MOLECULAR EPIDEMIOLOGY OF LEPTOSPIRA SPECIES AMONG AGROPASTORAL COMMUNITIES LIVING IN KATAVI-RUKWA ECOSYSTEM, TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Leptospirosis is an emerging zoonotic infectious disease which affects humans and animals worldwide as it causes febrile illness in humans. The disease has been reported in a number of human-livestock-wildlife interfaces of Northern and Eastern Tanzania. Very little is known of many zoonotic disease conditions in the research naive areas of Western and Southern Tanzania. This study aimed at detecting the prevalence of *Leptospira* species among agro-pastoralists at the human-animal interface areas of Katavi-Rukwa ecosystem. Microscopic agglutination test (MAT) was used to detect antibody against six *Leptospira* antigens including local serogroups Icterohaemorrhagiae, Ballum, Grippotyphosa, Sejroe and reference serogroups Hebdomadis and Lora. Samples with MAT titers ≥ 1:160 were scored as positive while MAT titers between 1:20 and 1:80 were scored as exposed to *Leptospira* and absence of agglutination titers was scored as negative. Of the 267 samples tested 80 (30%) were positive, 57 (21.3%) were negative and 130 (4 8.6%) were exposed to leptospiral infection. Infection rate in adults was higher 51 (63.75%) compared to children 29 (36.25%), $P<0.05$. Circulating serogroups were; Hardjo (15.7%); Icterohaemorrhagiae (8.98%), Grippotyphosa (4.87%), Hebdomadis (3.37%), Australis (1.49%) and Ballum (1.12%). Samples that were positive or scored as exposed by MAT were further tested using polymerase chain reaction (PCR) targeting *16S ribosomal* gene. Pathogenic *Leptospira* was detected in 33 (15.5%) out of 212 while no saprophytic *Leptospira* species was detected. Sequencing alignment based on *16S ribosomal* gene suggested *Leptospira interrogans, kirshinei* and uncultured *Leptospira* clone species as circulating species among agro-pastoralists of Katavi-Rukwa ecosystem. These findings suggest that in the Katavi leptospirosis in man is likely acquired from environment, probably by indirect contact with contaminated water or soil. This study also revealed that

serological diagnosis of leptospirosis should be considered in the diagnosis on non-malarial febrile illness in agro-pastoralists living in Katavi-Rukwa ecosystem, Tanzania.

DECLARATION

I, **Shabani Kililwa Muller**, do hereby declare to the Senate of Sokoine University of Agriculture, that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

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DEDICATION

To my parents for their love and encouragement

To my wife Eliza Dedan for her love and trust in me

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ABREVIATIONS AND SYMBOLS

µl	microliter
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CDC	center for disease control
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMJH	Ellinghausen, McCullough, Johnson and Harris
EtBr	ethidium bromide
FBS	fetal bovine serum
GIDEON	global infectious diseases epidemiological network
GLEAN	global leptospirosis environmental action network
IgG	immunoglobulin G
IgM	immunoglobulin M
IHA	indirect hemagglutination assay
KIT	royal tropical institute
L	leptospira
LAMP	loop-mediated isothermal amplification
LFA	lateral flow assay
MAT	microscopic agglutination test
MSAT	macroscopic slide agglutination test
NBS	national bureau of statistics
PBS	phosphate buffered saline

PCR	polymerase chain reaction
P-Value	probability (testing statistical significance)
RBC	red blood cells
RDT	rapid diagnostic test
RNA	ribonucleic acid
RT PCR	real time polymerase reaction
SOP	standard operating procedures
Spp.	species (plural)
Sp.	species (singular)
SUA	Sokoine university of agriculture
UK	United Kingdom
USA	United States of America
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Leptospirosis is a neglected zoonotic disease of worldwide public health importance which affects humans, domestic animals and wildlife (Feresu, 1987). The disease is caused by different serovars of *Leptospira interrogans* which belong to the order of Spirochaetales, family *Leptospiraceae*, genus *Leptospira* (Sitprija and Saovabha, 2011). Leptospirosis causes direct economic burdens to humans such as loss of productivity due to illness, sufferings, increased treatment costs and contribute to poverty in the affected countries (Pavli and Maltezou, 2010). Developing countries including Tanzania have significant burden of the disease with more than 500, 000 humans cases per year and a mortality rate ranging from 5 to 10% (GLEAN, 2013). The disease may represent up to 20% of febrile illness of unknown origin (GLEAN, 2013).

Leptospirosis is spread to human through direct contact with blood, tissues, organs and urine of infected animal reservoirs or indirect contact with contaminated water and soil (Smythe *et al.,* 2002). Humans are susceptible to infection with a variety of serogroups of *L. interrogans* but certain serovars show some degree of host specificity for instance; Serogroups Icterohaemorrhagiae mostly infect humans, serogroups Sejroe (serovars Hardjo) infect cattle, Canicola infect dogs and serogroups Pomona mostly infect pigs (Kessy *et al.,* 2010).

Symptoms of the disease are variable ranging from a mild febrile illness to severe disease forms such as potential lethal pulmonary syndrome, jaundice, hemorrhage, and acute renal failures (Bhattacharya *et al*., 2007; Boonsilp *et al*., 2011).

Leptospirosis occurs mostly in rural areas probably due to inadequate sanitation and poor housing which increase higher risk of exposure to animal reservoirs (Mohammed *et al*., 2011). The disease has been an occupational risk for farmers, livestock keepers, sewage workers and people exposed to contaminated water, soil and animals (Abela *et al*., 2010). Livestock farming plays a major occupational risk for human leptospirosis and cattle are among the source of infection to pastoralists (Swai and Schoonman, 2012). Rodents are the major reservoirs for leptospirosis; they maintain the infection in nature and serve as sources of infection to humans and animals. *Leptospira interrogans* maintain themselves in the kidney of infected rodents and other reservoir hosts for a long time from where they shed with urine to the environment (Levett, 2001). Climatic factors and environmental conditions such as alkaline soils, mud, swamps, streams and rivers favor the survival of *Leptospira* outside the host (Mahlaba *et al*., 2012). Microscopic agglutination tests (MAT) is regarded as the standard serological method for diagnosis of leptospiral infection which detects antibodies against different leptospiral serogroups in humans and animals (Abela-Ridder *et al*., 2010; OIE, 2014). However, MAT is time consuming, tedious and is subjected to cross-reactions with different serogroups (Pavli and Maltezou, 2010). The diagnosis has been improved by developing molecular techniques such as Polymerase Chain Reaction (PCR) and strain typing which are more robust, specific and sensitive compared to MAT (Mohammed *et al.,* 2011).

Previous studies of leptospirosis conducted among febrile patients and in farmers living in Moshi and Tanga reported serological prevalence of 8.8% and 15% respectively, (Biggs *et al*., 2011; Swai and Schoonman, 2012). However, there are limited reports of leptospirosis studies conducted in the Katavi-Rukwa ecosystem where interaction between humans, animals and wildlife is high.

1.2 Problem Statement and Study Justification

Leptospirosis is an emerging zoonotic disease which affects humans and animals worldwide (Bertherat *et al.,* 2014). The disease is responsible for serious suffering, death and imposes financial burdens on the affected societies. It is one of the febrile illnesses that are often confused with many other diseases that are endemic and epidemic in similar environmental and climatologic conditions such as dengue, rickettsiosis, enteric fevers and malaria (Hartskeerl and Ellis, 2011). This resemblance makes the disease to be misdiagnosed and the most underestimated and neglected disease in Tanzania (Biggs *et al.,* 2011).

Although decreased prevalence of malaria has been reported, cases of febrile illness continue to be reported. The increase in cases of febrile illnesses may be due to increased incidence of other causes of febrile illnesses that are not routinely diagnosed as the standard of care including leptospirosis. There is limited information of leptospirosis in human subjects in South-western part of Tanzania including Katavi-Rukwa ecosystem. Lack of knowledge on the circulating pathogenic *Leptospira* serogroups predisposes the Katavi community to serious febrile illness and socioeconomic impacts. The present study was carried out in order to understand the prevalence and molecular epidemiology of leptospirosis among Agro-pastoral communities living in Katavi-Rukwa ecosystem.

1.3 Research Objectives

1.3.1 Overall objective

To assess molecular epidemiology of leptospiral infection among agro-pastoral communities of Katavi-Rukwa ecosystem

1.3.2 Specific objectives

(i) To determine seroprevalence of leptospiral infection among agro-pastoral communities in Katavi-Rukwa ecosystem.

(ii) To determine the major *Leptospira* serogroups among agro-pastoral communities in Katavi ecosystem.

(iii) To determine *Leptospira* spp. circulating in Katavi-Rukwa ecosystem using molecular techniques.

1.3.3 Research questions

(i) What are the circulating *Leptospira* serogroups among agro-pastoralists of Katavi-Rukwa ecosystem?

(ii) What is the prevalence of leptospiral infection among agro-pastoralists of Katavi-Rukwa ecosystem using serology and molecular techniques?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Leptospirosis

Leptospirosis has been recognized for more than a century as a direct zoonotic disease caused by pathogenic spirochetes of the genus *Leptospir*a (De Vries *et al*., 2014). The disease is characterized by sudden onset of fever chill, jaundice, myalgia and renal failure. Leptospirosis has been known as an emerging global public health problem because of its increasing incidence in both developing and developed countries, commonly in tropical countries with heavy rainfall (Abela *et al*., 2010). The burden of leptospirosis is estimated to be more than 1.7 million severe cases of human leptospirosis which occurs annually with a fatality rate of about 8% (GLEAN, 2013).

In animals the disease is seen in almost all mammals and frequently in rodents because of their alkaline condition in urine. They harbor *Leptospir*a in the proximal renal tubules and may be leptospiruric throughout their lifetime (Galton *et al*., 1958). Other animals includes, cattle, pigs, dogs, goats, hamsters, voles, gerbils, coypus, shrews, hedgehogs, horses and bats have been also reported to carry the organism (Holt *et al*., 2006; Ahmed *et al*., 2013; Mgode *et al*., 2005). Humans commonly became infected through occupational, recreational or domestic contact with infected urine or animal fluid, or direct contact with contaminated water or soil (WHO, 2010).

2.2 *Leptospira* Species and Serogroups

Taxonomically, the bacterial agent is classified in the order of *Spirochaetales*, family; leptospiraceae, genus; *Leptospira* and species; *interrogan.* Surface antigens are used to define serological variation or serovars and genome studies are used to define species, each *Leptospira* sp. has its own ecology with some variations worldwide. Serovars is the

least taxonomic unit used to classify *Leptospira*. A group of serovars sharing the same antigenic structures are grouped as serogroups (Mgode *et al*., 2004). Serovars can serve in identification of the host reservoir which is important in epidemiology and it is the lowest unit of taxonomic importance (Schoonman and Swai, 2009).

According to their serological characteristic, *Leptospir*a are classified into more than 240 serovars, which differ on the basis of their structural heterogeneity in the carbohydrate component of lipopolysaccharide. The most common serovars are: serovars Hardjo, Pomona, Grippotyphosa, Canicola and Icterehemorragea (Romero and Yasuda, 2006; Bourhy *et al*., 2010; Mohammed *et al.,* 2011; Sitprija and Saovabha, 2011).

Based on their deoxyribonucleic acid (DNA) *Leptospira* spp. are divided into 20 species. Nine species including *Leptospira interrogans, L. kirschneri, L. noguchii, L. alexanderi, L. weilii, L. genomos- pecies1, L. borgpetersenii, L. santarosai and L. kmetyi are pathogenic. Five* species *(L. inadai, L. fainei, L. broomii, L. licerasae and L. wolffii)* are intermediate group. Six species including *L. biflexa, L. meyeri, L. wolbachii, L.genomospecies 3, L. genomospecies 4 and L. genomospecies* 5 are nonpathogenic (Sitprija and Saovabha, 2011; Ahmed and Grobusch, 2012).

2.3 *Leptospira*

Leptospira are obligate aerobic spirochetes. Their size ranges between 5 to 20mm long and 0.1mm wide with a hook at one or both ends of the cell which differ them from other spirochetes (Fig.1). *Leptospira* are highly motile and use corkscrew motion with movement in the direction of a straight end, which is best seen using a dark field microscope. The bacteria grow well at the temperature of 28 to 30.8°C in serum-containing semisolid medium such as Fletcher medium, Stuart' medium and Ellinghausen,

McCullough, Johnson and Harris (EMJH) medium, and use fatty acids or fatty alcohols with 15 or more carbon atoms for carbon and energy sources (Smith *et al*., 1994). These bacteria do not resist drought or hypertonicity but they support alkalization to pH 7.8 (Smith *et al*.,1994).

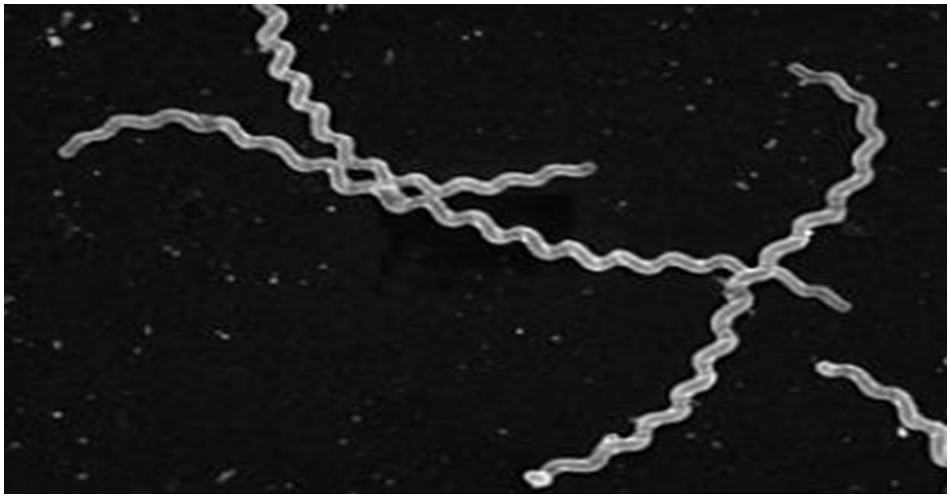

Figure 1: Morphological appearance of *Leptospira Interrogans* observed under electronic microscope (Source: WHO, 2010).

2.4 Transmission of Leptospirosis

Leptospiral infection is transmitted to humans through direct or indirect contact of mucous membranes or skin abrasions with urine from infected animals or contaminated freshwater surfaces, including mud or water in lakes, rivers, and streams. Ingestion or inhalation of contaminated water or aerosols may also result in infection. Infection has been occasionally reported following animal bite, laboratory accident, blood transfusion, organ transplantation, breast feeding, sexual intercourse, and through congenital transmission. Transmission between humans is very rare (Pavli and Maltezou, 2010).

