Vol. 17(10), pp. 230-245, October 2023
DOI: 10.5897/AJMR2023.9715
Article Number: FE0C09D71267
ISSN: 1996-0808
Copyright©2023
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJMR

ACADEMIC JOURNALS
expand your knowledge

African Journal of Microbiology Research

Full Length Research Paper

Molecular detection of pathogenic *Leptospira* from cattle in peri-urban areas of Addis Ababa, Ethiopia

Marrigje J. Kreuger[1]*, Woldaregay Erku[1], Antoine Stuitje[2], Zewdu Terefework[3], Gemechu Chala[1] and Siobhan M. Mor[4,5]

[1]Department of Medical Microbiology, Parasitology and Immunology, Addis Ababa University, Addis Ababa, Ethiopia.
[2]MRC Holland, Amsterdam, The Netherlands.
[3]MRC-ET Advanced Laboratory, Addis Ababa, Ethiopia.
[4]Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, United Kingdom.
[5]International Livestock Research Institute, Addis Ababa, Ethiopia.

Received 15 July, 2023; Accepted 18 September, 2023

Leptospirosis is a bacterial, zoonotic disease caused by pathogenic *Leptospira*. Rodents are known to carry pathogenic *Leptospira,* but livestock are also important hosts. The disease is economically important in cattle, causing abortion, decreased fertility and decline in milk yield. Pathogenic *Leptospira* are shed in cattle urine and can survive in the environment. Only a few studies have been performed in Ethiopia to investigate the presence of *Leptospira*. The aim of this study was to determine the prevalence of pathogenic *Leptospira* in cattle in peri-urban areas of Addis Ababa. A cross-sectional study was undertaken in peri-urban areas of Addis Ababa. Urine was collected from cattle. DNA was extracted and real-time PCR with melting curve analysis was performed to detect pathogenic *Leptospira*. Knowledge, attitudes and practices of the cattle-keeping households were assessed by a questionnaire and household level risk factors investigated using logistic regression. In total, 168 urine samples were collected from 168 cattle in 70 households. Pathogenic *Leptospira* were found in 3 of the 168 (1.8%) urine samples. Although potential exposure pathways were widely present in the households, no significant risk factors were detected in regression analysis. This study has shown that pathogenic *Leptospira* are present in cattle in peri-urban areas of Addis Ababa, which could be a potential threat for humans. These findings emphasize the need for large-scale studies concerning pathogenic *Leptospira* in Ethiopia, especially in communities with high human-animal interaction.

Key words: Pathogenic leptospira, PCR, Ethiopia, cattle, leptospirosis.

INTRODUCTION

Leptospirosis is a globally important zoonotic disease caused by bacteria of the genus *Leptospira*, which are thin, tightly coiled, spiral-shaped spirochetes (WHO, 2003; Picardeau, 2017). Pathogenic, intermediate and saprophytic *Leptospira* have been described. Saprophytic *Leptospira* are present in the environment and usually do not cause disease, while intermediate and pathogenic *Leptospira* species can cause disease in both humans and animals. Rodents are considered the main reservoir of leptospirosis, but a wide variety of wild and domestic

*Corrresponding author. E-mail: mariskakreuger@gmail.com.

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

animals including cattle, sheep,goats, horses and pigs can be infected. Once infected, animals can develop long term kidney colonization and shed *Leptospira* in urine. Transmission to other animals and humans occurs when *Leptospira* in urine-contaminated soil or water enters the body through mucous membranes, small cuts and abrasions (Levett, 2001; WHO, 2003; Allan et al., 2015).

Leptospirosis is found worldwide, but warm and humid tropical regions favour the survival and perpetuation of the spirochetes (Evangelista and Coburn, 2010; Hartskeerl et al., 2011). Globally, leptospirosis is estimated to cause 1 million clinical infections and 60,000 deaths each year in humans (Costa et al., 2015). However, information from the African continent is very sparse (De Vries et al., 2014; Costa et al., 2015) and limits a more accurate estimation of the global burden of leptospirosis. In humans, infections are often asymptomatic or a mild "flu-like" illness, while some patients develop severe illness with hemorrhage, hepatic and renal failure, called Weil's disease (WHO, 2003). Detection of *Leptospira* is challenging, as direct detection with dark-field microscopy is not reliable and culture is too slow (weeks-months) (Vijayachari et al., 2001; Musso and La Scola, 2013; Karpagam and Ganesh, 2020). The gold standard test, namely the microscopic-agglutination-test (MAT), is labour-intensive, needs a panel of live leptospires and cannot differentiate well between current and past infections, but is able to differentiate serovars (Musso and La Scola, 2013; Karpagam and Ganesh, 2020). Serological tests, such as ELISA, and molecular assays are more practical. Importantly, molecular testing of urine provides a non-invasive option for diagnosis during early and late stages of infection and gives information on genotypic *Leptospira* species, which has largely replaced the traditionally-used serological classification (Musso and La Scola, 2013; Esteves et al., 2018; Vincent et al., 2019; Karpagam and Ganesh, 2020; Di Azevedo and Lilenbaum, 2021). Early detection and start of treatment affect the outcome of the disease in a positive way (WHO, 2003; Levett, 2001).

