# ADDIS ABABA UNIVERSITY
# COLLEGE OF HEALTH SCIENCES
# SCHOOL OF MEDICINE

**Department of Medical Microbiology, Parasitology and Immunology**


# Molecular identification and characterization of pathogenic *Leptospira* from asymptomatic humans, livestock and water sources in peri-urban areas of Addis Ababa: A One Health Concept

**A Thesis Submitted for the Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology**


**By: Marrigje Jacoba Kreuger, MD, MSc**

**Advisor: Dr. Woldaregay Erku Abegaz, Associate Professor**



**July 2021**

**Addis Ababa – Ethiopia**

# ACKNOWLEDGEMENT

This Master Thesis was a very interesting learning experience and I am grateful to have experienced what it is to do a small research in Ethiopia, through big lessons in patience and creativity. This Master Thesis could not be finished without the help of many important people and without the help of God. I would like to thank my advisor Dr. Woldaregay Erku Abegaz for his unlimited support, patience and advices provided during the development of the idea and fulfillment of this study. I am very grateful for Dr. Zewdu Terefework from MRC-ET providing his support and offering me his laboratory to perform my research. Toon Stoutje and Jan Schouten from MRC-Holland have done an incredible work in providing materials and their enthusiasm in helping me to analyze the data. Dr. Marga Goris and Ahmed Ahmed in the Netherlands have been a great help with their leptospirosis-specific advices and the supply of laboratory materials. I would like to thank the Addis Ababa University for this special opportunity to study and perform research in Ethiopia. My classmate Gemechu Chala has been a special help during data collection and a good motivator before and afterwards. All animal health assistants, veterinary medicine students and veterinary doctors from the different woredas of Addis Ababa have been a great help in the data collection and connecting me to the right households. Siobhan Moore has helped me during the challenging part of analysing the results. My colleagues from Nordic Medical Centre have been giving me the freedom and support to fulfill this research. My father with his veterinary background and interest for zoonotic diseases should get the honour for inspiring me to focus on a zoonotic diseases in Ethiopia from One Health perspective.

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# ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AAU** | Addis Ababa University |
| **CAAT** | Cross-agglutination absorption test |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **FAO** | Food and Agriculture Organization of the United Nations |
| **GHSA** | Global Health Securities Agenda |
| **KAP** | Knowledge, attitude and practice |
| **LPS** | Lipopolysaccharide |
| **MAT** | Microscopic agglutination test |
| **NOHSC** | National One Health Steering Committee. |
| **OHCN** | National One Health Communication Network |
| **OIE** | World Organisation for Animal Health |
| **PCR** | Polymerase Chain Reaction |
| **WHO** | World Health Organization |

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# ABSTRACT

**Background**

Leptospirosis is a neglected zoonotic disease caused by spirochetes, pathogenic *Leptospira*. It can give a severe disease in humans. Many domestic animals can carry pathogenic *Leptospira* and *Leptospira* can survive in environmental water sources. There are barely studies done to investigate the presence of *Leptospira* in humans, animals or water sources in Ethiopia.

**Objectives**

Identification of the presence of pathogenic *Leptospira* in asymptomatic humans, livestock and environmental water sources and assessment of knowledge, attitude and practice concerning leptospirosis among households in peri-urban areas of Addis Ababa.

**Methodology**

Urine was collected from asymptomatic humans and livestock in peri-urban areas of Addis Ababa. Water samples were taken from the same areas. After DNA-extraction, real-time PCR and melting curve analysis were performed. Knowledge, attitude and practice (KAP) was assessed with help of a questionnaire.

**Results**

In total, 105 human urine samples, 194 animal urine samples and 32 water samples were collected from 85 households. Pathogenic *Leptospira* were found in 3 of the 194 animal urine samples (1.5%), characterized as *Leptospira borgpetersenii* and detected in urine of cattle. No pathogenic *Leptospira* were found in the human urine samples and in the water samples. Majority (97.6%) of the respondents had never heard about leptospirosis, but knew that water, animals' urine and rat urine could contain pathogens. Multiple risk factors for the presence of *Leptospira* were found in the studied households: walking through wet areas without adequate protection (48.2%), rats in (66.3%) or around (63.8%) the house and garbage stored inside the compound (56.1%). No significant relationship was found between risk factors and the positive *Leptospira* samples in this study.

**Conclusion**

This study has shown that pathogenic *Leptospira* are present in peri-urban areas of Addis Ababa. Risk factors for the presence of pathogenic *Leptospira* and other zoonoses were widespread in the study area. These findings highlight the need to create awareness among livestock-keeping households concerning leptospirosis using a "One Health" concept.



**Keywords:** *Leptospirosis; One Health; pathogenic Leptospira; PCR; Zoonosis*

# 1. INTRODUCTION

## 1.1 Background

Leptospirosis is a globally important zoonotic disease caused by infection with pathogenic *Leptospira,* which are bacteria belonging to the phylum *Spirochaetes* (WHO, 2003; Torgerson *et al.*, 2015). Humans are usually infected by contact with urine of an infected reservoir host, contaminated drinking water or soil, or infected animal tissue. Rodents are notorious reservoirs, but also are a variety of wild and domestic animals and insectivores (WHO, 2003). Pathogenic leptospires enter the body through mucous membranes, conjunctivae, small cuts, abrasions, and possibly wet skin and can cause severe illness with haemorrhage, jaundice and renal failure. Severe leptospirosis is called Weil's disease and has a high mortality. Though with early detection and treatment, leptospirosis is a treatable disease (Levett, 2001; WHO, 2003).

Although leptospirosis can be found worldwide, it is especially common in warm and humid tropical regions where the environment is favourable for the survival and perpetuation of the spirochetes (Evangelista and Coburn, 2010; Hartskeerl *et al.*, 2011). Known endemicity of human leptospirosis is focused on Asia, Middle- and South America, Oceania and Europe. In many low- and middle-income countries, also in Africa, leptospirosis is a neglected disease (Allan *et al.*, 2015; Costa *et al.*, 2015; Torgerson *et al.*, 2015). Neglected in different ways: lack of information, lack of diagnostic possibilities and lack of attention to the population vulnerable for leptospirosis. Especially from African countries, data is scarce. Most epidemiological data on leptospirosis in East Africa comes from Kenya, Uganda, Madagascar and Tanzania; and Ethiopia lags behind in available data on leptospirosis (de Vries *et al.*, 2014; Allan *et al.*, 2015; Asante *et al.,* 2019). Ethiopia has an estimated population of more than 114 million people (UN, 2019) and a rapidly growing and urbanizing capital city, Addis Ababa. There has not been a recent population census of Addis Ababa, the population was estimated to be 3.2 million in 2013 (CSA, 2013) and more than 4.7 million inhabitants currently (UN, 2019). Ethiopia is highly dependent on agriculture, has many (poor) livestock keepers and many households have direct contact with animals, important factors related to zoonotic diseases like leptospirosis (Grace *et al.*, 2012; Shapiro *et al.*, 2017).

Zoonoses, such as leptospirosis, are nowadays preferably approached by the "One Health" concept, which is a major movement of the 20$^{th}$ century and implies an integrated approach to health that focuses on the interactions between animals, humans and their diverse environments

(ECDC, 2018; WHO, 2017; WHO, FAO and OIE, 2019). According to the "One Health" approach, not only the presence of the disease in humans should be studied, also the presence of pathogens in reservoirs and favourable factors for transmission should be determined (Allan *et al*., 2015; Cleaveland *et al*., 2017; WHO, FAO and OIE, 2019).

### 1.2 Statement of the problem

There is insufficient information about the occurrence of leptospirosis in Ethiopia (de Vries *et al.*, 2014; Pieracci *et al.*, 2016). However, the circumstances for leptospirosis are favourable, with the presence of a huge livestock distributed among smaller farms, low coverage of animal health services, many people living in close contact with animals, and the presence of rainfall suitable to the pathogen (Grace *et al.*, 2012; Shapiro *et al.*, 2017). When the first prioritization process for zoonotic diseases in Ethiopia started a few years ago, leptospirosis was ranked as 4$^{th}$ in the list of zoonoses of concern for the country (Pieracci *et al.*, 2016). This implies that not paying attention to leptospirosis will negatively impact human and animal health. Despite that, diagnostic tests to detect leptospirosis are not easily available in Ethiopia. Also no recent studies are published about human leptospirosis in Ethiopia. Only one serological study was performed in 2003, among a limited number of symptomatic patients (Yimer *et al.*, 2004). The presence of leptospirosis in animals has also been hardly investigated (Moch *et al.*, 1975; Obeck & Berhanu, 1976; Tsegay *et al*., 2016; Marami *et al.*, 2021). The few previous studies done revealed positive serology for leptospirosis and gave an indication of the presence of leptospirosis in the country. Therefore, the burden of animal and human leptospirosis in Ethiopia is not known. While highest estimates of disease morbidity and mortality of leptospirosis are observed in resource-poor and low-surveillance areas, including East Sub-Saharan Africa (Costa *et al.*, 2015). Not knowing the extent of human leptospirosis in daily clinical practice can lead to misdiagnosis and mistreatment, as acute febrile illness of unknown origin is common in Ethiopia (Tadesse & Tadesse, 2013; Feleke *et al.*, 2015; Zerfu *et al.*, 2018). Additionally, being unaware about the magnitude of leptospirosal infections in livestock is undesirable in a country with the largest number of livestock in Africa, low livestock productivity and an increasing demand for livestock and livestock products (FAO, 2019).

Integrated studies and approaches from human, animal and environmental sectors, which are the components of "One Health", lack in Ethiopia for many zoonotic diseases, including leptospirosis (Pieracci *et al.*, 2016). As Ethiopia has committed itself to work on prevention and

control of leptospirosis (Pieracci *et al.*, 2016), it needs to gain information about the presence of leptospirosis and pathogenic *Leptospira* first, investigating the disease from "One Health" perspective.

### 1.3 Justification of the study

Since the data on leptospirosis in Ethiopia are scarce, regarding both the epidemiology and the level of public awareness of the disease, and since leptospirosis was recently marked as a disease of concern which needs attention in Ethiopia (Pieracci *et al.*, 2016), this study focuses on the detection of *Leptospira,* the causative agent of leptospirosis, from asymptomatic humans, livestock and water used by humans in the peri-urban areas of Addis Ababa. The fact that leptospirosis is a potential severe disease, but treatable and preventable, is another reason that leptospirosis is the subject of this study. Knowledge about the presence of pathogenic *Leptospira* can benefit health of both humans and animals, and can guide the strategy of awareness creation, prevention and treatment. Peri-urban areas of Addis Ababa were chosen as study area, because the circumstances in these areas are expected to favour survival and transmission of the spirochetes. In general, African peri-urban settings are characterized by crowded environments, poverty, poor housing and sanitation, close contact with animals, dependency on natural resources and are therefore prone to zoonoses (Waldman, 2015). Asymptomatic humans were chosen to get an impression of the baseline occurrence of *Leptospira* in the investigated community and it can be positive in case of active asymptomatic infection, recent infection or asymptomatic and even seronegative urine shedders (Ganoza *et al.*, 2010; Podgoršek *et al.*, 2020). Livestock was selected because livestock has been shown to be an important host of *Leptospira* in Africa (Allan *et al.*, 2015). Molecular assays were selected as diagnostic method, to be able to give reliable information about *Leptospira* and their characterization. Leptospiral DNA can be accurately detected in urine of humans and reservoir animals, and from water sources, by molecular assays (Levett, 2001; Mussa & La Scola, 2013).

While leptospirosis is considered as a neglected zoonosis, intersectoral approach and collaboration as guided by the "One Health" concept is fundamental and recommended in investigating the disease (WHO, 2014). Information provided on leptospirosis from the three pillars of "One Health", can help in developing "One Health" measures to prevent and intervene on the disease (Cleaveland *et al.*, 2017; Asante *et al.*, 2019).

## 1.4 Significance of the study

This study gives, to our knowledge, for the first time in Ethiopia, information about the occurrence of pathogenic *Leptospira* in asymptomatic humans, livestock and environmental water sources at the same time.

The findings derived from this study can assist healthcare and (public) health professionals as well as veterinary practitioners, in information provision about pathogenic *Leptospira* in the environment and households, to recognize the potential threat of leptospirosis, and to help them improve and protect human health.

The findings additionally help households to get to know existing risk factors for leptospirosis in their immediate environment, which they can use for healthy livestock keeping and protection of their own health.

Furthermore, the data obtained from the current study can be used as baseline information for further extensive researches concerning leptospirosis and pathogenic *Leptospira* to be conducted in the future, especially in line with the "One Health" concept and application. It can help parties involved in "One Health" to prioritize and to develop prevention and management directions.

# 2. LITERATURE REVIEW

## 2.1 Characteristics of *Leptospira*

Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*, the family *Leptospiraceae* and the order Spirochaetales (Paster *et al.*, 1991). *Leptospira* are thin, tightly coiled, spiral-shaped spirochetes, 6–20 μm long and 0.1-0.15 μm in diameter. *Leptospira* have endoflagella that reside within the periplasmic space (periplasmic flagella), which are inserted at each pole of the cell and extend towards the middle of the cell without overlapping. The two periplasmic flagella determine the motility of *Leptospira* and give the organism a spiral-shaped end morphology when the endoflagellum is rotated anticlockwise and a hook-shaped end when the endoflagellum is not rotated or rotated clockwise (Levett, 2001; Picardeau, 2017). The periplasmic space also contains periplasmic filaments with a diameter of 8 nm along the entire cell length, including in the middle of the cell, where endoflagella are absent (Picardeau, 2017). Leptospires have a double membrane structure in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane. Lipopolysaccharide (LPS) is the major component of the outer membrane and structurally similar to LPS from Gram-negative organisms. *Leptospira* are obligate aerobes with an optimum growth temperature of 28-30 °C and are killed by high temperatures, chemicals, a pH < 6.5 and > 8.4, drying and high salt levels (Levett, 2001; WHO, 2003; Picardeau, 2017).

Traditionally, the genus *Leptospira* was divided into two species: *Leptospira interrogans* comprising pathogenic strains requiring a host for multiplication and *Leptospira biflexa* comprising saprophytic strains capable of living in the environment. Further division within these species was made based on serology. Structural heterogeneity in the carbohydrate component of the LPS of the spirochetes determined antigenic diversity (Picardeau, 2017). A serovar was determined with the help of the cross-agglutination absorption test (CAAT): agglutination after cross-absorption with homologous antigen defined the serovars (Faine 1982; Levett, 2001; Marquez *et al*., 2017). Two strains with more than 10% heterogeneity were assigned to different serovars. A 0-10% difference in antibodies remaining after absorption represented strains belonging to the same serovar (Faine, 1982; Marquez *et al.*, 2017). Antigenically related serovars are grouped into serogroups, eg. serogroup *Icterohaemorrhagiae, Autumnalis, Bataviae.* Although serogroups are not considered valid in formal taxonomy anymore, they are still used for clinical and epidemiological purposes. More than 300 serovars have been established, of which more than 250 are pathogenic (Levett, 2001;

Picardeau, 2017). The genus *Leptospira* has been reclassified later with genetic techniques and the two species *Leptospira interrogans* and *Leptospira biflexa* have been separated in 22 genomospecies nowadays (Marquez *et al.*, 2017; Picardeau, 2017).

The phylogenetic classification does not correlate well with the serological classification (Levett, 2001; Levett, 2015; Thibeaux *et al.*, 2018). Phylogenetic classification was primarily based on the 16S rRNA gene and DNA-DNA hybridization, and later on whole genome sequences. This classification separated the genus into saprophytes, intermediates and pathogens (Picardeau, 2017; Thibeaux *et al.*, 2018). Genomes of pathogenic species (like *L. interrogans* and *L. borgpetersenii*) and saprophytic species (like *L. biflexa*) of leptospirosis are compared to each other to determine what makes a bacterial genus pathogenic. To correlate the genetic differences with biological differences is a challenge; there are many pathogen-specific genes with unknown function (Adler *et al.*, 2011). LipL32 is a subsurface lipoprotein in the cell wall, binding to extracellular matrix components, and is unique to pathogenic and intermediate species (Adler *et al.*, 2011; Picardeau, 2017). The leptospiral immunoglobulin-like protein family (LigA, LigB, LigC) is also found only in pathogenic leptospires; these proteins are indicated as virulence factors involved in cell adhesions (Fouts *et al.*, 2016). Another lipoprotein is Loa22, a lipoprotein in the cell wall without clear function. It was the first described virulence factor in pathogenic *Leptospira* species, although it is also found in saprophytic species, and this suggests that it is involved in survival rather than direct virulence (Adler *et al.*, 2011; Picardeau, 2017). Genes that are required for the synthesis of vitamin B12 and sialic acid are present only in pathogenic species (Fouts *et al.*, 2016; Picardeau, 2017). The Virulence Modifying proteins, a large novel protein family of unknown function, is found uniquely in pathogenic *Leptospira* (Fouts *et al.*, 2016). Contrariwise, genes found in saprophytic species are absent from the pathogenic species; these are mainly involved in environmental sensing and nutrient acquisition (Adler *et al.*, 2011; Picardeau, 2017). Next to pathogenic species and saphrophytic species, intermediate *Leptospira* species exist, which are phylogenetically close to pathogenic species but lack some of the genes. Examples of pathogenic species are *Leptospira interrogans, Leptospira kirschneri, Leptospira borgpetersenii, Leptospira santarosai* and *Leptospira weilii* (Fouts *et al.*, 2016).

