WHO, 2011).

2.3.4.2 Areas of concern

Kenya, as with most developing countries, lacks the diagnostic capacity needed to adequately control this disease. This coupled with the fact that it is a tropical country makes it hard to distinguish leptospirosis from malaria and other febrile illnesses which are endemic to this region. This therefore contributes to the under-recognized public health concern the disease poses. The disease has already been demonstrated among pastoralist communities of Kenya and is likely contributing to an unknown disease burden in their key economic resource, their animals.

The effects that a changing climate has on the incidence of disease are also exacerbated with poverty. Anthropogenic activities that drive disease are also leading to increased

disease incidence, a challenge that many small-holder farmers who are poor may not have capacity to handle (Grace & Bett, 2014). This therefore means that leptospirosis may handicap the health and livelihoods of many in communities in the country, some of whom do not have access to primary healthcare.

There is also lack of adequate epidemiological studies that can inform about local serotypes as well as the roles of different animal hosts in different parts of the country, all of which are important in control of the disease. For instance, very little is known about this disease in goats in developing countries (Ellis, 2015), which is a common and important domestic animal in Kenya.

2.4 Aims

To determine the prevalence of leptospirosis in rodents collected from Tana River and Garissa counties of Kenya

To determine differences in presence of leptospiral bacteria in rodent carriers from irrigated and pastoral areas of sampling

3.0 Animal leptospirosis

3.1 Aetiology

Leptospirosis is caused by pathogenic leptospires. The bacteria are in the phylum *Spirochaetes*, class *Spirochaetes*, order *Spirochaetales*, family *Leptospiraceae* and genus *Leptospira* (Garrity, Bell & Lilburn, 2004). The family *Leptospiraceae* initially contained two genera; *Leptospira* and *Leptonema* (Hovind-Hougen, 1979). A third genus, *Turneriella*, was later added to this family due to its genetic relatedness with other members of the family (Levett, Morey, Galloway, *et al.*, 2005). The *Leptospira* genus was formerly divided into *Leptospira interrogans* and *Leptospira biflexa* species, with the former containing all pathogenic strains of the bacteria and the later comprising the saprophytic or free-living strains found in the environment. These two were differentiated using various biochemical tests (Johnson & Faine, 1984; Faine & Stallman, 1982). Genotypic classification has now been established, though both methods of classification are currently in use.

3.1.1 Serological classification

Leptospira bacteria were initially classified into species based on serological approaches. This led to the simplest taxa of *Leptospira* being the serotype, which can also be called serovar. Serotypes are determined by cross-absorption with homologous antigens. If at least 10% of the homologous titre remained in at least one of the two antisera in repeated tests, then serological relatedness would be established (Dikken & Kmety, 1978). Strains that belonged to different serotypes but showed antigenic relatedness have been grouped into serogroups. Serogroups are useful for epidemiological studies as well as for interpreting results from the microscopic agglutination test (MAT), even though they have no place in

the taxonomy of the bacteria (Levett, 2015). Serological classification is therefore more familiar to epidemiologists and clinicians and continues to function independently from the genetic classification (Bharti, Nally, Ricaldi, *et al.*, 2003; Smythe, Adler, Hartskeerl, *et al.*, 2013). So far, there are more than 260 pathogenic serotypes and more than 60 saprophytic ones identified (Adler & de la Peña Moctezuma, 2010).

A summary of the common serogroups of *L. interrogans sensu lato* can be found in Table 2 below.

Table 2: A summary of common serogroups of *L. interrogans sensu lato* along with some associated serotypes, adapted from Levett, (2015)

Serogroup	**Serotype(s)**
Icterohaemorrhagiae	Copenhageni Icterohaemorrhagiae Lai
Grippotyphosa	Grippotyphosa Canalzonae Ratnapura
Autumnalis	Autumnalis Bim Fortbragg
Cynopteri	Cynopteri
Mini	Mini Georgia
Australis	Australis Bratislava Lora
Serjoe	Hardjo Serjoe
Panama	Panama Mangus
Hebdomadis	Hebdomadis Jules
Ballum	Ballum Arborea
Bataviae	Bataviae
Louisiana	Louisiana Lanka
Canicola	Canicola
Celledoni	Celledoni
Djasiman	Djasiman
Hurstbridge	Hurstbridge
Javanica	Javanica
Manhao	Manhao
Pomona	Pomona
Pyrogenes	Pyrogenes
Ranarum	Ranarum
Sarmin	Sarmin
Shermani	Shermani
Tarassovi	Tarassovi

3.1.2 Genotypic classification

Classification of leptospires based on genetic relatedness was later adopted and was based on DNA-DNA hybridization studies. This method has led to the identification of 20 species (Table 3).

A species was defined by Brenner *et al.* (1999) using three criteria from taxonomic DNA hybridization studies: 70% or more relatedness at 55°C renaturation temperature; 5% or less divergence within sequences that are related; and 60% or more relatedness at 70°C stringent renaturation temperature.

The previously unnamed genomospecies 1, 3, 4 and 5 were later assigned *L. alstonii*, *L. vanthielii*, *L. terpstrae*, and *L. yanagawae* respectively (Smythe, Adler, Hartskeerl, *et al.*, 2013). Both the *L. interrogans* and *L. biflexa* names remained in the genotypic classification, though they refer to the genetically identified species *L. interrogans sensu stricto* and *L. biflexa sensu stricto* (Levett, 2015). *L. idonii* is the latest entrant identified in using this classification (Saito, Villanueva, Kawamura, *et al.*, 2013).

Identification based on sequences of key genes has made species identification of leptospires possible without the need of DNA-DNA hybridization studies. The 16S rRNA (rrs) gene is widely used for phylogenetic classification of leptospires (Morey, Galloway, Bragg, *et al.*, 2006) and has led to the bacteria being grouped into three major clusters; pathogenic, intermediate and saprophytic species. Other genes have also been proposed for this purpose as well, viz., gyrB (Slack, Symonds, Dohnt, *et al.*, 2007), secY (Ahmed, Engelberts, Boer, *et al.*, 2009), rpoB (Scola, Bui, Baranton, *et al.*, 2006), among many others.

Table 3: All the species of *Leptospira* accepted so far based on molecular classification, adapted from Levett, (2015)

16S rRNA phylogenetic cluster	Species
Pathogenic species	*L. alexanderi* *L. alstonii* *L. borgpetersenii* *L. interrogans* *L. kirschneri* *L. kmetyi* *L. noguchii* *L. santarosai* *L. weilii*
Intermediate species	*L. broomii* *L. fainei* *L. inadai* *L. licerasiae* *L. wolffi*
Free-living (saprophytic) species	*L. biflexa* *L. idonii* *L. meyeri* *L. terpstrae* *L. vanthielii* *L. wolbachii* *L. yanagawae*

3.2 History

The clinical syndrome now termed leptospirosis was first described in modern history by Weil in 1886 (Adolf, 1886). The causative agent was later on described by Stimson (1907), who named the causative agent *Spirocheta interrogans* due to the morphological resemblance of the bacteria to a question mark (Figure 1). *Leptospira* however were first isolated from clinical cases by Inada *et al.* (1916). Rats were soon established as carriers of the disease, a detail that became vital in understanding leptospiral epidemiology. The chemical composition of water and soil was also seen as important in proliferation of the bacteria in the environment (Ido, Hoki, Ito, *et al.*, 1917).

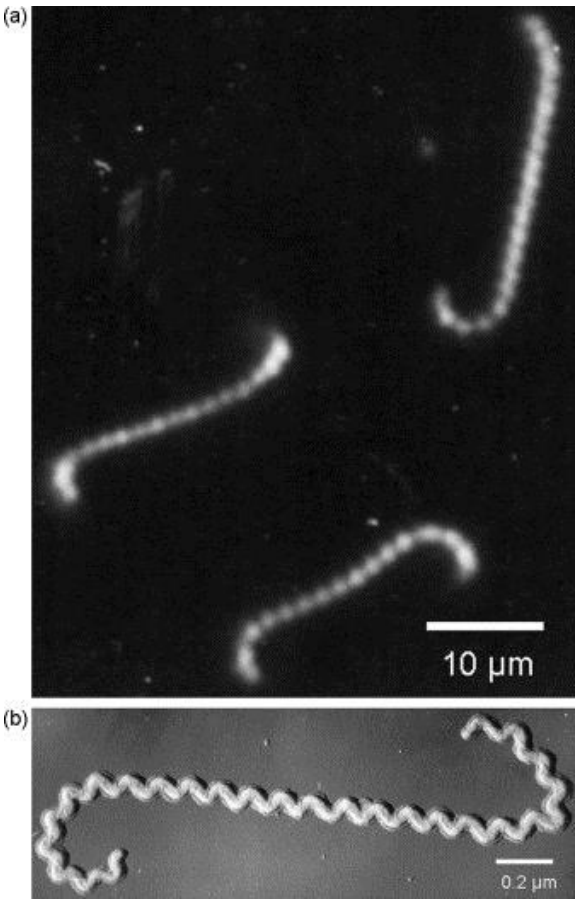

Figure 1: (a) Dark field and (b) Shadowed electron photomicrographs of leptospires (Adler & de la Peña Moctezuma, 2010) [1]

A strain of this bacteria, Hond Utrecht IV, was first isolated in dogs in 1933 (Klarenbeek & Schuffner, 1933). The disease was first reported in cattle as "infectious yellow fever of cattle" in 1940 (Semskov, 1940). The disease was recognized as an infectious disease of most mammalian species, with the role of domestic animals considered important in the spread of the disease among humans. It is now well known that leptospirosis contributes significant economic impact in livestock productivity, especially because of abortion storms and decreased milk yield from sick animals (Schneider, Jancloes, Buss, *et al.*, 2013)

[1] Reproduced from Adler, B. & de la Peña Moctezuma, A., (2010). Leptospira and leptospirosis. Veterinary Microbiology, 140(3-4), pp.287–296., Copyright (2016), with permission from Elsevier

Certain animal hosts have been seen to be common reservoirs to some serotypes of the bacteria, as highlighted in Table 4 below.

3.3 Microbiology

Leptospires are spirochaetes with particularly coiled bodies that give them a helical morphology. They are approximately 0.1μm diameter and 6 - 20μm in length. They are also highly motile and have characteristic hooked ends (Figure 1). They have two periplasmic flagellae that exhibit rotational and translational movements (Goldstein & Charon, 1988; Levett, 2015) and pathogenic strains have a double membrane consisting of an outer and inner membrane (Faine, Adler, Bolin, *et al.*, 1999). The inner membrane is closely associated with the peptidoglycan cell wall and is separated from the outer membrane with a periplasmic layer. The outer membrane consists of surface proteins (e.g. LigA, LigB and Loa22), transmembrane proteins, porin L1 and lipopolysaccharide. LipL32 is an important subsurface protein within the outer membrane as well (Fraga, Carvalho, Isaac, *et al.*, 2015).

The bacteria are obligate aerobes that grow optimally at 28 - 30°C and at a pH of 7.2 to 7.6. They are catalase and oxidase positive and grow in media enriched with long-chain fatty acids, ammonium salts and vitamins (Johnson & Faine, 1984). Media most commonly used in contemporary practice for growth of these bacteria are based on the Ellinghausen-McCullough-Johnson-Harris (EMJH) medium initially described by (Ellinghausen & McCullough, 1965) and (Johnson & Harris, 1967). Leptospires are gram-negative but silver staining and immunostaining techniques can offer better results and can be useful for post-mortem diagnosis using fixed or unfixed tissues (WHO, 2003).