Leptospirosis is also an economically important disease of cattle. In adult animals, infection is often sub-clinical and the development of clinical signs depends on the infecting species. Cattle are maintenance host of *Leptospira borgpetersenii*, serovar Hardjo (Hardjobovis), which is associated with infertility, abortions, stillbirths, weak offspring and drop in milk production, but gives a more subtle clinical picture than infection with non-hardjobovis leptospires (WHO, 2009; Lilenbaum and Martins, 2014; Ellis, 2015). Acute leptospirosis can also occur in calves and is associated with fever, anorexia, diarrhea, icterohaemorrhagic syndrome and conjunctivitis (WHO, 2009; Ellis, 2015; Yadeta et al., 2016). Cattle are an important source of infection for humans because they can shed large numbers of *Leptospira* in urine over several months, although survival in the environment varies with species (Hairgrove, 2004; Barragan et al., 2017; Rocha et al., 2017; Hamond et al., 2022; Monti et al., 2023). In addition to urine, *Leptospira* can also be found in aborted fetuses, birth products and uterine discharges of infected animals, which can contribute to environmental contamination (Yadeta et al., 2016).

Ethiopia has amongst the largest livestock populations in Africa with many zoonotic diseases being endemic and has a high dependency on agriculture with many households having direct contact with animals (Grace et al., 2012; Shapiro et al., 2017; Management Entity, 2021). There is a paucity of research on leptospirosis in Ethiopia with a few studies performed in animals and only one small study in humans, all suggesting *Leptospira* being prevalent in the country (Moch et al., 1975; Yimer et al., 2004; De Vries et al., 2014; Tsegay et al., 2016; Desa et al., 2021; Marami et al., 2021). One recent study on leptospirosis in cattle in South-West Ethiopia has been published, which found a 24.5% seroprevalence of hardjo-specific antibodies using indirect ELISA (Desa et al., 2021). Other recent serological investigations in horses (44%) (Tsegay et al., 2016) and dogs (15%) (Marami et al., 2021) also reflected high levels of lifetime exposure in animals. There is a need to better understand the epidemiology of leptospirosis in cattle and species circulating in Ethiopia, because Ethiopia has the highest cattle population in Africa with a high livestock density in and around urban areas and the (peri-)urban dairy sector is targeted for development to meet the growing demand for milk and milk products (Management Entity, 2021; Shapiro et al., 2015; FAO, 2020). Therefore, the aim of this study was to investigate the prevalence of pathogenic *Leptospira* in cattle in peri-urban areas of Addis Ababa and to assess the knowledge, attitudes, practices and household level risk factors of cattle-keeping households regarding leptospirosis.

MATERIALS AND METHODS

Study design and study population

A cross-sectional study was conducted from February to October 2019 in peri-urban areas of Addis Ababa. Cattle-keeping households in four peri-urban sub-cities of Addis Ababa were eligible to participate. The four sub-city administrations recommended certain livestock-keeping woredas (districts of Ethiopia) within their sub-city. Animal health assistants of these woredas directed us to the households based on their lists of cattle-keeping households. We aimed to sample every adult animal in a household, with a maximum of 10 samples per farm. In practice, this was not always possible, as not all cattle were able to produce urine during the time of the visit. The minimum sample size for estimation of prevalence was determined using the single population proportion formula according to Thrusfield (2005): $n = (Z\alpha/2)^2 \times P\ (1\text{-}P) / d^2$.

As there were no studies published from Ethiopia investigating leptospirosis in cattle using molecular methods, other recent studies in East Africa using a PCR assay and performed among cattle were considered in the sample size determination (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). Using this expected prevalence (P) of 8.8%, 5% precision, and a 95% confidence interval, a minimum sample size of 123 cattle was calculated. Taking into consideration field logistics, such as transport of the samples and availability of cattle in the households, a final sample size of 168 cattle was achieved.

Field data collection

A semi-structured questionnaire was used to collect data on household socio-demographics, livestock husbandry and water sources as well as knowledge, attitudes and practices (KAP) of the households in relation to leptospirosis and zoonoses more generally ("S1 Appendix"). Respondents were the household head or other adult aged >18 in the household. All interviews were conducted verbally in the local languages (Amharic and Oromo) by the lead investigator and with the assistance from veterinarians/ veterinary students or animal health assistants.

At least 15 mL of urine was collected from each animal (cow/heifer/bull/calf) in the household. A mid-stream urine sample was obtained during spontaneous urination or by gentle perineal massage performed by the investigator and animal health assistant, and collected in sterile bottles. The urine was neutralized immediately after collection with phosphate buffered saline 10x (Lucchesi et al., 2004). Urine samples were transported on the day of collection from the households to the laboratory, with a maximum transport time of 3 h.