A large-scale sequencing project entitled ‘Leptospira Genomics and Human Health’ has generated whole-genome sequences for numerous strains belonging to 20 *Leptospira* species

from diverse origins and geographical areas. Global analysis of these genomes has led to the identification of a core genome of 1764 genes, of which 737 genes are specific to *Leptospira*. Considering the pathogenicity, 369 genes are identified to be specific to species with some degree of pathogenicity (pathogens and intermediates), and 416 genes specific to pathogenic species (Fouts *et al.*, 2016; Guernier *et al.*, 2017).

### 2.2 Sources and Transmission of *Leptospira*

Leptospirosis as a disease is the result of a complex interaction between humans, animal reservoirs and the environment (WHO, 2003; Ullmann & Langoni, 2011). Various animal species including rodents, insectivores, companion animals and livestock, as well as wildlife, have been shown to be reservoirs for leptospires. Rodents were the first recognized reservoir animals and still considered as the largest group of reservoirs for the bacteria. Among domestic animals, cattle, dogs, sheep, goats, horses and pigs may act as a reservoir (Levett, 2001; WHO, 2003; Allan *et al.*, 2015). A certain serovar of leptospirosis is usually associated with one or more animal species and an animal infected with the host-adapted serovar of the organism is called a reservoir host or a maintenance host (WHO, 2003). For instance, rats are reservoir host for the serovar Icterohaemorrhagiae, Ballum and Copenhageni, dogs are reservoir for the serovar Canicola, pigs for the serovar Bratislava and cattle for the serovar Hardjo. Additionally, one animal species may act as a host to different serovars and a serovar may adapt to new host species (Levett, 2001; WHO, 2003; Ullmann & Langoni, 2011). An incidental or accidental host is a susceptible animal or human that becomes infected with a non-host-adapted serovar (WHO, 2003; Yadeta *et al.*, 2016).

Animals, as well as humans, spread leptospires in their urine because of colonization of the proximal renal tubules by leptospires. The urine is the source of contamination for other animals and humans (WHO, 2009; Monahan *et al.*, 2009). Transmission occurs when water or soil contaminated with urine of reservoir animals comes in contact with exposed mucosa, abrased skin or when ingested (Levett, 2001; Grace *et al.*, 2012). Accidental hosts, such as humans, shed the leptospires usually for a limited time in their urine, while many reservoir hosts shed the spirochetes for a prolonged time (months – years). Rodents are able to shed a large amount of leptospires throughout their lifespan in the urine, without clinical manifestations, and are therefore a permanent carrier of a particular serovar of leptospirosis (Levett, 2001; WHO, 2009; Ellis, 2015; Yadeta *et al.*, 2016). Leptospires can survive outside the host for more than six

months in moist, warm conditions. Therefore, pathogenic leptospires are widespread in nature and their life cycles keep them in the environment (WHO, 2009; Thibeaux *et al.*, 2018). Studies have shown periodes of heavy rain triggering outbreaks of leptospirosis, and a hypothetical mechanism is that virulent leptospires survive in soils, washed by the rain, putting particles including leptospires, in surface water (Bierque *et al.*, 2020). In general, occupational, behavioral or environmental factors exposing people to a wet environment contaminated with leptospires, or exposing them to the excretions or tissues of carrier animals, increases the risk of leptospiral infection. People who work with water and water supply, mine workers, fishermen and farmers have been described as being occupational at high risk for developing leptospirosis, but environmental factors like waste disposal in urban slums, flooding and heavy rainfall can also pose a risk to leptospiral infection (Levett, 2001; Ullmann & Langoni, 2011; Grace *et al.*, 2012; de Vries *et al.*, 2014).

### 2.3 Leptospirosis in animals

A wide range of wild and domestic animals can get infected with leptospirosis. Various Leptospirosa serovars have been isolated from rodents and from domestic animals, such as cattle, pigs, horses, dogs, rodents, goats and sheep. Numerous wild animals such as bats, marsupials, deers, buffaloes, foxes and coyotes have been shown to carry pathogenic *Leptospira* as well (WHO, 2003; Ellis, 2015; Hamond *et al.*, 2014).

Animals get infected mostly through environmental exposure or direct contact with urine from other animals, although venereal transmission is also common in some mammal species (Ellis, 2015). Clinical signs of leptospirosis in animals depend on the type of animal, the infecting serovar and leptospiral load. Asymptomatic infections are common, especially when reservoir animals get infected with host-adapted serovars (Yadeta *et al.*, 2016). Chronically infected rats and mice are asymptomatic (Gomes-Solecki, Santecchia & Werts, 2017). Illness occurs especially when the animal gets infected with a non-host-adapted serovar. An example of this incidental infection is when cattle gets infected with serovar Pomona (Evangelista & Coburn, 2010; Yadeta *et al.*, 2016). Leptospires can be found in body fluids of the infected animals, such as urine, blood and milk. In urine, the leptospires can be found from 5-10 days after the infection until months or even years. An intermittent pattern of urine leptospiral shedding has been described in animals (Barragan *et al.*, 2017; Rocha *et al.*, 2017). Additionally, leptospires

can be found in aborted fetuses, afterbirths or uterine discharges of infected animals, which can in turn lead to environmental contamination (Yadeta *et al.*, 2016).

Clinical signs in animals are usually related to reproduction, the kidney and the liver. Food producing animals, like cattle, sheep, goats and pigs, are relatively susceptible to clinical infections, resulting in production losses including reduced milk yield, abnormal milk yield, mastitis, reproductive failure, abortions, premature birth and stillbirth. Acute leptospirosis with fever, anorexia, diarrhea, icterohaemorrhagic syndrome and conjunctivitis is more often found in young animals such as calves, lambs and young goats, than in adult animals (WHO, 2009; Ellis, 2015; Yadeta *et al.*, 2016). Leptospirosis in adult cattle is often without clinical illness. When symptoms are present, they can include infertility, a temporary drop in milk production and abortions, depending on the infecting serovar (Lilenbaum & Martins, 2014). Most infections in sheep and goats are subclinical, and if symptomatic, symptoms are similar to those found in cattle. Severe equine leptospirosis is also uncommon, even though many horses carry high titers against certain serovars. Abortions, opthalmia and acute respiratory distress are observed in clinical equine leptospirosis. Acute and chronic infections in dogs can present similar to that in humans, while clinical disease in cats is uncommon (WHO, 2009; Grace *et al.*, 2012; Ellis, 2015; Yadeta *et al.*, 2016).

Licensed vaccines against leptospirosis are on the market for decades and are used in cattle, horses, dogs and pigs. They may contain one or more serovars. The major drawback of the vaccines is that their effect is short-term and that gained immunity is serovar specific (WHO, 2003; Ellis, 2015).

### 2.4 Leptospirosis in humans

Humans are an accidental dead-end host of leptospires (WHO, 2003; De Brito *et al.*, 2018). Humans can get infected via intact mucous membranes, via abrasions or cuts in the skin and additionally by prolonged immersion in, or swallowing of, contaminated water (Levett, 2001; De Brito *et al.*, 2018). After entering the port d'entree, the leptospires enter via lymphatic vessels into the bloodstream. Once inside the host, leptospires persist in the bloodstream during the leptospiremic phase of the illness, which is just before the onset of symptoms until the end of the first week (Haake & Levett, 2015). Hematogenous dissemination causes leptospires to find their way to the organs (Zuerner, 2015; De Brito *et al.*, 2018). The incubation period is

usually between one and two weeks, but might be between 3 and 30 days (WHO, 2009; Helmerhorst *et al.*, 2012).

The spectrum of symptoms in humans is very broad. Although an infection presents often as asymptomatic or as a mild 'flu-like illness', the most predominant health impact of the disease is attributed to more severe infections (5-15% of all clinical infections), multi-organ failure and pulmonary haemorrhage (WHO, 2003; Costa *et al.*, 2015). A common presentation of symptomatic leptospirosis infection is an acute febrile illness with headache, myalgia, prostration and any of the following symptoms: conjunctival suffusion, anuria or oliguria, jaundice, cough, haemorrhages, meningeal irritation, skin rash, cardiac arrhythmia or cardiac failure. Often there is a biphasic pattern of the fever (WHO, 2003). The prototype of clinical illness due to leptospiral infection is Weil's disease, although this represents only the severe presentation with (pulmonary) haemorrhage, jaundice, cardiovascular problems, hepatic and renal failure and a mortality of 20-50% without adequate treatment (WHO, 2003; Picardeau, 2017). Another severe presentation of leptospirosis, suggested to be different from Weil's disease, is severe pulmonary haemorrhagic leptospirosis which is characterized by intra-alveolar haemorrhage and respiratory failure with a mortality of more than 50% (Helmerhorst *et al.*, 2012).

In mild cases of leptospirosis, the disease is self-limiting. Antibiotic treatment is indicated for the more severe cases of leptospirosis, although antimicrobial therapy is given also for milder cases to reduce symptoms. The recommended antibiotics are penicillines, tetracyclines and cephalosporins (WHO, 2003; Haake & Levett, 2015). Development of severe outcomes depends on epidemiological conditions, host susceptibility and pathogen virulence. Mortality rates are related to the severity of the disease, and an altered mental status, oliguria, older age and pulmonary involvement are identified poor prognostic signs (Haake & Levett, 2015). A case fatality rate of 5.7% is described for leptospirosis worldwide (Costa *et al.*, 2015), while the case fatality rate of mild cases due to underdiagnosis is most likely much lower (Goris, 2016). With proper treatment and supportive care, most patients recover completely. Nevertheless, late sequelae including fatigue, myalgia, headache and uveitis may occur (Haake & Levett, 2015). The bacteria can be present for months in the kidneys of humans, but also in the anterior chamber of the eye (WHO, 2003; Ganoza *et al.*, 2010; Torgerson *et al.*, 2015).

Vaccinations against leptospirosis are available for humans, although not widespread, and only certain high-risk occupational groups get vaccinated. Current licensed human leptospirosis vaccines are monovalent or polyvalent whole cell-inactivated leptospiral vaccines and are used in a few countries, like Cuba, France, Japan and China. The vaccines might not protect against all circulating serovars in a community (Teixeira *et al.*, 2017).

### 2.5 Pathogenesis

Pathogenic *Leptospira* have the capacity to infect a broad range of hosts and the course of the infection is dependent on the host species and the bacterial serovar involved. Despite the many serovars of *Leptospira* and host species, the key steps in pathogenesis of the disease are similar (Zuerner, 2015).

Damaged skin or mucous membrane is penetrated by *Leptospira* that migrate through the dermis and across the endothelial barrier of blood vessels, spreading through the bloodstream and establishing infection in target organs. *Leptospira* use their flagella for moving between cell layers. After reaching the bloodstream, *Leptospira* attach to endothelial cells of blood vessels and extracellular matrix. *Leptospira* bind to fibroblasts, macrophages, endothelial cells and kidney epithelial cells. They also produce adhesins for binding to complement proteins, vascular VE-cadherin, thrombin, fibrinogen and plasminogen. Pathogenic *Leptospira* express surface-exposed immunoglobulin-like proteins (LigA, LigB and LigC) that promote binding and interaction with host cell receptors. Secretion of proteases contributes to their ability to invade tissues. Haemolysins secreted by *Leptospira* can degrade the host cell membrane (Picardeau, 2017; de Brito *et al.*, 2018). See Figure 1 for the processes that find place when *Leptospira* invade the body.

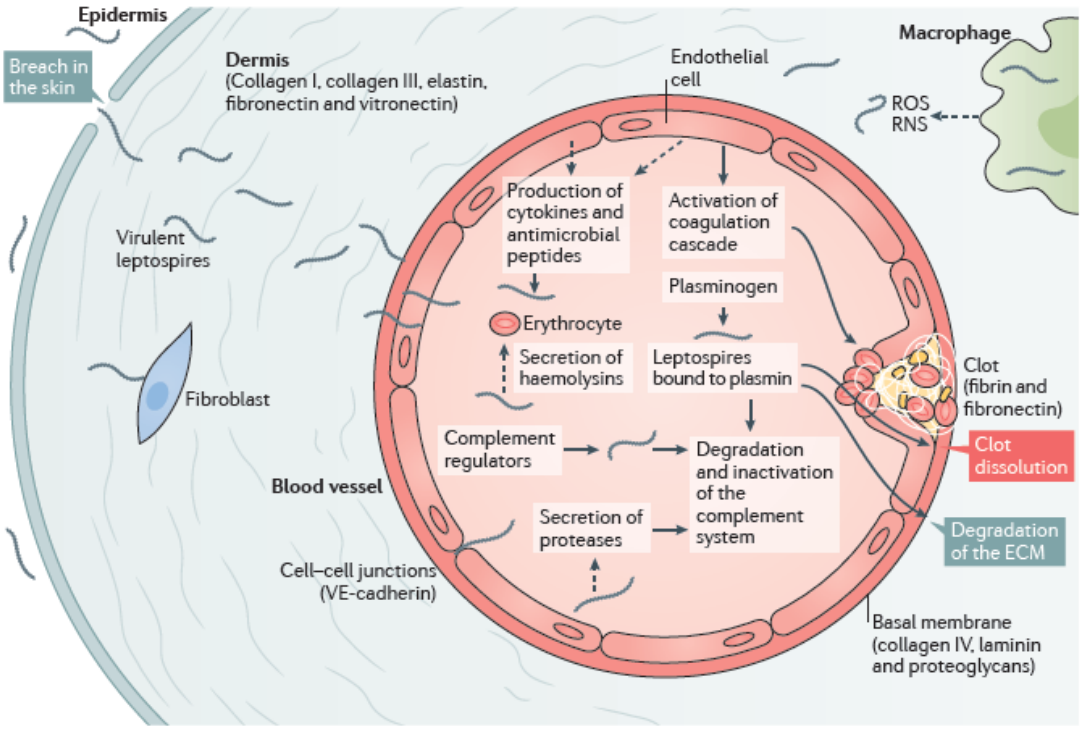


*Figure 1 – Initial Stages of Infection by Leptospira (Picardeau, 2017)*

Detection by the immune system can find place by pathogen recognition receptors, mainly the Toll-like receptors (TLRs) and Nod-like receptors, present on the plasma membrane surface. These receptors activate pathways that are crucial to innate and adaptive immune responses (Zuerner, 2015). Endothelial cells of capillaries in the human body are activated through *Leptospira* and produce inflammatory cytokines and antimicrobial peptides against the bacteria, which regulate the coagulation cascade and movement of white blood cells. *Leptospira* are phagocytosed by macrophages, but seem to be able to survive and replicate in macrophages. Activation of the alternative pathway of the complement system is one of the important response mechanisms of the innate immune system to *Leptospira*. Saprophytic *Leptospira* are highly susceptible to the lytic action of the complement system, while pathogenic strains have evolved strategies to avoid complement-mediated killing (Zuerner, 2015; Picardeau, 2017). The acquired immune response against leptospires depends on the production of antibodies and the activation of the classical pathway of the complement system. Produced antibodies are mainly directed against the LPS (Evangelista & Coburn, 2010; Zuerner, 2015).

Hematogenous dissemination brings *Leptospira* to all organs, mainly affecting liver, lungs and kidneys. After invading spaces between the hepatocytes, damage to hepatocytes and its intercellular junctions can cause leakage of bile into the bloodstream (de Brito *et al.*, 2018).

Changes in the lungs are common with toxin-mediated vascular injury involving the lung parenchyma and pleura. *Leptospira* can reach and damage the alveolar septum and interstitium. Kidney injury develops through nephrotoxic action of the *Leptospira* and secreted toxins. These processes trigger acute interstitial nephritis (Haake & Levett, 2015; de Brito *et al.*, 2018).

### 2.6 Detection of *Leptospira*

Detection of *Leptospira* by laboratory methods is challenging. Leptospires might be visible with dark-field microscopy in the acute stage of the disease, and need approximately $10^7$ leptospires/L for one cell per field to be visible. It is possible to culture leptospires in specific culture media, from blood and CSF fluid, and the leptospires can be cultured from the urine until several weeks after the onset of the disease. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium contains bovine serum albumin and is the most widely used medium to culture *Leptospira*. But the practical use of culture is limited, because *Leptospira* are slow growing organisms which need an incubation time of weeks to months to yield cultures and identification. Culture has therefore a low sensitivity, technically high demand and needs biosafety level II (WHO, 2003; Musso & La Scola, 2013; Marquez *et al.*, 2017).

The gold standard, the microscopic agglutination test (MAT), is based on serology. The test consists of mixing the test serum with a culture of leptospires and evaluating the degree of agglutination using a dark-field microscope. In addition to being highly labour-intensive and time-consuming, it needs a large panel of live leptospires (Levett, 2001; WHO, 2003; Marquez *et al.*, 2017). MAT cannot exactly differentiate between agglutinating antibodies due to current, recent or past infections, and interpretation of the test depends on the prevalence of leptospirosis in the area. Sera are screened at a 1:100 dilution and those showing agglutination are then serially diluted further to determine a titer endpoint. Very high antibody titers (≥1600) or a fourfold rise in titer are suggestive of recent in leptospirosis infection (WHO, 2003; Cornell University, 2016). Enzyme-linked immunosorbent assay (ELISA) is widely used, has a high sensitivity for acute leptospirosis, and detects antibodies reacting with a broadly reactive genus-specific antigen. ELISA depends on the accumulation of detectable amounts of anti-*Leptospira* antibodies, which takes around 5 days to develop, after onset of symptoms. Rapid tests are available and have very variable sensitivity. Specificity of IgM detection by ELISA is affected

by the antigen used in the assay, and by the presence of antibodies due to previous exposure or other diseases (Musso & La Scola, 2013; Rosa *et al.*, 2016).