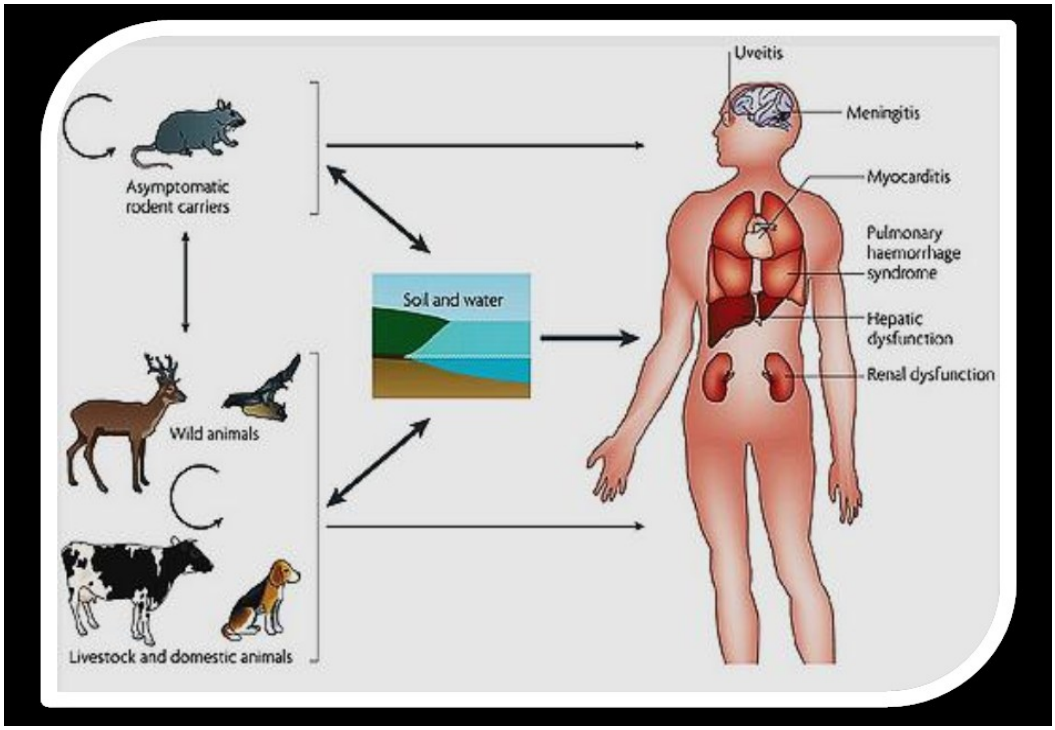

Figure 2: ***Leptospira*** **life cycle and pathogenesis (source: GLEAN, 2013)**

2.5 Geographical Distribution of Leptospirosis

2.5.1 Geographical distribution of leptospirosis in the world

Leptospirosis is distributed worldwide in both developed and undeveloped countries; however the most affected parts are tropical countries due the favorable conditions which allow survival of the pathogenic *Leptospir*a in the environment (De Vries *et al*., 2014).

In Latin and Central America; several outbreaks have been reported in humans. Brazil is more affected with about 70% of cases of leptospirosis occur in the urban areas. Romero and Yasuda (2006) managed to characterize forty clinical samples suspected of leptospiral infection using real time PCR in Brazil. Thirty-nine of these were determined to belong to serogroup Icterohaemorrhagiae (97.5%) and one to serogroup Sejroe (2.5%) (Romero and Yasuda, 2006). Nicaragua accounted 1,980 cases of leptospirosis between the year 2004 and 20010 (GLEAN, 2013).

In Sri Lanka leptospirosis was found to be a frequent cause of acute febrile illness in patient admitted to southern Sri Lanka hospitals. Of 889 patients with paired specimens, 120 had acute Leptospirosis and 241 had past leptospirosis, however serological method was used to diagnose *Leptospira* in this study (Reller *et al*., 2008). France reported the highest number of cases in Europe in 2005. The disease rates varied from 1.7 to 7.9 cases per 100 000 in part of France. The principal serogroups are Icterohaemorrhagiae (30% of cases), Australis, Grippotyphosa (23% of cases), Sejroe, Ballum, Hebdomadis, Canicola, Bataviae and Pomona (GLEAN, 2013).

In Africa limited reports have been documented and more than 50 % of reports are from Southwest of Indian Ocean islands. In Mauritius the burden of leptospirosis has been reported to be 2.5 cases per 100 000 inhabitants. Seychelles Islands were considered in the nineties as the hottest spot of leptospirosis in the world with an incidence of 101 cases per 100 000. Currently, the burden of leptospirosis has decreased with an incidence rate of 17 per 100 000 inhabitants in 2013. However, fatality remains high estimated to be 16% (De Vrie *et al*., 2014). Mayotte is currently consider as the high spot for leptospirosis in the Southwest Indian Ocean islands with an incidence rate ranging from 59 to 87 cases for 100 000 inhabitants in the last years (Bourhy *et al.,* 2010; De Vrie *et al*., 2014). Multiple exposure of the general population to *Leptospira* spp. during activities of daily living including farming and livestock keeping are among the reasons of high prevalence of leptospirosis in Mayotte (Lernout *et al*., 2012). In Nigeria and Ghana; West Africa the countrywide prevalence ranges between 20 and 35 % respectively (De Vrie *et al.*, 2014). A seroprevalence of 15.7% in healthy people was demonstrated in five villages in the northeast of Gabon Central Africa (De Vries *et al*., 2014).

In East Africa, human infection was first reported in Kenya by Forester *et al.* (1969). In 1987, 7.4% of 353 healthy people in Nyanza province and 16.9% of 130 in Coast province were found to have leptospiral antibodies. A recent outbreak investigation of malaria-negative fever in 21 patients in Malindi in Coast province of Kenya could not confirm leptospirosis (De Vries *et al*., 2014).

In Ethiopia a serological survey conducted in 2004 showed that 47.5% of 59 febrile patients in Wonji tested positive for leptospiral antibodies with the rapid diagnostic test LeptoTek Dri-Dottest (De Vries *et al.,* 2014). In most African countries there is scarce information of human leptospirosis.

2.5.2 Geographical distribution of leptospirosis in Tanzania

There is limited knowledge of leptospirosis in human subjects in Tanzania. Substantial works have been done in animal subjects. In 2005, new pathogenic *Leptospir*a serogroups Icterohaemorrhagiae (serovars Sokoine) was isolated from cattle in Morogoro, Tanzania posing health threat to farmers and livestock keepers in Morogoro (Mgode *et al*., 2005). A survey on zoonotic diseases in trade cattle in Tanga Region, northeastern part of Tanzania showed that leptospirosis was a public health concern accounting 51% prevalence in cattle slaughtered at the abattoir (Swai and Schoonman, 2012).

The first study on leptospirosis in humans from selected areas of Tanzania including Mtibwa, Katavi, Lushoto, Lower Moshi, Dar es Salaam, Mbeya, Mwanza, Morogoro and Sangasanga reported a prevalence of 0.3 %. Reactivity was observed to samples from Dar-es-salaam which reacted to serogroups Grippotyphosa only (Machang'u *et al.*, 1997).

Schoonman and Swai (2009) recounted a prevalence of leptospiral infection of 15.1% among the occupational group including agro-pastoralists living in Tanga (Schoonman and Swai, 2009). A recent report on leptospirosis among hospitalized febrile patients in Kilimanjara and Moshi reported a prevalence of 8.8 %, and stressed that leptospirosis was a major cause of febrile illness (Biggs *et al*., 2011). The predominant infecting serogroups were: Mini, Australis, Autumnalis, Grippotyphosa, Canicola, Celledoni and Djasiman. These reports showed that leptospirosis is a public health threat in Tanzania but still the disease is neglected and under estimated.

2.6 Clinical Signs of Leptospirosis in Human

The clinical signs vary from mild to severe fatal symptoms; in human the presentation of the illness is associated with an acute biphasic febrile illness with or without jaundice. The primary stage is characterized by presence of spirochete in blood and bodily fluid of the patient, this stage will last from three to seven days. Clinical symptoms during this section include; headache, high fever, chill, muscle pain, cough, nausea and innate reflex (Bhattacharya *et al*., 2007; Boonsilp *et al*., 2011).

This section is followed by immune section, throughout this second section the microorganism is cleared within the blood however; they would possibly move to eyes, urinary organ and cerebral body fluid. The severe forms of zoonosis area unit characterized by multi-organs failure together with liver and urinary organ and sever hemorrhages resulting in death. Rodents are considered as major maintenances host while humans are accidental host. Infection is through contact with urine or tissue from the sick animals (Adler *et al*., 2011; Mohammed *et al*., 2011).

2.7 Detection and Identification of Leptospiral Infection

Different techniques have been applied to diagnose *Leptospira* infections in humans and animals; the commonly used include; direct microscopic demonstration of clinical specimens, culture and isolation of the organism in EMJH media, serological tests (ELISA, cross absorption and agglutination test, lateral flow and Microscopic Agglutination Test) and molecular techniques including PCR, restriction length fragment polymorphism, RT-PCR and sequencing (González *et al*., 2011; Picardeau *et al*., 2014; Thaipadungpanit *et al*., 2011). The choice depends on whether the aim is surveillance or confirmation of the disease and whether the disease is acute or chronic (Dorjee, 2007). However; Microscopic Agglutination titer (MAT) is one of the serological test mostly used and is considered as the gold standard method for diagnosis of leptospirosis (Mahlaba *et al*., 2012). This work has based on MAT, Polymerase Chain Reaction (PCR) and sequencing which were within the scope of the dissertation.

2.7.1 Serological techniques

2.7.1.1 Microscopic agglutination test (MAT)

Microscopic agglutination test is a serial dilution of sera kept in contact with an equal volume of a well grown suspension of *Leptospira* serovars at a certain temperature for a certain period of time. The mixture is read microscopically by estimating 50% agglutination as the end point titer of the reaction (Korver, 1988). It works on the principle that the test sera react with antigens on the surface of the bacteria and agglutinate them. This method was first described by Schuffner and Mochtar in 1926 and improved by others (Cole *et al*., 1973).

MAT detects antibody against *Leptospira* usually after seven to ten days after the onset of the disease. In general it detects IgG and IgM present in sera of people who have been

exposed or currently infected *Leptospira* spp. higher agglutination titer is presumptive of current infection. MAT is considered as the diagnostic standard for leptospirosis (OIE, 2014).

2.7.1.2 Enzyme-linked immunosorbent assay (ELISA)

Enzymes linked immune sorbent assays are among the traditional serological techniques used for the diagnosis of leptospirosis based on the principle of antibody-antigen interaction. Numerous commercial IgM ELISAs have been developed for detection of antibodies against whole cell *Leptospira*. In general; the antigens used for ELISA may not recognize the diversity of circulating strains, and the overall sensitivity of these tests is poor. A conclusive serological diagnosis of leptospirosis cannot be made by ELISA alone but needs laboratory confirmation through MAT, PCR, or culture (Picardeau *et al*., 2014). However; ELISA can detect *Leptospira* antibodies earlier in the course of the disease than MAT. This is because anti-*Leptospira* IgM antibodies are not detectable before 4 to 5 days after onset of symptoms. They appear earlier than IgG and agglutinating antibodies (Radostitis, 2006).

2.7.1.3 Lateral flow assay (LFA)

LFA is usually made of colloidal gold labelled anti- IgM antibody specific for human or animal patient. The assay can be performed at the bedside of the patient using a whole blood (finger prick) and results can be obtained in 10 minutes. An LFA is based on whole cell antigen extract from the saprophyte *L.biflexa*. It enables rapid detection of *Leptospira* specific IgM antibodies in human sera (Erol *et al*., 2014). The assay is available as a commercial test but showed performances that are similar to ELISA and has been tested in the field (Picardeau *et al*., 2014).

2.7.1.4 Indirect hemagglutination assay (IHA)

IHA uses red blood cells sensitized with an extract of an erythrocyte-sensitizing substance from *L. biflexa* serovar Patoc, (Erol *et al*., 2014). IHA detects both IgM and IgG antibodies. Heat-inactivated serum is mixed with sensitized red blood cells, and agglutination is examined by the naked eye. Estimates of the sensitivity of the IHA in populations in which leptospirosis is endemic have varied from good to poor , possibly because of differences in case ascertainment and study design, including inclusion of epidemiological distinct populations and the unavailability of prospective unbiased samples (Ahmed *et al.,* 2014).

Other serological techniques like immunofluorescence, indirect heamagglutination assays and the macroscopic slide agglutination test (MSAT) have also been used for diagnosis of leptospiral infection however, their sensitivities and specificities are poor compared to MAT and molecular techniques (Picardeau *et al*., 2014).

2.7.2 Molecular based techniques

Molecular techniques have been used to supplement serological techniques. Most of them detect part of *Leptospira* DNA or they whole genome in clinical or environmental samples.

2.7.2.1 Genome of *Leptospira*

Deep sequencing method has revealed the genome of *Leptospira*. The genome of pathogenic *Leptospira* consists of two chromosomes. The size of the genomes of *L. interrogans* serovars Copenhageni and Lai is approximately 4.6. However, the genome of *L. borgpetersenii* serovar Hardjo is only 3.9 megabytes in size with a large number of pseudogenes, gene fragments, and insertion sequences relative to the genomes of *L. interrogans* (Bulach *et al.,* 2006). *L. interrogans* and *L. borgpetersenii* share 2708 genes

from which 656 are pathogenic specific genes. The guanine plus cytosine (GC) content ranges between 35% and 41% (Adler *et al*., 2011). *L. borgpetersenii* serovar Hardjo is usually transmitted by direct exposure to infected tissues, whereas *L. interrogans* is often acquired from water or soil contaminated by the urine of carrier animals harboring *Leptospira* in their kidneys. The high number of defective genes and insertion sequences in *L. borgpetersenii* Hardjo together with the poor survival outside of the host and difference in transmission patterns compared to *L. interrogans* suggest that *L. borgpetersenii* is undergoing insertion-sequence mediated genomic decay, with ongoing loss of genes necessary for survival outside of the host animal (Adler *et al*., 2011, Bulach *et al*., 2006).

2.7.2.2 Polymerase chain reaction (PCR)

PCR is an extremely powerful technique developed in 1983 by Mullis. It allows amplification of any specific sequence of genomic materials. Conventional PCR was introduced as a promising molecular detection method of *Leptospira* spp. in biological materials in 1989 (Thaipadungpanit *et al*., 2011). Initially this technique used a set of primers taken from genomic libraries to specifically detect serovars Hardjo Bovis (Van Eys *et al*.,1989). Later other primers pairs designed from varieties of genes including *rrs, rrl, flaB, gyrB*, and *ompl* genes have been used for detection of pathogenic leptospirosis (Ahmed *et al*., 2012). To increase sensitivity and specificity other primer pair targeting repetitive element and nested PCR were introduced (Ahmed *et al*., 2012). Comparative studies between culturing and PCR techniques in detection of leptospiral infection found that PCR was more sensitive (62%) than culture (48%) (Romero *et al*., 2013). Other studies compared conventional PCR over MAT, but its high sensitivity was not clearly explained due to cross-contamination of MAT (Romero *et al*., 2013).

Real time PCR which is a PCR based amplification of DNA that is monitored during the amplification process, has been introduced as a rapid and sensitive alternative to conventional PCR method. This method targets a variety of genes including *rRNA*, housekeeping genes, specific leptospires sequences and genes confined to pathogenic *Leptospira* (Ahmed *et al*., 2012). In 2009, Stoddard and others have developed a real-time polymerase chain reaction PCR assay using a TaqMan probe targeting lipL32 for detection of pathogenic species (Stoddard *et al*., 2009). The technique was highly sensitive and specific and was proved to be a rapid diagnosis of leptospirosis.

Several other real time PCR methods have been validated for clinical diagnosis of leptospirosis and showed high accuracy on blood samples at the early acute phase of the diseases. For instance Reller *et al*. (2014) has recently reported unsuspected leptospirosis as a cause of acute febrile illness in Nicaragua where 44 (6.3%) out of 704 patients were confirmed as *Leptospira* positive by using real time PCR. Another study targeting *rrs* genes and *rpoB* genes have been used to study geographic distribution of *Leptospira* in different areas of Russia by using real time PCR (Voronina *et al*., 2014).