Laboratory analysis

DNA was extracted from 140 µl of urine on the day of collection and stored at -20°C. DNA from the pellet was extracted using the QIAamp Viral RNA Mini Kit (Qiagen, 2020). The DNA extraction method was tested by the main investigators on 140 µl of urine of known leptospirosis patients. Extracted DNA was subjected to quantitative real-time PCR using *Leptospira* specific lipL32 and lfb1 PCR detection assays (Bourhy et al., 2011). Lfb1 PCR involves an Evagreen real-time PCR assay, in which a lfb1 PCR product is revealed by a specific melting curve with a Tm of more than 80°C, also allowing species 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 while lipL32-47Fd and lipL32-301Rd primers were used to amplify the *lipL32* gene (Bourhy et al., 2011). 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. Amplification was performed on a CFX96 real-time PCR system (BIO-RAD) with initial denaturation at 95°C for 1 min, followed by 45 cycles of 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 10 s. These conditions were used for both primer sets ("S2 Appendix"). After PCR, the samples were heated from 40 to 95°C with continuous data acquisition. Purified leptospiral DNA control samples were provided by the Expertise Centre for Reference and Research on Leptospirosis/OIE Reference Laboratory for Leptospirosis (AMC, Department of Medical Microbiology and Infection Prevention, the Netherlands), and included the following species: *Leptospira interrogans*, *L. borgpetersenii* and *Leptospira santarosai*. For quality control purposes, extracted DNA was analysed in both the MRC-ET Advanced laboratory in Addis Ababa and the MRC-Holland laboratory in the Netherlands under identical conditions. The extracted DNA was transported frozen to the Netherlands.

Data management and analysis

Prevalence of pathogenic *Leptospira* was described as the number of animals with positive PCR detection for pathogenic *Leptospira* divided by the total number of animals tested. Melting curve plots were generated and analysed using CFX Manager Software v3.0.1 (BIO-RAD) to determine the average melting temperature for each positive sample in reference to control DNA, and thus suggest the species of positive samples. Data from questionnaires was entered into spreadsheets and Epi Info™ 7 statistical software. Demographic characteristics and knowledge, attitudes and practices were described using frequency counts and proportions. The association between household-level *Leptospira* status (outcome) and KAP questionnaire responses (9 explanatory variables) was explored using univariable logistic regression analysis, using Epi Info™ 7 statistical software. A p-value < 0.05 was considered as statistically significant. Given the small number of positive *Leptospira* detections and non-significant findings in univariable models further multivariable analysis was not pursued.

Ethical considerations

Ethical approval was obtained from the Department Research Ethical Review Committee (DRERC) of the Department of Microbiology, Immunology and Parasitology, College of Health Sciences of Addis Ababa University. Permission to conduct the study was also provided by the Addis Ababa City Administration Health Bureau and the Addis Ababa City Livestock and Agriculture Bureau. Permission for transport of DNA extracts to the Netherlands was obtained in 2020 from the Addis Ababa University, College of Health Sciences, Institutional Review Board (CHS/RTTD/257/2020).

Household respondents were asked to provide oral consent after they were informed (in the local language) about the purpose of the study, voluntary participation, right to withdraw at any time and that the data obtained would be treated as confidential.

RESULTS

Study population

Of the 76 households contacted, 70 households agreed to participate in the study. Reasons for non-participation were: unwillingness to provide urine samples (n=4) or cattle being absent at the time of visit (n=2). Demographic characteristics of respondents and household characteristics are shown in Table 1. The median household size of the studied households was 6 persons (ranging from 1-17 persons), with a mean of 6.6 persons ± 3.3 standard deviation (SD). Most of the respondents were either primarily farmers (40.0%) or unemployed (35.7%), while almost one-fourth (24.3%) were involved in private or governmental work. All households had access to piped water for drinking purpose, food preparation, hand washing, and cleaning. The majority of households faced frequent shortages of water; only a few (14.3%) households reported never or rarely having interruptions to their water supply. Of the 70 studied households, 60.0% also owned other domestic animals such as sheep, goats or horses. The number of cattle owned by households ranged from 1 to 60.

Prevalence of leptospirosis in cattle

Urine samples were collected from 168 cattle across the

Table 1. Demographic characteristics of the investigated peri-urban households in Addis Ababa and their respondents (N=70), 2019.

Demographic characteristics	Category	Number	Percentage
Gender	Male	42	60.0
	Female	28	40.0
Age	Below 30 years old	15	21.4
	30-60 years old	37	52.9
	More than 60 years old	18	25.7
Marital status	Married	43	61.4
	Single	16	22.9
	Divorced/Widowed	11	15.7
Education	No formal schooling	14	20.0
	Elementary school	29	41.4
	High school	14	20.0
	College	8	11.4
	University	5	7.2
Occupation	Primarily farmer	28	40.0
	Unemployed / Retired / Student	25	35.7
	Private / Government job	17	24.3
Animal husbandry	Only cattle	28	40.0
	Two or more livestock species	42	60.0
	Sheep	22	31.4
	Goat	8	11.4
	Equine	22	31.4
	Pigs	0	0
Available facilities	Electricity	70	100
	Telephone	69	98.6
	Radio or Television	69	98.6
	Piped water	70	100
	Toilet		
	Pit latrine with cement slab	54	77.1
	Pit latrine without cement slab	7	10.0
	Flush	3	4.3
	No toilet	6	8.6

70 households. Of these, three were positive for pathogenic *Leptospira* by real-time PCR (1.8%) (Figure 1). The three positive samples were all found in Yeka sub-city. Two positive samples came from different cattle within the same household, but the samples were taken on a different day. The positive samples were considered to be *L. borgpetersenii*, based on the melting curve, when compared with the positive controls (Figure 2), although it is difficult to distinguish species solely on the basis of the *lfb1* gene amplification product (Bourhy et al., 2011).