Molecular testing has an advantage when antibodies are not yet detectable or not anymore detectable. PCR can also detect nonviable organisms. The bacterial load in serum/blood ranges from $10^5$ to $10^9$ leptospires/L and PCR detects DNA in the blood from the first days of disease up to approximately 15 days and in urine from the first days of the disease for at least three weeks (Mussa & La Scola, 2013). Therefore, the window of opportunity to acquire leptospires by PCR is longer in urine than in other body fluids, which is useful knowledge, because prolonged or even asymptomatic urinary shedding in the urine has been confirmed both in animals and humans (Ganoza *et al.*, 2010; Chow *et al.*, 2012; Sivasankari *et al.*, 2016; Barragan *et al.*, 2017; Rocha *et al.*, 2017). PCR is based on the detection of genes restricted to pathogenic *Leptospira* species (lipL32, lfb1, ligA, ligB) or genes universally present in bacteria (16S rRNA gene, gyrB etc.); and primers have been designed from a variety of these genes (rrs, rrl, flaB, gyrB, lfb1, lig, lipL32, lipL21 and secY) (Mussa & La Scola, 2013; Peláez Sánchez *et al.*, 2017).

Both conventional PCR and quantitative real-time PCR are used in molecular detection of *Leptospira* and quantitative real-time PCR seems to have more diagnostic accuracy (Esteves *et al.*, 2018). Real-time quantitative PCR (qPCR) can be performed using SYBR Green or Evagreen, which provides sensitive detection but is less specific than detection using fluorescent probe technology such as TaqMan probe (Mussa & La Scola, 2013).

Most PCR assays could not identify the infecting species, which is not significant for individual patients, but it is important for epidemiological purposes. Molecular techniques have been developed to overcome this obstacle: melting-curve analysis, restriction endonuclease digestion of PCR products, DNA-DNA hybridization, restriction fragment length polymorphisms (RFLPs), pulsed-field gel electrophoresis, ribotyping, multilocus sequence typing (MLST) and other PCR-based typing methods (Levett, 2001; Bourhy *et al.*, 2011; Guernier *et al.*, 2017; Peláez Sánchez *et al.*, 2017). High-resolution melting analysis is an emerging diagnostic technique to detect *Leptospira* species and the melting temperatures can reflect species-specific sequence variation in PCR products. It is a fast, accurate, non-damaging and sensitive method to determine species and there is no need for prior isolation or sequencing (Merien *et al.*, 2005; Peláez Sánchez *et al.*, 2017; Esteves *et al.*, 2018).

Both typing without isolation by culture and typing after isolation by culture have been done. Typing without isolation is performed especially for animal carriers of infection and environmental samples, but also for human samples. Previous studies of typing pathogenic *Leptospira* species without isolation used most frequently the 16S rRNA *rrs* gene, and the translocase pre-protein *secY* housekeeping gene, and also the *lfb1* gene, *lipL32* gene and *flaB* gene, which have all shown to be senstitive to detect pathogenic species (Bourhy *et al.*, 2011; Guernier *et al.*, 2017; Podgoršek *et al.*, 2020).

Water and soil can be investigated for pathogenic *Leptospira* with molecular methods, but there is no universally accepted and validated DNA-based methology for environmental samples. Saphrophytic species, as well as pathogenic species, are found in the environment, and differentiation was made by primers targeting pathogenic species, by multiplex PCR or by using a combination of primers. Primers targeting a gene fragment of the lipL32 lipoprotein were used often in previous studies, as the *lipL32* gene is found only in pathogenic species. Amounts of water collected ranged from 40-1000 mL and both filter paper and DNA extraction kits were used to extract the DNA from water sources (Wójcik-Fatla *et al.*, 2014; Wynwood *et al.*, 2014; Riediger *et al.*, 2016; Bierque *et al.*, 2020).

### 2.7 Zoonoses and "One Health"

Leptospirosis is an endemic zoonotic disease, but has also emerged as a health threat in new settings due to the influence of globalization and climate (WHO, 2009; Costa *et al.*, 2015). Public awareness of outbreaks of emerging zoonoses, such as Ebola virus or highly pathogenic avian influenza virus, is very high. While endemic zoonoses like leptospirosis grimace a much greater burden of mortality and morbidity, they attract less international concern (Grace *et al.*, 2012; Cleaveland *et al.*, 2017). Initiatives like the Global Health Securities Agenda (GHSA), the World Health Organization's "International Health Regulations" and the World Organization of Animal Health's (OIE) "Performance of Veterinary Services Pathway" pay attention to endemic zoonoses. But considering that almost two third of infectious organisms affecting human health are from zoonotic origin (Taylor, Latham and Woolhouse, 2001; Woolhouse & Gount, 2007), the international focus for endemic zoonoses, which cover majority of this two third, is less than expected (Grace *et al.*, 2012; GHSA, 2018).

In fact, it is not primarily the zoonotic diseases which are neglected, rather it is the population affected by zoonotic diseases (WHO, 2014; Cleaveland *et al.*, 2017). Zoonoses are related to poverty and livestock; both are found in low-income and low-resource settings (Grace *et al.*, 2017, WHO, 2014). Measures for prevention, treatment and control of endemic zoonoses are often already well-established in high-income countries and the disease burden is much lower in high-income countries than in neglected communities, such as poor livestock keepers in Asia and Africa. The risk of sustained transmission of endemic zoonoses in human populations is low, which affects the international concern regarding transboundary spread from low-income to high-income countries (Halliday *et al.*, 2015; Cleaveland *et al.*, 2017; EFSA, 2018). Another factor contributing to the neglect of populations affected by zoonoses is "disease visibility" – the fact that many endemic zoonoses present with non-specific clinical signs in humans and animals and that they frequently are misdiagnosed or overlooked (Halliday *et al.*, 2015). In the poor and marginalized communities at risk of zoonoses, the level of community awareness is often low, well-validated point-of-care diagnostic tests rarely available and treatment availabilities left behind (WHO, 2014; Halliday *et al.*, 2015). All these factors contribute, together with ineffective reporting systems, to underreporting and underrecognition of the burden of endemic zoonoses in low-income countries. This impacts the perceived need to prioritize investments for control and prevention of endemic zoonotic diseases in low-income settings (Cleaveland *et al.*, 2017).

Zoonotic diseases threaten the health of animals, they threaten the livelihood of people who are dependent on livestock for their income, and they threaten the health of people. A "One Health" approach tries to combine these issues by addressing closer cooperation between human, veterinary and environmental health sectors (Cleaveland *et al.*, 2017; WHO, 2017). Many endemic zoonoses are entirely or largely preventable through "One Health" measures targeting animal reservoirs and environmental sources. The "One Health" approach requires professionals with a range of expertise in these three sectors, adequate logistics and surveillance, and sufficient budget, which makes operationalization of "One Health" in low-income countries challenging (WHO, 2014). Many sub-Saharan African countries lack information on the distribution of zoonoses, while the link between humans, animals and the surrounding environment is very close in these countries (de Vries *et al.*, 2014; Guernier *et al.*, 2018; Asante *et al.*, 2019). Detection systems, disease surveillance and the ability to respond is still in the beginning phase, although efforts to implement "One Health" approaches in sub-Saharan Africa are also on the way (Fèvre, 2015; Pieracci *et al.*, 2016).

Among the sub-Saharan African countries, Ethiopia is highly vulnerable for zoonotic diseases. Ethiopia is one of the top ranked countries at the interface of poverty, emerging livestock systems and zoonoses (Grace *et al.*, 2012, FAO, 2019). Ethiopia has a large human population which has a high rate of direct contact with animals and high economical dependency on agriculture (Pieracci *et al.*, 2016). There are more than 11 million livestock-keeping households in Ethiopia and at least one fourth of them are considered by the government of Ethiopia to live below the poverty line (Shapiro *et al.*, 2017). The Food and Agriculture Organization of the United Nations (FAO) in Ethiopia emphasized in the recent years the need for increased multisectoral and multidisciplinary collaboration in "One Health" (FAO, 2017; FAO, 2019). Ethiopia became a member of the Africa One Health University Network, established in 2010 (AFROHUN, 2020). In addition, FAO Ethiopia and the National One Health Steering Committee (NOHSC) initiated the establishment of a multi-stakeholder and intersectoral National One Health Communication Network (OHCN) in Ethiopia at the end of 2017 (FAO, 2017).

### 2.8 Relevance and epidemiology of leptospirosis

When zoonotic diseases were ranked globally based on human mortality & morbidity, impact on livestock sector, amenability to agriculture-based control, severity of disease or emergence, high ranking was given to leptospirosis (Grace *et al.*, 2012; Salyer *et al.*, 2017). Leptospirosis causes globally an estimated number of nearly 1 million clinical infections and 60.000 deaths yearly (Costa *et al.*, 2015). Information from the African continent is very sparse and limits a more accurate estimation of the global burden of leptospirosis. The estimated annual cases in East Africa are 91.100 (Costa *et al*., 2015), but East Africa is prominent in lack of recent and available data (de Vries *et al.*, 2014; Allan *et al.*, 2015; Costa *et al*., 2015). However, the available information demonstrated seroconversion or the presence of *Leptospira* in reservoir animals and (symptomatic) humans, although numerous serosurveys done in Eastern Africa date from the 20$^{th}$ century. Data scarcity is even more prominent in studies assessing the prevalence of pathogenic *Leptospira* in water or other environmental samples in East Africa (de Vries *et al.*, 2014; Allan *et al.*, 2015).

Ethiopia, and especially Addis Ababa, possesses multiple factors which can promote the survival of *Leptospira* in the environment and presence of reservoir animals: heavy rainfall, livestock holding, farming, rodents, poor hygiene, inadequate waste disposal, and overcrowding

(Maciel *et al.*, 2008; JSI, 2015; Shapiro *et al.*, 2017). Leptospirosis cases in humans can be difficult to recognize and acute febrile illness of unknown origin is a common diagnosis in Ethiopia (Tadesse & Tadesse, 2013; Feleke *et al.*, 2015; Zerfu *et al.*, 2018). Leptospirosis was recently selected as one of the five prioritized zoonotic diseases for Ethiopia (Pieracci *et al.*, 2016). Despite that, no recent studies have been published about human leptospirosis in Ethiopia. Only one pilot study concerning human leptospirosis was published from Ethiopia, in which serological rapid tests were done in febrile patients negative for malaria, from a town 110 km southeast of Addis Ababa (Wonji), in 2003. This study revealed a 47.5% positivity for leptospiral antibodies in 59 patients, using the Lepto Dri-Dot rapid test (Yimer *et al.*, 2004).

Seroprevalence studies, suggesting infections in the past, like the study performed in Wonji, have been also performed in other countries in East-Africa. These studies gave prevalences of 15.1% (MAT with threshold titer 1:160) in a coast city in Tanzania (Schoonman & Swai, 2009), 13.4% among slaughterhouse workers in western Kenya using ELISA (Cook *et al.*, 2017), 35% (MAT with threshold titer 1:100) in 359 adults in health centers in Uganda (Dreyfus *et al.*, 2016) and a seroprevalence of 49.7% by MAT with a threshold titer of 1:200 in Egypt (Samir *et al.*, 2015). Studies suggesting current or recent infections gave lower prevalences: 5.0% (threshold titer 1:320 by MAT) in the previously mentioned study in Tanzania (Schoonman & Swai, 2009) and 1.9% (threshold titer 1:800 by MAT) in Uganda (Dreyfus *et al.*, 2016). A surveillance study in Egypt using culture and PCR of blood samples from 175 asymptomatic humans yielded no positive results (Samir *et al.*, 2015). Another community study in northern Tanzania using PCR on venous blood gave a prevalence of 2.3% in 128 people (Chilongola *et al.*, 2020). These researches indicate that human leptospirosis is prevalent throughout the region and that lack of data from Ethiopia can not be justified.

The presence of leptospirosis in animals has been also hardly investigated in Ethiopia. No recent studies are published on pathogenic *Leptospira* in cattle are published, while cattle is dominant in the livestock sector and an important source of human leptospirosis in Africa (Mgode *et al.*, 2015; Maze *et al.*, 2018). Serological investigation of 418 horses from different districts in Ethiopia showed that 44% of the horses were seropositive for at least one serovar (antibody titers ≥1:100), most commonly serovar Bratislava (Tsegay *et al.*, 2016). A study in the West Shewa Zone of Ethiopia found a seroprevalence of 15% in 385 dogs (Marami *et al.*, 2021). Other studies performed on *Leptospira* in animals were done more than 40 years ago (Moch *et al.*, 1975; Obeck & Berhanu, 1976). Prevalences found in the few other recent studies with a

significant sample size from East Africa targeting livestock are: 19.27% by MAT with a titer ≥1:100 and 2.5% by MAT with an antibody titer ≥1:400 in beef and dairy cattle in Uganda (Dreyfus *et al.*, 2017) and 27.8% by MAT with an antibody titer ≥1:100 in slaughtered cattle in Kampala, Uganda, with a prevalence of 8.8% for renal carriage and/or urinary shedding by real-time PCR (Alinaitwe *et al.*, 2019). Other prevalences found are: 7.08% in cattle, 1.20% in goats and 1.12% in sheep in Tanzania when tested with qPCR from kidney tissue samples (Allan *et al.*, 2018), and 0.68% for cattle and 0.85% for pigs, tested by culture in another study performed in Tanzania (Mgode *et al.*, 2015). As leptospirosis can affect livestock productivity and animals act as a source of infection to humans (WHO, 2009; Lilenbaum & Martins, 2015), these studies indicate that leptospirosis is a realistic threat in East-Africa, also for Ethiopia with its largest livestock of Africa.

There are no studies found describing the prevalence of *Leptospira* in water sources in Ethiopia or neighbouring countries. A "One Health" approach to investigate leptospirosis includes environmental sources as water and soil. Despite that, most studies from the African continent regarding *Leptospira* did not include water sources. Only two recent studies were found, from Egypt and South Africa, which were not able to detect pathogenic *Leptospira* in water samples (Saif, 2013; Samir *et al.*, 2015), although prevalences of 20 to 50% were also found in water samples in high-endemic areas in Thailand and South America (Ganoza *et al.*, 2006; Mason *et al.*, 2016; Kurilung *et al.*, 2017).

# 3. STUDY OBJECTIVES

## 3.1 General objectives

- To identify the presence of pathogenic *Leptospira* in asymptomatic humans, livestock and environmental water sources and to assess the knowledge, attitude and practice concerning leptospirosis among households in peri-urban areas of Addis Ababa.

## 3.2 Specific objectives

- To detect pathogenic *Leptospira* in urine samples of asymptomatic humans in peri-urban areas of Addis Ababa.
- To detect pathogenic *Leptospira* in urine samples of livestock in peri-urban areas of Addis Ababa.
- To detect pathogenic *Leptospira* in samples of environmental water sources in peri-urban areas of Addis Ababa.
- To characterize pathogenic *Leptospira* found in peri-urban areas of Addis Ababa.
- To describe the knowledge, attitude and practices among the peri-urban households of the study area regarding leptospirosis.

# 4. MATERIAL & METHODS

## 4.1 Study Area

This study was conducted in peri-urban areas of Addis Ababa, the capital city of Ethiopia. Addis Ababa is rapidly growing, and had an estimated population of at least 3.2 million inhabitants in 2013 (CSA, 2013), which is believed to have more than 4.7 million inhabitants currently (UN, 2019). Addis Ababa is situated approximately 2400 meters above sea level and the average annual rainfall of the city is estimated to be 1200 mm, with rainy seasons from June until September and from February until April. Average annual mean temperature is 17˚C and relative humidity is about 60% (Fazzini *et al.*, 2015). The city is divided in ten sub cities, which are subdivided in woredas, the lowest administrative unit (CSA, 2013). Among the ten sub cities of Addis Ababa, five involve peri-urban areas: Akaki Kaliti, Gullele, Bole, Yeka and Nefas Silk Lafto. These five peri-urban areas contained, according to the City Livestock and Agriculture Sector, around 11.000 livestock keeping households in 2018 (unpublished data). Study households were selected from these peri-urban sub cities of Addis Ababa only.

## 4.2 Study Design

A cross-sectional study design was employed, and sample collection took place from February 2019 until October 2019, to investigate pathogenic species of *Leptospira* in the urine of asymptomatic humans, in the urine of livestock and in environmental surface water sources. At the same time, factors related to transmission and prevention of leptospirosis were investigated through assessing the knowledge, attitude and practice (KAP) of the households using a questionnaire.

## 4.3 Target Population

All livestock-keeping households dwelling in peri-urban areas of Addis Ababa, owning one or more livestock species, were considered as the target population for the current study.

## 4.4 Study Population

The study population consisted of representative livestock-keeping households in four of the five peri-urban sub cities of Addis Ababa.

### 4.5 Sampling technique

The Livestock and Agriculture Sector of the Addis Ababa Sub City Administrations in Akaki Kality, Yeka, Bole and Nefas Silk Lafto identified and recommended certain woredas within their sub cities with a high density of livestock-keeping households. Households within these woredas were selected by a non-probability convenient sampling method, according to the availability of livestock. A non-probability convenient sampling method was used to select the households which were asked for their willingness to participate in the study. Although a simple random sampling method was aimed, a non-probability convenient sampling method was used based on availability and reachability of the households and knowledge of the animal health assistants about the households.

### 4.6 Target Sample Size

In this study, the required sample size was calculated separately for asymptomatic humans, livestock and environmental water sources. The following formula was used to calculate the target sample size, for a very large population (N > 10.000): $N = Z^2 p (1-p) / d^2$.

- N = minimum required sample size
- p = estimated prevalence in the target population
- d = degree of accuracy (estimated error) = 5% or 0.05
- Z = the standard normal value at confidence interval of 95% = 1.96

There was no available data which shows previous prevalence of leptospirosis in Ethiopia in asymptomatic humans and water sources. Considering animals, the only recent studies on prevalence of leptospirosis in animals in Ethiopia were done in horses and dogs (Tsegay *et al.*, 2016; Marami *et al.*, 2021), while cattle, sheep and goats are the most prevalent among the Ethiopian livestock. Therefore, prevalences from comparable studies performed in other East African countries were considered in the calculation of the sample size.