2.7.2.3 Sequencing techniques and phylogenetic analysis

Introduction of the sequenced based determination techniques has created an important contribution to new insights of the molecular medicine of *Leptospira* and therefore the taxonomy of spirochetes (Ahmed *et al*., 2012). For identification and characterization of *Leptospira* spp.; sequencing based on *16SrRNA* gene followed by phylogenetic analysis have been widely used by many researchers to study the genome and evolutionary relationship of *Leptospira* (Rettinger *et al*., 2012; Zakeri *et al.*, 2010). The *rrs* gene is widely conserved in the genus of *Leptospira* and it has been used for classification of eubacteria (Amhed *et al*., 2012). The phylogenetic analysis based on the same gene has

classified *Leptospira* genus into tree major clades including pathogenic, intermediate and saprophytic species (Ahmed *et al*., 2011). Although presently many additional variable genes are targeted for spirochetes classification, the *rrs* gene is still the most typically used for identification of *Leptospira* spp. using either direct sequencing or deep sequencing technique (Ahmed *et al*., 2011).

Direct sequencing or partial sequencing technique has been used by many researchers to complement serology techniques by clearly distinguished pathogenic from nonpathogenic *Leptospira* spp. using either clinical or environments samples. For instance Boury *et al.* (2013) used partial sequencing technique to identify five pathogenic *Leptospira* spp. circulating in French West Indies which could not easy be identified by serology. Deep sequencing or whole genome sequencing methods have contributed in understanding genetic and morphologic properties of the genus *Leptospira* including, pathogenesis, genome size, protein composition, protein functions, transmission and adaptability of *Leptospira* spp. in environment and different host species. Other sequencing related technique like ribotyping and multiple-locus variable number of tandem repeats analysis have been also used for identification and characterization of *Leptospira* spp. (Ahmed *et al.*, 2011).

2.7.2.4 Isothermal methods

Isothermal amplification is an attractive alternative to PCR-based methods since thermocyclers are not required. It simply requires a heating device to maintain a constant temperature between 60°C and 65°C, making it particularly suited to resource-limited settings. The techniques applied to leptospirosis include; nucleic acid sequence–based amplification, loop-mediated isothermal amplification (LAMP), helicase-dependent

amplification, rolling circle amplification, and strand displacement amplification (Sonthayanon *et al.*, 2011). In LAMP the amplified DNA can be detected by visual inspection of fluorescence or turbidity, without the need for gel electrophoresis (Mori and Notomi, 2009). The method may also enable direct amplification from clinical specimens, thereby eliminating the need for an additional nucleic acid purification step. The specificity of LAMP assays was moderate to low, and the limit of detection was determined between 2 and 100 *Leptospir*a per reaction mixture (Picardeau *et al*., 2014).

2.8 Social economic Importance of Leptospirosis

Leptospirosis, as a neglected zoonotic disease, imposes direct economic burdens to humans such as, death and loss of productivity due to illness particularly in poor rural communities and treatment costs (WHO, 2010). The disease reduces livestock productivity by causing losses primarily because of its effects on reproductive performance such as abortion, stillbirth, and birth of reduced viability weak offspring as well as mortality. It also decreases milk production in livestock. These effects cause food insecurity and poverty to the communities (Radostitis, 2006; WHO, 2010). Delayed disease detections and inadequate collaboration between sectors result to catastrophic socio economic impacts.

2.9 Treatment, Control and Prevention of Leptospirosis

Leptospira can be killed by a wide range of antibiotic such as penicillin. Doxycycline would usually be an appropriate initial antimicrobial treatment for an individual with either suspected leptospirosis. Azithromycin could be considered as an alternative treatment whenever doxycycline allergy is suspected. A human vaccine against leptospirosis was developed in the 1960s in France as a response to the sewer workers in Paris being strongly affected by this zoonosis. The vaccine, made of one inactivated

bacterial strain (serogroup Icterohaemorrhagiae, strain *verdun*), received its market authorization in 1979. It can be used in population highly at risk; however diversity of serogroups affect efficacy of the vaccines (GLEAN, 2013). Good hygienic conditions, elimination of rodents, proper disposal of infected animal carcass are different ways of preventing leptospirosis.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Katavi-Region (Katavi Rukwa ecosystem) specifically in Isinde, Kapalala, Mtakuja II, Mtakumbukwa, Mtandarani, Mamba, Nsimbo, Sitalike and Songambele villages (Fig. 3). All villages where the study was conducted belong to Mlele and Nsimbo districts (Fig. 3). Mlele and Nsimbo districts border two protected areas of Katavi National Park and Rukwa Game Reserve. Geographically, Katavi is a research naive area located between latitude 60^0 30' 00''South and longitude 31^0 30' 00''East (Banda *et al*., 2008). It experiences tropical climatic condition and heavy rain fail which may favor survival of *Leptospira* spp.

The main economic activities are agriculture, livestock keeping, honey production and fishing. The population size of Katavi is about 564 604 (NBS, 2012). There are also increased number of populations of livestock immigrants from Shinyanga and Tabora who are pastoralists and are intensively involved in livestock and farming activities next to the national park and game reserve, hence creating high interaction between humans, domestic animals and wildlife. This interaction exposes the community at higher risk of zoonotic infections including leptospirosis.

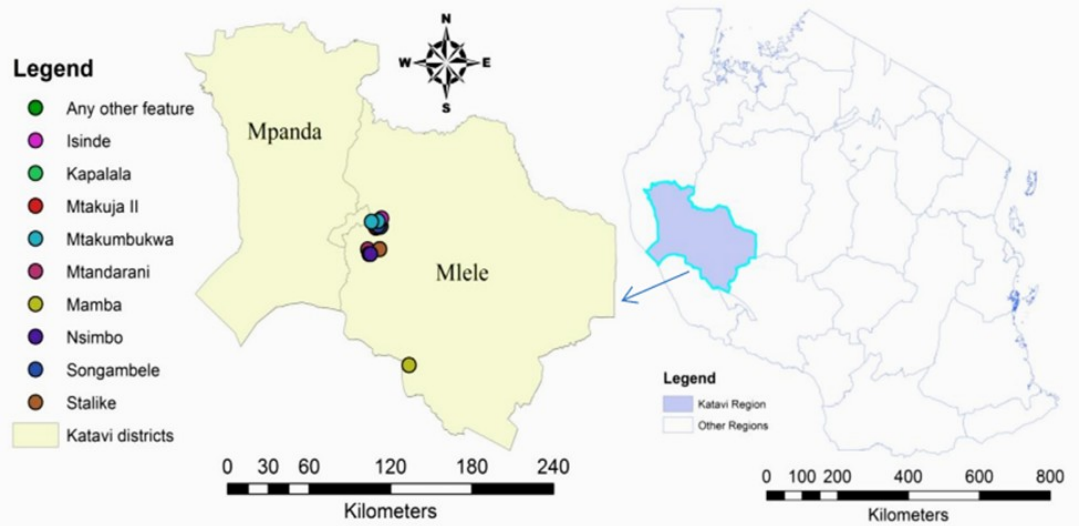

Figure 3: Map of Katavi region showing the study villages including Mlele and Mpanda districts.

3.2 Research Design

The research design for the present study was cross-sectional epidemiological study, in which a multistage cluster sampling was carried out. Villages were randomly selected as the primary unit, from which a total of 138 households were chosen from the list of agro-pastoralists using random numbers. Members of the selected households were subjected to a random selection to obtain a total of 267 humans who were readily available, regardless of their health status. The target was households with domestic animals (Assenga *et al.*, 2015).

The study was carried out between September 2012 and April 2013 in the Katavi region, southwest Tanzania, which is an agro-pastoral community with a wide range of domestic animals and wildlife (Assenga *et al*., 2015).

The expected sample size was calculated using EpInfo software version7 population survey and descriptive study. The expected prevalence was assumed to be 22% at 95%

confidence interval. Study participants were grouped according to age including children (age 2 to < 13 years), and adults (age ≥13). Children were enrolled only after the informed assent was obtained from either parents or guardians and adults consented themselves. Samples with MAT titers ≥ 1:160 were scored as positive; titers between 1:20 and 1:80 were scored as exposed to leptospiral infection and absence of agglutination titers was scored as negative (Machang'u *et al*., 2004; Machang'u *et al*.,1997; Schoonman and Swai, 2009).

3.3 Ethical Clearance

This study was cleared by Institutional Review Board of Sokoine University of Agriculture and was a part of the ongoing Afrique One project at SUA Morogoro, whose main objective was to study the non-malarial causes of febrile illness. Ethical clearance was provided by the Medical Research Coordinating Committee of the National Institute for Medical Research (Permit number: NIMR/HQ/R8a/Vol.1X/1627). Recruited subjects participated in the study after obtaining informed consent.

3.4 Sample Collection

A total of 5 to 10 ml of peripheral blood was collected into a plain and EDTA vacutainer tube (Becton Dickson, Dublin, Ireland) from each participant for serological and molecular diagnosis respectively. Each sample was labeled using a unique identification number. Samples were transported in cool boxes within 12 hours after collection to Mpanda District Hospital (MDH) for processing and storage. Clotted blood in the plain tubes was centrifuged at 3000 rpm for 10 min to separate clear sera. Eluted sera and blood in EDTA were finally stored in liquid nitrogen and transported to Sokoine University of Agriculture laboratory and stored at -80 °C upon arrive for further laboratory use.

3.5 Serological Study

3.5.1 Detection of *Leptospira* antibodies

A MAT test was performed to detect *Leptospira* antibodies by following WHO guidelines for diagnosis of leptospirosis, surveillance and control (WHO, 2003). Sera were tested for the presence of anti-leptospira antibodies in which six live *Leptospira* serogroups including local *Leptospira* serovars Sokoine (serogroup Icterohaemorrhagiae), serogroup Ballum (serovar Kenya), Austalis, Grippotyphosa, reference serogroups Sejroe and Hebdomadis were used antigens. Reference serogroups were kindly provided by Royal Tropical Institute (KIT) *Leptospira* reference center, The Netherlands, (Table 1).

Table 1: Characteristics of antigen used for MAT panel

Serogroup	Serovars	Species	References
Icterohaemorrhagiae	Sokoine(local isolate)	*L.kircsheri*	Mgode *et al.*, 2006
Australis	Lora (local isolate)	*L.interrogans*	Faine *et al.*, 1999
Ballum	Kenya(local isolate)	*L.borgpetersenei*	Machangu *et al*, 2004
Grippotyphosa	RM4 (local)	unknown	unpublished
Sejroe	Hardjo	*L.interrogans*	Faine *et al*., 1999
Hebdomadis	Hebdomadis	*L.santarosae*	Faine *et al*., 1999

Briefly, 10 µl of sera were titrated in a U-bottom microtitre plates (Sigma-Aldrich, St Louis, USA) containing phosphate buffered saline (PBS) to obtain initial titration ranged between 1:20 to 1:160. Equal volumes of antigens grown in liquid Ellinghausen, McCullough, Johnson and Harris (EMJH) medium to a density of approximately $3x10^8$ leptospires/ml on the MacFarland scale were added and then all plates were incubated for 3 hours at 30^oC. After incubation, sera mixed with antigen were examined for agglutination under the dark field microscope. A serum was considered positive if 50% or

more of the microorganisms in the microtitre well agglutinated at titer 1:160 (Schoonman and Swai, 2009). These positive sera were subsequently diluted to 1:20,480 in PBS and tested again with the antigen to establish the antibody titer.

3.6 Molecular Technique

3.6.1 DNA extraction from blood

DNA was extracted from blood using the Purelink Genomic DNA extraction kits (Invintogen, Leuden, The Netherlands) following manufacturer instructions (Appendix 3).

Briefly, 200 µl of blood were mixed with 20 µl of proteinase K (1.25µg/ml) and 20 µl of Rnase A. afterwards, the blood-proteinase K-Rnase. A mixture was incubated at room temperature for two minutes followed by addition of 200 µl of lysis buffer. Afterwards, the resulting mixture was incubated at 55° C for 10 minutes to digest protein followed by chemical protein precipitation by addition of 200 µl of absolute ethanol. Precipitated protein and lysed membrane were pelleted by centrifugation and the supernatant was allowed to pass through a silica column. DNA was trapped within the silica column after a slow speed centrifugation (6,000g for 1 minute). DNA within the silica column was washed using buffers to remove PCR inhibitors and finally freed from ethanoic buffers using high speed (10 000g for 5 minutes). The interaction between DNA and the silica column was disturbed using 100 µl low ionic strength elution buffer and the DNA eluted into an Eppendorf tube. DNA was stored at -20°C until use. The presence of genomic DNA was visualized after extracted DNA on a 2% agarose gel.

3.6.2 Molecular discrimination between pathogenic from non-pathogenic *Leptospira* species

The PCR were performed using two different set of primers. Selection of primers was based on primers targeting a 330 base pairs fragment of the conserved *16sRNA* gene, primers that can discriminate pathogenic from nonpathogenic *Leptospira* sp. as described by Mgode *et al*. (2005).

PCR amplification was performed using in a PTC-100 thermal cycler (Mj Research, Watertown, MA, USA) using a Fast *Taq* DNA polymerase (Invitrogen, Leuden, the Netherlands). DNA of pathogenic *Leptospira* including serovars Kenya, (serogroup Ballum) and saprophytic specie (serovars Patoc, serogroup Semaranga) were used as positive controls. Nuclease free water was used as negative control.

Table 2: Primers used to discriminate *Leptospira*

Type of *Leptospir*a	Primers	Sequences (5'→3')	Target gene	Expected PCR fragment size (bp)
Pathogenic*	Lepat1	GAGTCTGGGATAACTTT	*16SrRNA*	330
	Lepat2	TCACATCGCTTGCTTATTTT	*16SrRNA*	
Non-pathogenic*	Sapro1	AGAAATTTGTGCTAATACCGAATT	*16SrRNA*	240
	Sapro2	GGCGTCGCTGCTTCAGGCTTTCG	*16SrRNA*	

The PCR mix was prepared as indicated bellow (Table 3). The PCR amplification conditions for pathogenic Leptospira were: initial denaturation at 93^{o}C for 3 min then 35 cycles of denaturation at 93^{o}C for 1 min, primer annealing at 48^{o}C for 1 min, DNA

extension at 72^{o}C for 1 min, and further 10 min extension after the last cycle. Saprophytic PCR condition were: heat denaturation at 93^{o}C for 3 min, then 35 cycles of heat denaturation at 93^{o}C for 1 min, primer annealing at 63^{o}C for 1.5 min, DNA extension at 72^{o}C for 2 min and after the last cycle extension continued for further 10 min.

After DNA amplification, PCR products were visualized after performing agarose gel electrophoresis. Briefly, 2% agarose gel was prepared and pre-stained using Gel Red dye (Bio-Rad, California-USA). Electrophoresis of DNA was performed at 100 V for 30 minutes followed by visualization of PCR product on a gel documentation system (Applied Biosystem, California, USA).

Table 3: PCR mix for the detection of *Leptospira*

Reagents	**Volumes(µl)**
PCR master mix	10
Forward primer(10nM)(Lepat1 or Sapro1)	0.3
Reverse Primer (10nM)(Lepto2 or Sapro2)	0.3
Nuclease free water	9.4
DNA template (100ng)	5
Total	**25**

3.6.3 DNA sequencing

Four representative samples of unpurified PCR products were sent in Netherlands for DNA sequencing. DNA sequencing was performed at Macrogen laboratory (Amsterdam, the Netherlands) using Lepat1 and Lepat2 primers as forward and reverse primers respectively.