Knowledge, attitudes and practices

The majority (97.1%) of respondents had never heard about leptospirosis. Nevertheless, 85.9% of the households knew that animals could be a source of human infection and were able to mention how: by direct contact with animals, eating raw milk and meat, touching urine or birth products of cattle, or by rats. More than 60% of the households knew that urine from cattle could contain pathogens which can affect humans. Similarly,

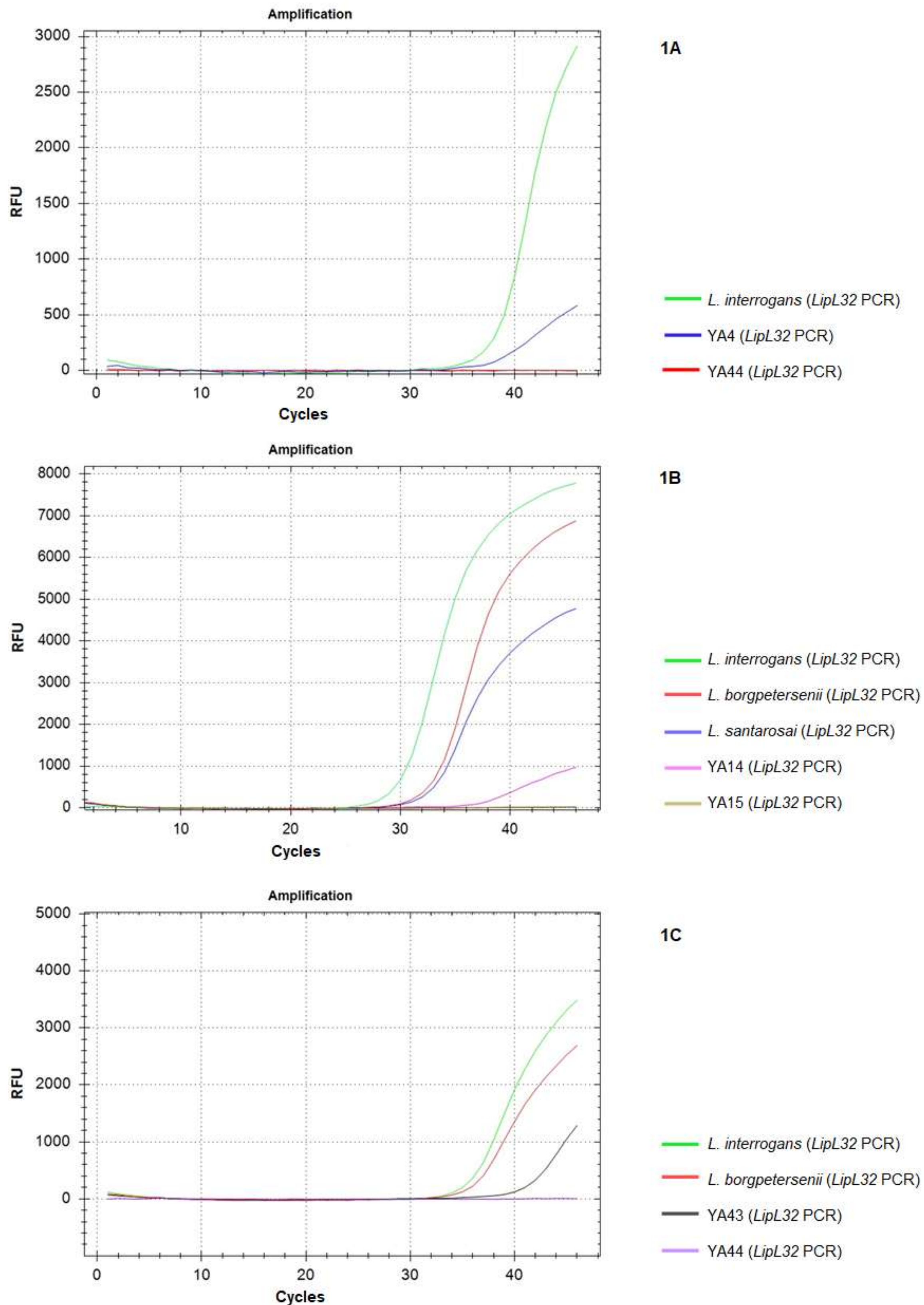

Figure 1. Leptospiral DNA detected in urine from 3 cows in peri-urban areas of Addis Ababa, Ethiopia, after being subjected to the lipL32 PCR reaction with TaqMan probe. Hydrolysis of the lipL32 specific TaqMan probe confirms the presence of *Leptospira* species in sample YA4 (1A), YA14 (1B) and YA43 (1C). RFU = Relative Fluorescence Unit.