3.4 Transmission

Animal infections usually happen in areas with the common risk factors for leptospirosis, like moist and warm climates, as well as areas with high rodent infestation and poor sanitation (Ellis, 2015). The infection is maintained in the sylvatic cycle through transmission between rodent carriers. Animals acquire the infection from contact with rodent species or infected animals in the herd. The pathogens are transmitted to humans by direct contact with reservoir animals or through contaminated soils and water (from urine of infected animals). *Leptospira*-infected animals shed the bacteria largely through urine (though saliva and blood also may contribute) into the water and soil (Ko, Goarant & Picardeau, 2009). This process has been summarized with Figure 2 below.

Leptospires also demonstrate preference to animal hosts, with evidence of certain serotypes often being isolated from these animal species as illustrated in Table 4 below.

3.5 Pathogenesis

The bacteria infect most animals through entry via mucous membranes of the eye or ear, mouth, genital tract and broken skin. The bacteria incubate for about 4-20 days, after which they will be present in the blood stream of the animal and persist for 7-10 days. The bacteria can be isolated from blood, cerebrospinal fluid (CSF) and many organs of the animal (liver, lungs, central nervous system, kidney, genital tract) where they are replicating during this leptospiremic phase. Agglutinating antibodies which are now in circulation at this phase are also demonstrable. This bacteremic phase usually coincides with the acute leptospirosis. Signs and symptoms of the acute disease vary with the host and the infective serotype, but many include renal damage, hemolytic disease, hemoglobinuria, icterus and death. Buffalo, cattle and sheep may present with agalactia.

Table 4: Animal reservoir hosts commonly associated with certain serotypes, adapted from Bharti *et al.,* (2003)

Reservoir host	**Serotype(s)**
Bats	Cynopteri Wolffi
Cattle	Hardjo Pomona
Dogs	Canicola Bratislava (Ellis, 2015)
Horses	Bratislava
Marsupials	Grippotyphosa
Mice	Ballum Arborea Bim
Pigs	Pomona Tarassovi Bratislava (Ellis, 2015) Kenniwicki (Ellis, 2015)
Racoon	Grippotyphosa
Rats	Icterohaemorrhagiae Copenhageni
Sheep	Hardjo

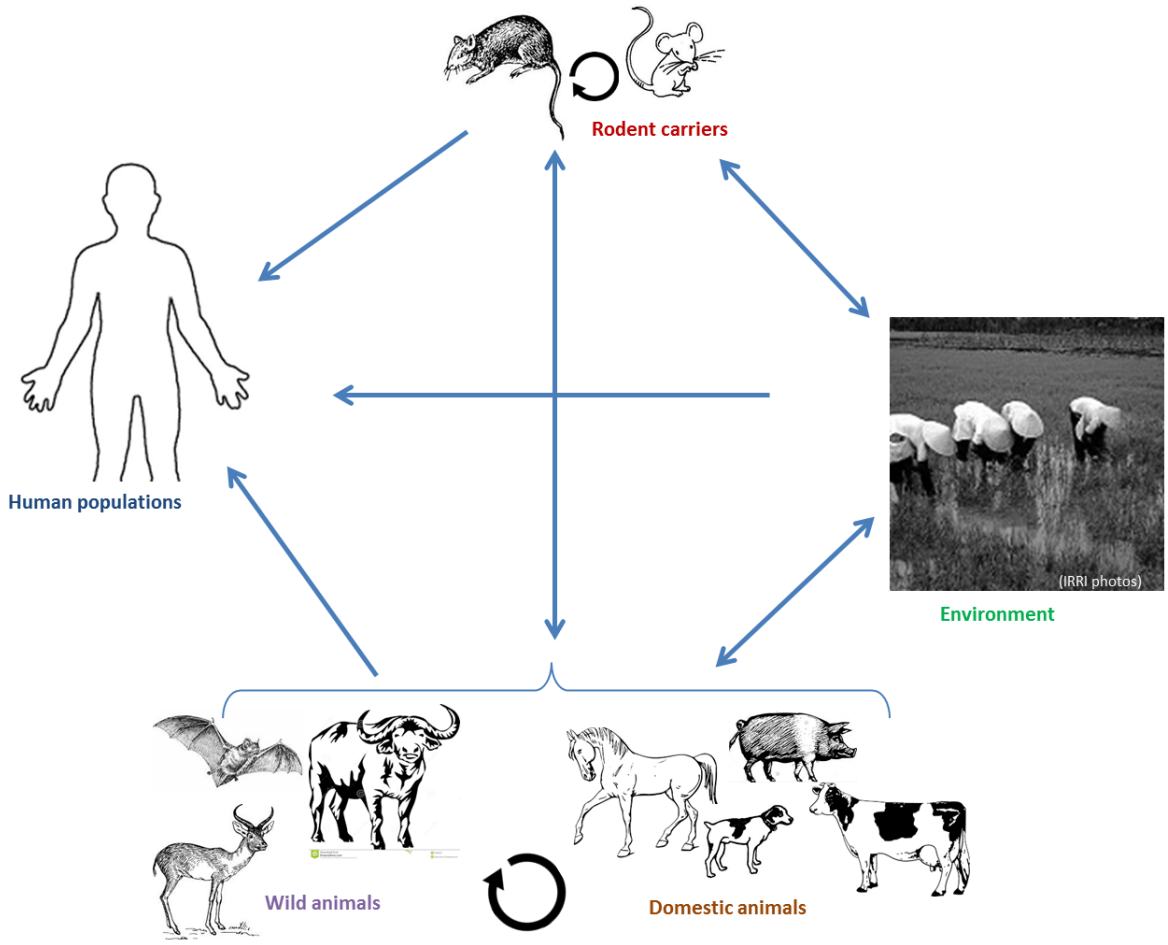

Figure 2: The transmission cycle of leptospirosis, considering the environment, rodent hosts, domestic and wild animals and human beings

After this leptospiremic phase, the bacteria sequester in the proximal tubules of the kidney nephron where they multiply and get shed into the urine of the infected animal. Leptospiuria may persist for varying periods, depending on the species, individual animal and the infective serotype. The bacteria may also localize in the uterus of pregnant females to cause intrauterine infections which, especially late in the gestation period, may result in stillbirths, abortions and neonatal disease. Infertility may also arise from chronic colonization of the uterus and oviducts. Immune response from the host usually clears the

bacteria from the blood and tissues, eventually eliminating it from the host. This however may not be the case in renal tubules and even the vitreous humor of the eye, which are immune-privileged. This may lead to continued shedding of the pathogen even after the illness ends (Merckvetmanual.com, 2016; Ellis, 2015; Ko, Goarant & Picardeau, 2009).

3.6 Diagnosis

There exists a wide array of diagnostic methods for detection of the presence of leptospires currently in use, each with their own specificity and sensitivity. The Microscopic Agglutination Test (MAT) first described by (Martin & Pettit, 1918) is regarded as the gold standard for diagnosis of leptospirosis in humans and animals (Goris & Hartskeerl, 2005). The test has been modified from the original description and has high diagnostic specificity (WHO, 2003). It is performed by incubating patient serum with a panel of *Leptospira* serotypes. The MAT titer is obtained by testing for reactivity between the positive serotype and the test sera at different dilutions. Agglutination is visualized by dark-field microscopy. Serotypes that show reactivity with patient sera are thought to be the infective serotypes, even though evidence shows that cross-reactivity between serotypes is possible. Due to this cross-reactivity, this method may not always identify the infective serotype. Antibodies against the infective serotype in a patient's serum may not be detected if the infective serotype is not represented in the panel of antigens used in the test used: in this case, there will be no MAT titre. Low MAT titers can also be found when the infectious serotype is not represented in the panel but agglutination with serotypes that are antigenically similar to the infective one happen. Therefore, the presence of low or no MAT titre does not exclude presence of the disease in this case (WHO, 2003). Knowledge of the epidemiology of leptospirosis therefore greatly aids in the choice of leptospires to include in a panel in

any lab, as locally circulating serotypes should necessarily be included. When local serotypes are unknown, serotypes representing all serogroups should be included. This method also relies on the use of live bacteria, a risk that other diagnostic methods may not pose to laboratory staff (Chirathaworn, Inwattana, Poovorawan, *et al.*, 2014; WHO, 2003). MAT can detect both IgM and IgG antibodies. Standardization of the test however is not possible because of the many differences in the live antigens used by different labs all over the world (WHO, 2003). This method is also expensive and time consuming, a challenge that led to development of many other rapid diagnostic tests, especially when dealing with clinical cases which are usually time-sensitive.

Enzyme Linked Immuno-sorbent Assays (ELISAs) are useful for detection of genus-specific antibodies but are not recommended for serotype identification. They are relatively simpler to conduct than the MAT and have high sensitivity and specificity, hence their preference in many epidemiologic surveys (WHO, 2003). Some ELISA kits used currently are Panbio® *Leptospira* IgM ELISA (Panbio Diagnostics, Montpellier, France) for diagnosis in humans as well as the Linnodee *Leptospira* ELISA Kit™ (Linnodee Animal Care, Ballyclare, Northern Ireland) for diagnosis in cattle. IgM antibodies have been shown to be detectable even after 3 years after onset of illness in humans (Ari, Guracha, Fadeel, *et al.*, 2011) and perhaps may be useful in determining both acute and chronic cases in seroprevalence studies.

Other serological diagnostic tests that can be used include; indirect fluorescent antibody test (IFAT), counter-immunoelectrophoresis (CIE), complement-fixation test (CFT), dipstick tests like LEPTO Dipstick and LeptoTek Lateral Flow, dried latex agglutination test (LeptoTek Dri-Dot), latex agglutination test (LA), indirect haemagglutination test (IHA),

macroscopic slide agglutination test (SAT), microcapsule agglutination test, patoc-slide agglutination test (PSAT) and sensitized erythrocyte lysis test (SEL) (WHO, 2003).

Polymerase Chain Reaction test (PCR) can be used for detection of the bacteria in clinical samples. The test detects the presence of the bacteria through enzymatic amplification of a gene of choice in leptospiral DNA. Amplicons can be detected through intercalating dyes that associate with the amplicons and gel electrophoresis for conventional PCR, or during real-time PCR. Being a molecular technique, PCR can detect very minute levels of the bacteria and is therefore useful in diagnosis of early cases of the illness. The technique can also be used to detect all pathogenic leptospires when primers for conserved regions of the genome are used. The technique however requires valid controls, specialized skills and laboratory equipment in a lab.

3.7 Treatment, prevention and control

Treatment of acute animal leptospirosis is usually done by administration of an antibiotic therapy as well as clinical management of symptoms. The control of the disease has not changed much through the years as well. Though there exists few clinical trials to determine the role of various antibiotics in the treatment of leptospirosis, most will go for penicillin and streptomycin to treat acute illness. Treatment of herds of cattle, sheep and pigs may involve use of vaccination alongside the antibiotic therapy to obviate reproductive wastage. In chronic illness, antibiotic therapy may be used to mitigate abortion storms in pigs (Ellis, 2015).