A330 base pair of *16SrRNA* gene was used for differentiation of *Leptospira* sp. The amplified PCR products were purified at Macrogen laboratory by using DNA purification kit before sequencing process. Sequencing was performed in both directions for each PCR product by using the dideoxy chain termination procedure (Chemistry V3.1; Applied Biosystems, Foster City, CA) and the 3730XL DNA analyzer (Applied Biosystems) at the service center of Macrogen Company (The Netherlands).

3.7 Statistical Analysis

Descriptive analyses were performed with Epi info^{TM} version7 (CDC, USA) and Medcalc software version 12.1 (Osteen, Belgium). Proportions were compared using $\chi 2$ test. Microsoft excel was used to compile raw data from field and laboratory works.

3.8 Molecular Analysis

Raw sequences data or chromatograms were visualized and trimmed using sequence scanner software version one (Applied Biosystem CA, USA). Then, the forward and the reverse complement nucleotide sequences delimited by forward and reverse primers of several 16sRNA genes PCR products of *Leptospira* were aligned to obtain a consensus sequence. The consensus sequences of the amplified products were compared with reference sequences stored in GeneBank using blast analysis (www.ncbi.nlm.nih.gov) browsed on March 8, 2015. Alignment and sequence similarities of all sequences were done using ClustalW algorithm implemented within Molecular Evolutionary Genetics Analysis version six (MEGA6); (Tamura *et al*., 2013).

3.9 Phylogenetic Analysis

Phylogenetic analysis was done by using MEGA 6 software (Tamura *et al*., 2013). Selected and identical *Leptospira* spp. representing pathogenic, nonpathogenic and

intermediate species were retrieved from GenBank for comparative analysis (Table 4). Phylogenetic trees were constructed based on partial nucleotide sequences of *16sRNA*gene of *Leptospira* spp. stored in GenBank and those sequences obtained from this study. The tree topologies were evaluated by using bootstrap test of phylogeny in the maximum likelihood method and the bootstrap P-values were obtained after 1000 replicates of the dataset. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the isolates analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentages of replicate trees in which the associated isolates clustered together in the bootstrap test were shown next to the branches.

Table 4: *Leptospira* species used for phylogenetic tree construction

Species	Pathogenicity	GenBank Accession number
L. interrogans	Pathogenic	NR074481.1
Linterrogans	Pathogenic	JQ988859.1
L.interrogans	Pathogenic	KC441522.1
L.interrogans	Pathogenic	JQ988857.1
L.interrogans	Pathogenic	JQ988856.1
L.interrogans	Pathogenic	JQ988845.1
L.interrogans	Pathogenic	JQ988846.1
L.intrrogans	Pathogenic	JQ988842.1
L.interrogans	Pathogenic	JQ988841.1
L.interrogans	Pathogenic	JQ988854.1
Linterrogans	Pathogenic	JQ785630.1
L.interrogans	Pathogenic	JF910147.1
L.interrogans	Pathogenic	AM050565.1
L.interrogans	Pathogenic	Q204296.1
L.kirshineri	Pathogenic	JQ906659.1
l.kirshineri	Pathogenic	JQ988856.1
L.kirshineri	Pathogenic	AM050567.1
L.kirshineri	Pathogenic	JK154572.1
Uncultured *L.* species	Pathogenic	GU254502.1
Uncultured *L.*species	Pathogenic	Gu254504.1
Uncultured *L.*species	Pathogenic	KJ150300.1
L.fainei	Intermediate	LFU60594.1
L.fainei	Intermediate	JQ988851.1
L.fainei	Intermediate	U60594.1
L.inadai	Intermediate	LIU94975.1
L.bromii	Nonpathogenic	AY796065.1
L.biflexia	Nonpathogenic	JQ988840.1
L.meyeri	Nonpathogenic	AF157985.1
OG: Out Group/*L.biflexia* cdn gene	Nonpathogenic	AF430837.1

CHAPTER FOUR

4.0 RESULTS

4.1 Serological Study

4.1.1 Relationship between, leptospirosis, age, sex and location

A total of 267 participants were enrolled in the present study from nine different villages of Nsimbo and Mlele districts (Appendix 1). The majority of participants were adults 159(59.5%); children were 108 (40.4%). The results obtained from the present study showed that 80 out of the 276 study participants (30%) enrolled were seropositive for leptospiral infection. Adults were more infected with and exposed to *Leptospira* compared to children participants (Table 5). However, there was no statistical significance between the proportions of infection with regard to sex (Table5).

The seroprevalence of leptospirosis varied from one study village to another with highest seropositivity in Mtakuja II (53.3%) and lowest seropositivity in Mamba (7.6%) (Table 5). In all villages the number of participants who were exposed and infected with *Leptospira* was bigger than those who were tested negative.

Table 5: Prevalence of *Leptospira* with age and sex

	MAT		
	Positive	**Negative**	**Exposed**
Total (n)	80	57	130
Age median, range	18.5(2-83)	13(3-70)	16(1-80)
Age group			
Adult	51(63.7%)	29(50.8%)	79(59.8%)
children	29(36.2%)	28(49.1%)	51(39.2%)
P value	**0.03**	0.8	**0.03**
Sex			
Male	45(56.2%)	29(50.8%)	63(48.4%)
Female	35(43.7%)	28(49.1%)	67(51.5%)
P value	0.3	0.8	0.8

Table 6: Prevalence of *Leptospira* with villages

	Number of subjects	Positive (%)	MAT Negative (%)	Exposed (%)
Isinde	55	19(34.5)	11(20)	25(45.4)
Kapalala	10	4(40)	3(30)	3(30)
Mamba	26	3(11.5)	10(38.4)	13(50)
Mtakuja II	15	8(53.3)	2(13.3)	5(33.3)
Mtakumbukwa	46	18(39.1)	3(6.52)	25(54.3)
Mtandarani	38	11(28.9)	5(13.15)	22(57.8)
Nsimbo	9	2(22.2)	4(44.4)	3(33.3)
Songambele	47	11(23.4)	12(25.5)	24(51)
Stalike	21	4(19)	7(33.3)	10(47.6)

4.1.2 Prevalence of *Leptospira* serogroups in the study population

The circulating leptospiral serogroups in the study population included Hardjo, Sokoine, Grippotyphosa, Hebdomadis, Lora and Ballum (serovars Kenya). A total of 13 individuals were cross agglutinated with more than one serogroups. With one of the participant being cross-agglutinated with three serogroups: Hardjo, Sokoine and Grippotyphosa (Table 6).

Table 7: Leptospiral infection and cross-agglutination with more than one serogroups

Serogroups	Number of positive (n=267)	%
Lora	4	1.49
Kenya(serogroups Ballum)	3	1.12
Single agglutination		
Hardjo	42	15.7
Sokoine	24	8.9
Grippotyphosa	13	4.87
Hebdomadis	9	3.37
Dual-agglutination		
Hardjo and Grippotyphosa	2	0.75
Hardjo and Sokoine	3	1.12
Hardjo and Hebdomadis	1	0.37
Hebdomadis and Sokoine	2	0.75
Kenya and Hardjo	1	0.37
Sokoine and Grippotyphosa	3	1.12
Trial-agglutination		
Sokoine, Hardjo Grippotyphosa	1	0.37

Positive agglutination titers ranging between 1:160 and 1:1024 were detected for Sokoine and Hardjo. No titer above 1:160 was detected for serovars Hebdomadis. The titers and the number of subjects for each serogroup are also shown (Table 7).

Table 8: Tested serogroups and its positive agglutination titers

Serogroups	Titers							Total
	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	
Sokoine	2	2	1	9	4	4	2	24
Hardjo	18	9	5	2	5	2	1	42
Hebdomadis	9	0	0	0	0	0	0	9
Grippotyphosa	2	3	3	4	0	0	1	13
Kenya	3	0	0	0	0	0	0	3
Lora	3	0	1	0	0	0	0	4
Total	**37**	**14**	**10**	**15**	**10**	**6**	**4**	**96**

4.2 Molecular Discrimination Between Pathogen and Saprophytic *Leptospira*

In the present study, DNA was extracted from a total of 210 samples that were positive or exposed based on serological results. After extraction, the presence of genomic DNA was ascertained by performing agarose gel electrophoresis (Fig. 4).

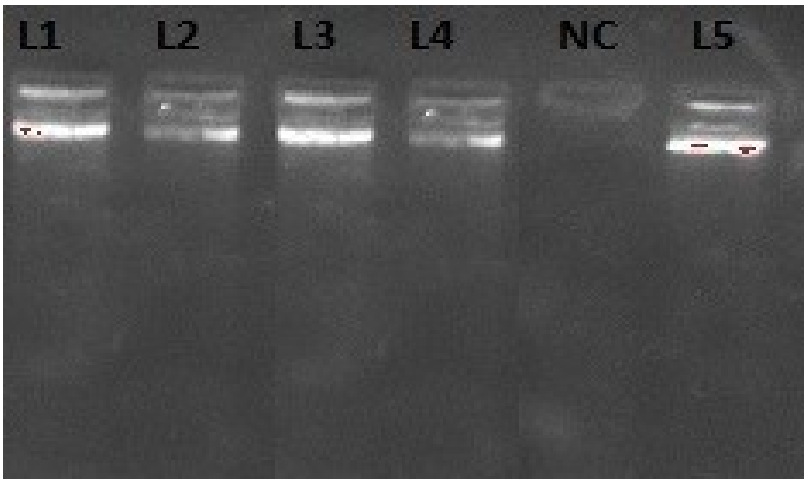

Figure 4: Electrophoresis of genomic DNA after extraction. Genomic DNA is seen as sharp band in the tested samples L1 to L5). No DNA is seen in the negative control (NC).

4.2.2 PCR results

A total of 210 samples that were either MAT positive or exposed to leptospiral infection were subjected to PCR. Out of this 80 (38 %) were from children and 130 (61.9%) were from adults. Thirty three out of 210 (15.7%) were infected with pathogenic *Leptospira* spp. Among the PCR positive samples, 20 (60.6%) were adults and 13 (39.39%) were children.

A total of 108 (51.4%) male participants that were MAT positives and exposed to leptospiral infection were tested for PCR. Of this, 19 (57%) were infected with pathogenic *Leptospira*. None of the samples were PCR positive with saprophytic *Leptospira* (Fig.5 and Fig. 6).

Figure 5: PCR products of non-pathogenic participants amplified with Sapro1 and Sapro2 primers (L1: 168, L2:48; L3:258; L4:110; L5:114; L6: 258; L7: 242; L8: 240; L9: 216). M: DNA ladder (100-bp-invitogen). PC: Positive control from saprophytic *Leptospira* serovars Patoc, serogroup Semaranga. NC: negative control

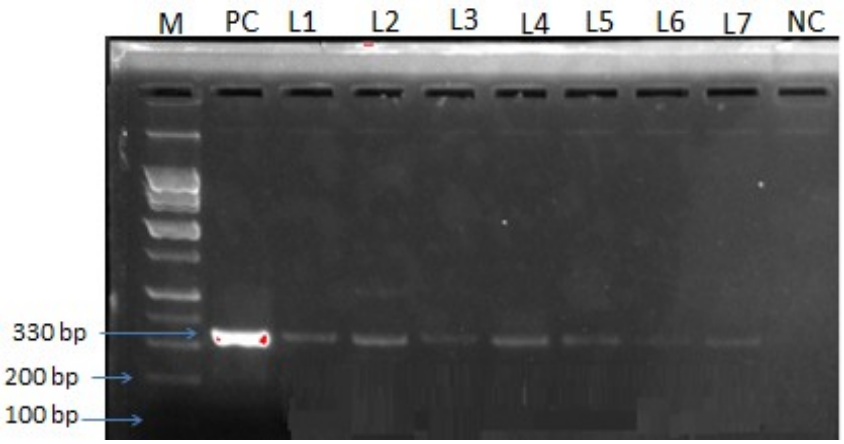

Figure 6: PCR products of DNA from pathogenic participants. (L1:168, L2:48; L3:258; L4:110, L5:114; L6:258 and L7: 242) with Lepat1 and Lepat2 primers. M: DNA ladder (100-bp-Invitrogen) PC: Positive control from pathogenic *Leptospira* serovars Kenya, serogroup Ballum. NC: negative control.

4.3 Molecular Identification of *Leptospira* species by Sequencing Technique

Leptospira nucleotide sequences from Katavi were submitted to GenBank and provided with accession numbers KP 313246 and KP 313247. Alignment of the *16sRNA* gene nucleotide sequences from Katavi showed the sequences from Katavi were not 100% similar. *Leptospira* KP313247 was 100% similar to pathogenic *Leptospira* spp. retrieved from GenBank while KP313246 were 97% similar to same species retrieved from GenBank. Sequence KP313246 had eight nucleotide variable sites compared to the rest sequences included in the alignment (Fig. 7). When translated to amino acids two variables sites were observed (Fig. 8). Majority of *Leptospira* spp. identical to those from Katavi were *Leptospira interrogans* and *Leptospira kirshnei.*

The phylogenetic trees showed all the three clades: clade I pathogenic; clade II intermediate and Clade III nonpathogenic *Leptospira.* *Leptospira* spp. from Katavi clustered in the same clade with other pathogenic species retrieved from GenBank (Fig. 9). However, one sequence from Katavi KP313246 was not 100% identical to other pathogenic *Leptospira* spp. (Fig. 10).

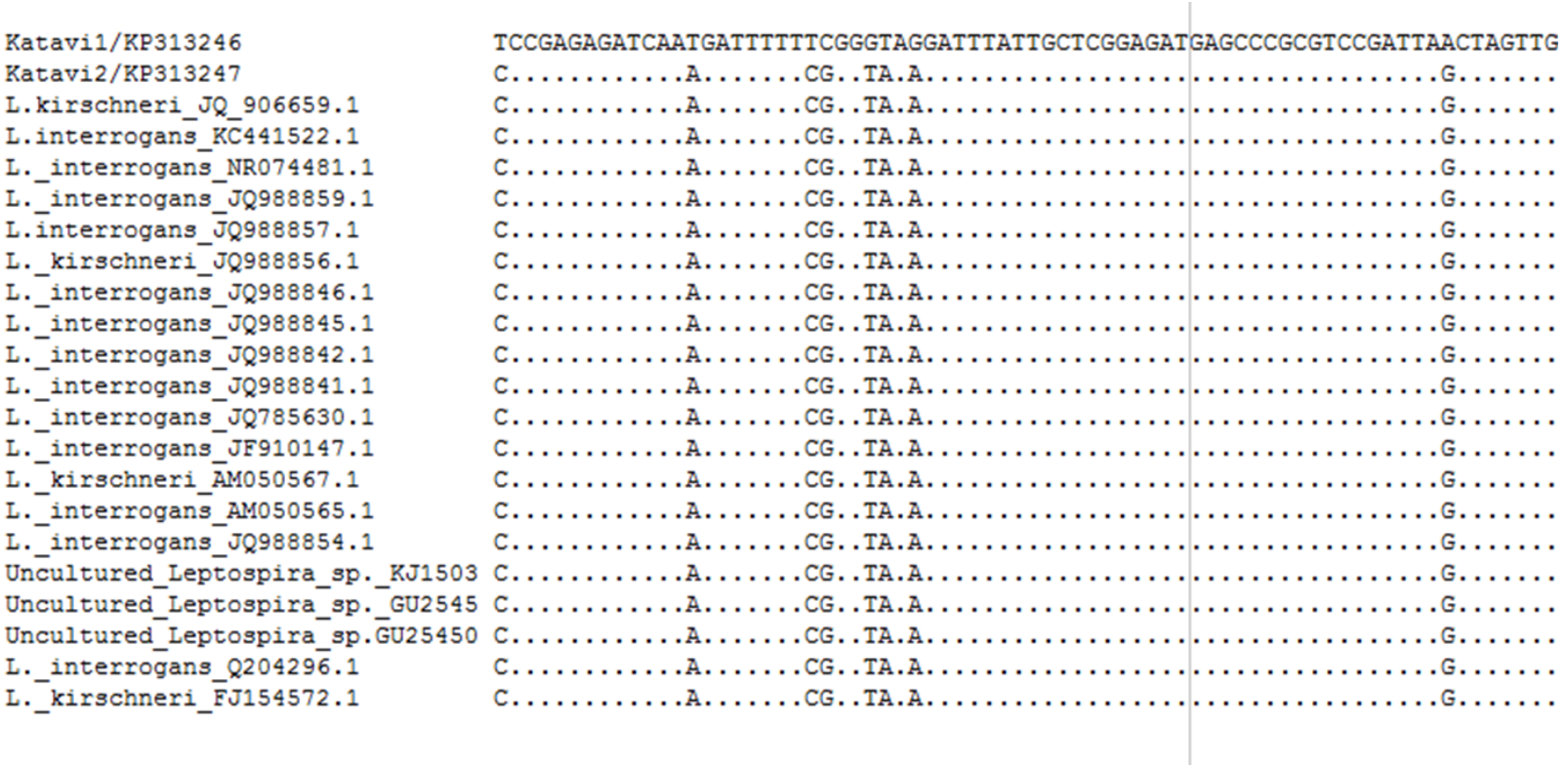

Figure 7: Nucleotide sequence alignment of *Leptospira* species showing nucleotides variable sites among pathogenic *Leptospira* species obtained from Katavi and those retrieved from GenBank. KP 313247 is 100% identical to other *Leptospira* species while KP313246 is 97% identical to other species Dots: represent identical nucleotides in all species. A: adenine; G: guanine; C: cytosine;T: thymine.