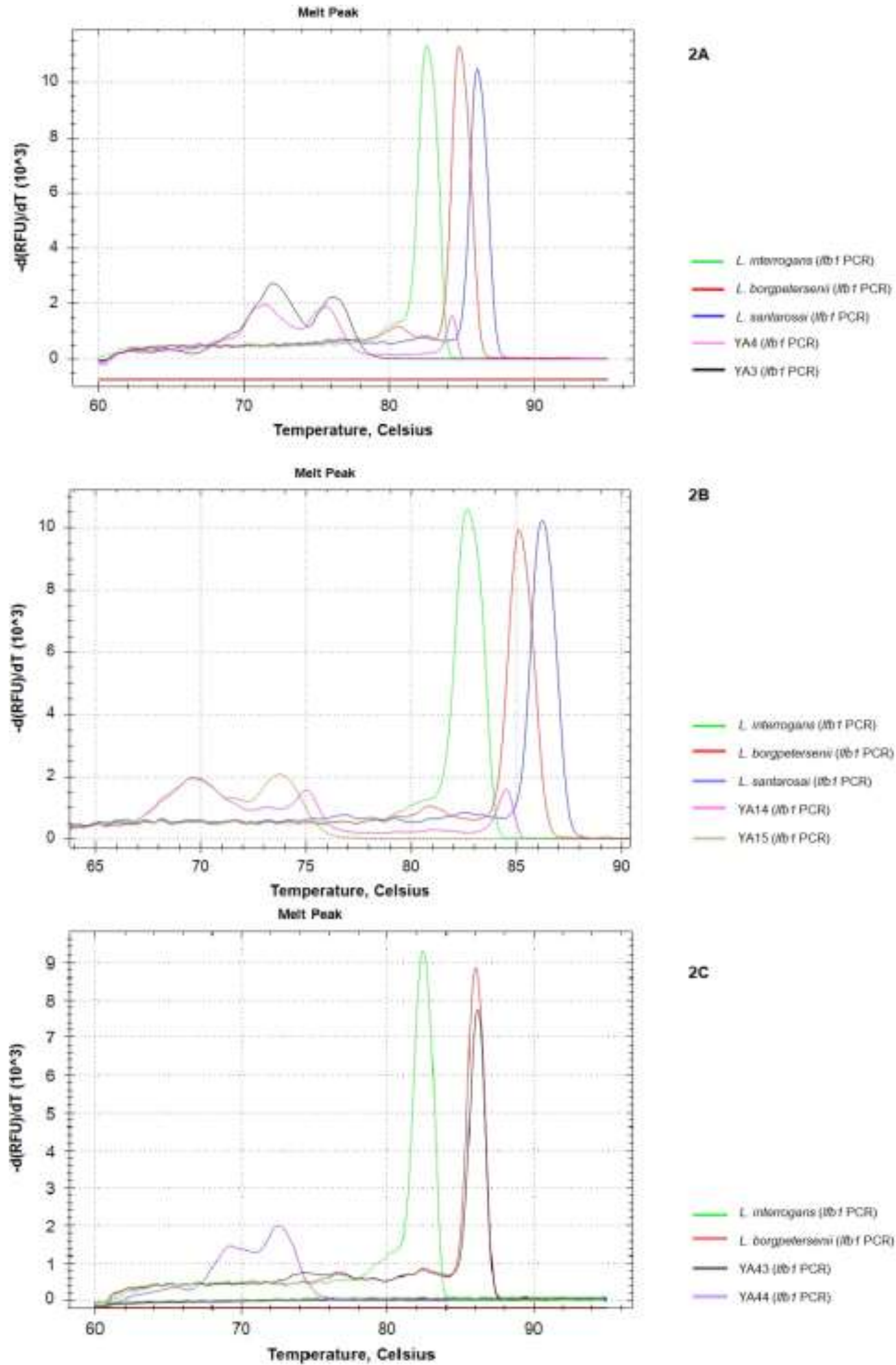

Figure 2. Melting curve analysis of DNA detected in urine from 3 cows sampled in peri-urban areas of Addis Ababa, Ethiopia, after being subjected to the previously described PCR reaction, using lfb1 primers in the presence of Evagreen. The melting curves of the *Leptospira interrogans*, *Leptospira borgpetersenii* and *Leptospira santarosai* positive control DNA samples are shown. The positive urine DNA samples YA4 (2A), YA14 (2B) and YA43 (2C) have a similar melting temperature with the *Leptospira borgpetersenii* control DNA sample. The fact that the melting peaks of urine DNA sample YA4 (2A) and YA14 (2B) are lower than the *Leptospira borgpetersenii* control DNA sample can be explained by the lower concentration of *Leptospira* in sample YA4 and YA14 than in the positive DNA control sample. RFU = Relative Fluorescence Unit.

Table 2. Univariate logistic regression analysis of explanatory variables for *Leptospira* positivity in peri-urban households of Addis Ababa, 2019.