Among serosurveys done among asymptomatic humans in East Africa (de Vries *et al*., 2014), only a few are done during the 21st century and most of them do use non-comparable methods giving information about past infections. The highest prevalence among studies that detect current or very recent infection was found in a study from Tanzania (Schoonman & Swai, 2009), which gave a prevalence of 5%. Taking this prevalence of 5%, the calculated required sample size from the human population was: $N = (1.96)^2 * 0.05 * (1-0.05) / (.05)^2 = 73$ humans.

Considering animal prevalences, the study from Tsegay *et al.* (2016) and Marami *et al.* (2021) were the only recent studies performed in Ethiopia with a prevalence of 44% and 15%, respectively. These studies were performed with an assay different from PCR and horses and dogs as study subjects, while cattle, goats and sheeps were expected to be the most common encountered livestock in the study area. Therefore, other recent studies in East Africa using a PCR assay, performed among cattle, sheep and goats were considered in the sample size determination (Mgode *et al.*, 2015; Dreyfus *et al.*, 2017; Allan *et al.*, 2018; Alinaitwe *et al.*, 2019). The highest prevalence, 8.8%, was found among cattle in the capital city of Uganda (Alinaitwe *et al*, 2019). Therefore, the calculated required sample size from the animal population was: $N = (1.96)^2 * 0.088 * (1-0.088) / (.05)^2 = 123$ animals.

Because households use common water sources, the amount of surface water samples was determined after interviewing the households about the (common) water sources they use for the households' daily activities and drinking water for animals in the study area. Therefore, about 6-10 sites were selected per sub city to collect water samples, which made the total number of water samples 32. The amount of water samples was limited because methods detecting *Leptospira* from water are not as validated as methods detecting *Leptospira* from urine, so it was preferred to spend the extraction kits and PCR materials on urine samples.

### 4.7 Inclusion & Exclusion criteria

Human participant inclusion criteria:

 Adult persons (18 years and above) belonging to a household in peri-urban sub cities of Addis Ababa, owning livestock, which consist of cattle, horses, donkeys, sheep, goats or pig.

Human participant exclusion criteria:

 Persons unwilling to participate in the research.

 Persons who had symptoms and signs of an acute febrile illness at the moment of the visit.

Animal inclusion criteria:

 Cattle, horses, donkeys, sheep, goats or pigs belonging to the selected households in peri-urban sub cities of Addis Ababa, whereof urine could be collected.

Animal exclusion criteria:

 Animals less than 1 month old.

Water inclusion criteria:

 Rivers or pools of water in the environment around the households, water sources used for human consumption, washing and cleaning, and from water sources used in animal husbandry. Water sources used for irrigation purposes, found in close proximity to where cattle is raised or dairy farming is performed.

Water exclusion criteria:

 Water collections, pools or rivers in the environment of the households, but not (yet) used by the household or their animals. Water sources which were not reachable by the investigator for sample taking.

### 4.8 Sample Collection and Transportation

In order to obtain the required samples, livestock-keeping households were visited and oral consent was obtained from participants. Questions about knowledge, attitude and practice (KAP) were discussed with one person from the household. The animal and human samples were taken at the same visit as the KAP assessment, while the water samples were taken later, after assessing the commonly used water sources of different households in a certain area. The samples were transported in cold boxes to the laboratory. Animal health assistants from the woreda were hired to help in finding the livestock-keeping households, to explain the procedures and to collect the samples.

 Human samples:

At least 15 ml of urine was collected from one or more voluntary persons in a livestock-keeping household. The urine was collected in sterile bottles and was neutralized immediately after collection with phosphate buffered saline 10x, according to recommendations (Lucchesi *et al.*, 2004).

 Animal samples:

At least 15 mL of urine was collected from cattle, sheeps, goats, donkeys or horses. When more animals were available in the visited household, urine from several animals was obtained, depending on the ability to get urine from the animal. Urine was obtained during spontaneous urination or by perineal massage performed by the investigator and animal health assistant, and collected in sterile bottles. Similar to the human urine, the animal urine was neutralized immediately after collection with phosphate buffered saline 10x (Lucchesi *et al.*, 2004).

 Surface water samples:

At least 50 ml of water were collected from streams or standing water or other water sources around the visited households. The samples were taken from water sources or collections used for human consumption, washing and cleaning, and from water sources used in animal husbandry. Water sources used for irrigation purposes, found in close proximity to where cattle is raised or dairy farming is practiced, were also used to take a sample. Because *Leptospira* distribution is dependent on rainfall, sample collection was done after the rainy season (September and October). Standing water sources and water sources having connection with sewage were taken into account for selection of the water sources because of their association with pathogenic *Leptospira,* after assessment of the water sources used by study participants (Casanovas-Massana *et al.,* 2017). In accordance with previous studies (Ganoza *et al.,* 2006; Mason *et al.,* 2016; Kurilung *et al.,* 2017), 50 ml of surface water was taken from one source and collected in sterile polypropylene bottles. Phosphate buffered saline 10x was added on the place of collection. Samples were stored at 4˚C for not more than 24 hours before processing.

### 4.9 Knowledge, attitude and practice (KAP) questionnaire

The semi-structured questionnaire used for KAP used validated questions and examples from previous studies conducted by Sakinah *et al.* (2015), Mohan & Chadee (2011) and Zahiruddin *et al.* (2018). There were open questions regarding socio-demographic information and regarding the livestock and water sources. The closed questions concerned the knowledge, attitude and practice of the household in relation to leptospirosis. The questionnaires were prepared in English, Amharic and Afaan Oromo. All interviews were done verbally by the investigator and a veterinary doctor or a veterinary medicine student or an animal health assistant. A few persons in the peri-urban areas of Yeka sub city, owning livestock, were asked to complete the questionnaire as a pre-test, and modifications were made afterwards for questions which were unclear or subject to interpretation in multitude ways.

### 4.10 Sample Processing

The general steps for the processing of the samples were the same for water and the urine samples: DNA extraction followed by quantitative real-time PCR, targeting the *lipL32* and *lfb1* gene (Bourhy *et al.*, 2011). The detailed description of the procedures is found in Appendix III and IV. DNA from the pellet was extracted using the QIAamp Viral RNA Mini Kit, which was recommended by the AMC Medical Microbiology, WHO/FAO Collaborating Centre for

Reference and Research on Leptospirosis, because the buffer AVL supplied with this kit is optimized to inactivate PCR inhibitors found in urine. QIAamp Viral RNA Mini Kit does not exclusively extract RNA because it is not designed to separate viral RNA from cellular DNA (Qiagen, 2020). Cellular DNA including any possible leptospiral DNA are also extracted and purified using this kit. The WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis in the Netherlands also recommended, based on the centrifuge available in MRC-Ethiopia (Eppendorf 5430), to distribute the water samples in portions of 1.5 mL, to centrifuge these at 8000 rpm and to add the pellets together.

DNA was extracted at the day of collection and stored at -20˚C. Human and animal urine samples were analysed in the MRC-ET laboratory in Addis Ababa and repeated in the MRC-Holland laboratory in the Netherlands. The extracted DNA was transported frozen to the Netherlands. Surface water samples were analysed in the MRC-Holland laboratory in the Netherlands. Amplification of the DNA was done using a CFX96 real-time PCR detection system (BIO-RAD). The primers, probes, buffers and positive controls were provided by the MRC-Holland laboratory and the AMC Medical Microbiology, a WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis in the Netherlands. All PCRs were run in triplicate. The samples were stored in -20˚C so that further phylogenetic analysis could and can be done later.

PCR detection of leptospiral DNA was done with *Leptospira* specific lipL32 and lfb1 PCR decetion assays. Lfb1 PCR involves an Evagreen real-time PCR assay, in which a possibly correct lfb1 PCR product is revealed by a specific melting curve with a Tm of more than 80˚C, also allowing serotype identification. The lipL32 PCR detection involves a TaqMan probe hydrolysis assay that specifically detects the real-time formation of a lipL32 PCR product. LFB1 F/R primers were used to amplify the *lfb1* gene and lipL32-47Fd and lipL32-301Rd primers to amplify the *lipL32* gene. The 25 µl PCR reactions contained 19.7 µl of master mix, 0.3 µL of Salsa polymerase and 5 µl of extracted bacterial DNA, to a final volume of 25 µl.

We performed the following amplification protocol in the CFX96 real-time PCR instrument (BIO-RAD): denaturation at 95 °C for 1 minute, followed by 45 cycles of 95 °C for 10 seconds, 58 °C for 30 seconds, and 72 °C for 10 seconds. These conditions were used for both primer sets. After PCR cycling, the samples were heated from 40 °C to 95 °C with continuous data acquisition.

Six pathogenic *Leptospira* reference cultures, provided by the Dutch Reference Laboratory for Leptospirosis (AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis), were used as positive controls: 4 strains belonging to *L. interrogans*, *L. borgpetersenii*, *L. santarosai* and *L. weilii* and 2 human isolates belonging to *L. interrogans* and *L. kirschneri*. Melting curve plots were generated and analysed using CFX Manager Software v3.0.1 (BIO-RAD) to determine average melting temperature (Tm) for each *Leptospira* spp.

### 4.11 Data Analysis

 Data acquired by the CFX96 real-time PCR detection system (BIO-RAD) and BIO-RAD software was presented with descriptive statistics, describing the presence or absence of pathogenic *Leptospira*. A differentiation between viable and non-viable *Leptospira* could not be made with the quantitative real-time PCR.

 Melting curve plots were generated with melting curve analysis and analysed using specific software belonging to the CFX96 real-time PCR detection system to determine average melting temperature (Tm) for each found *Leptospira*. Reference strains (positive controls) were used to define the species and differential melting curves.

 Data from the questionnaires was entered into spreadsheets and the Epi Info™ 7 statistical software. Descriptive statistics were used for demographic characteristics and KAP. Association between KAP & demographic characteristics and the positive *Leptospira* samples was studied with help of logistic regression.

### 4.12 Operational Definitions

**Asymptomatic humans**: Any person from a household in the selected area, who shows no symptoms which can be related to leptospirosis or an acute febrile illness (such as fever, chills, bleeding tendency or jaundice).

**Livestock**: Cattle, horses, donkeys, sheep, goats and pigs.

**Household**: a group of people living together in the same house or shelter.

**Surface water:** Water from streams or standing water or other water sources in the environment of the livestock-keeping household. The household should be using the water for human consumption, washing, cleaning, animal husbandry or irrigation (in proximity to the presence of livestock).

### 4.13 Result dissemination

Study results are presented and submitted to the Department of Microbiology, Immunology & Parasitology, School of Medicine, College of Health Sciences, Addis Ababa University, School of Medicine. The findings will be also distributed to the Addis Ababa Public Health Research and Emergency Management Core Process, as well as to the AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis in the Netherlands and the MRC-ET & MRC-Holland laboratory. Efforts will be made to disseminate the findings to the One Health Steering Committee in Ethiopia and other parties involved in "One Health" in Ethiopia. Preferably the findings will also be published in reputable peer reviewed journals.

### 4.14 Ethical considerations

Ethical approval for the study was obtained from the Department of Microbiology, Immunology and Parasitology ethical review committee. The study was subsequently done after permission from the Addis Ababa Public Health Research and Emergency Management Core Process and, for the animal samples, the Addis Ababa City Livestock and Agriculture Sector. Participants of the study were asked to provide oral consent after they were informed (in local language) about the purpose of the study, voluntary participation, possibility of withdrawal at any time and that the investigators were dealing confidentially with the personal information and the obtained data.

During the study, the transport of DNA extracts to the Netherlands seemed to be inevitable because of technical difficulties conducting the molecular assays in-country. Therefore, the original research proposal had to be adjusted. This has been reviewed and the amendment was approved by the Department Ethical Review Committee (DRERC) of the Department of Microbiology, Immunology and Parasitology on February 4, 2020. Subsequently, permission from the College of Health Sciences Institutional Review Board (IRB) was asked and approved on June 15, 2020. The National Research Ethical Review Committee (NRERC) approved the adjusted proposal on February 5, 2021, after getting permission from the Biodiversity Institute on February 4, 2021.

# 5. RESULTS

## 5.1 Description and demographic characteristics

In the selected peri-urban sub cities in Addis Ababa, 85 households in livestock-keeping woredas, which fulfilled the inclusion criteria and whose household head agreed to participate in this research, were visited. From these 85 households, 105 human urine and 194 animal urine samples were collected (Figure 2). In the areas surrounding these households, 32 water samples were collected. The households were considered as one entity concerning the assessment of knowledge, attitude and practices.

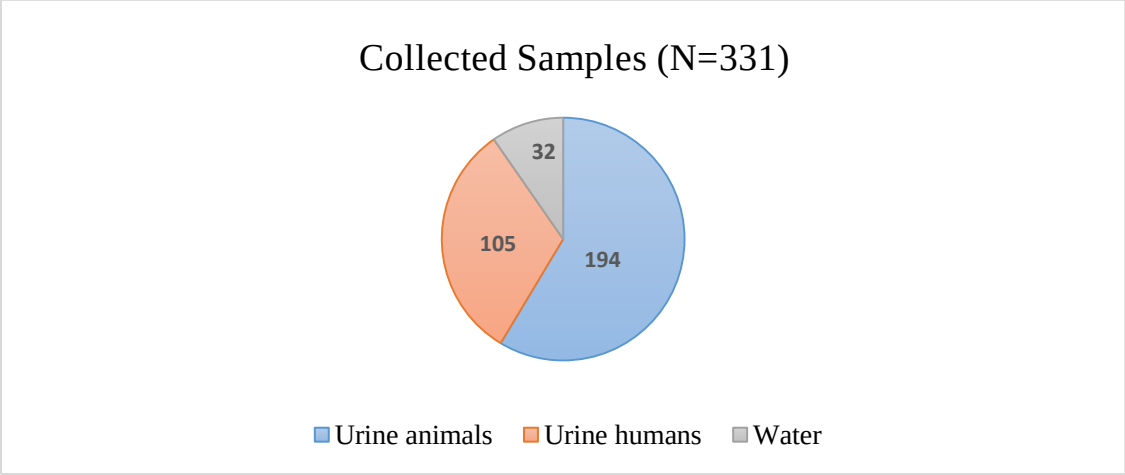


*Figure 2 – Distribution of Collected Samples*

Urine was collected from 105 asymptomatic humans; 20.0% of the urine samples were from inhabitants from Yeka subcity, 49.5% from Akaki subcity, 20.0% from Bole subcity and 10.5% from Nefas Silk Lafto subcity. A total of 194 animal urine samples were collected, where 24.2% were from Yeka subcity, 25.2% from Akaki subcity, 38.7% from Bole subcity and 11.9% from Nefas Silk Lafto subcity. Majority of the collected animal urine samples (168; 86.6%) were collected from cattle, and only a minority could be collected from other animals: sheep (22; 11.3%), goat (2; 1.1%), horse (1; 0.5%) and donkey (1; 0.5%). The 32 water samples were collected with the assumption that they would be representative for the woredas and sub cities where the urine samples were taken from. The water was collected from places where animals were grazing and from drinking water sources: Bulbula river, Akaki river, small rivers and pools in Akaki, Yeka, Bole Bulbula and Nefas Silk Lafto. The water samples were collected just after the rainy season, in September and October.

Demographic characteristics of the households are listed in Table 1. The median household size of the studied households was 5.5 persons (ranging from 1-17 persons), with a mean of 6.6 persons ± 3.3 standard deviation (SD). Of the interviewed persons from the households, 51.8% were male and 48.2% female. Overall, almost a quarter (24.8%) of the respondents had not received any formal schooling and more than a quarter (28.8%) had only finished elementary school. Most of the respondents were either primarily farmers (34.1%) or unemployed (35.3%), while almost one-fourth (24.7%) were involved in private work or working for employers. All households had access to piped water for drinking purpose, food preparation, hand washing and cleaning. Majority of the households faced a shortage of water throughout the year, only a few (13.0%) households experienced never or rarely interruption of the water supply. Surface water – collected from rain, pools, small canals or rivers – was used by almost a quarter (23.5%) of the households to clean the animal pen or to give the animals to drink.

Of the 85 studied households, majority (87.1%) owned cattle. The number of animals found belonging to the households or small farms ranged from 1 to 400. Forty percent of the households had a laborer taking care of the animals or helping the family with caring for the animals, the other households took care of animals with their own family members (husband, wife, children).

*Table 1 – Demographic Characteristics - Households (N=85)*

| DEMOGRAPHIC CHARACTERISTICS | CATEGORY | PERCENTAGE |
|---|---|---|
| **GENDER** | Male | 51.8% |
| | Female | 48.2% |
| **AGE** | Below 30 years old | 20% |
| | 30-60 years old | 52.9% |
| | More than 60 years old | 27.1% |
| **MARITAL STATUS** | Married | 58.8% |
| | Single | 22.4% |
| | Divorced/Widowed | 18.8% |
| **EDUCATION** | No formal schooling | 24.8% |
| | Elementary school | 28.8% |
| | High school | 17.6% |
| | College | 12.9% |
| | University | 5.9% |
| **OCCUPATION** | Farmer | 34.1% |
| | Unemployed / retired | 35.3% |
| | Private / government job | 24.7% |
| | Student | 5.9% |
| **ANIMAL HUSBANDRY** | Cattle | 87.1% |
| | Sheep | 35.3% |
| | Goats | 9.4% |
| | Horse(s) or donkey | 25.9% |
| | Pig | 0% |
| | Chicken | 40.0% |
| | Only cattle | 37.6% |
| | Only sheep | 7.1% |
| | Two or more livestock species | 49.4% |
| **AVAILABLE FACILITIES** | Electric supply | 100% |
| | Telephone | 98.8% |
| | Radio or Television | 97.6% |
| | Refrigerator | 74.1% |
| | Piped water | 100% |
| | Toilet | |
| | Pit latrine with cement slab | 78% |
| | Pit latrine without cement slab | 9.4% |
| | Flush | 4.7% |
| | No toilet | 7.1% |

### 5.2 Knowledge, attitude and practices concerning leptospirosis and zoonotic diseases

The majority (97.6%) of the respondents had never heard about leptospirosis. Nevertheless, 83.3% of the households realized that they could get diseases from water and 64.2% knew that they could get diseases from urine of their livestock. Rat's urine as a source of diseases for humans was recognized by 66.3% of the households. The households were asked whether symptoms that could be attributed to leptospirosis were observed in their household during the last month, for which their responses were as follows: 29.1% had seen fever in their household, while kidney failure, jaundice and bleedings were rarely seen (7.8%, 1.3% and 3.9%, respectively).