Name	Group	-	-	-	-	-	-	-	-	S	E	R	S	Y	D	F	F	G	*	-	F	I	-	-	G	D	E	P	A
1. Katavi1/KP313246		-	-	-	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.	-	.	.	-	-	.	.	.	.	.
2. Katavi2/KP313247		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
3. L.kirschneri JQ 906659.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
4. L.interrogans KC441522.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
5. L. interrogans NR074481.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
6. L. interrogans JQ988859.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
7. L.interrogans JQ988857.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
8. L. kirschneri JQ988856.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
9. L. interrogans JQ988846.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
10. L. interrogans JQ988845.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
11. L. interrogans JQ988842.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
12. L. interrogans JQ988841.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
13. L. interrogans JQ785630.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
14. L. interrogans JF910147.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
15. L. kirschneri AM050567.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
16. L. interrogans AM050565.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
17. L. interrogans JQ988854.1		K	G	K	L	I	L	D	G	P	.	.	.	?	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
18. Uncultured Leptospira sp. KJ150300		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
19. Uncultured Leptospira sp. GU254502.		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
20. Uncultured Leptospira sp.GU254500.		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
21. L. interrogans Q204296.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.
22. L. kirschneri FJ154572.1		K	G	K	L	I	L	D	G	P	.	.	.	*	.	.	S	.	K	-	.	.	-	-	.	.	.	.	.

Figure 8: Amino acid sequences of translated nucleotides showing variable sites (highlighted in yellow) among *Leptospira* from Katavi (Highlighted in green) and *Leptospira* species retrieved from GeneBank. Dots represent identical, Amino acid: K: lysine; G: Glycine; L: Leucine, I: Isoleucine,D: Aspartic acid P: proline,S: Serine; E: Glutamic acid; R: Argine,Y: Tyrosine; F: Phenylalanine,*: Termination; A: Alanine; V: Valine. bar: gaps

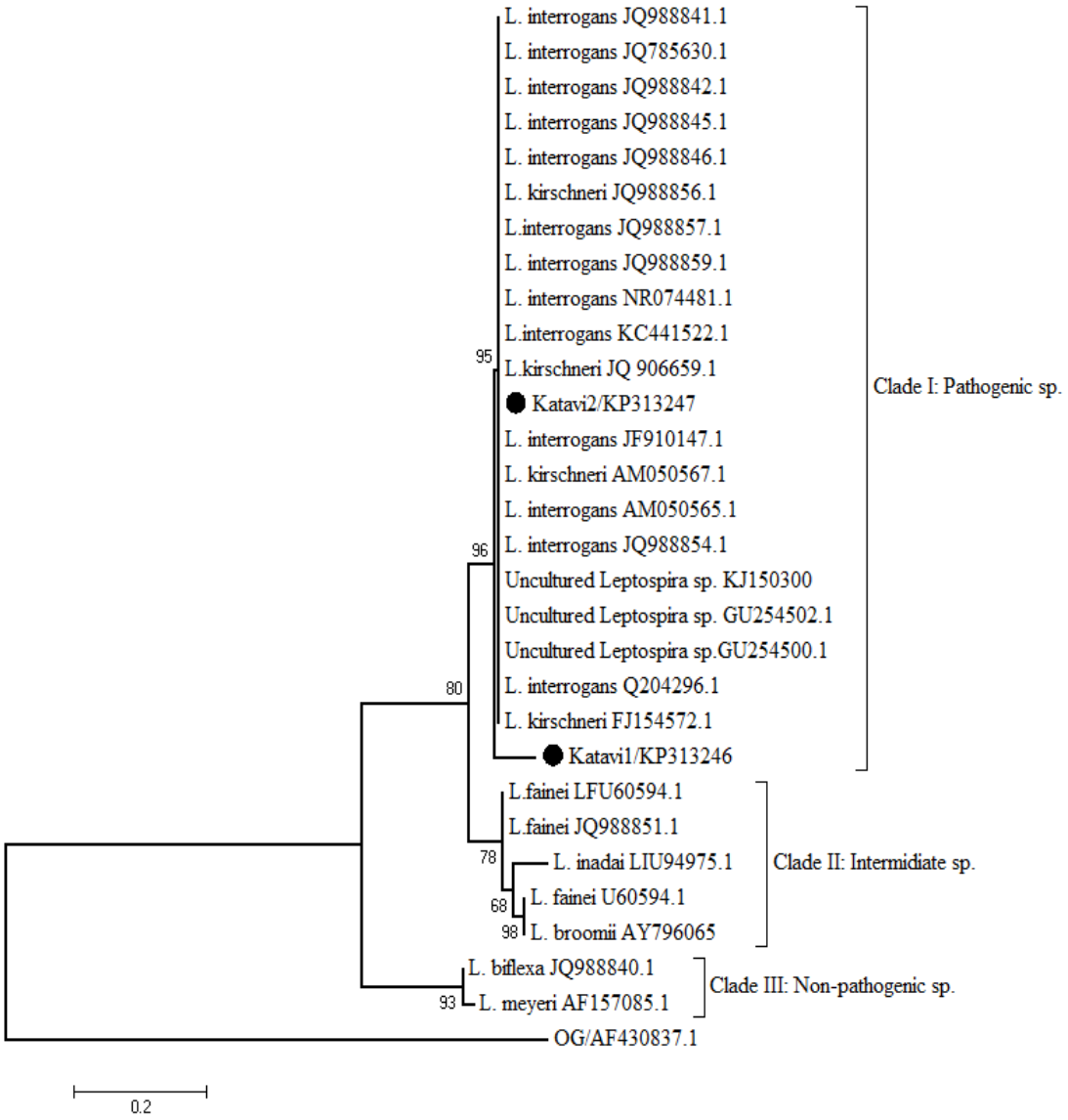

Figure 9: A maximum likelihood phylogenetic tree depicting the relationship of *Leptospira* species obtained from this study (indicated with circles) with other *Leptospira* retrieved from GenBank representing all *Leptospira* clades. *Leptospira* from Katavi are clustered within pathogenic clade but they not 100% identical. Phylogeny was inferred following 1000 bootstrap replications and values <50% were not shown. OG: outgroup

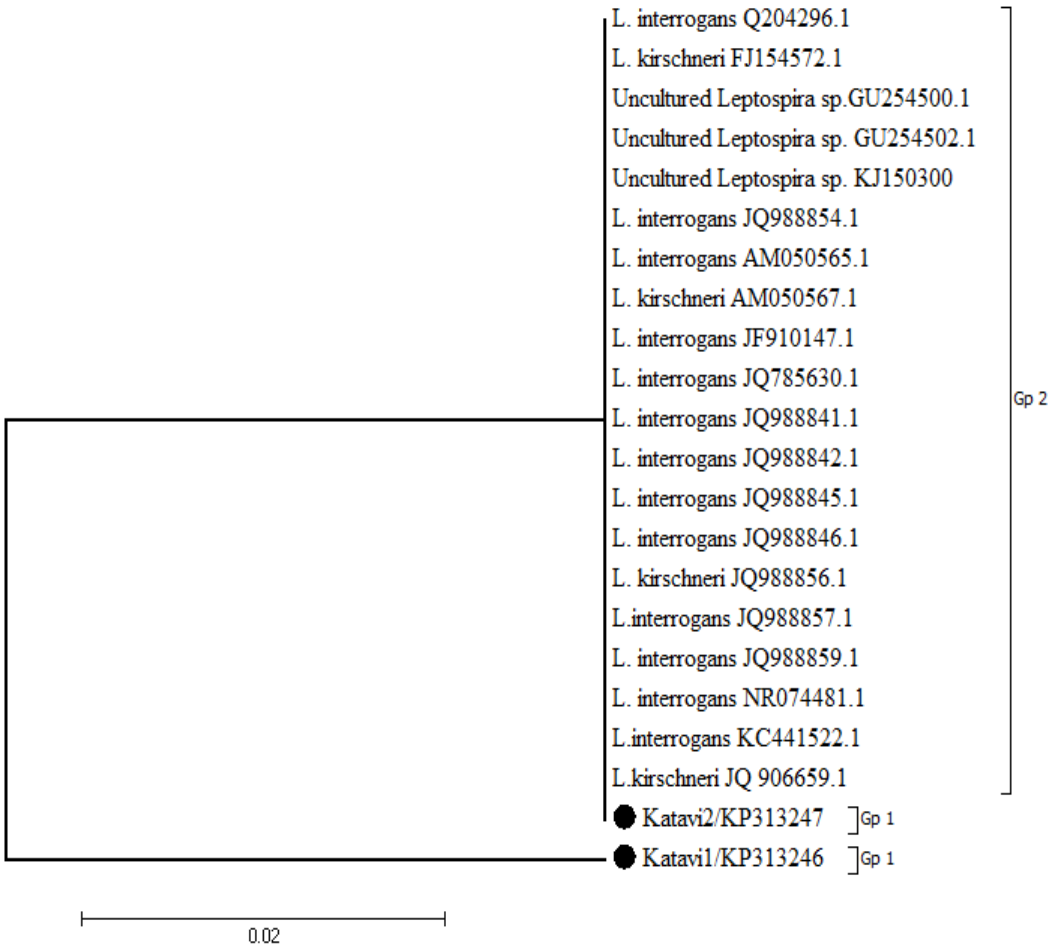

Figure 10: Phylogenic tree showing differences among pathogenic species *Leptospira* species clustered in clade I. The Katavi species KP313246 is slightly different from the rest. Phylogeny was inferred following 1000 bootstrap replications. Gp1: group1 species from Katavi; Gp2: group2 pathogenic species retrieved from GenBank

CHAPTER FIVE

5.0 DISCUSSION

The present study was undertaken to understand the epidemiology of *Leptospira* infection among agro-pastoral community of Katavi-Rukwa ecosystem. The results obtained from the present study showed that an overall seroprevalence of 30% and *Leptospira* infection was found in the study participants, suggesting that leptospirosis is endemic in the study area. In addition findings showed that *Leptospira interrogans*, *Leptospira kirshineri* and uncultured *Lesptospira* clone species are the major circulating *Leptospira* spp. in Katavi-Rukwa ecosystem.

Previous study in Katavi did not find any leptospiral infection (Machang'u *et al*., 1997). The emergence and endemicity of *Leptospira* in Katavi-Rukwa ecosystem may have resulted from the influx and increased pastoral population over the recent years. Leptospirosis diagnosis and management is not the standard point of care in Katavi probably leading to endemicity of leptospirosis among the study population. In addition, leptospirosis may be misdiagnosed and mismanaged as malaria or brucellosis due to its clinical presentation with acute febrile illnesses (Levett, 2001; Hernadez and Quintero, 2011; Crump *et al*., 2013).

In this study serologic data showed multiple serogroups likely circulate in Katavi-Rukwa ecosystem. However, proper interpretation of these data is complex because of probable cross-reactivity among different serogroups. Swai and Schoonman (2012) have shown prevalence of serogroups by using a panel of seven standard serogroups in MAT was as low as 15% (Schoonaman and Swai, 2012). Biggs *et al.* (2011) reported similar findings but a slightly lower prevalence of 8.8% in clinical samples by using more than seven

serogroups. Therefore, it seems evident that a range of serogroups are causing human leptospiral infection in Tanzania.

Prevalence of serogroups Sejroe (serovars Hardjo), Hebdomadis, Grippotyphosa, Lora, Ballum (serovar Kenya) and Icterohaemorrhagiae (serovars Sokoine) in the present study might be an indication of the important role of rodents, cattle/buffaloes in human leptospirosis in Katavi-Rukwa ecosystem.

These findings are consistent with the studies conducted in northern part of Tanzania which confirmed serogroup Hardjo, Hebdomadis, Grippotyphosa and Ictererrohaemoragiae (which are in the same serogroup with Sokoine) as major circulating serogroups in Tanzania (Machangu *et al.*, 1997; Biggs *et al.,* 2011; Swai and Schoonman, 2012). However serogroups used in northeastern part of Tanzania were all reference serogroups. Using local serogroups in the list of antigen portrays the real picture of the genuine serogroups circulating at the local level and is one of the recommendations in the diagnosis of leptospirosis (WHO, 2010; GLEAN, 2013).

In this study serogroups Sejroe (serovars Hardjo) was the most prevalent. These results are comparable to what was reported by Swai and Schoonman (2012) that serovars Hardjo were among the major circulating serovars in Tanga Region where a prevalence of 20% was observed. It is possible that serovar Hardjo in our study was transmitted to human from cattle as cattle are considered as the main reservoir for the serogroups Sejroe (Radostitis *et al*., 2006). The difference in proportion between these two studies might be explained by the differences in climatic and environmental conditions. Swai and Schoonman (2012) conducted their research in a more temperate area of the coastal region

where most likely there might be many cattle harboring serogroups Sejroe in their renal tubules than in the Katavi.

Serogroup Icterohaemorrhagiae (serovars Sokoine) was similarly reported in Tanga as the most prevalent serovars with prevalence of 36%. The same ***serogroup*** was reported in Kilimanjaro by Biggs *et al*. (2011) with 10 % of prevalence. The main reservoir of serovars Sokoine is rodents; human is incidental host (Mgode *et al*., 2004; Radostitis *et al*., 2006). The higher prevalence in Tanga might be contributed to differences in route of transmission, health status of participants and participants 'immunity. Biggs *et al*. (2011) reported prevalence in patients who were hospitalized and most probably their immunity was lower due to multiple infection including malaria and HIV-AIDS.

Serogroups Grippotyphosa was reported in Dar es Salaam in 1997 with a lower prevalence of 0.3% than reported in this study (Machangu *et al.,* 1997). Grippotyphosa mostly affect rodents, its main reservoir are rodents and buffalo. Grippotyphosa has been isolated in cattle and Horses as well (Erol *et al*., 2014; Romero and Yasuda, 2006, Radostitis *et al*., 2006). Increased number of cattle and climatic condition in Katavi might justify the higher prevalence of the serogroup Grippotyphosa reported in this study.