Explanatory variable	N = 70 households		p-value
	Positive (%)	Negative (%)	
Knew that animals can be source of diseases	2 (100)	53 (77.9)	0.998
Knew about diseases transmitted by rats' urine	1 (50)	42 (61.8)	0.718
Knew about diseases transmitted by cattle's urine	0 (0)	42 (61.8)	0.998
Wet areas around the house	1 (50)	43 (63.2)	0.701
Walking through the water with animals	0 (0)	16 (23.5)	0.998
Two or more livestock species	2 (100)	40 (58.8)	0.998
Rats inside the house	1 (50)	46 (67.6)	0.586
Using rat traps or poison	0 (0)	30 (44.1)	0.998
Protective measures after dealing with diseased animals	2 (100)	55 (80.9)	0.998
Washing hands after dealing with animal excretions	1 (50)	44 (64.7)	0.535

rat's urine as a source of infection for humans was recognized by 62.3%. Nearly 70% of households reported that they frequently saw rats inside their houses and rat poison/traps were used by almost half (43.5%) of the households. Because leptospirosis is known to be transmitted by standing water contaminated by urine from domestic animals and rats, households were also asked about wet areas around their houses. More than half (61.4%) of the households stated that they had areas around the house that were wet, with more households (76.8%) reporting this during the rainy season. More than half (52.2%) of the respondents walked frequently in the wet areas around the house without shoes or with open-toed shoes. Walking with the animals through water happened in almost a quarter (23.2%) of the households. Water as a potential source of diseases was recognized by 82.9% of the households. Households were asked for symptoms that could be attributed to leptospirosis: 26.9% had seen fever in one of the household members during the last month, while kidney diseases, jaundice and bleedings were seen less often (9.0, 1.5 and 4.4%, respectively).

Finally, participants were asked how they managed sick animals. Animal health issues were resolved with veterinary assistance (92.4%) and/or by the households themselves (37.7%). Washing hands after dealing with animal excretions was practiced by around two third of the households (70.3%). Protective measures such as gloves or hand washing after dealing with diseased animals were reportedly practiced by 89.1% of the households.

Household-level risk factors for leptospirosis

Table 2 summarizes variables included in the univariable analysis. None of the 10 selected explanatory variables were significantly associated with cattle *Leptospira* positivity (p-value > 0.05).

DISCUSSION

This study is the first of its kind to detect pathogenic *Leptospira* by molecular methods in cattle in Ethiopia. The study confirms the presence of pathogenic *Leptospira* in peri-urban areas of Addis Ababa. Considering the large cattle population and high human-animal interaction in Ethiopia, this study provides important information on a potential threat for public health, as presence of pathogenic *Leptospira* in urine implies spread into the environment. Environmental contamination and exposure to animal excretions is the cause of leptospirosis infections in both animals and humans (WHO, 2003; Hartskeerl et al., 2011). The presumable identification of *L. borgpetersenii* is consistent with *Leptospira* species identified in cattle in Africa (Allan et al., 2015; Dreyfus et al., 2017). This study also confirms that a molecular assay targeting the *lipL32* and *lfb1* gene can be used to detect the presence of *Leptospira* in the urine of cattle.

The prevalence of leptospirosis in cattle in this study was 1.8% (3 positive samples out of 168 cattle tested). Comparing this study with previously published studies on *Leptospira* in Ethiopia is difficult because these studies used serological tests (Tsegay et al., 2016; Desa et al., 2021, Marami et al., 2021). The present study detected *Leptospira* DNA directly which reflects current or recent infection rather than cumulative exposure. Cattle may shed *Leptospira* intermittently in urine for up to 18 weeks (Rocha et al., 2017; Hamond et al., 2022) and thus PCR of urine is considered a useful, non-invasive diagnostic modality, particularly when understanding environmental contamination is of interest. One of the largest recent studies on urinary shedding of pathogenic *Leptospira* in cattle was done in New Zealand, where the urine of 4000 cattle was tested by real-time PCR and found a prevalence (2.4%) similar to ours, although majority of their cattle population was vaccinated and both environmental and cattle characteristics differ from the Ethiopian or African context (Yupiana et al., 2020).

The few published studies using molecular assays in cattle in African countries have shown similar or slightly higher prevalences than ours. In Egypt, leptospiral DNA was detected in 1.1% of 625 cows (urine or blood) (Samir et al., 2015). In Eastern Africa, *Leptospira* prevalences of 8.8% (kidney and/or urine) and 5.8% (only urine) were observed in Uganda (Alinaitwe et al., 2019) and 7.1% (kidney tissue) in northern Tanzania (Allan et al., 2018). A substantial higher prevalence was detected in South Africa, where *Leptospira* DNA was detected in kidney tissue samples of 26.9% (slaughtered) cattle (Dogonyaro et al., 2023), which could be related to the testing of kidney tissue instead of urine and to environmental factors.