Avoidance of free-ranging of animals as a preventative measure against acquiring the disease is difficult since rodent reservoirs can be found in all environments and can

therefore transmit the illness. The most valid preventative measure therefore is vaccination using polyvalent inactive vaccines (Merckvetmanual.com, 2016). Vaccination has also been used in humans for preventative purposes as it is quite difficult to employ other measures like occupational hygiene (Adler & de la Peña Moctezuma, 2010). Vaccines are commercially available for dogs, pigs and cattle, but vaccination efforts have shown to be only partly effective due to the serotype-limited immunity elicited by the vaccine used, as well as the possibility of local serotypes being present in the area targeted by the vaccination program that are not in the vaccine supplied. The success of vaccination efforts therefore requires knowledge of the epidemiology of the disease before execution (Adler & de la Peña Moctezuma, 2010). Vaccines have also been shown to confer cross-protection against different serotypes of the bacteria in various experimental studies (Sonrier, Branger, Michel, *et al.*, 2000; Rosario, Arencibia, Suarez, *et al.*, 2012; Dib, Gonçales, de Morais, *et al.*, 2014).

Human prevention efforts, as with animal ones, also depend on understanding the epidemiology and the local risk factors of the disease. Human leptospirosis is strongly linked to poverty and efforts that prevent contact of rodents with humans in their living spaces greatly reduce the risk of transmission of the disease. Flood-control projects that prevent flooding of residential areas also prevent outbreaks of the disease. The use of personal protective equipment (PPE) for workers at high-risk can also prevent infection by offering a physical barrier to points of entry of the bacteria. Avoidance of water sports as well as walking bare foot in endemic areas can also reduce risk of infection. Diagnosis and treatment as well as immunization of agricultural or companion animals also plays a key role in prevention of the disease in humans (Haake & Levett, 2015)

3.8 The zoonotic potential of leptospirosis

Humans are considered dead-end hosts and there is no documented human-to-human transmission. Humans also do not shed enough of these pathogens to be important reservoir hosts (Ko, Goarant & Picardeau, 2009). Leptospirosis has been considered an occupational hazard, especially for people who work with water and sewage systems (miners, sewer workers and fish farmers) (Waitkins, 1986) and those that are exposed to animals and their bodily fluids of animals. Veterinary staff, dairy workers and livestock and pig farmers, butchers, and rodent control workers are at risk of occupational exposure (Merckvetmanual.com, 2016; Vijayachari, Sugunan & Shriram, 2008). There also exists exposure from certain recreational activities, including water sports (Vijayachari, Sugunan & Shriram, 2008). These may include swimming in lakes, hunting, canoeing, biking through infected water, rafting, hiking, caving, trail biking, kayaking, hunting and fishing (Pavli & Maltezou, 2008; Antony, 1996). This is because of the associated risk factors that come with these activities which include but not limited to; immersion in water, walking barefoot, contact with floodwater, and drinking river water, especially when having skin wounds.

4. Materials and Methods

4.1 Study Area

The study was done in Bura and Hola irrigation schemes of Tana River County as well as Ijara and Sangailu divisions of Ijara district in Garissa County (Figure 3) and (Figure4).

Tana River County is located in the former Coast Province of Kenya located between coordinates 1°30′ S, 40°0′E and 1.5° S, 40° E (Koech, Kinuthia, Karuku, *et al.*, 2016). The county is 38, 436.9 sq. km. (KenInvest, 2016) and is named after the Tana River which supplies the surrounding area with water. The county is composed of three constituencies; Garsen, Galole and Bura (Kenya Open Data Portal, 2016). The population according to the 2009 population and housing census of Kenya was 240,075 people (Kenya National Bureau of Statistics, 2010) and more than 70% of people here live below the absolute poverty line (Koech, Kinuthia, Karuku, *et al.*, 2016). The area is generally hot and dry, receiving low and erratic conventional rainfall of a bimodal pattern and a long-term annual average of 220 to 500mm of rainfall. Rainy seasons are usually from April to June and November to December (Koech, Kinuthia, Karuku, *et al.*, 2016). Farming and pastoralism are the main economic activities in this semi-arid area. The area has seen development of irrigation schemes, the biggest of these having been set up by the Kenyan government. The biggest irrigation schemes in this area are the Hola Irrigation Scheme, Bura Irrigation Scheme and the Tana Delta Irrigation Project and are used for maize and rice production (Tana River County, 2016).

Ijara is a sampling site located in Garissa County of Kenya. Garissa County is located in the former North Eastern province of Kenya. In the last census it had a population of 623,060 people (Kenya National Bureau of Statistics, 2010). The county has 6 constituencies; Fafi,

Garissa Township, Lagdera, Dadaab, Ijara and Balambala. The county is regarded as arid and the main economic activity is pastoralism. The Tana River is a major source of water for this dry area. The climate of the county is semi-arid, with temperatures ranging from 21°C to 39°C and bimodal rainfalls averaging 250 to 300mm annually. Rainy season is usually from March to May and September to October. The county has a large number of nomadic pastoralists who live in some of the harshest climates on earth (Gore-Langton, Mungai, Alenwi, *et al.*, 2015)

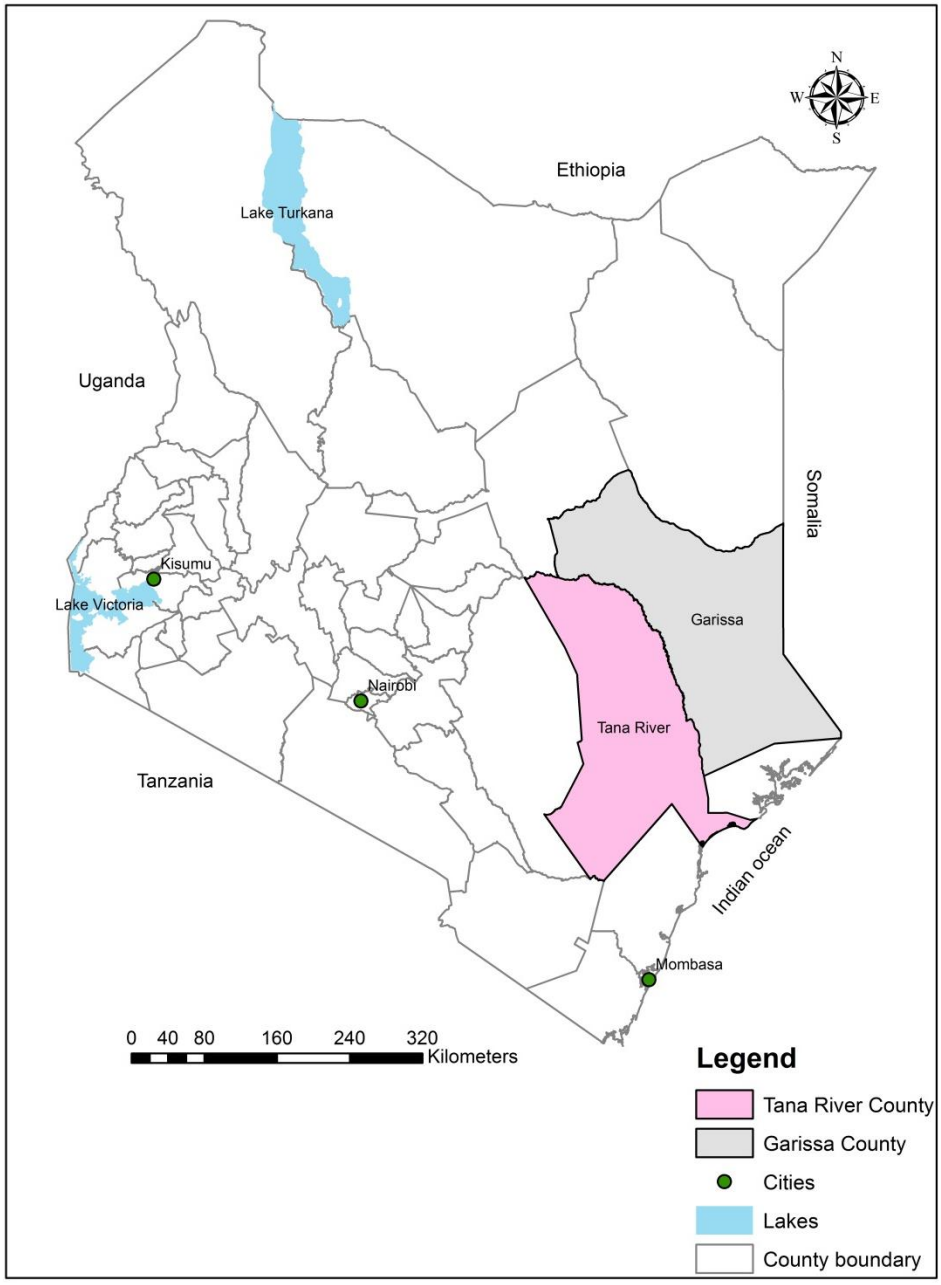

Figure 3: A map of Kenya with counties involved in the study highlighted

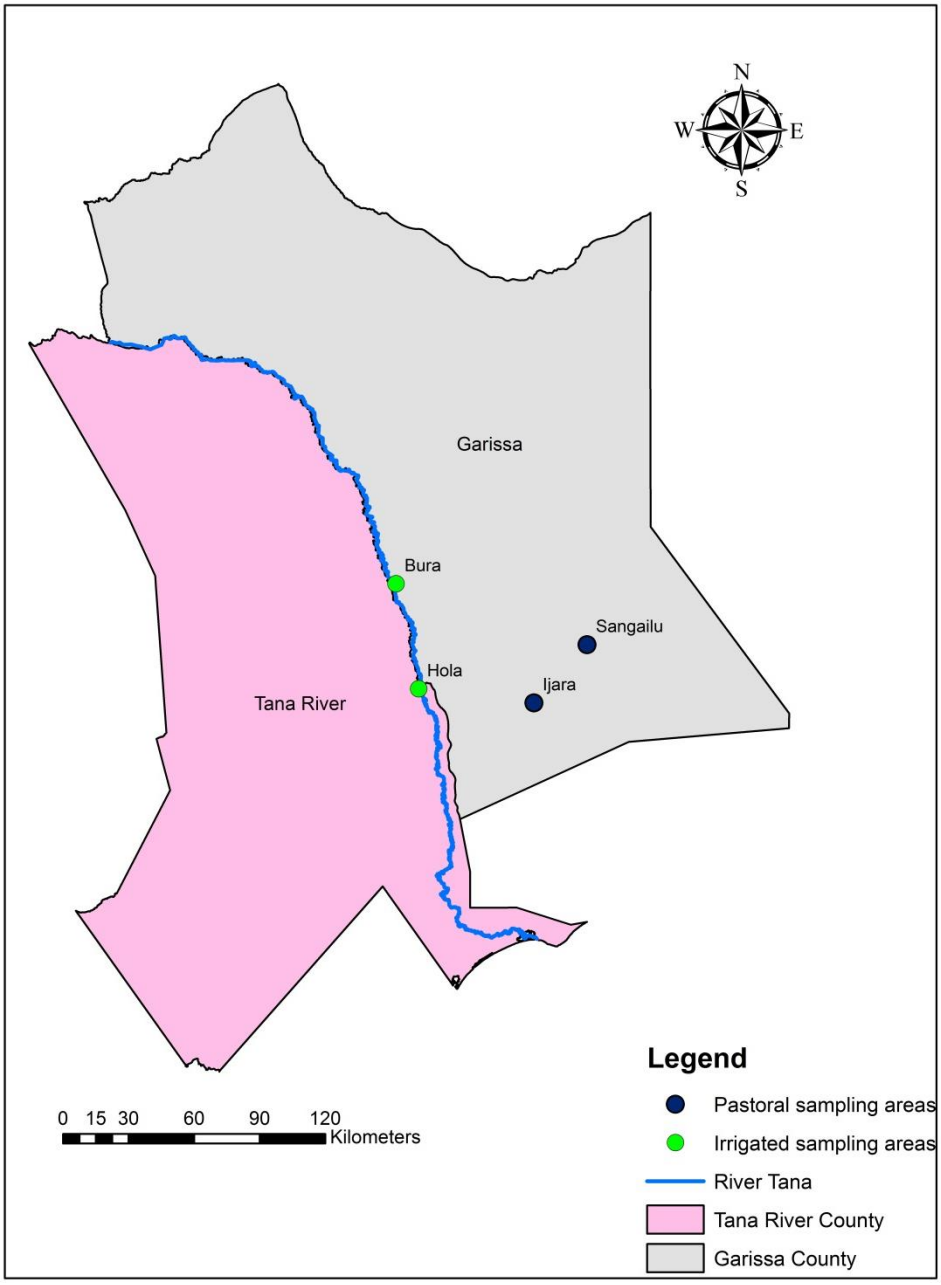

Figure 4: Tana River and Garissa Counties with towns involved in the study highlighted