In this study the prevalence to serogroup Hebdomadis was higher compared to what was reported in Kilimanjaro (2.5%) by Biggs *et al*. (2011). Rodent diversities harboring different serogroups between these two areas of studies might be the main reason for serogroups diversities (Radostitis *et al*., 2006).

Serogroup Ballum (serovar Kenya) was isolated for the first time in giant rats in 2004 in Morogoro, Tanzania. Its main reservoir is rodents and wild animals (Machang'u *et al*.,

2004). Serogroup Lora has been widely used in animal subjects in Tanzania where its prevalence ranges between 2 to 4% (Mgode, 2014). Serogroup *Australis* was also observed in our study, this observation was not surprising as there is an increases interaction between humans, domestic and wild animals in Katavi region. In northern Tanzania study conducted by Biggs *et al*. (2011) prevalence of serogroups *Australis* was also reported. And in this part of the country interactions between humans, domestic and wild animals are also known to be high. The same human-animal interactions might contribute to transmission of the serogroups Kenya and Lora to humans in Katavi-Rukwa ecosystem.

In the current study the higher seroprevalence was observed in adults compared to children, the difference was found to be significant ($P<0.05$) Similar findings were reported by Swai and Schoonman (2012) who reported seroprevalence of leptospirosis amongst at-risk groups in Tanga, Tanzania. Likewise literatures confirm that adults are more infected than children. However, exposure to infection starts at early age (Radostitis *et al*., 2006). This observation is likely due to the fact that in Katavi-Rukwa ecosystem adults are more involved in livestock, herding animals and farming activities than children. Therefore adults may have high rate of leptospiral infection compared to younger age.

The findings of the current study summarize prevalence of leptospirosis in all nine villages. However, the proportion of infection at Mamba village is lesser compared to other villages. Its geographic location might be the reason for this difference. Mamba is a peri-urban village located next to Majimoto, the town of Mlele District. However, all villages are equally exposed to leptospirosis. Such observation is supported by studies

conducted elsewhere which confirmed that rural areas are mostly affected by leptospirosis (Bertherat *et al*.; 2014; Biggs e*t al*., 2011; Zakeri *et al*., 2010). As stated by Karimuribo *et al*. (2008); limited knowledge on leptospirosis, livestock activities low level of education and environmental conditions might contribute to transmission of the disease in villages.

The present study found *Leptospira* infection of 15.7% by PCR technique. This relatively high infection rate represents a hazard to public health. To my knowledge this is the first study to confirm pathogenic *Leptospira* spp. in human subjects by PCR technique in Tanzania. Primers used Lepat 1 and Lepat2 are genus specific for detection of pathogenic. These primers were used in Morogoro in characterization of Serogroup Kenya from captive African giant pouched rats in Morogoro, (Machang'u *et al*., 2004). Mgode *et al*. (2004) used the same primer to detect pathogenic *Leptospira* spp. in animal blood.

In this study failure of PCR to detect high number of *Leptospira* DNA in suspected cases could be justified by the absence of the organism in blood samples or low level of DNA yield or quality (Bourhy *et al.*, 2012; De Abrue *et al.*, 2006; Francesca *et al*., 2010). Participants exposed and positive to leptospirosis by MAT were tested for PCR; negative samples were not included. PCR detects presence of bacteria in blood at the onset of disease while MAT detects raise of *Leptospira* antibody in sera from seven to fifteen days after the exposure of the disease. However, it is likely that *Leptospira* spp. from western Tanzania have different molecular characteristic from what have been reported elsewhere.

In this study, BLAST report of the sequenced *16SrRNA* genes from Katavi-Rukwa ecosystem confirms PCR results by identifying 100% of similarity to reference sequence of the uncultured pathogenic clone sp. CNS 909 (Accession number: KJ150300.1.). The

uncultured clone CNS 909 was isolated from cerebrospinal fluid of human subject belonging to environmental sample in Laos; Thailand (KJ150300.1). This result suggests that transmission of leptospirosis in human subjects of Katavi-Rukwa is through direct or indirect contact with infected animals, water or soil. These leptospiral clones have been responsible for causing outbreak in Asia. For instance in 2000, a dominant pathogenic *Leptospira* clone isolated from human being was responsible for leptospirosis outbreak in Thailand (Thaipadungpanit *et al*., 2007).

Sequence analysis showed identity ranging between 97% and 100% with published sequences of leptospires available in GenBank. The most prevalent were *L. interrogans* and *L. kirshineri*. This observation confirmed that in the Katavi-Rukwa ecosystem leptospirosis is originated from rodents, domestic and wild animals since *L.interrogans and L. kirshineri* are hosted in those reservoirs. However, this does not rule out the possibility of having more than one species causing leptospirosis among agro-pastoral communities in Tanzania.

Sequences comparison between KP313246 and KP313247 showed that KP313246 has more variable nucleotide sites compared to KP313247. However, most of these sites are nonsense codons since only one site was translated to protein. This remark suggests that KP313246 species may be a different strain.

Similarly, phylogenetic analysis (Fig. 9) of 30 species based on *16sRNA* gene nucleotide sequences revealed that species from Katavi belong to clade I and cluster in the same group with *Leptospira interrogans* and *Leptospira kirshineri* which are all pathogenic species. However, clustering of KP313246 branched into separate taxa under a different

pathogenic group (Fig. 10). This suggests that KP313246 is probably a different strain from other pathogenic species.

Failure to obtain many good quality sequences from pathogenic might be contributed to several factors including sequences noises, decreased DNA concentration after PCR purification and most likely PCR products contaminations. However, the resulting sequence from this study provides proper information and knowledge of the genetic material of the pathogenic species circulating in human subjects from Tanzania.

This study has some limitations. First, we were not able to identify a clear case definition of MAT titer for positive samples. For examples, in the Democratic Republic of Congo and India MAT titers ≥ 1:100 were considered as positive (Bertherat *et al*., 2014; Shivakumar *et al.*, 1990). In Northern part of Tanzania MAT titer of ≥ 1:400 was considered positive; laboratory work was done at Center for Disease Control, USA. In this study we have applied the cut-off value set by the reference *Leptospira* laboratory at SUA Morogoro (Machang'u *et al*., 2004; Mgode *et al*., 2005) where reactivity titer of ≥ 1:160 is considered as seropositive. This cut off value has been used in South Africa (De Vrie *et al*., 2014). Decision on MAT cut off value to consider as positive also depends upon type of the reference laboratory used.

Second; the choice of primers used for PCR differs from one study to another depending of the objective of the study and length of DNA of target. Lepat 1 and Lepat 2 have been widely used at Sokoine University of agriculture for detection of pathogenic *Leptospira* spp. in blood and animal sera targeting an amplicon of 330 bp.

In this study sequencing of two samples were obtained from Macrogen Inc Company (Netherlands) which may not generalize all species circulating in Katavi-Rukwa ecosystem. However, this study expands our knowledge on the genetic information of *Leptospira* spp. circulating in Tanzania. The obtained sequences were comparable to other sequences available on the GeneBank and gave similarities ranging between 97% and 100%. This information is useful for public health intervention such as vaccine and diagnostic tool development.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMENDATIONS

6.1 Conclusions

This study found widespread leptospiral infection among agro-pastoral communities from Katavi-Rukwa ecosystem, southwest Tanzania. Antibodies against *Lepstospira* serogroups were detected by MAT test in sera of agro-pastoralists; the overall serological prevalence was 30%. PCR technique detected 15.7% of positivity in blood of study participants and sequencing identified *Leptospira Kirshineri*, *Leptospira interrogans and* uncultured *Leptospira* clone species as major circulating species in Katavi region.

The present study showed that in the Katavi-Rukwa ecosystem adults are more infected with *Leptospira* compared to children. The major circulating serogroups were serogroups Sejroe (serovars Hardjo) 15.7%; Icterohaemorrhagiae (serovars Sokoine): 8.98%; Grippotyphosa 4.87%; Hebdomadis 3.37%; Lora 1.49% and Ballum 1.12%. Non pathogenic *Leptospira* speci es were not found suggesting that the study area is contaminated with pathogenic *Leptospira*.

Serological reactivity to the six serogroups tested and identification of the pathogenic *Leptospira* spp. by sequencing may suggest that leptospiral infection to humans in Katavi-Rukwa ecosystem may be caused by multiple serogroups and species and that transmission may be through indirect contact with water and soil contaminated by urine of the infected hosts. Also rodents, wildlife and livestock animals are most likely host reservoir of *Leptospira* spp. in the Katavi-Rukwa ecosystem. The influx of livestock population and the high interaction between humans, domestic and wild animals

contribute to high prevalence of leptospiral infection among agro-pastoral communities of Katavi Region.

In all nine villages the number of individual infected and exposed to *Leptospira* is bigger than individuals who are negative from *Leptospira*. Climatological condition, influx of livestock, interaction between humans, domestic and wildlife animals might contribute to the high prevalence of leptospiral infection reported in this study.

Sequence alignment of *Leptospira* spp. revealed 97 to 100% similarity with pathogenic *Leptospira* spp. stored in GenBank. In General, pathogenic *Leptospira* spp. circulating in the Katavi-Rukwa ecosystem are public health threats to the community living in study area.

6.2 Recommendations

With evidence of leptospiral infection among agro-pastoralists using both serological and molecular techniques, this study therefore recommends that:

Further work on molecular epidemiology of leptospirosis should be carried out in Katavi-Rukwa ecosystem and other areas of Tanzania by using both clinical and non-clinical settings. A large epidemiological study will allow estimation of the actual prevalence of leptospirosis in Tanzania.

We also recommend in future study to use other techniques like culture, and advance molecular technique like RT PCR, microarray and Multi-locus sequencing techniques to complement MAT and conventional PCR. Advance molecular techniques will help to

evaluate the use of PCR in diagnosis of human leptospirosis in Tanzania and it will finally determine the importance of Tanzania strains in clinical disease of humans.

Furthermore, cut off value used at SUA to consider positive samples for MAT should also be reviewed since leptospirosis seemed to be endemic in Tanzania.

A multidisciplinary approach should be used for effective intervention of the leptospirosis in Tanzania. Active disease surveillance involving, the Ministry of Health and Social Welfare, the Ministry of Livestock and Fisheries should be thought of in order to save lives of agro-pastoralists, create baseline information on inter-epidemic serovars transmission and areas at risk. Also physicians and laboratory staffs should be informed on leptospirosis for good patient management and prevention.

Moreover, the Ministry of Health and Social Welfare should consider including serological diagnosis of leptospirosis in the diagnosis of non-malarial febrile illness in health facilities particularly communities living in rural parts of the country such as the agro-pastoralists living in Katavi region (Katavi-Rukwa ecosystem).

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APPENDICES

Appendix 1: Bio data of participants involved in this study

Subject ID	Village	District	Sex	Age (year)	Positive	Titers	Serovars	Pathogenic	Nonpathogenic
NS1	KAPALALA	Nsimbo	Male	70	-	-		ND	ND
NS2	KAPALALA	Nsimbo	Female	68	-	1:80	H	-	-
NS3	KAPALALA	Nsimbo	Female	12	-	-		ND	ND
NS4	KAPALALA	Nsimbo	Female	45	-	1:80	H	-	-
NS5	KAPALALA	Nsimbo	Female	25	+	1: 640; 1:1280,1:20, 1:40	S, G,Hd, L	+	-
NS6	KAPALALA	Nsimbo	Female	14	+	1:1280,1:40, 1:80,1;40	S,L,Hd,H	+	-
NS7	KAPALALA	Nsimbo	Female	20	+	1:320, 1:2560,1:80	S, H,L	+	-
NS8	KAPALALA	Nsimbo	Female	40	-	1:80	L	-	-
NS9	KAPALALA	Nsimbo	Female	13	-	-		ND	ND
NS10	KAPALALA	Nsimbo	Female	32	+	1:160, 1:80, 1:160,	S,Hd,H	-	-
NS11	MTAKUJA II	Nsimbo	Male	83	+	1:320,1:40	H,L	+	-
NS12	MTAKUJA II	Nsimbo	Male	40	+	1:2560; 1:640;1:640	S, G,H	-	-

NS13	MTAKUJA II	Nsimbo	Female	53	+	1:1280; 1:160,1:80	G,H,L	-	-
NS14	MTAKUJA II	Nsimbo	Male	20	-	1:40,1:80	L,S	-	-
NS15	MTAKUJA II	Nsimbo	Male	10	+	1:320, 1:80,1:180	G,S,H	-	-
NS16	MTAKUJA II	Nsimbo	Male	11	+	1:2560,1:20,1:80	L	-	-
NS17	MTAKUJA II	Nsimbo	Male	4	-	-		ND	ND
NS18	MTAKUJA II	Nsimbo	Male	6	+	1:160,1:40,1:40,	H,L,S	-	-
NS19	MTAKUJA II	Nsimbo	Female	6	-	1:80, 1:40	L,Hd	-	-
NS20	MTAKUJA II	Nsimbo	Female	6	-	1:40	L	-	-
NS21	MTAKUJA II	Nsimbo	Female	9	-	-		ND	ND
NS22	MTAKUJA II	Nsimbo	Female	13	-	1:20	l	-	-
NS23	MTAKUJA II	Nsimbo	Male	10	+	1:5120	H	-	-
NS24	MTAKUJA II	Nsimbo	Male	7	-	1:20, 1:80	L,H	-	-
NS25	MTAKUJA II	Nsimbo	Male	8	+	1:5120,1:80	S,Hd	-	-
NS26	SONGAMBELE	Nsimbo	Female	30	-	1:80	Hd	-	-
NS27	SONGAMBELE	Nsimbo	Male	16	-	1:80, 1:40	Hd,H	-	-
NS28	SONGAMBELE	Nsimbo	Female	11	-	1:20	L	-	-
NS29	SONGAMBELE	Nsimbo	Male	30	+	1:160, 1:2560	Hd, S	-	-

NS30	SONGAMBELE	Nsimbo	Female	11	-	-		ND	ND
NS31	SONGAMBELE	Nsimbo	Female	16	+	1:640, 1:20, 1:40,1:20	G,L,S,H	-	-
NS32	SONGAMBELE	Nsimbo	Male	80	-	1:40	S	-	-
NS33	SONGAMBELE	Nsimbo	Female	23	-	-		ND	ND
NS34	SONGAMBELE	Nsimbo	Female	40	+	1:160, 1:160, 1:80	L,Hd,H	-	-
NS35	SONGAMBELE	Nsimbo	Female	11	-	-		ND	ND
NS36	SONGAMBELE	Nsimbo	Female	2	-	1:80		-	-
NS37	SONGAMBELE	Nsimbo	Male	4	-	-		ND	ND
NS38	SONGAMBELE	Nsimbo	Female	3	+	1:640,1:180, 1:180, 1:180,	H,Hd, K,L	-	-
NS39	SONGAMBELE	Nsimbo	Male	3	+	1:160, 1:40,1:40	Hd,G,H	-	-
NS40	SONGAMBELE	Nsimbo	Female	1	-	1:40, 1:20	L, H	-	-
NS41	SONGAMBELE	Nsimbo	Male	12	-	1:40	L	ND	ND
NS42	SONGAMBELE	Nsimbo	Male	44	+	1:160,1:80	S,H	-	-
NS43	SONGAMBELE	Nsimbo	Male	20	-	1:40	H	-	-
NS44	SONGAMBELE	Nsimbo	Male	21	-	-		ND	ND
NS45	SONGAMBELE	Nsimbo	Male	16	-	1:40	L	-	-
NS46	SONGAMBELE	Nsimbo	Male	19	+	1:80, 1:160, 1:1280	L,Hd,S	-	-