Risk factors for leptospirosis in animals are not well characterized in Africa, although some common risk factors have been described. This includes exposure to rats, presence of other (reservoir) animals, pasture grazing and walking through rivers (Schoonman and Swai, 2010; Ngugi et al., 2019; Desa et al., 2021). These risk factors were also present in our study area. Studies concerning knowledge, attitudes and practices of people regarding leptospirosis have mainly been performed in South-America and Asia in areas where leptospirosis is known to be endemic and peoples' awareness might be higher than in Africa (De Araújo et al., 2013; Ricardo et al., 2018; Palma et al., 2022). Despite this, it is remarkable that majority of the respondents in our study area had not heard about the disease leptospirosis. However, people in a majority of the studied households were aware that contact with environmental water, rat's urine or cattle urine and excretions could transmit diseases. The majority of the households took protective measures, like using gloves, washing hands and asking for veterinary assistance. The presence of some knowledge regarding transmission of diseases and the presence of many risk factors reflects the gap between knowledge and daily practice concerning leptospirosis. This is consistent with previous reports from Ethiopia on leptospirosis and zoonotic diseases in general (Desa et al., 2021; Alemayehu et al., 2021). No significant relationship was found between the positive households and the investigated risk factors for zoonotic diseases and leptospirosis, although many of the commonly recognized risk factors were present in the households. The absence of statistical significance does not imply that non-significant potential factors pose no risk as the low prevalence observed in this study resulted in low statistical power for the logistic regression analysis.

Strengths and limitations of the study

This study is the first to investigate pathogenic *Leptospira* in peri-urban areas of the capital city of Ethiopia, where there is little knowledge about animal reservoirs of pathogenic *Leptospira* spp. The study responds to previous calls to investigate the presence of leptospirosis and pathogenic *Leptospira* in Ethiopia (Pieracci et al., 2016; Tulu, 2020). Even though the estimated prevalence was low (1.8%), this study has implications for public health given the zoonotic potential of pathogenic *Leptospira*. The detection of pathogenic *Leptospira* by a molecular assay is also the first of its kind in Ethiopia for detection of *Leptospira* in any species. In cattle, PCR on urine samples is more useful than serological tests, given that many shedders and carriers will not be detected through serology (Monti et al., 2023). This study confirms 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*. The DNA isolation method was tested by the investigators prior to this study with urine samples of known leptospirosis patients, which revealed positive PCR-results with both the lfb1 and lipL32 assay. Even at relatively low bacterial load levels (Ct values > 32), a distinct leptospiral specific PCR product with a melting peak of > 80°C was observed. Additionally, this study provided insights into the lack of awareness of leptospirosis in Ethiopian cattle-keeping households and presence of potential exposure pathways in peri-urban areas of Addis Ababa. These findings indicate that animal and human exposure to pathogenic *Leptospira* species is likely in peri-urban areas of Addis Ababa.

Nevertheless, there are several limitations which should be mentioned. The prevalence estimated in this study may underestimate the true prevalence given the low urine volume examined which may have affected the quantity of DNA and the fact that every animal was only sampled once, potentially missing intermittent shedding (Monti et al., 2023). Additionally, although the DNA isolation method was tested by the main investigators prior to the study on human urine samples, bovine urine samples were not available to test the effectiveness of our DNA extraction method. Furthermore, serological tests like microscopic agglutination test (MAT) would have provided additional information on *Leptospira* exposure of cattle in Addis Ababa and culture techniques would have been useful for providing further information on the leptospiral serovar. The use of more than one type of assay to detect *Leptospira* in urine from naturally infected cattle could have revealed a higher prevalence (Nally et al., 2020). Differentiation based on melting temperature (Tm) allows to differentiate between the most common pathogenic species, but is not able to unambiguously differentiate between *L. borgpetersenii* and other pathogenic species (Bourhy et al., 2011). Further proof of identification would require isolation of the bacteria and characterization by sequencing of the 16S ribosomal RNA gene.

Conclusion

Overall, this study confirmed the presence of pathogenic

Leptospira in cattle in peri-urban areas of Addis Ababa. Analysis of knowledge, attitude and practice among the households revealed that knowledge about leptospirosis is low and that exposure pathways for leptospirosis are widely present.

Further studies should investigate the presence of pathogenic *Leptospira* in cattle on a wider scale, as Ethiopia has the highest cattle population in Africa and cattle is the dominant species in most households. The presence of pathogenic *Leptospira* in cattle's urine indicates contamination of environment and potential exposure of humans. Further studies should therefore take other components of the "One Health" approach into consideration to understand the human and environmental burden in Ethiopia. This study highlights the need to educate cattle-keeping households and responsible veterinary and health professionals about the presence of pathogenic Leptospira.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank all farmers, animal health assistants, veterinary medicine students, and veterinarians from the different sub-cities for their incredible help and support in data collection and logistics. They also thank Jan Schouten from MRC-Holland for his support during this study. Marga G. A. Goris (in memoriam) and Ahmed A. A. Ahmed from the AMC, Department of Medical Microbiology and Infection Prevention, Expertise Centre for Reference and Research on Leptospirosis/OIE Reference Laboratory for Leptospirosis in The Netherlands have contributed in an invaluable way by providing control samples, expertise and advices.