4.2 Study design

The work was designed as a pilot study that could check for the presence of the disease in rodent carriers. This would inform fuller observational studies in estimating prevalence for sample size calculations. It would also show the need for further studies that would elucidate leptospirosis in the study area. Execution of the project would demonstrate the time, feasibility, methodology and adverse events in future studies. The study was done on a small-scale and meant to demonstrate the presence of the pathogen in areas sampled by a larger project; the Dynamic Drivers of Disease in Africa: Ecosystems, livestock/wildlife, health and wellbeing: RVF case study in Kenya project (REF. NE/J001422/1). Ethical approval for this project was done by the Ethics and Scientific Review Committee (ESRC) of the Africa Medical and Research Foundation (AMREF) (REF: AMREF-ESRC P65/2013).

No sample size was calculated for this pilot study before animal sampling was done.

4.3 Setting of rodent traps

Biological samples used in this study originated from rodents collected during the months of October and November, 2013 as well as March 2014. Collection of rodents had been done in and around human dwellings as well as in the surrounding environment, using the method briefly described here. Sherman rodent traps (H. B. Sherman Traps Inc., Tallahassee, FL) from the National Museums of Kenya (NMK) were set in Ijara district as well as Bura sub-county and Hola town.

Traps in Bura and Hola towns were set in October, 2013 and March 2014. These were close to the areas that had animals tested as part of the field work for the DDDAC project. The

traps were placed near human settlements, in wild shrub land forests and on irrigation farms. Traps were placed close to irrigation canals, near holes, on trees, and putative rat trails. In human settlements, traps were placed in kitchens and on top of shelves where food is stored as well as in living places. In Hola town, traps were laid in Matanya village and the forest surrounding the National Youth Service (NYS) garage.

In Bura town, the traps were set in temporary structures and human settlements within the Bura Irrigation Scheme, farms and bushes as well as in the Bura Country Club.

In Ijara, traps were set in Sangailu and Ijara towns and were set strategically inside and outside houses, bushes, inside and outside shops as well as in human settlement areas. Traps were set in December 2013 and March 2014.

A total of 119 samples (Table 5) had been collected from the field.

Table 5: Distribution of rodents collected with the towns in the study sites

Species collected	Bura	Hola	Ijara	Sangailu	Grand Total
Acomys wilsoni	0	7	1	0	8
Arvicanthis niloticus	1	0	0	0	1
Gerbilluscus robustus	0	0	1	0	1
Mastomys natalensis	18	3	1	11	33
Mus musculus	26	27	11	10	74
Rattus rattus	0	2	0	0	2
Grand Total	**45**	**39**	**14**	**21**	**119**

4.4 Sample collection

Collected rodents had been euthanized humanely using an intraperitoneal injection of Ketaminol 50mg/ml. The rodent species were identified by mammologists from the NMK. Blood samples were obtained where possible, as well as internal organs, and put in cryovials. Urine and the urinary bladder were also obtained where possible. Rodent morphometric data was also recorded and included weight, total length, tail length, hind foot length and ear length. The sex and estimate age (whether adult, juvenile or sub-adult) of the rodents were also noted as well as autopsy comments.

All samples collected were stored in cryovials that have unique barcode identifiers. They were put in dry ice and transported to the International Livestock Research Institute (ILRI) headquarters in Nairobi, Kenya where they were archived in liquid nitrogen in the Azizi Biorepository before analysis in this study.

In total, 69 rodent samples had blood and kidney tissue available for PCR tests for this study. DNA for two of these rodents got degraded and therefore did not yield results. The total number of rodents used for the analysis therefore was 67.

4.5 Sample analysis

4.5.1 DNA extraction

Extraction was done using automated DNA extraction as well as manual methods. The automated extraction was carried out on all blood samples using the MagNA Pure LC machine (Roche Diagnostics, Risch-Rotkreuz, Switzerland). The kit used was the high performance MagNA Pure Total Nucleic Acid isolation kit (Roche Diagnostics, Risch-Rotkreuz, Switzerland, Cat. No. 03038505001). Kidney tissue from the rodents was used in

the extractions. These underwent manual extraction using the DNeasy® blood and tissue kit (Qiagen, Hilden, Germany). Manufacturer's instructions guided the extraction in both protocols. Extracted nucleic acids were stored at -80°C until use.

4.5.1.1 MagNA Pure LC

The MagNA Pure LC can extract 32 samples at a go. The steps used in this procedure were as follows:

i. The MagNA Pure 3.0 software was initialized.

ii. Under sampling order, the identifiers for the samples and controls were keyed in. The extraction protocol "Total NA HS200.bk" was used. No post-elution settings were set (we selected none). The MagNA Pure LC kit lot number was also keyed in. The sample volume was set at 200µl, elution volume at 50µl and dilution volume at 0µl as these were thought to be comparable.

iii. Under the stage setup menu, the reagent tubs for wash buffers I, II and III, lysis/binding buffer, proteinase K, magnetic glass particles (MGP) and elution buffers were set in their respective places in the machine. The volumes specified by the machine were also added to the tubs – these volumes are automatically calculated by the MagNA 3.0 software depending on the number of samples being analyzed. Tip trays were also filled with the appropriated tips.

iv. The extraction was initialized and was performed automatically for the samples chosen.

v. After extraction, the DNA eluate was held on the cool block

Extracted DNA was quantified and transferred into 1.5ml Eppendorf tubes for safe storage at -80°C. Tubes were duly labelled using a permanent marker with the sample identification number.

4.5.1.2 Qiagen DNeasy® manual extraction method

DNA extraction using the DNeasy® blood and tissue kit from Qiagen was done as per the manufacturer's instructions (Qiagen, 2011). A negative extraction control (NEC) was included in every batch of extractions performed. This comprised of extraction reagents only and no sample.

The procedure was as follows:

1. Lysis was done for blood and tissue samples as follows.
   i. The whole kidney was used in this extraction. Tissues were cut into small pieces using sterile surgical blades on a cutting surface (changed between different samples) and placed into 1.5ml Eppendorf tubes. 180µl of ATL buffer was added and 20µl proteinase K after this. This was mixed by vortexing and incubated at 56°C until complete lysis happened; occasional vortexing was done during the incubation
   ii. For the blood samples, 20µl proteinase K was put into a 1.5ml Eppendorf tube and 5 -10µl of the blood was put, depending on the available volume. The volume was adjusted to 220µl using Phosphate Buffered Saline (PBS). 200µl of AL buffer was added, mixing done by vortexing and incubation of the blood sample was done at 56°C for 10 min
2. 200µl of absolute ethanol was added to every tube and mixing was done by vortexing

3. The mixture was transferred into the DNeasy Mini spin columns placed in 2ml collection tubes supplied with the kit. Centrifugation was done at 1000 rpm for 1 min and the collection tubes with the flow-through liquid were discarded.
4. The spin column was placed into a new collection tube and 500µl of AW1 buffer was added. Centrifugation at 1000rpm for 1 min was done. The collection tubes with the flow-through were discarded
5. The spin column was placed into new collection tubes and 500µl of AW2 buffer was added. Centrifugation was done at 14000 rpm for 3 min. The flow-through liquid in the collection tube was discarded.
6. The spin column was put in a new 1.5ml Eppendorf tubes and elution was achieved by adding 200µl of AE buffer into the centre of the spin column membrane. Incubation for 1 min at room temperature was done after which centrifugation for 1 min at 1000 rpm was done to complete the extraction

Tubes having the extracted DNA were well labelled and aliquoted into working aliquots and stock aliquots. Working aliquots underwent the dilution step and the stock aliquots were stored at -80°C.

Concentrations and purity of the working aliquots were determined using the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Concentrations above 100ng/µl were diluted down to 100ng/µl so as to be suitable for PCR. No DNA eluates were set aside from the PCR because of lack of DNA. The lowest concentration obtained was 0.62ng/µl. DNA purity was determined using the A_{260}/A_{280} and A_{260}/A_{230} ratios. Extracted DNA was visualized on an agarose gel as shown in Figure 5 below.

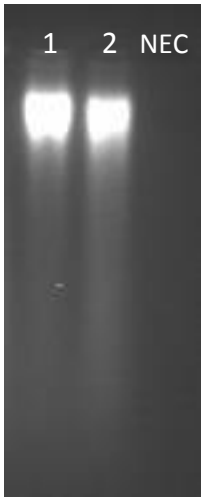

Figure 5: Gel photo of genomic DNA extracted using the DNeasy® protocol, with lanes 1 and 2 showing DNA from kidney tissue and lane 3 showing the negative extraction control

4.5.2 Polymerase chain reaction (PCR)

A conventional (endpoint) PCR test to detect leptospiral DNA was done on the nucleic acids isolated from the rodent blood and tissue samples. The thermocycling conditions are as Table 6 illustrates. Annealing temperature for the primers was determined using the Eppendorf MasterCycler® Nexus Gradient thermocycler (Thermo Fisher Scientific, Waltham, MA, USA). The GeneAmp® PCR system 9700 Thermocyler (Thermo Fisher Scientific, Waltham, MA, USA) was used for the tests after optimization of the PCR conditions was done.

Table 6: Conditions optimal for amplification of leptospiral DNA using G1/G2 primers

Step	**Temperature in degrees Celsius (°C)**	**Time in minutes (min)**	
Initial Denaturation	95	3	
Denaturation	95	0.5	40 cycles
Annealing	55	1	
Extension	72	1	
Final Extension	72	10	
Hold	10	∞	

DreamTaq Green PCR master mix and Nuclease Free Water provided in the kit from ThermoFischer Scientific (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. K1081) were used. G1/G2 primers (Inqaba Biotec East Africa Ltd.) that detect *L. interrogans sensu lato* with the exception of *L. kirschneri* were used. These primers were first described by (Gravekamp *et al.*, 1993) and target the sec-Y gene that codes for the translocase SecY protein. A lack of positive control that could be used for B64-I/B64-II primers that target the flagellin gene of *L. kirschneri* meant that we could not detect the group, even though many studies have demonstrated the species in rodents (Mayer-Scholl *et al.*, 2014; Halliday *et al.*, 2013). The G1/G2 and B64I/B64II primers are usually used as a set for the detection of pathogenic leptospires (Gravekamp *et al.*, 1993).