Because leptospirosis is known to be transmitted by standing water contaminated by rat's urine or domestic animals' urine, households were asked about wet areas around their houses. More than half (56.6%) of the households had often wet areas around the house and this was more common during the rainy season (68.7%). Almost half (48.2%) of the respondents walked frequently in the wet areas around the house without shoes or with open shoes. Almost a quarter (22.9%) mentioned that they themselves or their family members walked with the animals through the water. Rats were frequently seen at daytime around the house (63.8% of the households) and seen inside the house (66.3% of the households). Garbage, which might attract rats, was stored inside the compound at more than half of the households (56.1%), and not removed daily in almost half (48.2%) of the households. Rat poison or traps were used by 42.2% of the households.

Family health issues were discussed with family members or friends by all households and most households also visited local health service centers concerning health issues. Households realized in general (86.1%) that animals can be a source of human diseases and were able to mention how: by direct contact with animals, eating raw milk and meat, touching urine of animals, or rats entering the house. Animal health issues were solved with veterinary assistance (93.1% of the households) and also by the households themselves (36.2% of the households). Protective measures as gloves or hand washing after dealing with diseased animals was practiced by 96.8% of the households.

### 5.3 Prevalence of pathogenic *Leptospira* in various sources

In total, 331 samples were analyzed from the 85 livestock-keeping households. Of these, 3 samples were found positive for pathogenic *Leptospira* by real-time PCR. These positive samples were all animal urine samples (3/194), collected from cows. The urine samples containing *Leptospira* all came from the same subcity - Yeka subcity. Two positive samples came from the same household, but the positive samples were taken on a different day. The positive samples were considered to be *Leptospira borgpetersenii* based on the melting curve, compared to the other positive controls (Figure 3, 4 and 5).

Figure 3, 4 and 5 show DNA isolated from urine samples after being subjected to the previously described PCR reaction, using lfb1 primers in the presence of Evagreen.

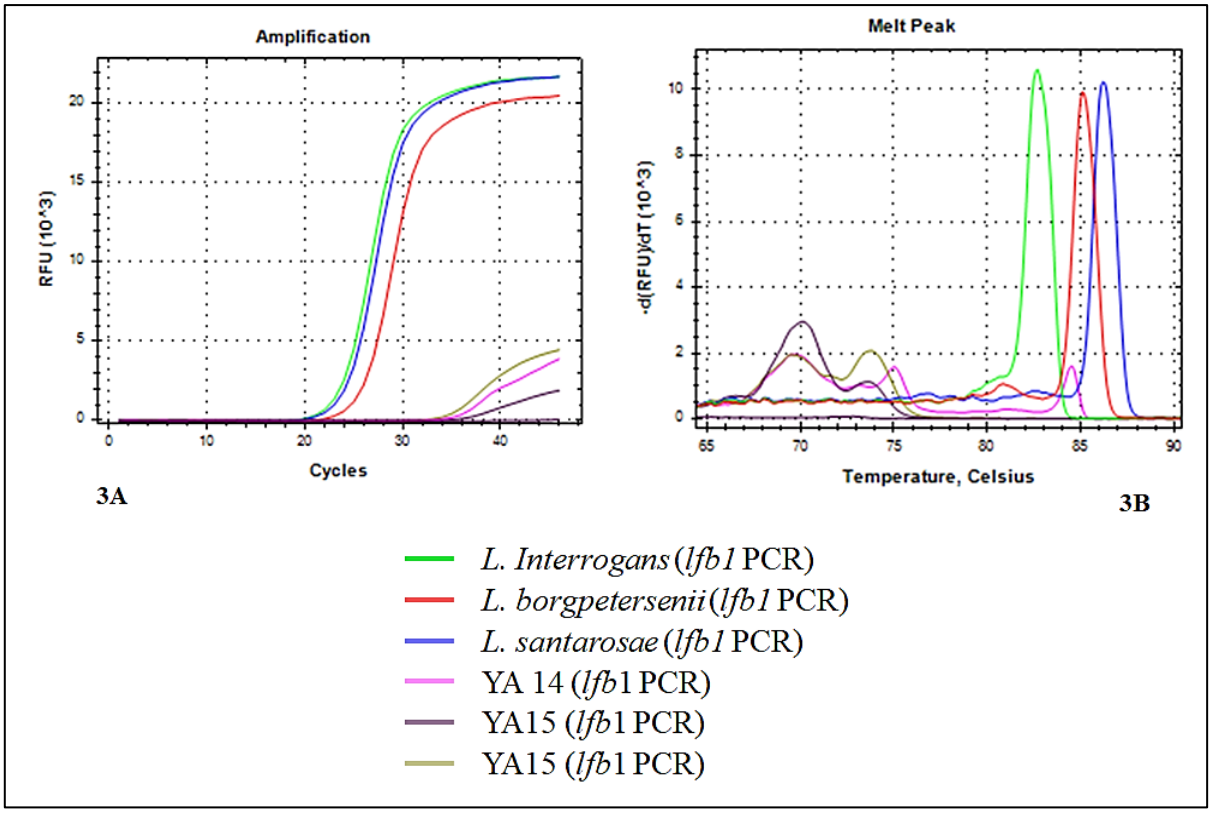


*Figure 3A & 3B – Lfb1 PCR – Amplification & Melting Curve YA14*

Figure 3A shows amplification curves of a *Leptospira borgpetersenii*, *L. interrogans* and *L. santarosai* control DNA sample, urine DNA sample YA14 (positive) and urine DNA sample YA15 (negative). Figure 3B shows the melting curve of the *L. interrogans*, the *L. borgpetersenii* and *L. santarosai* control DNA sample, the melting curve of the YA14 urine DNA sample and the melting curve of the YA15 urine DNA sample. This figure displays a similar melting

temperature for the YA14 (positive) urine DNA sample and *L. borgpetersenii* control sample. The fact that the melting peak of urine DNA sample YA14 is lower than the *L. borgpetersenii* control sample can be explained by the lower concentration of *Leptospira* in YA14 than in the positive control.

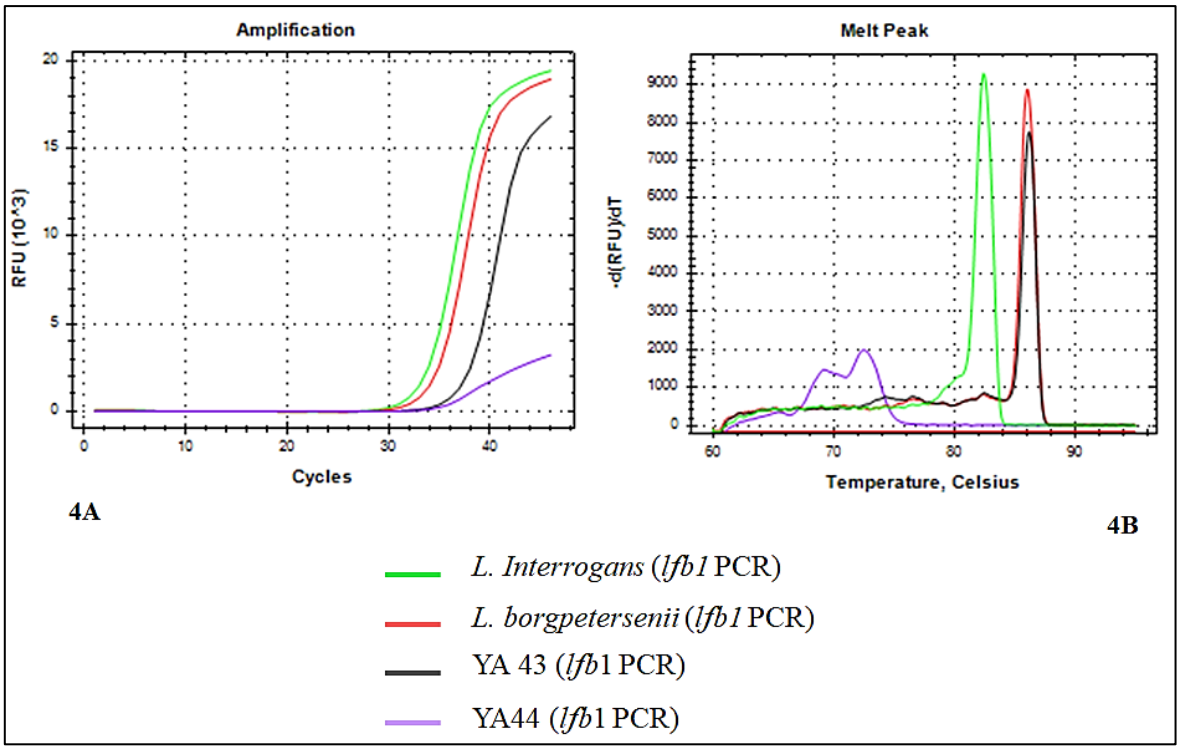


*Figure 4A & 4B - Lfb1 PCR – Amplification & Melting Curve YA43*

Figure 4A shows amplification curves of *Leptospira borgpetersenii* and *Leptospira interrogans* control samples, urine DNA sample YA43 (positive) and urine DNA sample YA44 (negative). Figure 4B shows the melting curve of the *L. interrogans* and *L. borgpetersenii* control DNA sample, the melting curve of the YA43 (positive) urine DNA sample and the melting curve of the YA44 (negative) urine DNA sample. Melting temperature and melting peak of the *L. borgpetersenii* control sample and urine DNA sample YA43 are overlapping, suggesting that the urine DNA sample YA43 contains *L. borgpetersenii*.

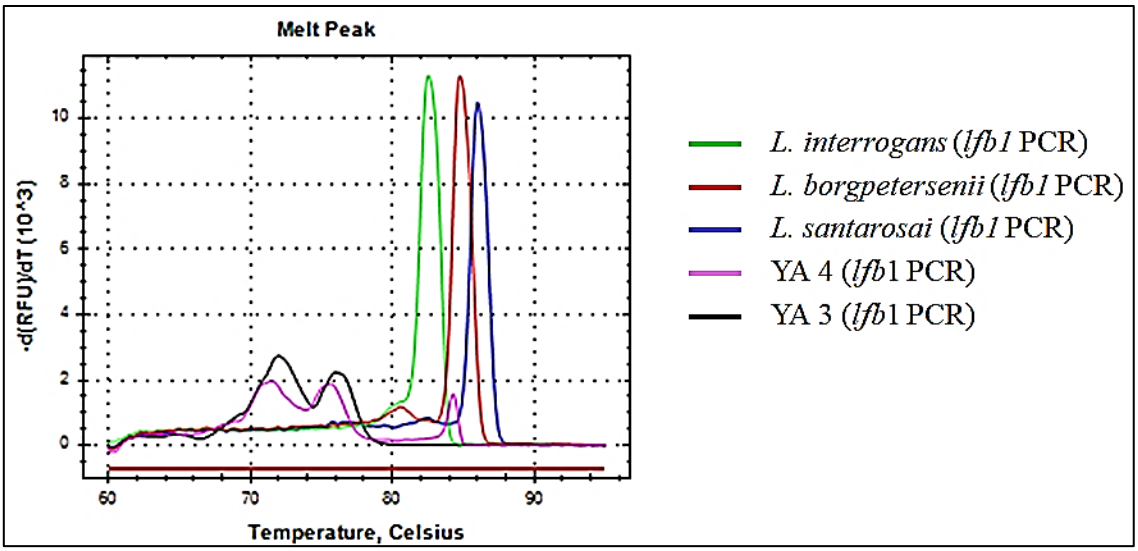


*Figure 5 – Lfb1 PCR – Melting Curve YA4*

Figure 5 shows the melting curve of *Leptospira borgpetersenii*, *Leptospira interrogans* and *Leptospira santarosai* control samples, urine DNA sample YA4 (positive) and urine DNA sample YA3 (negative). Melting temperature of the urine DNA sample YA4 is equivalent to the *L. borgpetersenii* sample, which indicates the presence of *L. borgpetersenii* in urine DNA sample YA4.

Figure 6 shows DNA isolated from urine samples after being subjected to the *lipL32* PCR reaction with TaqMan probe. Hydrolysis of the *lipL32* specific TaqMan probe confirms the presence of *Leptospira* species in YA4, YA14 and YA43, but species identification is not possible.

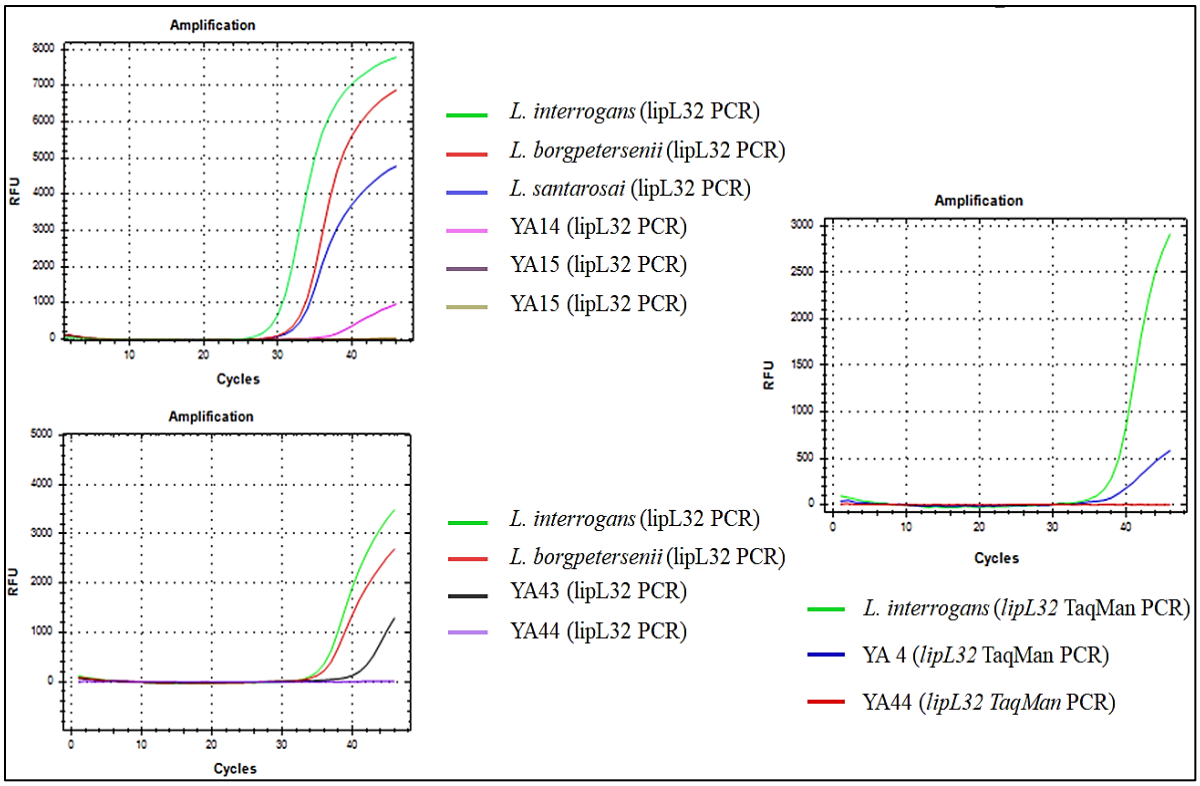


*Figure 6 - LipL32 PCR 1*

Melting peaks of the different *Leptospira* species were verified using positive controls (heat-inactivated *Leptospira*), urine and blood samples of patients with proven leptospirosis, provided by the AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis.

### 5.4 Analysis of zoonotic risk factors associated with prevalence of pathogenic *Leptospira*

Analysis of the 12 explanatory factors obtained from the questionnaire was undertaken to explore the potential risk factors for *Leptospira* positivity of animal samples. Accordingly, a univariable logistic regression analysis was run for animal *Leptospira* positivity (outcome variable) and any of the 12 selected explanatory factors. Table 2 summarizes variables included in the univariable analysis. The results of the univariable logistic regression analysis showed that none of the 12 selected explanatory variables were significantly ($p < 0.05$) associated with *Leptospira* positivity. Multivariable analysis was not pursued because there were no significant findings in the univariable analysis.