NS47	SONGAMBELE	Nsimbo	Male	13	-	-		ND	ND
NS48	SONGAMBELE	Nsimbo	Male	10	+	1:80, 1:1280	L,H	+	-
NS49	SONGAMBELE	Nsimbo	Male	13	-	-		ND	ND
NS50	SONGAMBELE	Nsimbo	Male	5	-	1:80	G	-	-
NS51	SONGAMBELE	Nsimbo	Female	3	-	-		ND	ND
NS52	SONGAMBELE	Nsimbo	Male	70	-	-		ND	ND
NS53	SONGAMBELE	Nsimbo	Male	30	-	-		ND	ND
NS54	SONGAMBELE	Nsimbo	Male	19	-	1:80	Hd	-	-
NS55	SONGAMBELE	Nsimbo	Male	7	-	1:80	G	-	-
NS56	SONGAMBELE	Nsimbo	Male	20	-	1:20, 1:40	L,H	-	-
NS57	SONGAMBELE	Nsimbo	Male	13	-	1:80,1:80	L,Hd	-	-
NS58	SONGAMBELE	Nsimbo	Male	16	-	1:40	L	-	-
NS59	SONGAMBELE	Nsimbo	Male	52	-	1:40	L	-	-
NS60	SONGAMBELE	Nsimbo	Female	40	-	1:40	L	-	-
NS61	SONGAMBELE	Nsimbo	Male	22	+	1:80,1:160	L,H	+	-
NS62	ISINDE	Nsimbo	Female	80	-	1:80	Hd	-	-
NS63	ISINDE	Nsimbo	Female	36	+	1:10240	S	-	-

NS64	ISINDE	Nsimbo	Female	30	+	1:20, 1:1280, 1:20	L,G,H	+	-
NS65	ISINDE	Nsimbo	Male	10	-	-		ND	ND
NS66	ISINDE	Nsimbo	Female	33	-	1:20, 1:80	L, S	-	-
NS67	ISINDE	Nsimbo	Male	23	+	1:40, 1:160, 1:160	L,Hd,H	+	-
NS68	ISINDE	Nsimbo	Male	20	-	1:40, 1:80	L,Hd	-	-
NS69	ISINDE	Nsimbo	Male	16	-	1:80	S	-	-
NS70	ISINDE	Nsimbo	Male	35	+	1:80, 1:160	L,Hd	-	-
NS71	ISINDE	Nsimbo	Male	15	-	-		ND	ND
NS72	ISINDE	Nsimbo	Male	19	-	1:40	H	-	-
NS73	ISINDE	Nsimbo	Male	7	+	1:40, 1:1280	L, S	+	-
NS74	ISINDE	Nsimbo	Male	24	-	1:80,1:80	s,H	-	-
NS75	ISINDE	Nsimbo	Female	65	+	1:80,1:80,1:320	L,Hd,H	+	-
NS76	ISINDE	Nsimbo	Male	36	+	1:80, 180,1:160	L,Hd,H	-	-
NS77	ISINDE	Nsimbo	Female	19	-	1:20	L	ND	ND
NS78	ISINDE	Nsimbo	Male	25	-	1:80, 1:80	L,H	-	-
NS79	ISINDE	Nsimbo	Male	8	+	1:20,1:80,1:5120, 1:80	L,Hd,S,H	-	-
NS80	ISINDE	Nsimbo	Female	13	-	1:80	H	-	-

NS81	ISINDE	Nsimbo	Male	18	-	-		ND	ND
NS82	ISINDE	Nsimbo	Male	41	-	-		ND	ND
NS83	ISINDE	Nsimbo	Male	26	+	1:20, 12560	L,H	-	-
NS84	ISINDE	Nsimbo	Female	11	+	1:20,1:640	L,H	-	-
NS85	ISINDE	Nsimbo	Female	18	-	-		ND	ND
NS86	ISINDE	Nsimbo	Male	1	-	1:80, 1:20	L,H	-	-
NS87	ISINDE	Nsimbo	Male	58	-	1:20	L	-	-
NS88	ISINDE	Nsimbo	Male	11	-	1:40	L	-	-
NS89	ISINDE	Nsimbo	Female	6	-	-		ND	ND
NS90	ISINDE	Nsimbo	Female	6	-	1:20	L	-	-
NS91	ISINDE	Nsimbo	Female	32	-	1:40, 1:80	S, H	-	-
NS92	ISINDE	Nsimbo	Male	2	+	1:320	H	-	-
NS93	ISINDE	Nsimbo	Male	6	-	1:40	H	-	-
NS94	ISINDE	Nsimbo	Female	60	+	1:80, 1:40, 1:160,1:160	L,K,G,H	-	-
NS95	ISINDE	Nsimbo	Male	13	+	1:20, 1:320	L,G	-	-
NS96	ISINDE	Nsimbo	Female	16	-	1:80	L	-	-
NS97	ISINDE	Nsimbo	Female	13	+	1:160, 140, 1:10240,1:40	L,S, G,H	-	-

NS98	ISINDE	Nsimbo	Female	20	-	1:20	H	-	-
NS99	ISINDE	Nsimbo	Female	14	+	1:640, 1:80	G,H	-	-
NS100	ISINDE	Nsimbo	Female	11	+	1:160	H	-	-
NS101	ISINDE	Nsimbo	Female	9	-	1:20, 1:40, 1:80	L, G,H	-	-
NS102	ISINDE	Nsimbo	Female	6	-	1:80	H	-	-
NS103	ISINDE	Nsimbo	Female	4	-	-		ND	ND
NS104	ISINDE	Nsimbo	Female	4	-	1:20, 1:80,1:80	L,S,H	-	-
NS105	ISINDE	Nsimbo	Female	4	-	1:40	H	-	-
NS106	ISINDE	Nsimbo	Female	63	-	1:80	H	-	-
NS107	ISINDE	Nsimbo	Female	23	-	1:80	L	-	-
NS108	ISINDE	Nsimbo	Female	24	-	1:80,1:80	L,L	-	-
NS109	ISINDE	Nsimbo	Male	4	-	-		ND	ND
NS110	ISINDE	Nsimbo	Male	3	+	1:160		+	-
NS11	ISINDE	Nsimbo	Male	5	-	1:40		-	-
NS112	ISINDE	Nsimbo	Male	10	-	1:20, 1:80, 1:180, 1:20	L,HD,S,H	-	-
NS113	ISINDE	Nsimbo	Female	4	-	-		ND	ND
NS114	ISINDE	Nsimbo	Male	11	+	1:5120, 1:320	S,G	+	-

NS115	ISINDE	Nsimbo	Male	11	+	1:20, 1:160, 1:20	L,S,H	+	-
NS116	ISINDE	Nsimbo	Female	12	-	-		ND	ND
NS117	SONGAMBELE	Nsimbo	Female	45	+	1:160	H	+	-
NS118	SONGAMBELE	Nsimbo	Male	12	-	1:80	H	-	-
NS119	SONGAMBELE	Nsimbo	Male	17	-	1:80	H	-	-
NS120	SONGAMBELE	Nsimbo	Female	17	-	1:80	H	-	-
NS121	SONGAMBELE	Nsimbo	Female	6	-	1:80, 1:20	H, K	-	-
NS122	SONGAMBELE	Nsimbo	Male	8	-	-		ND	ND
NS123	SONGAMBELE	Nsimbo	Female	16	-	1:80	H	-	-
NS124	SONGAMBELE	Nsimbo	Female	33	-	1:40	H	-	-
NS125	SONGAMBELE	Nsimbo	Male	11	-	1:40, 1:20	H,L	-	-
NS126	SONGAMBELE	Nsimbo	Female	50	+	1:320, 1:80, 1:10240	H,K,S	+	-
NS127	SONGAMBELE	Nsimbo	Male	17	-	1:20,1:40	K,L	-	-
NS128	MTAKUMBUKWA	Nsimbo	Male	12	-	-		ND	ND
NS129	MTAKUMBUKWA	Nsimbo	Female	32	-	1:20	L	-	-
NS130	MTAKUMBUKWA	Nsimbo	Male	9	-	1:40	H	-	-
NS131	MTAKUMBUKWA	Nsimbo	Male	40	-	-		ND	ND

NS132	MTAKUMBUKWA	Nsimbo	Male	16	+	1:20, 1:320,1:80	K,S,H	-	-
NS133	MTAKUMBUKWA	Nsimbo	Male	22	+	1:20, 1:1280	K,S	+	-
NS134	MTAKUMBUKWA	Nsimbo	Female	16	+	1:80,1:20,1:640	K,L,H	+	-
NS135	MTAKUMBUKWA	Nsimbo	Female	20	+	1:80, 1:1280, 1:20	K,S,H	-	-
NS136	MTAKUMBUKWA	Nsimbo	Female	18	-	1:80,1:80	K,H	-	-
NS137	MTAKUMBUKWA	Nsimbo	Male	17	-	1:20,1:80	K,H	-	-
NS138	MTAKUMBUKWA	Nsimbo	Male	12	+	1:20, 1:20,1:160	L,K,H	-	-
NS139	MTAKUMBUKWA	Nsimbo	Male	5	-	1:20	L	-	-
NS140	MTAKUMBUKWA	Nsimbo	Male	32	-	1:20,1:40	K,H	-	-
NS141	MTAKUMBUKWA	Nsimbo	Male	4	+	1:80, 1:1280	K,H	+	-
NS142	MTAKUMBUKWA	Nsimbo	Male	3	-	1:20, 1:20	K,H	-	-
NS143	MTAKUMBUKWA	Nsimbo	Male	40	+	1:20, 1:160	K ,H	-	-
NS144	MTAKUMBUKWA	Nsimbo	Male	30	+	1:40,1:160	L, H	-	-
NS145	MTAKUMBUKWA	Nsimbo	Male	16	-	1:20	k	-	-
NS146	MTAKUMBUKWA	Nsimbo	Male	25	-	1:80	H	-	-
NS147	MTAKUMBUKWA	Nsimbo	Male	28	-	1:20, 1:40	K,H	-	-
NS148	MTAKUMBUKWA	Nsimbo	Male	6	-	1:20,	L	-	-

NS149	MTAKUMBUKWA	Nsimbo	Female	7	-	-		ND	ND
NS150	MTAKUMBUKWA	Nsimbo	Female	7	+	1:20, 1:2560	K,H	+	-
NS151	MTAKUMBUKWA	Nsimbo	Female	3	+	1:80, 1:2560	K,S	-	-
NS152	MTAKUMBUKWA	Nsimbo	Female	10	-	1:80	K	-	-
NS153	MTAKUMBUKWA	Nsimbo	Female	40	-	1:20, 1:180	K,H	-	-
NS154	MTAKUMBUKWA	Nsimbo	Female	60	-	1:20	K	-	-
NS155	MTAKUMBUKWA	Nsimbo	Female	30	-	1:40	Hd	-	-
NS156	MTAKUMBUKWA	Nsimbo	Male	52	+	1:20, 1:160	K,H	-	-
NS157	MTAKUMBUKWA	Nsimbo	Male	49	+	1:20,1:160	L,H	-	-
NS158	MTAKUMBUKWA	Nsimbo	Male	4	-	1:80,1:40,1:20	K,G,H	-	-
NS159	MTAKUMBUKWA	Nsimbo	Female	25	+	1:20,1:80, 1:320	K,S,H	-	-
NS160	MTAKUMBUKWA	Nsimbo	Male	52	+	1:640, 1:20,1:1280,1:160	L, K,G,H	+	-
NS161	MTAKUMBUKWA	Nsimbo	Female	54	-	1:80, 1:80, 1:80	K,S,H	-	-
NS162	MTAKUMBUKWA	Nsimbo	Female	10	-	1:40	H	-	-
NS163	MTAKUMBUKWA	Nsimbo	Male	30	-	1:20, 1:80	K,H	-	-
NS164	MTAKUMBUKWA	Nsimbo	Male	70	-	1:20, 1:80	L,Hd	-	-
NS165	MTAKUMBUKWA	Nsimbo	Male	60	-	1:80, 1:20	K,H	-	-

NS166	MTAKUMBUKWA	Nsimbo	Male	10	-	1:20, 1:40	L,H	-	-
NS167	MTAKUMBUKWA	Nsimbo	Male	12	-	1:40	K	-	-
NS168	MTAKUMBUKWA	Nsimbo	Male	14	-	1:20, 1:40, 1:20	L,K,H	+	-
NS169	MTAKUMBUKWA	Nsimbo	Male	8	+	1:80, 1:80, 1:2560	L,K,H	+	-
NS170	MTAKUMBUKWA	Nsimbo	Male	20	+	1:20, 1:20, 1:1280, 1:80	L,K,S,H	-	-
NS171	MTAKUMBUKWA	Nsimbo	Male	7	+	1:160: 1:80	L,K	+	-
NS172	MTAKUMBUKWA	Nsimbo	Male	18	+	1:80, 1:20, 1:1280, 1:40	L,K,S,H	-	-
NS173	MTAKUMBUKWA	Nsimbo	Male	6	-	1:40,1:80	K,G	-	-
NS174	MTANDARANI	Nsimbo	Male	12	+	1:20, 1:5120, 1:80	K,S,H	+	-
NS175	MTANDARANI	Nsimbo	Male	7	-	1:20	L	-	-
NS176	MTANDARANI	Nsimbo	Male	9	-	1:20, 1:20	L,Hd	-	-
NS177	MTANDARANI	Nsimbo	Male	6	-	1:40	H	-	-
NS178	MTANDARANI	Nsimbo	Male	14	+	1:20, 1:80, 1:80,1:640	K,G,S,H	-	-
NS179	MTANDARANI	Nsimbo	Female	35	-	1:80	H	-	-
NS180	MTANDARANI	Nsimbo	Female	18	-	1:80	K	-	-
NS181	MTANDARANI	Nsimbo	Female	3	-	1:80, 1:80	K,H	-	-
NS182	MTANDARANI	Nsimbo	Female	32	+	1:20, 1:160	K,H	-	-

NS183	MTANDARANI	Nsimbo	Female	3	+	1:20, 1:360	H	-	-
NS184	MTANDARANI	Nsimbo	Female	6	-	1:40, 1:80	K,H	-	-
NS185	MTANDARANI	Nsimbo	Female	13	-	1:80,1:80	L,H	-	-
NS186	MTANDARANI	Nsimbo	Female	8	+	1:160	H	-	-
NS187	MTANDARANI	Nsimbo	Female	24	+	1:80, 1:40, 1:40, 1:5120	L,K,F,H	+	-
NS188	MTANDARANI	Nsimbo	Female	63	-	1:20, 1:40	K,H	-	-
NS189	MTANDARANI	Nsimbo	Female	25	-	1:80, 1:80	Hd,H	-	-
NS190	MTANDARANI	Nsimbo	Female	9	-	1:40, 1:40, 1:40	L,Hd,K	-	-
NS191	MTANDARANI	Nsimbo	Female	9	-	1:80, 1:80	K,H	-	-
NS192	MTANDARANI	Nsimbo	Female	43	-	1:80	K	-	-
NS193	MTANDARANI	Nsimbo	Female	39	+	1:20,1:20, 1:80, 1:2560	Hd,K,S,H	+	-
NS194	MTANDARANI	Nsimbo	Male	18	-	1:80,1:40	K,S	-	-
NS195	MTANDARANI	Nsimbo	Male	13	-	1:20	Hd,	-	-
NS196	MTANDARANI	Nsimbo	Male	7	+	1:160, 1:20, 1:40	Hd,L,H	-	-
NS197	MTANDARANI	Nsimbo	Male	6	-	1:20	K	-	-
NS198	MTANDARANI	Nsimbo	Male	5	-	1:20, 1:20	K,H	-	-
NS199	MTANDARANI	Nsimbo	Male	12	-	-		ND	ND