A gradient PCR was performed to determine the best annealing temperature for the PCR reaction (Figure 6). An annealing temperature of 55°C was chosen because it could detect weakly positive samples, though it also caused non-specific amplifications in some samples

(Figure 7, Lane 2). It was therefore decided that DNA purification of the bands of interests was to follow to do away with all non-specific amplifications.

Controls used in every PCR run included a non-template control (NTC) and a positive amplification control (PAC). The non-template control included PCR reagents only and nuclease-free water was added in the place of DNA template. The positive amplification control used was a DNA construct with part of the secY gene.

Table 7: Master mix recipe used for G1/G2 reactions

PCR reagent	**Stock concentration**	**Working concentration**	**1 reaction (µl)**
DreamTaq Green PCR Mastermix	2x	1x	12.5
Nuclease Free Water (NFW)			8.5
Forward primer (10µM)	100µM	10µM	1.5
Reverse primer (10µM)	100µM	10µM	1.5
DNA template			1
Reaction Volume			**25**

A summary of the master mix recipe is highlighted in Table 7 above.

A summary of the primer and positive control construct sequences used can be found in Table 8.

PCR products were stored at -20°C until further use.

Table 8: Sequences of primers used to identify pathogenic *Leptospira* species (except *L. kirschneri*) as well as positive control construct used

Name	Sequence	Length and position in target gene
G1	5' CTGAATCGCTGTATAAAAGT 3'	276-295=20 bp
G2	5' GGAAAACAAATGGTCGGAAG 3'	11-30=20 bp
Leptospira spp. synthetic construct used as a positive amplification control (PAC). Binding sites are underlined.	5'TCTGCAGTACGGAAAACAAATGGTCGGAAGAAAAATGGTTCAGGCCAAGAGTCAATCTATTCCTTTCAAAGTAAACGGCGCGAACGTGATGCCGATCATTTTTGCTTCGTCTTTGATTTTATTTCCTCAGACGATTATTCAATGGTTATCTAATAGTAGTCAAGAATGGGCTGGATGGGCAGTGATTATGGATTTTTTTAATCCATTCTCTCAGATTTGGTATCATGCGTTATTTTATTTCGTAATTTATACCGCTTTAATTGTATTCTTTGCTTACTTTTATACAGCGATTCAGTTTAATCCTGCCCGGGTTTGGAATCTCCACCCCCGAACAAGCAAGTCAAATTGCCGGGTATGCGGACGGAATCATCATCGGATCTGCCATCCAAAGGGTCATCGAAGAGAATGGGCAAGACGCTTCCAAAGCAAAAAATGTTTTGGCGGACTACATAACAAAGATTCGGGCATCAATTTCCTAAATTTTTTTCCGAAATCACTCAAAAACATTTGAGTCTCTTTAAAAAATCCGAGAGAATGGTCGGAAATTCCTTAACGAATAAAGAGGCTCTATGTCCCCCGGGAAATGGACTGCGGATGCAGCTGCTAAAGGTCGTTTAATCAATGTTACTGAACTAACAACTGCAGGTAAATCAGGAGCGGCTTTAGTTGCTTTTAGATCGGCAGCTTTGGCTGGTGCTGCTACTTGTGCAAAAGATATCTTATCCAAGGAAAGTGAAGAGGCACAGCGCATTGCTTTCTCTCTACAT 3'	767bp Product = 285bp

4.5.3. Agarose Gel Electrophoresis

PCR products were run on 2% agarose gels and run for 30 minutes at 100Volts. 0.5X TAE buffer (procedure in Appendix 1) was used to prepare the gel as well as the running buffer. GelRed™ nucleic acid gel stain was used for illumination under ultraviolet light.

No loading dye was used as the master mix used is pre-laced with loading dye. 5µl of the PCR product was loaded to the gel and 3µl of O'GeneRuler™ 100bp ready-to-use DNA Ladder (ThermoFischer Scientific, Waltham, MA, USA; Cat No. SM1143) were used for the electrophoresis.

Gel photos were taken using ultraviolet (UV) light trans-illumination and gel photos labelled and saved. Bands of interest were at 285bp.

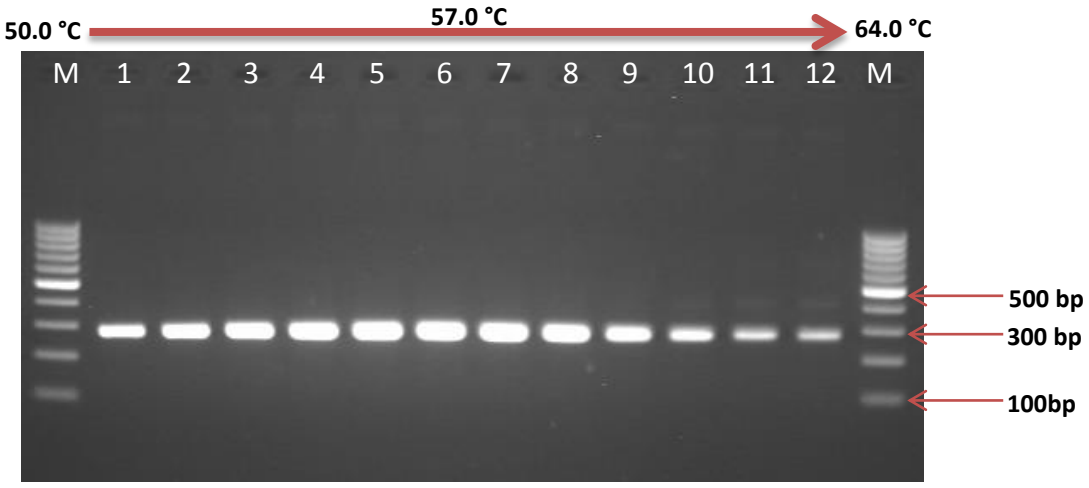

Figure 6: Gradient PCR to determine best annealing temperature of G1/G2 primers using 20ng/µl of the positive control. The key used is 1. 50.0, 2. 50.4, 3. 51.2, 4. 52.5, 5. 54.4, 6. 56.1, 7. 57.9, 8. 59.6, 9. 61.5, 10. 62.8, 11. 63.6, 12. 64.0 and M for Molecular Weight ladder (100bp)

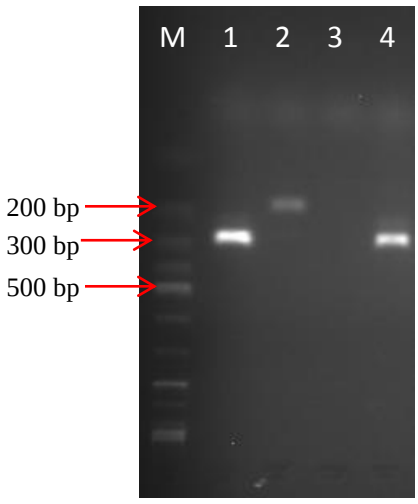

Figure 7: Gel electrophoresis of PCR products, with lane 1 showing a positive sample, lane 2 showing a band of non-interest as it is not in the required region of 285bp. Lanes 3 and 4 showing the non-template control (NTC) and positive amplification control (PAC) respectively.

4.5.4 Gel purification

Some samples exhibited multiple bands at the chosen annealing temperature of 55°C. Therefore, for a subset of samples that had the band of interest (285bp), the area containing the band was extracted from the gel and DNA purification. The commercial kit used for this step was the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, Madison, WI, USA; Cat. No. A9281/2/5). Purified DNA product in some samples was run on gel to confirm presence of band of interest before sending for sequencing (Figure 8)

The process was as follows:

1. Microcentrifuge tubes were labelled with the sample IDs and weighed.
2. Bands of interest on the agarose gel were excised on a UV illuminator using a clean blade and placed into the tubes.

3. The tubes were weighed with the gel slice in them. The weight of the slice was determined. The gel slice was dissolved by adding 10µl of membrane binding solution per 10mg of the gel slice, vortexing and incubating at 55°C until the gel was completely dissolved.
4. SV minicolumns were labelled and fitted into the collection tubes provided.
5. The dissolved gel mixture was transferred into the minicolumn assembly and incubated at room temperature for 1 minute.
6. The mixture was centrifuged at 16000 rcf for 1 min. The flow-through was discarded and the minicolumn inserted into a fresh collection tube.
7. 700µl of reconstituted membrane wash solution was added and the tubes centrifuged at 16000 rcf for 1 min. The flow-through was discarded and columns placed in fresh collection tubes as before.
8. This wash step in Step 7 was repeated with 500µl of the membrane wash solution and centrifugation done at 16000 rcf for 5 minutes.
9. The collection tubes were emptied and centrifuged again for 1 min at 16000 rcf with the lid of the microcentrifuge open to allow for evaporation of residual ethanol.
10. The minicolumns were transferred into clean and labelled 1ml microcentrifuge tubes in readiness for elution. 50µl of nuclease-free water was added and incubation done for 1 minute at room temperature. The columns were then centrifuged at 16000 rcf for 1 minute. The minicolumns were discarded and eluted DNA stored at 4°C in readiness for sequencing.

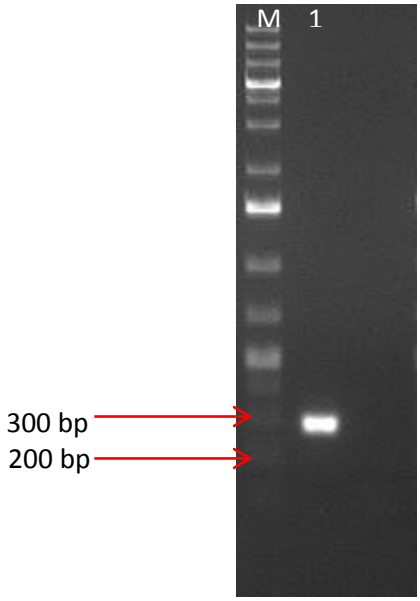

Figure 8: PCR positive kidney sample (Lane 1) after gel purification of band of interest at 285bp. Lane 2 is the non-template control used in the PCR reaction. Lane M is the molecular weight ladder (O'GeneRuler 1kb Plus DNA ladder, ThermoFischer Scientific, Walham, MA, USA, Cat. No. SM1343

4.5.5 Sanger sequencing and phylogeny

Some positive samples from both the irrigated and pastoral areas were sent to Macrogen Inc. for Sanger sequencing to ascertain that the targeted loci were being amplified by the PCR reaction. Consensus sequences (contigs) were developed from the forward and reverse sequences of the positive samples sent for sequencing using CLC Main Workbench software package version 6 (Qiagen, Aarhus, Denmark).

The contigs were subjected to a search on the Basic Local Alignment Search Tool for nucleotides searched (BLASTn) suite to check for similarities with other DNA sequences (NCBI, 2016).