*Table 2 – Univariate logistic regression analysis of explanatory variables for animal Leptospira positivity*

| **EXPLANATORY VARIABLES** | **N = 85 HOUSEHOLDS** | | **P-VALUE** |
|---|---|---|---|
| | **Positive (%)** | **Negative (%)** | |
| Wet areas around the house | 1 (50%) | 57 (68.7%) | **0.5841** |
| Walking through the water with animals | 0 (0%) | 19 (22.9%) | **0.9769** |
| Rats seen at daytime around the house | 1 (50%) | 53 (63.9%) | **0.6912** |
| Rats inside the house | 1 (50%) | 55 (66.3%) | **0.6376** |
| Using rat traps or poison | 0 (0%) | 34 (41.0%) | **0.9708** |
| Garbage stored inside the compound | 0 (0%) | 47 (56.6%) | **0.9688** |
| Garbage kept for several days inside the compound | 0 (0%) | 40 (48.2%) | **0.9694** |
| Knows that animals can be a source of disease | 2 (100%) | 68 (84.0%) | **0.9979** |
| Knows about diseases transmitted by rat urine | 1 (50%) | 55 (66.3%) | **0.6376** |
| Knows about diseases transmitted by animals urine | 0 (0%) | 53 (65.4%) | **0.9692** |
| Protective measures after dealing with diseased animals | 2 (100%) | 59 (96.7%) | **0.9799** |
| Washing hands after dealing with animal excretions | 2 (100%) | 58 (96.7%) | **0.9797** |

# 6. DISCUSSION

This study is the first of its kind in describing the presence of pathogenic *Leptospira* from "One Health" perspective in Ethiopia. Even in East-Africa, no studies have been published that investigated *Leptospira* prevalence from a "One Health" approach. The presence of pathogenic *Leptospira* in Ethiopia was confirmed with this study, albeit a few in number, by the positive samples which were found in urine of cattle. This is also the first study detecting pathogenic *Leptospira* by PCR in Ethiopia.

Pathogenic *Leptospira* were only found from urine of cattle, which were overrepresented in our sample. Nonetheless, among animals, cattle appear to be important hosts of *Leptospira* in Africa (Allan *et al.,* 2015; Allan *et al.,* 2018). The number of positive samples among animals was 3 out of 194, which is 1.5%. More specific regarding animal species, the number of positive samples among cattle was 3 out of 168, which gives a prevalence of 1.8%. As mentioned before, similar studies have not been done before in Ethiopia, and also not many in East Africa, which makes it difficult to compare the detected numbers in this study with others. Similar studies in East Africa using PCR assays in cattle showed higher percentages, such as 8.8% (Alinaitwe *et al.*, 2019) and 7.1% (Allan *et al.*, 2018), which can have a variety of possible explanations, such as transport of the samples, amount of urine taken for DNA extraction and the material on which PCR was performed (kidney samples versus urine). Studies that used the microscopic agglutination test (MAT) gave higher prevalences, but are difficult to compare with this study, because positive results, especially when a reactive MAT was determined by an antibody titer ≥1:100, also indicate past infections (de Vries *et al.*, 2014; Cornell University, 2016). One study performed in beef and dairy cattle in Uganda (Dreyfus *et al.*, 2017), using an antibody titer ≥1:400 by MAT, revealed a result (2.5%), which is close to the finding of our study. Another comparable study performed in Egypt (Samir *et al.,* 2015) using PCR assay found *Leptospira* in urine or blood in 7 out of 625 cows (1.1%), while antibodies were found more often, in 37.6% of the cows.

*Leptospira borgpetersenii* is one of the pathogenic species predominant in animals, mainly cattle, reported from Africa (Allan *et al.*, 2015) and was also the only species discovered in this study.

There were no *Leptospira* found among the human urine samples tested in this study, which can be explained by the low prevalence in the animals from the same households (1.5%), the low sample size and due to screening only of asymptomatic persons. Taking urine of symptomatic patients could have given a higher yield, but needed a different approach and screening whole households, including their humans, animals and water sources, was the aim of the study. Screening urine of humans for the presence of *Leptospira* has been selected as the method for this study, because it is non-invasive and *Leptospira* urinary shedding has been proven in humans, including from asymptomatic humans in endemic areas, although in low quantity (Ganoza *et al.*, 2010; Chow *et al.*, 2012; Sivasankari *et al.*, 2016; Barragan *et al.*, 2017*).* Previous studies performed in the region used methods revealing seropositivity, which can not be compared completely with the current study, but give an indication about the existence of pathogenic *Leptospira* infection. Studies elsewhere that have been investigating acute infections, using MAT, culture or PCR, gave much lower prevalences: 5.0% in Tanzania (Schoonman & Swai, 2009), 1.9% in Uganda (Dreyfus *et al.*, 2016) and no positive results in Egypt (Samir *et al.*, 2015). With zero positive results among 105 tested humans, these results are adequate to conclude at the 0.95 confidence level that the studied population is free from disease, at an expected minimum prevalence of 3% (Ausvet, 2021).

There were also no pathogenic *Leptospira* found in the water, but the number of investigated water samples was purposely kept small, because there is no universally validated method of DNA extraction from water samples and there was no capacity to extract DNA from large amounts of water during this study. The method used in this study to extract DNA from the water, the amount of water taken and the PCR assays used have been decided after consulting the AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis. Similar methods have been also able to detect pathogenic *Leptospira* during previous studies (Ganoza *et al.*, 2006; Mason *et al.*, 2016; Riediger *et al.*, 2016). Among the limited number of studies that investigated pathogenic *Leptospira* with molecular methods from water sources, only two studies were found from Africa. One study from South-Africa detected no pathogenic *Leptospira* in 77 water samples (Saif, 2013). Another study from Egypt detected also no pathogenic *Leptospira* among 45 water samples (Samir *et al.*, 2015). Several studies suggested that leptospiral DNA is found more in soil than in water (Bierque *et al.*, 2020), although prevalences up to 50% were also found in water samples in high-endemic areas in Asia and South-America (Ganoza *et al.*, 2006; Mason *et al.*, 2016).

Searching an explanation for the small amount of positive samples found in this study, one of the concerns was the validation of the method of isolating the DNA after urine collection. The small amount of urine taken for DNA extraction, 140 microliter, might be a potential factor in missing pathogenic *Leptospira*. The isolation method was developed together with MRC-Holland and the AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis and also tested during the study with urine samples of known leptospirosis patients (provided by the AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis), which revealed positive PCR-results with both the lfb1 and lipL32 assay. Even at relatively low infection levels (Ct values > 32), a distinct leptospiral specific PCR product with a melting peak of > 80°C was observed. Nevertheless, transport of urine samples to the laboratory, the amount of urine or water examined and the use of a manual DNA extraction method might be factors that could have negatively affected detection of the presence of *Leptospira* in the samples. However, with 3 positives out of 194 animal samples, it can be concluded with 95% confidence that the population is not free from *Leptospira*, but the prevalence is not higher than 5% in the study population (Ausvet, 2021).

In African countries, risk factors for leptospirosis and the presence of pathogenic *Leptospira* are not well characterized and studied. Studies concerning knowledge, attitude and practice of people regarding leptospirosis are mainly performed in South-America and Asia, in areas where leptospirosis is known to be endemic and peoples' awareness might be higher than in Africa. Respondents in urban endemic areas in Argentinia and Brazil (Navegantes de Araújo *et al.*, 2013; Ricardo *et al.*, 2018) knew about leptospirosis, its clinical presentation and risk factors, in contrast with our study population, which did not know about leptospirosis although keeping livestock is practiced, which could be a reservoir for pathogenic *Leptospira*. Despite that, people in about two-third of the studied households were aware that contact with environmental water, rat urine or urine of their livestock could give diseases. Additionally, the general awareness and behaviour considering zoonotic disease was good, which was similar to, or even better than, findings from other studies done recently in rural Ethiopia (Fesseha & Abeba 2020; Alemayehu *et al.*, 2021). Educational level and availability of information in the peri-urban settings might be the contributing factor to the high awareness found in the study population.

Many known risk factors for leptospirosis (Goarant, 2016), such as rats in the environment, close contact with animals, wet areas around the house and walking through the river with the animals, were found among many of the studied households in peri-urban Addis Ababa.

This is consistent with previous reports from Kenya (Halliday *et al.*, 2013) and Tanzania (Maze *et al.*, 2018), which indicate that human exposure to pathogenic *Leptospira* species is considerable in peri-urban areas of Addis Ababa. This reflects the gap between knowledge and daily practice concerning leptospirosis and zoonotic diseases.

Concerning the relationship between KAP and the positive samples, no significant relationship was found between the positive samples and risk factors for zoonotic diseases and leptospirosis. This is not an unexpected finding, as the positive samples were found in only two of the 85 investigated households and the sample size was limited. Therefore, the analysis was likely underpowered.

**Strenghts and limitations of the study**

One strength of this study is the detection of pathogenic *Leptospira* by a molecular assay, which is the first of its kind in Ethiopia. It shows that a PCR assay with melting curve analysis – targeting the *lfb1* and *lipL32* gene and performed on the DNA extracted from the urine of cattle – can be used as a diagnostic method to detect pathogenic *Leptospira*. This study is unique in Ethiopia and East-Africa by investigating the presence of pathogenic *Leptospira* from a "One Health" perspective. Additionally, it gave valuable insights into risk factors for leptospirosis and zoonotic diseases in peri-urban areas of Addis Ababa.

There are not many comparable studies done in East-Africa. The few studies performed using PCR revealed low prevalences, which made the target sample size of this study small. In addition, the costs of the PCR materials and the labour intensive process of collecting samples made it not feasible to increase the sample size more than the one used in the current study. These factors might have their influence on the absence of detected pathogenic *Leptospira* in the human samples and the low (1.5%) positivity rate in the animal samples. Another limitation is the lack of validated methods to investigate water samples for pathogenic *Leptospira* which influenced the decision to spend resources on the water samples. Furthermore, investigating different animal species simultaneously instead of selecting one species, can make it difficult to compare our study with others.

# 7. CONCLUSION & RECOMMENDATIONS

Overall, this study has proved the presence of pathogenic *Leptospira* in peri-urban areas of Addis Ababa, Ethiopia, and the presence of risk factors for leptospirosis. Urine samples of cattle were found to contain pathogenic *Leptospira,* which suggest the potential risk for humans in the current study area where contact with livestock and their environment is inevitable. Molecular characterization revealed that the detected pathogenic *Leptospira* belong to *Leptospira borgpetersenii*. Although there were no detected pathogenic *Leptospira* during this study from the human and water samples, analysis of knowledge, attitude and practice among the households revealed that knowledge about leptospirosis is insufficient and that risk factors for leptospirosis are widely present. Given the above conclusions, the following recommendations can be forwarded:

- The knowledge about the presence of pathogenic *Leptospira* in Ethiopia, obtained from the current study, can be used as baseline information for further researches concerning leptospirosis and pathogenic *Leptospira* in the future. Research in the future should continue to use the "One Health" concept and application, as the presence of pathogenic *Leptospira* in animals' urine also indicates contamination of the environment and infection of humans.
- This study marks the importance of informing livestock-keeping households and responsible veterinary and health professionals about the presence of pathogenic *Leptospira.* There is a need for awareness creation among the livestock-keeping households on recognizing the potential threat of leptospirosis.
- As risk factors for leptospirosis were widely available, households in peri-urban areas of Addis Ababa should focus on preventive practices that may contribute to reducing the risk of livestock acquiring pathogenic *Leptospira* and zoonotic diseases in general. These preventive practices may also minimize the risk of zoonotic diseases for humans.

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# APPENDICES

**Appendix I – Information Sheet**

**Participant Information Sheet & Consent Form**

**Title:** Molecular identification and characterization of pathogenic *Leptospira* from asymptomatic humans, livestock and water sources in peri-urban areas of Addis Ababa: A One Health Concept

**PART I – Information Sheet**

**Investigator**: Mariska Kreuger, medical doctor, Master in Medical Microbiology student, Addis Ababa University. mariskakreuger@gmail.com / 09 84184873
**Advisor:** Dr. Woldaregay Erku, associate professor Addis Ababa University

This Master thesis research is about leptospirosis, which is a cause of acute febrile illness and barely investigated in Ethiopia. I invite you to be part of this research and will give you some information. You can decide yourself whether or not you want to participate in this research.

Leptospirosis is a disease of animals and humans. In animals, it can cause abortions and problems with milk productions. In humans, it can cause fever, severe liver and kidney problems and internal bleeding. You can get the disease from water and from your animals. That is why I want to investigate animals, humans and water here in the environment.

This research will imply that you give one time urine, that you fill in a questionnaire and that we collect urine of one or more of your animals. We have small bottles for your urine and that of your animals. Participation in the research involves for you only a one-time investigation.

When you participate in this research, we will get new information about an unknown disease in Ethiopia. Because there is almost no information on leptospirosis in Ethiopia. And in this way, we can give the information about the disease to hospitals and important people in the medical field, which can give them more knowledge and help them in treating patients in the right way. So, finally it can help you in better hospital care.

Personal information like your name and names of your family will not be shared with others. We make the other information which we need, like living place, anonymous.

You can decide at any moment that you want to stop participating in this research, and this will have no any negative consequence.

**Part II – Certificate of Consent**

**I have heard or read the previous information and hereby give my permission to voluntarily participate in this study.**

**I understand that urine that I give will be examined, that urine of my animal(s) will be examined and that I fill in a questionnaire.**

**Name:** **Signature:**

**Date:**

**Name Researcher: Marrigje Jacoba (Mariska) Kreuger**

**Signature Researcher:**

**Date:**

## የተሳትፊዎች የጥናቱ መረጃ እና ፈቃደኝነት ቅጽ

**የጥናቱ ርእሱ**፡ ‘Molecular identification and characterization of pathogenic *Leptospira* from asymptomatic humans, livestock and water sources in peri-urban areas of Addis Ababa: A One Health Concept’

### ክፍል-1፡ የተሳትፊዎች የጥናቱ መረጃ

**ተመራማሪ**፦ ማሪስካ ክሪውገር (ህኪም እና በአዲስ አበባ ዩኒቨርሲቲ የሜዲካል ማይክሮ ባዮሎጂ ተማሪ)

**ስልክ**፦ 09 84 18 48 73 **ኢ.ማል** mariskakreuger@gmail.com

**አማካሪ**፡ ዶ/ር ወልደአረጋይ እርቁ (በአዲስ አበባ ዩኒቨርሲቲ ተባባሪ ፕሮፌሰር)

**ይህ የድህረ ምረቃ ጥናት ስለ ሌፕሮስፓይሮሰስ የሚያጠና ሲሆን ሌፕሮስፓይሮሰስ የአኩት ፈብራይል ኢልነስ አምጪ ተዋህስ ሲሆን ይህ ጥናት ከዚህ በፊት በኢትዮጵያ አልተካሄደም። ስለዚህ የጥናቱ ተሳታፊ እንዲሆኑ እየጋበዝኩት ስለ ጥናቱ መሰረታዊ መረጃዎችን እሰጥዎታለሁ። ጥናቱ ላይ መሳተፍ በእርሶ ሙሉ ፈቃደኝነት ላይ የተወሰነ ይሆናል።**

**ሌፕሮስፓይሮሰስ በሰው እና በእንስሳት ላይ ሊገኝ የሚችል በሽታ ነው። በእንስሳት ላይ ውርጃ እንዲሁም የወተት ምርት መቀነስ ሊያመጣ ይችላል። በሰው ላይ ትኩሳት፣ የጉበት በሽታ፣ የኩላሊት ችግር እና በሰውነት ውስጥ ደም መፍሰስ ሊያስከትል ይችላል።**

**በሽታው ወደ ሰው ሊተላለፍ የሚችለው ከተበከለ ውሃ እንዲሁም በሽታው ካለባቸው እንስሳቶች ነው። ስለዚህ የሚያስፈልገን ናሙና ከሰው፣ ከእንስሳት እንዲሁም ከውሃ ነው። ጥናቱ የሚያካትተው የአንድ ጊዜ ከሰው ሽንት መስጠት፣ መጠይቅ መሙላት እንዲሁም ከእንስሳት ሽንት መውሰድ ይሆናል። ከሰውም ሆነ ከቤት እንስሳት ሽንት ለመውሰድ የሚያስችለን ትናንሽ እቃዎችን አዘጋጅተናል። በጥናቱ ላይ የሚሳተፉትም አንድ ጊዜ ብቻ ነው።**

**በጥናቱ ላይ በመሳተፍዎ በኢትዮጵያ ውስጥ የሌፕሮስፓይሮሰስ መረጃ ስለሌለን የማይታወቁ አዲስ መረጃዎችን ማግኘት እንችላለን። የጥናቱ ውጤትም ለጤና ባለሙያዎችም ለሚመለከታቸው አካላት በመስጠት ስለ በሽታው ተጨማሪ መረጃ ማግኘት የሚቻል ሲሆን ታካሚዎችም የተሻለ ህክምና ማግኘት የሚችሉበት መንገድ ይፈጥርልናል።**

**ክፍል-2**

**በጥናቱ ሲሳተፉ የእርስዎም ሆነ የቤተሰብዎ ስም ለሌላ ወገን አይተላለፍም። የመኖሪያ አድራሻዎትም አይገለጽም። ተሳትፎዎትን በመሀል ማቆም ከፈለጉ ማንም የማያስገድድዎት እና ምንም አይነት ጉዳት የሚያደርስብዎት አካል እንደሌለ እንገልጽልዎታለን።**

**ከላይ የተሰጠኝን መረጃ ሙሉ በሙሉ ተረድቻለሁ። በሙሉ ፈቃዴም ጥናቱ ላይ ለመሳተፍ ተስማምቻለሁኝ።**

**የጥናቱ ባለበት ስም** _____**ፊርማ** _____

**ቀን** _____

**የጥናቱ ተሳታፊ ስም፥** _____**ፊርማ፥** _____

**ቀን** _____

**Odeeffannoo fi unka walii galtee qooda fudhattoota qorannoo**

Mata duree qorannoo: 'Molecular identification and characterization of pathogenic *Leptospira* from asymptomatic humans, livestock and water sources in peri-urban areas of Addis Ababa: A One Health Concept'

**Kutaa 1ffaa: Odeeffannoo qooda fudhattoota qorannoof qophaa'e.**

Qorataa: Mariska Kreuger: Ogeessa yaalaa, Koolleejjii Fayyaa Yuuniversiitii Addis Abaabaatti barataa digirii 2ffaa. Lakk. Moobaayilaa: 09 84184873; E-mail: mariskakreuger@gmail.com

Gorsaa: Dr. Woldearagaay Irquu, Associate. Professor

Qorannoon kun kan irratti xiyyeeffatu dhukkuba 'Leeptoospirosis' yoo ta'u, dhukkubni kuni kan ho'ina qaamaa dabaluun beekamuu yoo ta'u, Itoophiyaa keessatti hedduu hin qoratamne. Kanaafuu ati qorannoo kana irratti hirmaachuun odeeffannoo dabalataa akka naaf ibsituuf afferamteetta. Qorannoon kuni feedhii irratti kan hundaa'e waan ta'eef irratti hirmaachuufio dhiisuun mirga keeti.