NS200	MTANDARANI	Nsimbo	Male	13	+	1:1:20, 1:1280	K,S	-	-
NS201	MTANDARANI	Nsimbo	Male	9	+	1:80, 1:40, 1:1280, 1:10240	Hd,K,S,H	+	-
NS202	MTANDARANI	Nsimbo	Male	15	-	1:20,	K	-	-
NS203	MTANDARANI	Nsimbo	Female	22	-	1:40, 1:80	K,H	-	-
NS204	MTANDARANI	Nsimbo	Female	20	-	1:40	K	-	-
NS205	MTANDARANI	Nsimbo	Female	13	-	-		ND	ND
NS206	MTANDARANI	Nsimbo	Female	45	+	1:160, 1:20, 1:40	Hd,K,H	-	-
NS207	MTANDARANI	Nsimbo	Male	30	-	-		ND	ND
NS208	MTANDARANI	Nsimbo	Male	20	-			ND	ND
NS209	MTANDARANI	Nsimbo	Male	45	-	1:40, 1:40	K,H	-	-
NS210	MTANDARANI	Nsimbo	Female	35	-	1:40	H	-	-
NS211	MTANDARANI	Nsimbo	Female	8	-	-		ND	ND
NS212	STALIKE	Nsimbo	Female	20	+	1:40, 1:320	K,H	-	-
NS213	STALIKE	Nsimbo	Female	30	-	-		ND	ND
NS214	STALIKE	Nsimbo	Female	35	-	-		ND	ND
NS215	STALIKE	Nsimbo	Female	42	+	1:40, 1:160, 1:320	L,K,H	-	-
NS216	STALIKE	Nsimbo	Male	7	-	1:80	K	-	-

NS217	STALIKE	Nsimbo	Male	3	-	1:40	K	ND	ND
NS218	STALIKE	Nsimbo	Female	35	-	1:80, 1:80	S,H	-	-
NS219	STALIKE	Nsimbo	Male	43	-	1:20	K	-	-
NS220	STALIKE	Nsimbo	Male	1	-	1:80	K	-	-
NS221	STALIKE	Nsimbo	Female	6	-	1:20	K	-	-
NS222	STALIKE	Nsimbo	Female	28	+	1:80, 1:320	K,H	+	-
NS223	STALIKE	Nsimbo	Male	32	-	1:40, 1:40	K,S	-	-
NS224	STALIKE	Nsimbo	Male	15	+	1:40, 1:40, 1:320	Hd,K,H	+	-
NS225	STALIKE	Nsimbo	Male	9	-	-		ND	ND
NS226	STALIKE	Nsimbo	Female	27	-	-		ND	ND
NS227	STALIKE	Nsimbo	Female	8	-	1:80	H	-	-
NS228	STALIKE	Nsimbo	Female	4	-	1:20	K	-	-
NS229	STALIKE	Nsimbo	Female	5	-	1:80	S	-	-
NS230	STALIKE	Nsimbo	Female	23	-	1:80, 1:80	S,H	-	-
NS231	STALIKE	Nsimbo	Male	28	-	-		ND	ND
NS232	STALIKE	Nsimbo	Male	14	-	-		ND	ND
NS233	Nsimbo	Nsimbo	Male	33	-	1:80		-	-

NS234	Nsimbo	Nsimbo	Male	16	-	1:20, 1:80	K,S	-	-
NS235	Nsimbo	Nsimbo	Male	10	-	-		ND	ND
NS236	Nsimbo	Nsimbo	Female	11	-	-		ND	ND
NS237	Nsimbo	Nsimbo	Female	7	-	-		ND	ND
NS238	Nsimbo	Nsimbo	Female	30	-	1:40	H	-	-
NS239	Nsimbo	Nsimbo	Female	49	+	1:160	Hd	-	-
NS240	Nsimbo	Nsimbo	Male	12	+	1:160, 1:80, 1:80	K,S,H	+	-
NS241	Nsimbo	Nsimbo	Male	10	-	-		ND	ND
MB242	Mamba	Mlelele	Female	58	+	1:20, 1:160	K,H	+	-
MB243	Mamba	Mlelele	Female	52	+	1:160, 1:40	K,H	+	-
MB244	Mamba	Mlelele	Male	59	-	1:80	S	-	-
MB245	Mamba	Mlelele	Female	2	-	1:180	H	-	-
MB246	Mamba	Mlelele	Female	10	-	1:40, 1:80, 1:80	Hd,K,H	-	-
MB247	Mamba	Mlelele	Female	28	-	-		ND	ND
MB248	Mamba	Mlelele	Female	23	-	1:20, 1:80	K,H	-	-
MB249	Mamba	Mlelele	Female	22	-	1:20	K	-	-
MB250	Mamba	Mlelele	Male	12	-	-		ND	ND

MB251	Mamba	Mlelele	Male	42	-	-		ND	ND
MB252	Mamba	Mlelele	Female	9	-	-		ND	ND
MB253	Mamba	Mlelele	Male	49	-	1:80, 1:80	S,H	-	-
MB254	Mamba	Mlelele	Female	19	-	-		ND	ND
MB255	Mamba	Mlelele	Male	26	-	1:40, 1:80	L,H	-	-
MB256	Mamba	Mlelele	Female	16	-	1:80	H	-	-
MB257	Mamba	Mlelele	Female	16	-	1:80	H	-	-
MB258	Mamba	Mlelele	Male	10	+	1:40, 1:160	L,H	+	-
MB259	Mamba	Mlelele	Female	53	-	-		ND	ND
MB260	Mamba	Mlelele	Female	38	-	-		ND	ND
MB261	Mamba	Mlelele	Male	27	-	-		ND	ND
MB262	Mamba	Mlelele	Female	45	-	-		ND	ND
MB263	Mamba	Mlelele	Female	5	-	1:80, 1:40	S,G	-	-
MB264	Mamba	Mlelele	Female	9	-	1:40, 1:80	L,H	-	-
MB265	Mamba	Mlelele	Female	5	-	1:80, 1:20	L, H	-	-
MB266	Mamba	Mlelele	Female	5	-	-		ND	ND
MB267	Mamba	Mlelele	Female	28	-	1:20	L	-	-

Appendix 2: Field and laboratory photos

Photo1: Microscopic Agglutination Test assay performed at pest management center, SUA. 1: Dark field microscope; 2 Microtiter plates containing sera and testing antigen; 3: Glass slide for reading agglutination under dark field microscope; 4: Recording of agglutination titer

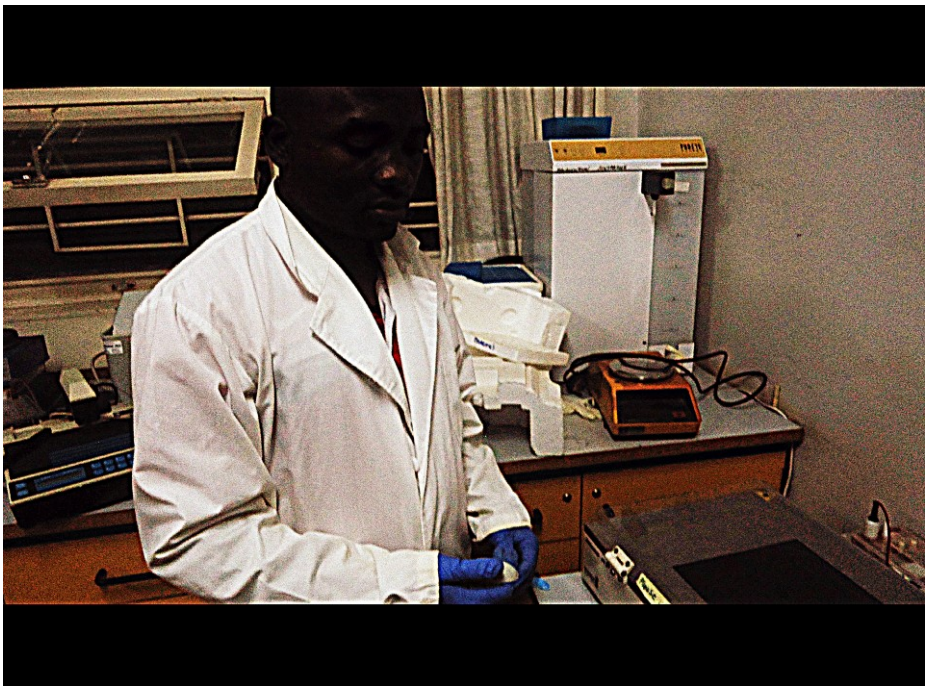

Photo 2: Shabani Kililwa Muller running gel ectectrophoresis at Sokoine University of Agriculture. Department of microbiology and parasitology.

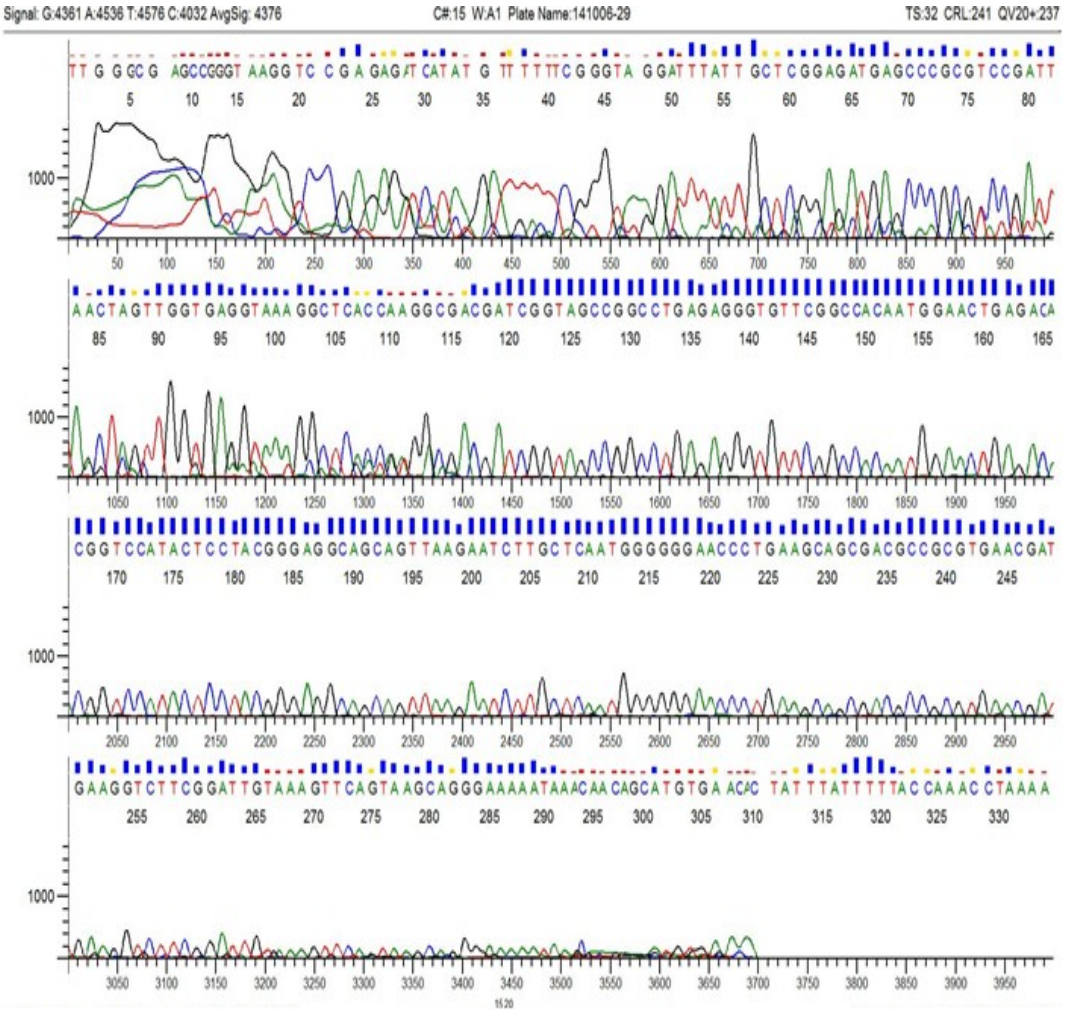

Figure 1: Raw data of the sequences from the pathogenic uncultured *Leptospira* clone species from Katavi-Rukwa ecosystem.

Appendix 3: Standard operating procedure for DNA extraction from Blood lysate

SOP version: 001

Title: Invitrogen DNA extraction from EDTA blood Document Number: VMLEPTO001			
Prepared by Muller Shabani	Name and title Laboratory scientist	Signature	Date:05/06/2014
Revised by			
Approved by			

1. Material/Equipment

96-100% ethanol

EDTA Blood samples

PBS

Proteinase K

RNase A

PureLink Genomic Lysis/Binding Buffer

PureLink Spin Column

Wash Buffer 1,

Wash Buffer 2

Genomic Eluting Buffer

Microcentrifuge capable of 10 000g

Water bath

-20 freezerpable of accurately dispensing 1 to 200ul volumes.

Calibrated pipettes capable of accurately dispensing 1 to 200ul volumes

2. Consumable

3. Procedures

1. Set the water bath at 55^0C
2. Add 200 ul of frozen blood in a sterile microcentifuge tube
3. Add 20 ul of Proteinase K to the sample
4. Add 20 ul RNase A to the sample, mix well by vortexing, and incubate at room temperature for 2 min
5. Add 200 ul of PurLink^{tm} genomic Genomic Lysis buffer/Bindig Buffer , mix well by vortexing to obtain a homogeneous solution
6. Incubate at 55 oC for 10 min to promote protein digestion
7. Add 200 ul 96-100%ethanal to the lysate. Mix well by vortexing for 5 second to yield homogenous solution

   Binding DNA

   Remove a Pure link^{TM} Spin column in a collection tube from the package
8. Add the lysate (about 640 ul) to the Purelink^{TM} Spin Column
9. Centrifuge the column at 10 000 xg for 1 minute at room temperature.

   Note: if you are processing >200 ul staring materials such as blood , buccal swabs,or Oragene^{TM} preserved saliva, you need to perform multiple loading of the lysate by transferring any remaining lysate to the same PureLink^{TM} Spin Column (above) and centrifuge at 10,000 xg for 1 minute.
10. Discard the collection tube and place the spin column into a clean PureLink^{TM} Collection Tube supplied with the kit.

Washing DNA

11. Add 500 ul Wash Buffer 1 to the column
12. Centrifuge column at room temperature at 10 000 xg for 1 min

13. Discard the collection tube and place the spin column into a clean PureLink™ collection tube supplied to the kit
14. Add 500ul Wash Buffer2 to the column
15. Centrifuge the column to the maximum speed for 3 minutes at room temperature, discard the collection tube

Eluting DNA

16. Place the spin column in a sterile 1.5ml microcentrifuge tube
17. Add 100 ul of PurelinkGenomic Buffer to the column
18. Incubate at room temperature for 1 minute. Centrifuge the column at maximum speed for 1 minute at room temperature. The tube contains purified genomic DNA.
19. NB: To recover more DNA, perform a second elution step using the same elution buffer volume as firs elution in another sterile 1.5ml microcentrifuge tube.
20. Centrifuge the column at maximum speed for 15 minutes at room temperature The tube contains purified DNA. Remove and discard the column.

Storing DNA

21. Store the purified DNA at -20 oC or use DNA for the desired downstream application