Construction of a phylogenetic tree (Figure 12) was done using the Molecular Evolutionary Genetics Analysis software version 6.0 (MEGA6) (Tamura, Stecher, Peterson, *et al.*, 2013) to check for evolutionary relatedness based on the secY gene. This gene has been used for

phylogenetic classification and has shown to be sufficiently discriminatory (Ahmed, Engelberts, Boer, *et al.*, 2009; Halliday, Knobel, Allan, *et al.*, 2013). The neighbor-joining method (Saitou N, 1987) was used to generate the tree and measures of support for each node (given in percentage) were generated using bootstrapping tests (Felsenstein, 1985) of 1000 replicates and was stated in beside each node. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were determined by using the maximum composite likelihood method (Tamura, Nei & Kumar, 2004).

4.6 Data analysis

Data was put in MS Excel platform to create a master-list containing the rodent ID, sampling details (town, coordinates, dates, species and sample IDs), rodent morphometric data and results from these analyses. Positive rodents were determined if either blood or kidneys or both showed positive PCR results. Distribution of the results with rodent species, town of collection, sex and estimated age were derived using MS Excel (MS Office suite 2007, Microsoft Corporation, Redmond, WA, USA). Prevalence data was determined using PCR results.

The Fisher's exact test was used to test the null hypothesis that there is independent association between PCR results and the sampling sites, rodent species and estimated age of the rodents. This was done using STATA version 14 (StataCorp LP, Texas, USA). The Fisher's exact test was chosen because of the small sample size used in this study. An Odds Ratio (OR) was also calculated using the same software to demonstrate the likelihood that rodents from pastoral areas had in carrying the bacteria than those from the irrigated area.

5. Results

Kidney and blood samples from 67 rodents were analyzed using the PCR test.

Results obtained from the analyses are as follows:

5.1 PCR test results

PCR tests from 67 rodents were analyzed. Positive results were obtained from 28 rodents (41.8% prevalence, 95% CI=29.8% - 54.5%) and the remaining 39 were negative results. Results were distributed with the species as represented in Table 9 below.

Prevalence figures were: **16%** (4/25) (95% CI 4.54 to 36.08) **in Bura**; **42%** (8/19) (95% CI 20.25 to 66.50) **in Hola**; **82%** (9/11) (95% CI 48.22 to 97.72) **in Ijara** and **58%** (7/12) (95% CI 27.67 to 84.83) **in Sangailu**. This is summarized in Figure 9 below.

Table 9: PCR results with the species of rodents collected

Species	Negative		Positives		Total count
	n	%	n	%	
Acomys wilsoni	0	0.0	1	100.0	1
Gerbilluscus robustus	0	0.0	1	100.0	1
Mastomys natalensis	12	57.1	9	42.9	21
Mus musculus	27	64.3	15	35.7	42
Rattus rattus	0	0.0	2	100.0	2
Total	**39**	**58.2**	**28**	**41.8**	**67**

Out of the rodents tested, 44% (16/36) of the females and 39% (12/31) of the males were PCR positive (Figure 10). The prevalence dependent on the age of rodents was as follows; 38.2% (21/55) among adult rodents, 50% (3/6) among sub-adults, 75% (3/4) among juveniles and 50% (1/2) in rodents where age was not recorded. Results of prevalence distribution with age are summarized in Figure 11 below.

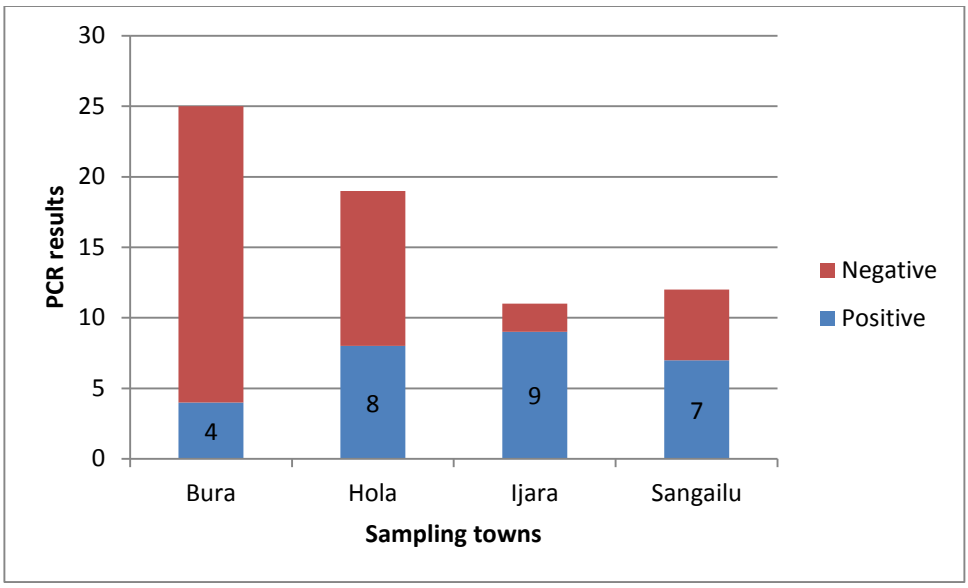

Figure 9: A stacked bar chart showing distribution of PCR results with the sampling sites

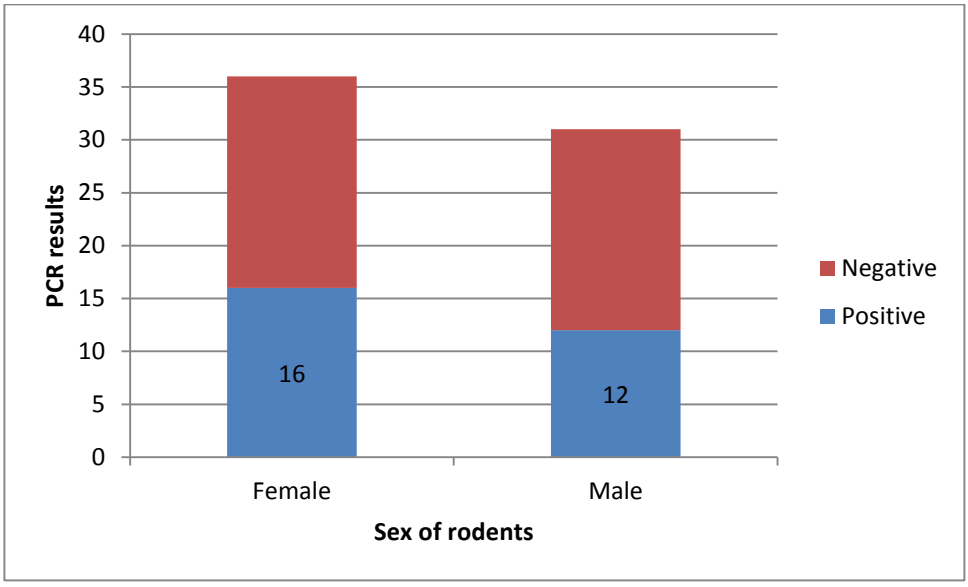

Figure 10: A stacked bar chart showing distribution of PCR results with sex of the rodents collected

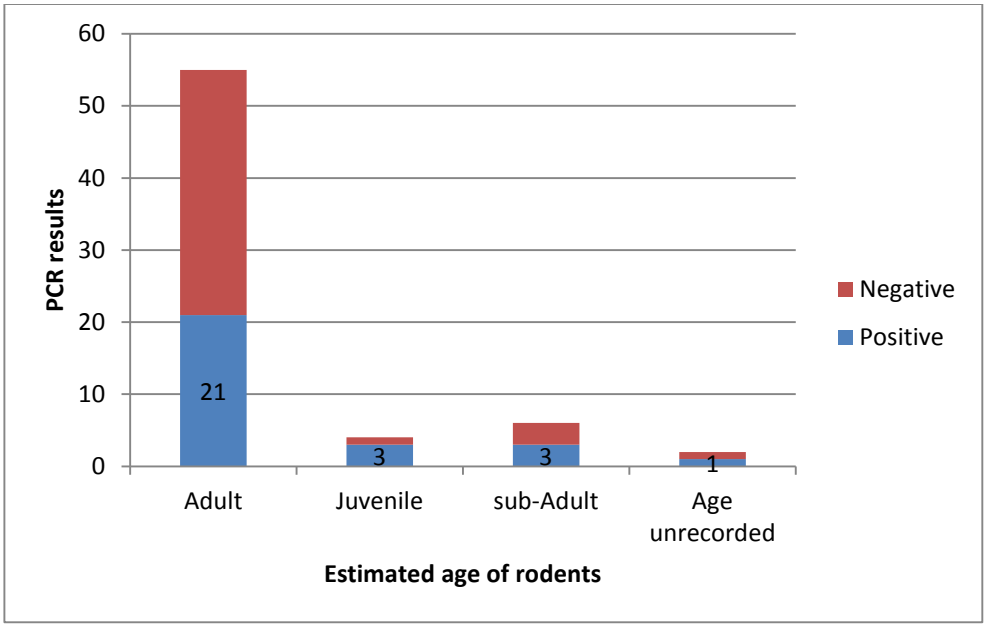

Figure 11: Distribution of prevalence with the estimate age of rodents collected

5.1.1 Hypothesis testing of PCR data with variables

The Fisher's exact test was performed on the town of sampling, rodent species and estimated rodent age.

Bura, Hola, Ijara and Sangailu were used in the Fisher's exact test and results are shown in Table 10 below. Since $p<0.005$, it was determined that sampling town does have an effect on the prevalence of leptospirosis among the rodents.

Ijara and Sangailu were grouped as pastoral areas, whereas Bura and Hola were grouped as irrigated areas, to determine the influence the sampling area more precisely. The test was performed and it was found that the pastoral areas had significantly higher prevalence ($p=0.002$) as shown in Table 11 below. Since $p<0.005$, the null hypothesis was rejected. An odds ratio was also calculated on the pastoral and irrigated areas and OR=6.095 (CI 95% 2.011-18.470) was obtained.

Table 10: *Leptospira* prevalence in different areas

Town	Total results		
	Positive	Negative	Total
Bura	4	21	25
	16.00%	84.00%	100.00%
Hola	8	11	19
	42.11%	57.89%	100.00%
Ijara	9	2	11
	81.82%	18.18%	100.00%
Sangailu	7	5	12
	58.33%	41.67%	100.00%
Total	28	39	67
	41.79%	58.21	100.00%

Fisher's exact = 0.001

Table 11: *Leptospira* prevalence in areas sampled, with irrigated areas (Bura and Hola) and pastoral areas (Ijara and Sangailu).

Group (town)	PCR results		Total
	Positive	Negative	
Irrigated	12	32	44
	27.27%	72.73%	100.00%
Pastoral	16	7	23
	69.57%	30.43%	100.00%
Total	28	39	67
	41.79%	58.21%	100.00%

Fisher's exact = 0.002

The test was also done with all the species as well as the two species with the most numbers in this study. When all species were compared, p=0.125 was obtained. Comparison with the most populace species, *Mastomys natalensis* and *Mus musculus*, p=0.595 was obtained. Since in both tests, p>0.005, we could not demonstrate that species is associated with the presence of the bacteria.

The Fisher's exact test performed on all ages as well as grouped ages, adults and non-adults (sub-adults and juveniles combined) is as represented in Table 12 and Table 13 below. Since $p>0.05$ in both, it seems that the estimate age doesn't have any influence on a rodent's ability to carry leptospiral bacteria.