Dhukkubni armaan oliitti caqasame kuni namootaafi beeyiladoota kan miidhu yoo ta'u, beeyiladoota irratti miidhaa baasaa yookiin gatachiisuu akkasumas hiri'ina aannanii kan fidudha. Namoota irratti immoo dhukkubni kuni olka'insa ho'ina qaamaa, dhukkubbii tiruufi kale cimaa akkasumas qaamaa keessatti dhiiguu kan fidudha. Dhukkubni kuni bishaan faalamaa akkasumas beeyiladootaafi bineensota biro irraa namatti nama qabuu ni danda'a. Sababiidhuma kanaaf eega qorannoon kuni kan nama, beeyiladaafi bishaan yookiin qabiyyee naannoo irratti kan xiyyeeffate. Qorannoo kana fiixaan baasuuf gargaarsa kee kan na barbaachisuufi kanuma dhugoomsuufis qoda qaruuraa fincaan maatii kee itti naaf gumaachitan qabadheen jira. Kana malees, fincaan xiqqoo breeyiladoota kee irraa fi akksumas bishaan itti gargaaramtan irraas xiqqoo ni fudhadha. Atis yoo fedhii guutuu qabaatte gaaffilee gaggabaaboo fi yoo baay'ate daqiiqaa 25 keessatti raawwatuuf deebii sitti fakkaate naaf deebisuun na gargaarta.

Bu'aan ati qorannoo kana irraa argattu, odeeffannoo haaraa qorannoon kuni gumaachu yoo ta'u akkasumas odeeffannoon as irraa argmu kuni karaa mootummaa xiyyeeffannoo argachuun dhukkubni kuni namoota akkasumas beeyiladoota irraa akka yeroon ittifamuufi to'atamuuf kan gargaaru ta'a. Kanaafuu bu'aan qorannoo kana irraa argamu ogeessota yaalaa hospitaala adda addaa keessa tajaajilaaniif ibsamuun siifi namoota biroof yeroo kamittuu tajaajilli ga'aaniif I yeroon ta'e akka isiiniif kennamuuf karaa bana. Qabxiileen ijoo eenyummaa kee ibsan kamiyyuu qorannoo kana keessatti icciitiin kan qabaman kan ta'uu fi kana gochuufis gabaajee fi mallattoolee adda addaatti gargaaramuun qindaa'a. Odeeffannoon eenyummmaa kee akkasumas maatiikee ibsu kamiyyuu namoota dhuunfaas ta'e qaama garabiraa kamiifuu kan qoodamu miti.

Yeroo barbaaddetti qooda fudhattummaa kee addaan kuttee hirmaannaa kee dhaabuu ni dandeessa; waan kana gooteefis miidhaan sirras ta'e maatii kee irra ga'u hin jiru.

**Kutaa 2ffaa: Unka walii galtee qorataafi qooda fudhataa qorannoo kanaa ibsu**

Odeeffannoo armaan oliitti ibsame dubbisee (naaf dubbifamee) hubadheera. Kanaaf qorannoo kana irratti fedhiin hirmaachuuf guutummaan walii galuukoo nan mirkaneessa.

Akkuma armaan oliitti ibsamuuf yaalamettiifi anis hubadhetti, fincaan koo fi kan beeyilada kook an qorannoof fudhatamaniifi akkasumas gaaffiiwwan qorannoo kanaa waliin wal qabatan deebisuuf walii galuu koo mallattoo kootiin nan mirkaneessa.

**Maqaa Qorataa:_____** **Mallattoo: _____**

**Guyyaa:_____**

**Maqaa Qooda fudhataa: _____** **Mallattoo: _____**

**Guyyaa: _____**

**Appendix II – Questionnaire**

**KAP Assessment Questionnaire**

This questionnaire will take approximately 20 minutes to answer. Any information you provide will be anonymous and no personal information collected will appear in any documents or reports based on this survey.

Interview Date: ___ /___/_____ (DD/MM/YYYY)

Interviewer Name: _____

Sub-city: _____ Woreda: _____ Household: _____

Respondent Status:

□ Female head of household □ Male head of household □ Other adult (>18)

**Questions related to demographic characteristics of the household**

1. Sex: □ Male □ Female
2. Marital status:

   □ Single □ Married □ Divorced □ Widowed
3. Age:

4. What is the highest educational level you have attained?

   □ No formal education □ Read and write □ Elementary
   □ High school □ College level □ University level
5. How many people (including children) are in your household? _____
6. How many children < 5 years of age live in your household? _____
7. What is your occupation?

   □ Employed: A) Government B) Private C) NGO □ Builder/carpenter
   □ Farmer □ Unemployed/retired/Housewife
8. Does your household have:

   □ Electricity □ Television □ Radio
   □ Refrigerator □ Telephone/Mobile phone
9. What kind of toilet facility do members of your household usually use?

   □ Flush, connected to latrines □ Pit latrine with cement slab
   □ Pit latrine, without cement slab □ Canal or open defecation/bush/field
   □ Other (please, specify)

10. Who is responsible to care for the animals?

□ Wife □ Husband □ Children □ Other (please, specify)

11. What is the main water source of the household for the following activities?

| | Piped water | Surface water | Ground/bore-hole | Rain water | River water | Bottled water | Other |
|---|---|---|---|---|---|---|---|
| Drinking water for household | | | | | | | |
| Drinking water for animals | | | | | | | |
| Water for food preparation | | | | | | | |
| Water for cleaning kitchen, utensils | | | | | | | |
| Water for hand washing and laundry | | | | | | | |
| Water for cleaning of animal pen | | | | | | | |

12. If the water source is surface water or river water, from which area do you get it?

_____

13. Are there any times during the year when water is not readily available?

□ Yes: (please specify when) □ No □ Don't know

_____

14. Do you have any of the following animal species?

| Animal species | Number of animals |
|---|---|
| Sheep | |
| Goat | |
| Cattle | |
| Pig | |
| Horse/Donkey/Mule | |
| Chicken | |
| Others (specify) | |

15. What is the source (s) of feeding for your animals?

□ Scavenging □ Household leftover □ Purchase feed

□ Graze by road side □ Bring in forage □ Swill

**Questions related to the knowledge, attitude and practices of households regarding zoonoses**

16. Whom do you talk to most regularly about your family's health issues?

□ Family doctor □ Other health professionals □ Neighbors/relatives
□ Family member/friends □ Local health service centers □ Other people

17. Do you think that animals can be a source of human diseases?

□ Yes □ No

18. Do you know how a human can get a disease from animals?

□ Direct contact with animals □ Eating raw or undercooked meat/milk products
□ Drinking raw or under boiled milk □ Touching urine of animals
□ Other (please, specify)

19. What can be done to avoid diseases?

□ Avoid contact with environmental water □ Protect rats from entering the house □ Avoid touching animals having abortions □ I don't know

20. If you suspect an animal having a disease, what do you do?

□ Seek veterinary assistance □ Sell the animal □ Slaughter the animal
□ Treat the animal self □ Do nothing □ Others (please, specify)

21. Do you take any specific action to protect yourself when dealing with a diseased animal?

□ Yes □ No

If yes, what kind of action (s) do you take?

□ Use gloves
□ Wash hands
□ Others (please, specify)

22. Do you wash your hands with soap after contact with animals or their milk, manure or urine?

□ Yes
□ Sometimes
□ No

**Questions related to the knowledge, attitude and practices regarding leptospirosis and risk factors for leptospirosis**

23. Have you heard of diseases that you can get from contact with water?

□ Yes (please specify) □ No

24. Have you heard of diseases transmitted by rat's urine to humans?

□ Yes □ No

25. Have you heard of diseases transmitted by urine of cattle, sheep or goat, to humans?

□ Yes □ No

26. Did you hear about a disease called leptospirosis?

□ Yes □ No

If yes, how did you hear about it?

……

27. How can humans get leptospirosis?

□ From mosquitos

□ From cattle, sheep or goats

□ Contaminated food

□ Contact with urine from rats

□ Contact with water

28. The following symptoms can occur in the disease leptospirosis, which you can get from contact with water or urine of rats or domestic animals. Which of the following symptoms did you see in you or your family during the last month?

| | Yes | No | I don't know |
|---|---|---|---|
| Fever | | | |
| Kidney failure | | | |
| Jaundice | | | |
| Bleeding | | | |

29. Indicate whether you agree with the statement:

| | Yes | No | Sometimes |
|---|---|---|---|
| The area around my house is wet | | | |
| The area around my house is wet during the rainy season | | | |
| I walk without shoes or with open shoes through wet areas around the house | | | |
| I walk through the water with the animals | | | |
| My family members walk through the water with the animals | | | |
| I see rats at daytime around the house | | | |
| Rats come inside the house | | | |
| The garbage is stored in the compound of the house | | | |
| There is a cover on the garbage | | | |
| The garbage is eliminated every day | | | |
| I have rat traps around my house | | | |
| I use rat poisoning | | | |

This is the end of the questionnaire. Thank you for agreeing to take part in this valuable study. Please feel free to mention any additional comments regarding the study or information you provided.

**<u>የጥናቱ ተሳታፊዎችን እዉቀት፤ ግንዛቤ እና ተግባራቸዉን ለመለካት የሚጠየቅ ጥያቄ</u>**

ጥያቄው በግምት ከ20 ደቂቃ የሚፈጅ ይሆናል፡፡ እዝህ ላይ የሚትሰጡት መልስም ሆኔ ሀሳብ በምስጥር የምየዝ ስሆን፤ ማንኛውም ስለ አንቴም ሆኔ ስለቤተሰቦችክ የሚገልጽ ማንኛዉም መረጃ በኮድ የምቀመጥ ስሆን እነኝ ዶኩመንቶች ያላንቴ ፍቃድ ከጥናቱ አባላት ውጪ የማይታይ/የማይገለጽ ይሆናል፡

:

ቀን: ___ /___/_____ (ቀን/ወር/ዓ.ም)

የአጥኝዉ ስም: _____

ክፍለ ከተማ: _____ወረዳ: _____ ቀበሌ: _____ ኮድ _____

የተጠያቂው ሁኔታ:

□ ሴት እና የቤቱ እማወራ □ ወንድ እና የቤቱ አባወራ □ ሌላ (>18)

**<u>የተሳታፊውን የኑሮ ሁኔታ የሚገልፁ ጥያቄዎች</u>**

01. ፆታ: □ ወንድ □ ሴት

02. የጋብቻ ሁኔታ:

□ ያላገባ/ች □ ያገባ/ች □ የፈታ/ች □ በሞት የተለያዩ

03. ዕድሜ (በዓመት):

04. ያጠናቀከው ከፍተኛ የትህምርት ደረጃ፡

□ ያልተማረ □ ማንበብ እና መፃፍ □ አንደኛ ደረጃ
□ ሁለተኛ ደረጃ □ ኮሌጅ ደረጃ □ ዩኒቨርሲቲ

05. ልጆችን ጨምሮ ቤታቹ ውስጥ ስንት ሰው ይኖራል? _____

06. ከ5 ዓመት በታች ስንት ልጆች ቤታቹ ውስጥ ይኖራሉ? _____

07. ስራክ/ሽ ምንድነዉ?

□ የመንግስት ሥራ □ ግንበኛ/አናፂ
□ የግል መስርያቤት □ ጡረተኛ/የቤት እመቤት
□ አርብቶ/አርሶአደር □ ስራ የሌለዉ

08. ቤትክ/ሽ ውስጥ ከታች የተዘረዘሩት መገልገያዎች አሉ?

□ ኤለክትርክ □ ተሌቪዥን □ ራዲዮ
□ ፍሪጅ □ የገመድ/ሞባይል ስልክ

09. ቤቴሰቦችክ/ሽ በምን አይነት ሽንት ቤት ይጠቀማሉ?

□ በውሃ የምሰራ እና ከጉድጓድ ጋር የተገናኘ □ የተቆፈሬ ጉድጓድ እና በስምነቶ የተደፈነ
□ የተቆፈሬውና በስምንቶ ያልተደፈነ □ ሽንት ቤት የለም
□ ሌላ (ግለፅ/ጭ)

10. ለእንስሳቶቹ ማን እንክብካቤ ያደርጋል?

□ ሚስት □ ባል □ ልጆች □ ሌላ ሰው (ግለፅ/ጭ)

11. ለምከተሉት ተግባራት የምትጠቀሙት የውሃ አይነት የቱ ነዉ?

| | የቧንቧ | የወንዝ/ኩሬ/ሐይቅ | የጉድጓድ | የዝናብ | የሀይላንድ | ሌላ ሚንጭ |
|---|---|---|---|---|---|---|
| የምጠጣ ዉሃ ለሰዉ | | | | | | |
| የምጠጣ ዉሃ ለእንስሳት | | | | | | |
| ለምግብ ዝግጂት | | | | | | |
| ለምግብ ቤት ዕቃ ፅዳት | | | | | | |
| ለእጅ እና ልብስ | | | | | | |
| የእንስሳትን በረት ለማፅዳት | | | | | | |

12. የውሃ ሚንጫቹ የወንዝ/ኩሬ/ሐይቅ ከሆኔ ከየት አከባቢ ነው የምታገኙተ?

_____

13. በአመት ውስጥ ውሃ የምታጡበት ወቅት ይኖራል?

□ አዎ፤(ግለፅ/ጭ) _____ □ የለም □ አላዉቅም

14. ከታች ከተዘረዘሩት እንስሳት ዉስጥ የትኛዉን ይኖርካል/ሻል?

| እንስሳት አይነት | ብዛት |
|---|---|
| በግ | |
| ፍየል | |
| ከብት | |
| አሳማ | |
| ፈረስ/አህያ/በቁሎ | |
| ዶሮ | |

| | |
|---|---|
| ሌላ (ግለፅ/ጭ) | |

15. ለእንስሳቶችክ/ሽ የምግብ ምንጭ ምንድናው?

□ ወድያ ወድህ ብሎ ያገኛሉ □ የቤት ትራፊ □ የምገዛ
□ መንገድ ዳር ይግጣሉ □ የታጨዱ ሳር □ በጉድጓድ ውስጥ የምዘጋጅ

**በጥናቱ ላይ ተሳታፊ የሆኑትን ሰዎች እዉቀት፤ አስተሳሰብ እና ተግባራቸዉን ለመገምገም የምዉል ጥያቄዎች**

16. ብዙዉን ግዜ ስለ ቤተሰብክ/ሽ የጤና ሁኔታ ከማን ጋር ትወያያለክ/ሽ?

· ለቤተሰቡ ሐኪም · ለሌሎች የጤና ባለሙያዎች · ለጎሮቤታችነ/ለዘመዳችን

· ለቤተሰብ/ጓደኛ · በአከባቢያችን ለምገኘዉ ጤና ጣብያ · ሌላ

17. እንስሳት ለሰዉ ልጅ የበሽታ መንስዐ ይሆናሉ ብለክ/ሽ ታስባለክ/ሽ?
· አዎ · አይደለም

18. ከምከተሉት በሽታዎች ዉስጥ የትኞቹ ከእንስሳ ወደ ሰዉ ልጅ ይተላለፋል ብለክ/ሽ ታስባለክ/ሽ?

19. የበሽታ ለማስወገድ/ለማጥፋት ምን መደረግ/መሠራት አለበት?
· ለአካባቢ የተጋለጠ ውሃ አለመጠቀም
· አይጦች ወደ መኖሪያ ቤት እንዳይገቡ
ማድረግ
· በእንስሳት ውርጃ ወቅት ንክኪ አለማድረግ
· አላውቅም

20. እንስሳት በበሽታ መያዙን ብታውቅ ምን ታደርጋልክ/ሽ?

· የእንስሳት ሐኪም ዕርዳታ መጠየቅ · እንስሳቱን መሸጥ · እንስሳቱን ማረድ

· በራስ እንስሳቱን ማከም · ምንም አላደርግም · ሌላ (ግለፅ/ጭ)

21. የታመሙትን እነስሳት ስታግዝ/ዥ ምን አይነት ጥንቃቄ ታደርጋለክ/ሽ?
· አዎ · አላደርግም

መልስክ/ሽ አዎ ከሆነ፤ ምን አይነት ጥንቃቄ ትወስዳለክ/ሽ?
· የእጅ ግላቭ በመጠቀም · እጅ በመታጠብ · ሌላ (ግለፅ/ጭ)

22. እንስሳትን ከነካቹ/ ከእንስሳት ጋር የተያያዘውን ስራ
ካከናወናላቹ በኋላ ምንያክል እጃቹን በሳሙና ትታጠባላቹ?

- አዎ
- አልፎ አልፎ
- አይደለም

**<u>በጥናቱ ላይ ተሳታፊ የሆኑትን ሰዎች ሌፕቶስፓይሮሲስ እዉቀት፤ አስተሳሰብ እና ተግባራቸዉን ለመገምገም የምዉል ጥያቄዎች</u>**

23. በውሃ ንክኪ ምክንያት ስለሚመጣ በሽታ ሰምተው ያውቃሉ?