Table 12: Fisher's exact test used on the estimated age

Estimated age	**PCR results**		**Total**
	Positive	Negative	
Adult	21	34	55
	38.18%	61.82%	100.00%
Juvenile	3	1	4
	75.00%	25.00%	100.00%
Sub-adult	3	3	6
	50.00%	50.00%	100.00%
Total	27	38	65
	41.54%	58.46%	100.00%

Fisher's exact = 0.297

Table 13: Fisher's exact test on age groups

Age groups	**PCR results**		**Total**
	Positive	Negative	
Adults	21	34	55
	38.18%	61.82%	100.00%
Non-adults (juvenile and sub-adults)	6	4	10
	60.00%	40.00%	100.00%
Total	27	38	65
	41.54%	58.46%	100.00%

Fisher's exact = 0.297

5.2 Sequencing results

Results from the BLASTn searches confirmed the contigs generated were leptospiral secY genes. 25 partial sequences of the secY gene from different leptospiral species were chosen and aligned using MEGA6 with the sequences from samples in this study. Phylogenetic analysis revealed that all 6 positives chosen for sequencing were *L. interrogans* as they clustered in this group. It was also observed that common leptospiral species clustered together. This is shown in Figure 12 below. Accession numbers were used as labels on the phylogenetic tree and descriptions of their representative sequences are shown in Table 14 below.

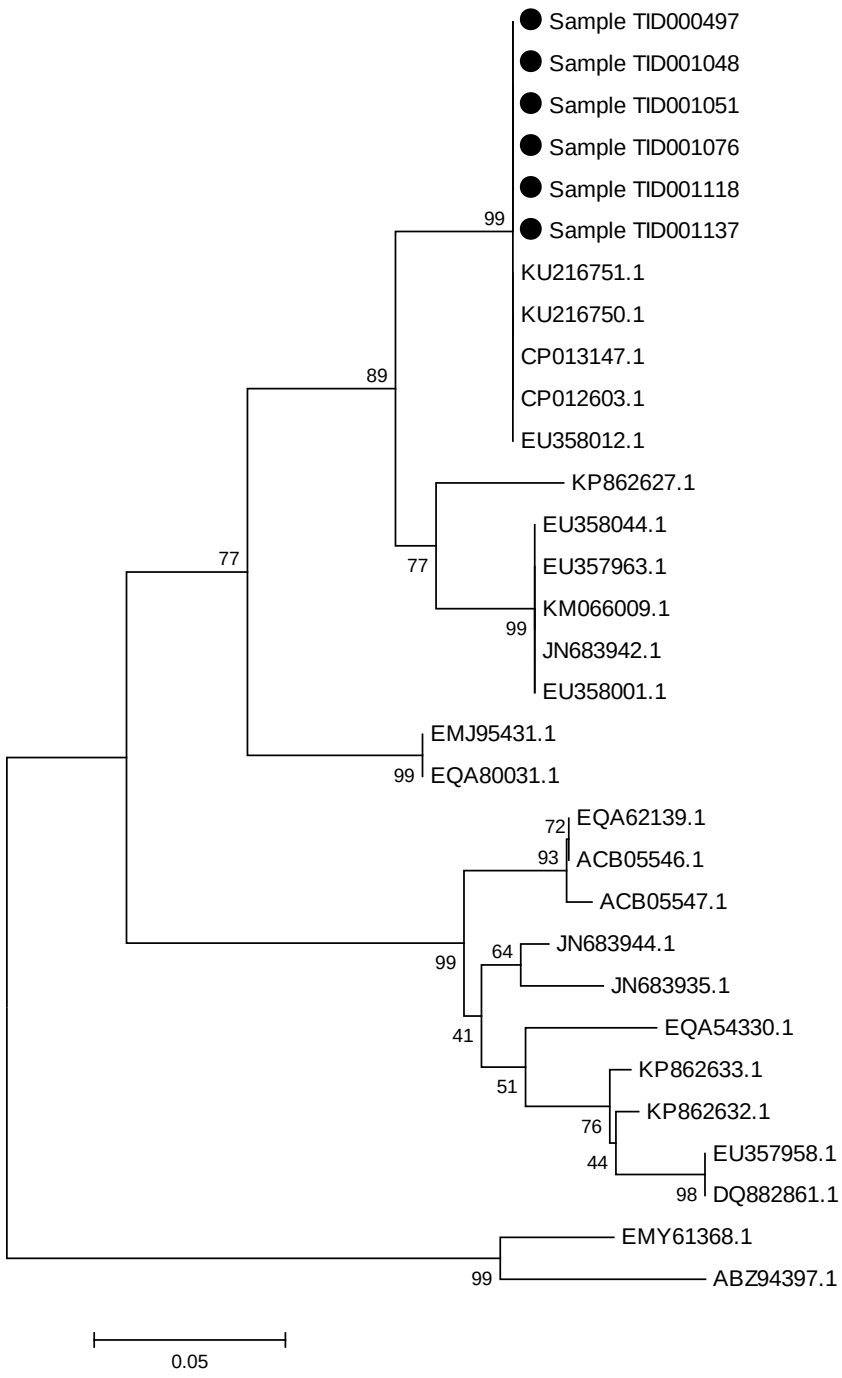

Figure 12: Phylogenetic tree of partial sequences of the secY gene of some positive rodent samples and 25 reference strains representative of various leptospiral species. The tree was drawn to scale. The length of branches is representative of evolutionary distances. Each node is supported by a probability next to it.

Table 14: Accession numbers used in construction of phylogenetic tree

Accession number	Description
KU216751.1	*Leptospira interrogans* serovar Hardjo-prajitno strain OMS protein translocase SecY (secY) gene partial cds
KU216750.1	*Leptospira interrogans* serovar Hardjo-prajitno strain Bolivia protein translocase SecY (secY) gene partial cds
CP013147.1	*Leptospira interrogans* serovar Hardjo-prajitno strain Hardjoprajitno chromosome 1 sequence
CP012603.1	*Leptospira interrogans* serovar Hardjo str. Norma chromosome I complete sequence
EU358012.1	*Leptospira interrogans* serovar Lai SecY (secY) gene partial cds
KP862627.1	*Leptospira noguchii* isolate 2013 U73 preprotein translocase (secY) gene partial cds.
EU358044.1	*Leptospira kirschneri* serovar Kunming strain K 5 SecY (secY) gene partial cds.
EU357963.1	*Leptospira kirschneri* serovar Kamituga SecY (secY) gene partial cds.
KM066009.1	*Leptospira kirschneri* strain 12-494 SecY (secY) gene partial cds.
JN683942.1	*Leptospira kirschneri* strain 201001687 SecY (secY) gene partial cds.
EU358001.1	*Leptospira kirschneri* serovar Ndambari SecY (secY) gene partial cds.
EMJ95431.1	*Leptospira alstonii* serovar Sichuan str. 79601 preprotein translocase SecY subunit
EQA80031.1	*Leptospira alstonii* serovar Pingchang str. 80-412 preprotein translocase SecY subunit
EQA62139.1	*Leptospira alexanderi* serovar Manhao 3 str. L 60 preprotein translocase SecY subunit
ACB05546.1	*Leptospira alexanderi* serovar Mengla partial preprotein translocase subunit SecY
ACB05547.1	*Leptospira alexanderi* serovar Manzhuang partial preprotein translocase subunit SecY
JN683944.1	*Leptospira borgpetersenii* strain 201002429 SecY (secY) gene partial cds.
JN683935.1	*Leptospira borgpetersenii* strain 200901118 SecY (secY) gene partial cds.
EQA54330.1	*Leptospira kmetyi* serovar Malaysia str. Bejo-Iso9 preprotein translocase SecY subunit
KP862633.1	*Leptospira santarosai* isolate 2013 U160 preprotein translocase (secY) gene partial cds.
KP862632.1	*Leptospira santarosai* isolate 2013 U152 preprotein translocase (secY) gene partial cds.
EU357958.1	*Leptospira santarosai* serovar Balboa strain 735 U SecY (secY) gene partial cds.
DQ882861.1	*Leptospira noguchii* serovar Panama SecY (secY) gene partial cds.
EMY61368.1	*Leptospira terpstrae* serovar Hualin str. LT 11-33 ATCC 700639 preprotein translocase SecY subunit
ABZ94397.1	*Leptospira biflexa* serovar Patoc strain apos Patoc 1 (Ames) apos Preprotein translocase SecY subunit

6. Conclusion

6.1 Discussion

There exist few studies that explore prevalence of leptospires in rodent carriers in North-Eastern Kenya, which is an ASAL and rural area of Kenya. A recent study of *Leptospira* in rodents in Kibera slum informed about the disease in urban settings of Kenya (Halliday, Knobel, Allan, *et al.*, 2013). Other major rodent studies of leptospirosis in the country date back to the sixties (Ball, 1966). This study is one of the few that demonstrated pathogenic leptospires in rodents in rural Kenya and, by extension, risk that it poses in these poor and marginalized communities.

Phylogenetic analysis showed that the sequences clustered in the *L. interrogans* group. The analysis also showed clustering of all sequences that were of the same species. Different species remained distinct from each other as well. The saprophytic species, *Leptospira terpstrae* and *Leptospira biflexa*, also clustered together and showed greater evolutionary distances from the rest of the species in the tree (these were pathogenic and intermediate species). This corroborates the utility of the secY gene in phylogenetic classification due to its ability to greatly discriminate *Leptospira* spp. This study therefore adds to the growing use of the gene in determination of leptospiral species in epidemiological studies. Determination of serogroups was not included in this study due to lack of funds. This however falls in areas of future exploration as it will aid in many control efforts for the disease.

Many studies done in urban settings have identified rats as posing great risk of transmission of human leptospirosis (Sarkar, Nascimento, Barbosa, *et al.*, 2002; Faria, Calderwood, Athanazio, *et al.*, 2008; Piedad, Londoño, Quiroz, *et al.*, 2009). Mice have also been shown

to pose disproportionate risk of human leptospirosis compared to other species in urban settings (Halliday, Knobel, Allan, *et al.*, 2013; Vanasco, Sequeira, Sequeira, *et al.*, 2003). This study however didn't show any association of the rodent species and age with the prevalence of the bacteria. The use of Sherman traps which have higher successes with trapping smaller and younger rodents may have left out larger rodents in this study, which have been shown to be more prevalent in carrying the bacteria than their smaller counterparts (Vanasco, Sequeira, Sequeira, *et al.*, 2003; Halliday, Knobel, Allan, *et al.*, 2013).

Rodent sightings have also been frequently reported among urban populations with high leptospirosis (Ko, Galvão Reis, Ribeiro Dourado, *et al.*, 1999; Halliday, Knobel, Allan, *et al.*, 2013). One of the inspirations of this study was the frequency of respondents to have sighted rodents during field work for the DDDAC project field work. The rodent population in the irrigated areas have also been noted by the DDDAC project to have increased, perhaps because of clearing of bushes as well as increased food in the farms as irrigation practices continue (Ontiri, Bett, Lindahl, *et al.*, 2014). While the risk posed by rodent sightings need case-control studies for better understanding, it is a recurring theme in areas with high leptospirosis prevalence. Though there are many channels of transmission of the bacteria to human and animal populations, transmission through rodents is effective because of the nature of the carriers – very abundant and diverse, widely distributed populations, having close contact with humans and their animals as well as interaction with wildlife (Meerburg, Singleton & Kijlstra, 2009).

This study shows that land use may have an effect in the prevalence of *Leptospira* in rodent carriers. Rodents collected from the pastoral regions (Ijara and Sangailu) had six times the

odds of carrying the bacteria than those from the irrigated regions (Bura and Hola). This could be attributed to the scarce water sources in the areas which allow for sharing among human populations, domestic animals, wild life and rodents. This may pose a greater risk of transmission of the bacteria. The close contact of humans and animals in pastoral areas also enhances transmission of the bacteria (Cacciapuoti, Nuti, Pinto, *et al.*, 1982) which could be picked up in animal carriers through urine contamination of scarce water points. The observation from the DDDAC project of higher seroprevalence in pastoralist communities when compared to those from the irrigated areas also corroborate this finding (Bett, Said, Sang, *et al.*, 2015). Collection of human samples in the DDDAC project was done in the same areas and time as those of this study. Results from this study however could be confounded by the fact that samples in the irrigated areas were collected when irrigation was on-going (wet season) and the pastoral areas during dry season. Future studies should factor in seasonality in the analysis of samples. There will also be great benefit to demonstrate the bacteria and local serotypes in domestic animals so as to elucidate the risk of transmission of the disease in the areas as well as know the circulating serotypes.

Land use changes have been linked to disease emergence in many parts of the world, with anthropogenic activities like irrigation, road construction, agricultural encroachment, wetland modification, deforestation, dam building, mining, coastal zone degradation and development of urban environments acting as drivers of disease (Patz, Daszak, Tabor, *et al.*, 2004). Maintenance of the bacteria in irrigated areas may be enhanced by the observed rise in populations of rodents as well as loss of tree cover and bush lands with increase in irrigated/cultivated land (Bett, Said, Sang, *et al.*, 2015; Ontiri, Bett, Lindahl, *et al.*, 2014). A rise in population of rodents habouring the bacteria could lead to increased disease

incidence among human and livestock populations. Investigation of land use changes and disease incidence in future will therefore greatly advise policy makers on the benefits and dangers of large-scale economic activities in rural populations and better inform control strategies that will cause no harm to human health and livelihoods.

Health inequities are also rife in these two counties. Key gaps in the health systems in Garissa include long distance to health facilities coupled with a lack of adequate transportation services and amenities, lack of hospital supplies and equipment, poor condition of hospital facilities, severe shortage of human resources for health (HRH) and limited services being offered. Basic diagnostic services are not available in the vast unpopulated area of the county except in the provincial general hospital in Garissa town (APHIAplusNAL, 2012). Similar inequities can be observed in Tana River County.

Because of this, there is need for strengthening of health systems in these areas so as to cater for emerging zoonotic diseases whose effect is felt the most by the impoverished. Proper diagnosis of such diseases at an early stage and early treatment with simple antibiotics will greatly curtail debilitating effects of leptospirosis and any sequelae. This also applies to animal leptospirosis which when diagnosed and treated early does not give rise to reproductive wastage in livestock. Studies such as this will inform public and animal health policy on the risk posed by such ‘silent diseases’ as leptospirosis.

Garissa has in the past had its fair share of insecurity, with the latest being the gruesome Garissa University College terrorist attack, and collection of samples for study is largely dependent on the prevailing peace. This, coupled with the remoteness of the area may explain the few studies that have been carried out in the area. This pilot study however has

not only demonstrated the disease in rodent carriers but also shown the feasibility of future studies in the counties.

6.2 Recommendations

There is need to explore more animal hosts from the study areas and demonstrate the circulating serotypes in the area. A one-health approach that considers human populations, domestic animals and wildlife will do the best in understanding the complicated transmission dynamics in areas that under the force of dynamic disease drivers.

PCR primers used in this study are known to confidently detect all pathogenic leptospires with the exception of *L. kirschneri* species. The G1/G2 primers used were not coupled with the *L. kirschneri*-specific B64I/B64II primers (Gravekamp, Van de Kemp, Franzen, *et al.*, 1993) due to lack of funding for this as well as a lack of positive control. The pathogenic leptospires may therefore be understated in this respect and future studies would better inform on prevalence of the disease if both primers are used.

Though this study utilized a small sample size, it is one of the few that has demonstrated the presence of the bacteria in rodent carriers in these remote areas of Kenya. This demonstrates the understated public health importance of leptospirosis in these areas. The use of a sample size that is more statistically representative of the number and species of rodents in the area will also reveal more on these rodent carriers of the disease.

7. References

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Appendix 1

1. **Preparation of 50X TAE buffer**

Needed

242g Tris Base (MW=121.1)
57.1ml Glacial Acetic Acid
100ml 0.5M EDTA

i. Measure Tris powder into reagent bottle and dissolve with 700ml of distilled water. Mix using a stir bar and a magnetic stirrer.
ii. Add glacial acetic acid and EDTA solution
iii. Top up the volume to 1L

To make 10L of 0.5X working solution, take 100ml of stock solution and top up to the 10L mark.

2. **Preparing a 2% 100ml agarose gel**

i. Assemble the gel tray and combs for creating the well. Ensure the platform for gel polymerization flat by using a leveler.
ii. Measure 2g of agarose powder and add 100ml of working solution of TAE buffer in a conical flask
iii. Melt the agarose and let it cool down
iv. Add 5µl of GelRed™ dye (Biotium, Hayward, CA, USA)
v. Pour into the gel tank and let it polymerize

Appendix 2

Table A1: Results of diagnostic analyses of samples analyzed using PCR test.

Sample ID	Sample type	Rodent ID	RESULTS	ng/µl	260/280	Selected for sequencing
TID001053	Blood	BRA IJ01	Negative	2.84	0.057	
TID001048	Kidney	BRA IJ01	Positive	41.04	0.821	*
TID001076	Kidney	BRA IJ02	Positive	155	3.1	*
TID001054	Kidney	BRA IJ03	Negative	8.61	0.172	
TID001021	Kidney	BRA IJ05	Weak Positive	13.41	0.268	
TID001012	Blood	BRA IJ06	Positive	5.78	0.116	
TID001031	Kidney	BRA IJ06	Positive	43.81	0.876	
TID001051	Kidney	BRA IJ07	Positive	54.59	1.092	*
TID001024	Kidney	BRA IJ08	Positive	25.48	0.51	
TID001137	Kidney	BRA IJ09	Positive	101.73	2.035	*
TID001118	Blood	BRA IJ10	Positive	117.18	2.344	*
TID001177	Kidney	BRA IJ10	Negative	31.69	0.634	
TID001213	Blood	BRA IJ11	Negative	7.53	0.151	
TID001123	Kidney	BRA IJ11	Negative	20.97	0.419	
TID001057	Kidney	BRA IJ12	Positive	26.07	0.521	
TID001011	Blood	BRA IJ13	Negative	5.93	0.119	
TID001211	Kidney	BRA IJ13	Weak Positive	19.23	0.385	
TID001281	Kidney	BRA IJ14	Negative	11.43	0.229	
TID001292	Kidney	BRA IJ15	Negative	36.08	0.722	
TID001187	Kidney	BRA IJ16	Weak Positive	26.4	0.528	
TID001282	Blood	BRA IJ18	Negative	4.75	0.095	

Sample ID	Sample type	Rodent ID	RESULTS	ng/µl	260/280	Selected for sequencing
TID001245	Kidney	BRA IJ18	Negative	39.93	0.799	
TID001330	Blood	BRA IJ19	Positive	17.52	0.35	
TID001229	Kidney	BRA IJ19	Negative	11.51	0.23	
TID001231	Blood	BRA IJ20	Negative	4.54	0.091	
TID001234	Kidney	BRA IJ20	Positive	71.1	1.422	
TID001251	Kidney	BRA IJ21	Negative	61.83	1.237	
TID000029	Blood	BRA1482	Negative	92.23	1.845	
TID000031	Kidney	BRA1482	Positive	333.48	6.67	
TID000037	Blood	BRA1483	Negative	15.97	0.319	
TID000044	Blood	BRA1484	Negative	130.95	2.619	
TID000099	Blood	BRA1485	Negative	365.64	7.313	
TID000504	Kidney	BRA1485	Positive	253.14	5.063	
TID000068	Blood	BRA1486	Positive	63	1.26	
TID000064	Blood	BRA1487	Positive	33.71	0.674	
TID000055	Blood	BRA1488	Negative	14.19	0.284	
TID000497	Kidney	BRA1489	Positive	235.42	4.708	*
TID000054	Blood	BRA1489	Negative	235.42	4.708	
TID000507	Blood	BRA1490	Negative	102.42	1.98	
TID000496	Blood	BRA1491	Negative	45.84	1.91	
TID000137	Blood	BRA1492	Negative	109.97	2.02	
TID000058	Kidney	BRA1493	Positive	280.17	5.603	
TID000479	Blood	BRA1494	Weak Positive	81.13	2.07	
TID000370	Blood	BRA1495	Negative	42.95	1.87	
TID000210	Blood	BRA1496	Negative	59.91	2.01	
TID000200	Blood	BRA1497	Negative	96.32	1.95	
TID000516	Blood	BRA1498	Negative	51.39	1.99	
TID000241	Kidney	BRA1499	Positive	8.19	1.22	

Sample ID	Sample type	Rodent ID	RESULTS	ng/µl	260/280	Selected for sequencing
TID000179	Blood	BRA1499	Negative	251.98	2.06	
TID000177	Blood	BRA1500	Negative	121.89	2.438	
TID000189	Blood	BRA1501	Negative	42.58	0.852	
TID000240	Blood	BRA1502	Negative	52.78	1.056	
TID000243	Blood	BRA1503	Negative	138.94	2.779	
TID000202	Blood	BRA1504	Negative	83.74	1.675	
TID000375	Blood	BRA1505	Negative	108.9	2.178	
TID000201	Blood	BRA1506	Negative	21.05	0.421	
TID000383	Blood	BRA1507	Negative	162.63	3.253	
TID000217	Blood	BRA1508	Negative	302.88	6.058	
TID000387	Blood	BRA1509	Weak Positive	1041.72	20.834	
TID000215	Blood	BRA1510	Negative	653.67	13.073	
TID000213	Blood	BRA1511	Negative	77.39	1.548	
TID000378	Blood	BRA1512	Negative	78.83	1.577	
TID000368	Blood	BRA1513	Negative	278.62	5.572	
TID000173	Blood	BRA1514	Negative	281.03	5.621	
TID000386	Blood	BRA1515	Positive	513	10.26	
TID000662	Blood	BRA1516	Positive	111.3	2.226	
TID000611	Blood	BRA1517	Negative	212.94	4.259	
TID000220	Blood	BRA1518	Negative	176.06	3.521	
TID000697	Blood	BRA1519	Negative	84.22	1.684	
TID000694	Blood	BRA1520	Negative	36.75	0.735	
TID000673	Blood	BRA1521	Negative	226.12	4.522	
TID000667	Blood	BRA1522	Negative	36.32	0.726	
TID000708	Blood	BRA1523	Negative	71.72	1.434	
TID000702	Blood	BRA1524	Positive	17.61	0.352	
TID000661	Blood	BRA1525	Negative	10.27	0.205	
TID001092	Kidney	BRA1527	Positive	5.26	0.105	

Sample ID	Sample type	Rodent ID	RESULTS	ng/µl	260/280	Selected for sequencing
TID001073	Blood	BRA1528	Positive	6.2	0.124	
TID001090	Kidney	BRA1528	Negative	16.73	0.335	
TID001038	Kidney	BRA1529	Weak Positive	10.95	0.219	
TID001022	Kidney	BRA1530	Negative	13.53	0.271	