 አዎ አውቃለው (ካለ ይጥቀሱ _____) አላውቅም

24. በአይጥ ሽንት አማካኝነት ወደ ሰው የሚተላለፍ በሽታ እንዳለ ሰምተው ያውቃሉ ?

 አዎ አውቃለው አላውቅም

25. ቀንድ ከብት፤ በግ እና ፍየል ሽንት አማካኝነት ወደ ሰው የሚተላለፍ በሽታ እንዳለ ሰምተው ያውቃሉ?

 አዎ አውቃለው አላውቅም

26. **ሌፕቶስፓይሮሲስ** ስለሚባል በሽታ ሰምተው ያውቃሉ ?

 አዎ አውቃለው አላውቅም

27. **ሌፕቶስፓይሮሲስ** በምነን ምክንያት ይመጣል ብለው ይገምታሉ ?

- ወባ
- ቀንድ ከብት፤ በግ እና ፍየል
- ከተበከለ ምግብ
- ከአይጥ ሽንት
- ውሃ ንክኪ

28. ከሚከተሉት ወውሰስጠጥ የ**ሌፕቶስፓይሮሲስ ምልክት የቱ ይመስልዎታል**?

| | ነው | አይደለም | አላውቅም |
|---|---|---|---|
| ያለ ህመም ማመርቀዝ | | | |
| ትኩሳት | | | |
| የኩላሊት ችግረ/ ህመም | | | |
| የአይን ቢጫ መሆን | | | |
| መድማት/ ደም መፍሰስ | | | |

29. ከዚህ በታች ለተዘረዘሩት ሃሳቦች አዎ፤ አይደለም ወይም አልፎ አልፎ በሚሉት አማራጮች ላይ ( ) ምልክት በማድረግ ይለፉ

| | **አዎ** | **አይደለም** | **አልፎ አልፎ** |
|---|---|---|---|
| የመኖሪያ አካባቢዬ እርጥበት አለበት | | | |
| የመኖሪያ አካባቢዬ እርጥበት አለበት | | | |
| እርጥበት በባለበተት መኖሪያ አካባቢዬ ያለጫማ እንቀሳቀሳለው | | | |
| የበቤተሰበብ አባሎቼ ከእንስሳቱ ጋር ወንዝ ያቋርጣሉ | | | |
| ቀን ላይ አይጦች በመኖሪያ አካባቢዬ ይንቀሳቀሰሳሉ | | | |
| አይጦች በመኖሪያ በቤተቴ ይገባሉ | | | |
| በኒቢዬ ውስጥ የቆሻሻ ማጠራቀሚያ አለ | | | |
| የቆሻሻ ማጠራቀሚያው መክደኛ አለ | | | |
| የተጠራቀመው ቆሻሸሻ በየቀኑ ይወገዳል | | | |
| በቤቴ አይጥ ወጥመድ ይገኛል | | | |
| የአይጥ መርዝ እጠቀማለው | | | |

ጥያቄው እዝህ ላይ ያልቃል፤እናም በዝህ አስፈላጊ ጥናት ላይ ለመሳተፍ ፈቃደኛ ስለሆኑ እጅግ በጣም አመሰግናሁኝ። እባክህ/ሽ ሌላ ማንሳት የምትፈልግ/ጊ ካለ ለመጠየቅ ትችላለክ።

_____

_____

**Gaaffiilee beekumsa, hubannoo (ilaalcha) fi gochaa horsiisee bultootaa madaaluuf qophaa'an**

Walumaagalatti gaaffileen kuni yoo baay'ate daqiiqaa 20 kan fudhatu ta'a. Adeemsa qorannoo kana keessattis ta'e gaafilee dhiyaatan keessatti deebiin yookiin odeeffannoon ati kennitu akkasumas maatii kee waliin kan wal qabatu jiru martu bifa icciitiin kan galmaa'uufi odeeffaannoon ati keennitu martuu nama sadaffaa garabiraatti kan hin beeksifamneefi qorannoo kana keessattis odeeffannoon akka maqaa fi kanneen dhimma dhuunfaa ilaallatan martuu bifa gabajeen yookiin immoo mallattoo adda addaatiin kan bakka buufaman ta'a.

Guyyaa gaaffiifi deebiin itti ta'u: _____/_____/_____ (Guyyaa/Ji'a/Waggaa)

Nama gaaffiilee gaafatu: _____

Kutaa Bulchiinsa Magaalaa _____Aanaa_____ Lakk. Gaafatamaa____

Maalummaa qooda fudhataa:

□ Abbaa manaa □ Haadha Manaa □ Nama gara biraa (ga'eessaa waggaa 18 olii)

**Gaaffillee waliigalaa**

1. Saala:
   □ Dhiira □ Dhalaa
2. Maalummaa maatii: Baaqqee kan fuute/kan heerumte kan hiike/te kan irraa du'e/te
3. Gidduugala umurii:
4. Sadarkaa barnootaa ol'aanaa
   Kan hin barannee dubbisuufi barreessuu kan danda'u sadarkaa 1ffaa sadarkaa 2ffaa
   Sadarkaa koolleejjii sadarkaa Yuuniversiitii
5. Maatiin keesssan nama meeqa qaba (ijoollota dabalatee)? _____
6. Ijoolleen umurii 5 gadii meeqatu maatii keessa jira? _____
7. Hojiin kee maali?
   # Hojjetaa mootummaa # Hojjetaa dhaabbata dhuunfaa
   # Hojjetaa dhaabbata-miti mootummaa
   # Qotee/horsiisee bulaa # Hojii dhuunfaa # Kan biro
8. Mana kee keessaa tajaajila kanneen gadii kam qabaa?
   # Humna elektrikii # Televitsiinii/Raadiyoo # Qabbaneessaa # Bilbila manaa/ # Moobaayilii
9. Maatiin keessan mana fincaanii akkamiitti gargaaramaa?
   □ Kan mana keessaa □ Boolla qabiyyeen isaa simmintoo
   □ Boolla qabiyyeen isaa biyyee □ Bo'ii/ tajaajila bakkeetti
   □ Garabiraa (adda baasi_____)

10. Maatii keessan keessaa eenyutu beeyiladoota kunuunsa?

□ Haadha manaa □ Abbaa manaa □ Ijoollee □ Kan biraa (adda baasi_____)

11. Maddi bishaan tajaajila adda addaa armaan gadiif oolu maali?

| | Tuubboo | Bishaan kuusaa | Boolla | Bokkaa | Jallisii | Pilaastika | Kan biraa |
|---|---|---|---|---|---|---|---|
| Dhugaatiif (namaaf) | | | | | | | |
| Dhugaatiif (beeyiladootaaf) | | | | | | | |
| Nyaata qopheessuuf | | | | | | | |
| Qulqullina bakka nyaata itti qopheessaniif | | | | | | | |
| Harkaa fi uffaata miicuuf | | | | | | | |
| Mana galmaa beeyiladootaa qulqulleessuf | | | | | | | |

12. Yoo maddi bishaanii keesaanii bishaan banaa yookiin bishaan yaa'aa ta'e, eessatti argama?

_____

13. Waggaa keessatti hanqinni bishaanii yeroon itti mul'atu ni jiraa?

□ Eeyyee: (ibsi_____) □ Lakki □ Hin beeku

14. Beeyiladoota armaan gadii keesssaa kam qabda?

| Gosa beeyiladaa | Baay'ina beeyiladaa |
|---|---|
| Hoolaa | |
| Re'ee | |
| Sa'a ykn Sangaa | |
| Booyyee | |
| Farda, Harree, Gaangee | |
| Lukkuu | |
| Kan biraa (ibsi) | |

15. Maddi nyaata beeyilada kee maali?

□ Oliifi gadi deemuun argatu □ Haftee nyaata namaa □ Nyaata bitamu

□ Daandii cinaa dheeduu □ Okaafi marga haamame □ Nyaata bulbulame

**Gaaffilee beekumsa, hubannaa fi gochaa qooda fudhattootaa madaaluuf qophaa'e**

1. Yeroo baay'ee waa'ee fayyaa maatii kee eenyuun marii'atta?

□ Ogeessa fayyaa maatii □ Ogeessa fayyaa kan biroo □ Ollaa/fira

□ Maatii/hiriyyaa □ Buufata tajaajila fayyaa naannoo □ Hin beeku

2. Bineeldi madda dhukkuba namaa ni ta'a jettee ni yaaddaa?

□ Eeyyee □ Lakki

3. Kanneen armaan gadii keessaa akka yaada keetti namni karaa kamiin bineelda irraa dhukkubaan qabamuu danda'aa?

□ Bineelda qaqqabuun □ Foon hin bilchaatiin nyaachuu

□ Aannan haalaan hindanfiin dhuguu □ Karaa biraa (ibsi)

□ Fincaan beyiladootaatti tuqu

4. Dhukkuba Leptoospaayiroosisii jedhamu kana dhabamsiisuuf maaltu godhamuu qaba?

□ Bishaan xuraa'aa naannoo irraa of eeguu □ Hantuuta mana irraa dhorkuu □ Beeyilada gatate tuttuquu dhiisuu □ Hin beeku

5. Horiin kee akka dhukkubsatte yoo shakkite maal goota?

□ Ogeessa fayyaa beeyiladaan soqa □ Nan gurgura □ Nan qala

□ Ofiin horicha yaala □ Homaa hin godhu □ Kan biroo (ibsi)

6. Yeroo horii dhukkubsate gargaartu ofeeggannoo adda ta'e ni taasiftaa?

□ Eeyyee □ Lakki

Yoo deebiin kee Eeyyee ta'e, maal goota?

□ Golgaa harkaan fayyadama □ Harka dhiqadha □ Kan biraa (ibsi)

22. Ati yookiin maatiin kee erga horii qe'ee yookiin bakka jireenya isaanii qaqqabatanii booda harka isaanii saamunaan ni dhiqatuu?

□ Yeroo maraa □ Darbee darbee □ Yeroo tokko tokko □ Tasumaa

**Gaaffii beekumsaa, ilaalchaa fi shaakkala leptospirosis fi sababii miidhaa leptospirosis**

23. Dhukkuba bishaaniin wal qabatee namatti darbu dhageessee beektaa?

□ Eeyyee (ibsi) □ Lakki

24. Dhukkuba karaa hantuutaa ykn fincaan hantuutaa namatti darbu dhageesseettaa?

□ Eeyyee □ Lakki

25. Waa'ee dhukkuboota fincaan loonii,hoolaa fi re'ootaan namootatti dadarbanii Quba qabdu?

 Eeyyee  Lakki

26. Waa’ee dhukkuba ‘Leeptoospaayiroosisii’ jedhamu dhageessee beektaa?

 Eeyyee  Lakki

Yoo deebin kee Eeyyee ta’e akkamiin dhageesse?

 Karaa oduu/miidiyaan  Barnootaan  Muuxannoon
 Nama dhukkubsatee beeku irraa  Karaa hojii idilee  Karaa biro (ibsi)

27. Namni akkamiin dhukkuba kanaan qabama jettee yaadda?

 Bookee irraa
 Beeyilada manaa irraa
 Nyaata faalame irraa
 Fincaan hantuutaaf saaxila bahuun
 Bishaan xuraa’aaf saaxila bahuun

28. Mallattoolee armaan gadii keesssaa kamtu mallattoo dhukkuba kanaati jetta?

| | Eeyyee | Lakki | Hin beeku |
|---|---|---|---|
| Mallattoo hin qabu | | | |
| Ho’ina qaamaa garmalee dabaluu | | | |
| Kaleen hojii dhaabuu | | | |
| Halluu keelloo qaama irratti mul’achuu | | | |
| Dhiiguu | | | |

29. Kanneen armaan gadii irratti walii galuufi dhiisuu kee beeksis:

| | Eeyyee | Lakki | Yeroo tokko tokko |
|---|---|---|---|
| Naannoon jireenya koo jiidhina qaba | | | |
| Naannoon mana koo yeroo waktii roobaa ni jiidha | | | |
| Dhoqqee naannoo mana koo keessa miila qullaan deema | | | |
| Beeyilada waliin bishaan keessa miilanan ce'a | | | |
| Maatiin koo beeyilada waliin bishaan keessa miilaan ce'u | | | |
| Hantuuta guyyaan naannoo mana kootti argeera | | | |
| Hantuutni mana keenya keessa ni seenti | | | |
| Kosiin mooraa manaa keessa kuufama | | | |
| Koosiin kuufamaan golgaa hin qabu | | | |
| Kosiin mooraa keessaa guyyuun gatama | | | |
| Kiyyoo hantuutaa mana keessaa ni qaba | | | |
| Qoricha summaa'aa hantuutaaf nan fayyadama | | | |

Gaaffiin keenya asuma irratti xumurama. Waan qorannoo bu'a qabeessa kana irratti hirmaatteef hedduu galatoomi. Hadaraa yaada dabalataa qorannoo kanas ta'e odeeffannoo naaf qoodde irratti qabdu kamiyyuu naaf dheeruu ni dandeessa.

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## Appendix III – DNA Extraction protocol

DNA is extracted with the QIAamp® Viral RNA Mini kit, according to the steps described in the QIAamp® Viral RNA Mini Handbook (Qiagen, 2020).

1. Take 140 µl of urine or water and pipet it in an Eppendorf tube
2. Add 560 µl of QIAamp RNA Viral Mini buffer which contains 5.6 µg of RNA carrier
3. Mix it well and keep it for 20 minutes at room temperature
4. Add 560 µl of ethanol 100% and mix it well
5. Centrifuge for 1 minute at 8000 rpm
6. Load 630 µl of the mix on the Qiagen column
7. Centrifuge it two times 1 min at 8000 rpm
8. Load the rest of the mix on the Qiagen column
9. Centrifuge it two times 1 min at 8000 rpm
10. Wash the column with 500 µl of buffer AW1
11. Centrifuge it 1 min at 8000 rpm
12. Wash the column with 500 µl of buffer AW2
13. Centrifuge it 1 min at 8000 rpm
14. Column eluted with 60 µl of H20
15. More than 1 minute incubation and centrifuge 1 min at 8000 rpm
16. Store the elute at -20˚C

**Appendix IV – PCR Protocol**

Developed by MRC-Holland and AMC Medical Microbiology, WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis.

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1. Lfb1 PCR involving an Evagreen Real-Time PCR assay, in which a possibly correct lfb1 PCR product is revealed by a specific melting curve with a Tm of more than 80˚C, also allowing serotype identification.
2. LipL32 PCR decetion involving a TaqMan probe hydrolysis assay that specifically detects the Real-Time formation of a lipL32 PCR product. No serotype identification possible.

<u>PCR reactions</u>

Per reaction:

1. 20 µl mix containing 0.3 µl Salsa polymerase and 19.7 µl of the mastermix
2. 5 µl of the extracted DNA sample is added to this 20 µl mix

Used Primers and Reverse Primers:
*Lfb1*
LFB1-F 5′-CATTCATGTTTCGAATCATTTCAAA-3′
LFB1-R 5′-GGCCCAAGTTCCTTCTAAAAG-3′
*LipL32*
LipL32-47Fd 5’-GCATTACMGCTTGTGGTG-3
LipL32-301Rd 5’-CCGATTTCGCCWGTTGG-3’
Controls:
A negative control with PCR-grade water was always used with the samples.
Purified leptospiral DNA control samples and patient urine, blood and serum samples were provided by the WHO/FAO Collaborating Centre for Reference and Research on Leptospirosis in the Netherlands.

**Lfb1 PCR reaction with Evagreen fluorescence melting curve detection**

| | µl |
|---|---|
| 10x SALSA PCR buffer | 2,5 |
| Test-2540 (100 µM) | 0,1 |
| Test-2541 (100 µM) | 0,1 |
| Evagreen | 1 |
| dNTPs (4mM) | 1,2 |
| DNA | 5 |
| SALSA TAQ polymerase | 0,3 |
| $H_2O$ | 14,8000 |
| Total volume | 25,0000 |

**LipL32 Reaction with TAQ-Man probe and degenerate primers**

| | µl |
|---|---|
| 10x biolabs buffer | 2,5 |
| Test-2872 (100 µM) | 0,175 |
| Test-2873 (100 µM) | 0,175 |
| Test-2551 (50 µM) | 0,075 |
| dNTPs (4mM) | 1,2 |
| DNA | 5 |
| SALSA TAQ polymerase | 0,3 |
| $H_2O$ | 15,5750 |
| Total volume | 25,0000 |

CFX96 real-time PCR detection system (BIO-RAD) with BIO-RAD software

Biorad PCR detection system settings:

Lid 105 °C. 4/10°C per cycle.

Step 1 95˚C for 1 min

Step 2 95°C for 0:10 min

Step 3 58°C for 0:30 min

Step 4 72°C for 0:30 min

Step 5 45 times

Step 6 40°C for 3 min

Step 7 40°C for 0:05 min

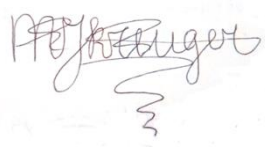
Step 8 95°C = END

# Declaration

I, Marrigje Jacoba Kreuger, declare that this thesis, title "Molecular identification and characterization of pathogenic *Leptospira* from asymptomatic humans, livestock and water sources in peri-urban areas of Addis Ababa: A One Health Concept", is my own original work, that it has not been presented for a degree in any other university, and that all sources of materials used for the thesis have been duly acknowledged.

Name: Marrigje Jacoba Kreuger

Date: July 02, 2021

Signature: 

Supervisor: Dr. Woldaregay Erku Abegaz

Date: July 02, 2021

Signature _____