REFERENCES

Alemayehu G, Mamo G, Desta H, Alemu B, Wieland B (2021). Knowledge, attitude and practices to zoonotic disease risks from livestock birth products among smallholder communities in Ethiopia. One Health 12:100223.

Alinaitwe L, Kankya C, Allan KJ, Rodriguez-Campos S, Torgerson P, Dreyfus A (2019). Bovine leptospirosis in abattoirs in Uganda: molecular detection and risk of exposure among workers. Zoonoses Public Health 66(6):636-646.

Allan KJ, Biggs HM, Halliday JEB, Kazwala RR, Maro VP, Cleaveland, Crump JA (2015). Epidemiology of Leptospirosis in Africa: A systematic review of a neglected zoonosis and a paradigm for 'One Health' in Africa. PlOS Neglected Tropical Diseases 9(9):e0003899.

Allan KJ, Halliday JEB, Moseley M, Carter RW, Ahmed A, Goris MGA, Hartskeerl RA, Keyyu J, Kibona T, Maro VP, Maze MJ, Mmbaga BT, Tarimo R, Crump JA, Cleaveland S (2018). Assessment of animal hosts of pathogenic *Leptospira* in northern Tanzania. PLOS Neglected Tropical Diseases 12(6):e0006444.

Barragan V, Nieto N, Keim P, Pearson T (2017). Meta-analysis to estimate the load of *Leptospira* excreted in urine: beyond rats as important sources of transmission in low-income rural communities. BMC Research Notes 10:71.

Bourhy P, Bremont S, Zinini F, Giry C, Picardeau M (2011). Comparison of Real-Time PCR assays for detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. Journal of Clinical Microbiology 49(6):2154–2160.

Costa F, Hagan JE, Calcagno J, Kane M, Torgerson PR, Matinez-Silveira MS, Stein C, Abela-Ridder B, Ko AI (2015). Global morbidity and mortality of leptospirosis: A systematic review. PLOS Neglected Tropical Diseases 17;9(9):e0003898.

De Araújo WN, Finkmoore B, Ribeiro GS, Reis RB, Felzemburgh RD, Hagan JE, Reis MG, Ko AI, Costa F (2013). Knowledge, attitudes, and practices related to leptospirosis among urban slum residents in Brazil. American Journal of Tropical Medicine and Hygiene 88(2):359-363.

Desa G, Deneke Y, Begna F, Tolosa T (2021). Seroprevalence and associated risk factors of *Leptospira interrogans* serogroup *Sejroe* serovar *Hardjo* in dairy farms in and around Jimma town, Southwestern Ethiopia. Veterinary Medicine International 6061685.

Di Azevedo MIN, Lilenbaum W (2021). An overview on the molecular diagnosis of animal leptospirosis. Letters in Applied Microbiology 72(5):496-508.

Dogonyaro BB, van Heerden H, Potts AD, Fasina FO, Casanovas-Massana A, Kolo FB, Lötter C, Byaruhanga C, Ko AI, Wunder EA Jr, Adesiyun AA (2023). Molecular characterization of *Leptospira* species detected in the kidneys of slaughtered livestock in abattoirs in Gauteng Province, South Africa. Pathogens 12(5):666.

Dreyfus A, Odoch T, Alinaitwe L, Rodriguez-Campos S, Tsegay A, Jaquier V (2017). Cross-sectional serological survey for *Leptospira* spp. in beef and dairy cattle in two districts in Uganda. International Journal of Environmental Research and Public Health 14(11):1421.

Ellis WA (2015). Animal leptospirosis. Current Topics in Microbiology and Immunology 387:99-137.

Esteves LM, Bulhões SM, Branco CC, Carreira R, Vieira ML, Gomes-Solecki M, Mota-Vieura L (2018). Diagnosis of human leptospirosis in a clinical setting: Real-Time PCR high resolution melting analysis for detection of *Leptospira* at the onset of disease. Scientific Reports 8(1):9213.

Evangelista KV, Coburn J (2010). Leptospira as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. Future Microbiology 5(9):1413-1425.

FAO (2020). The future of livestock in Ethiopia. Emerging public health risks in urban and peri-urban areas. Rome, Italy: Food and Agriculture Organization of the United Nations.

Grace D, Mutua F, Ochongu P, Kruska R, Jones K, Brierley L, Lapar L, Said M, Herrero M, Phuc PM, Thao NB, Akuku I, Ogutu F (2012). Mapping of poverty and likely zoonoses hotspots. Zoonoses Project 4. Report to the UK Department for International Development. Nairobi, Kenya: International Livestock Research Institute (ILRI).

Hairgrove TB (2004). Leptospirosis in cattle. The AABP Proceedings 37:36-39.

Hamond C, LeCount K, Putz EJ, Bayles DO, Camp P, Goris MGA, van der Linden H, Stone NE, Schlater LK, Sahl JW, Wagner DM, Nally JE (2022). Bovine leptospirosis due to persistent renal carriage of *Leptospira borgpetersenii* serovar Tarassovi. Frontiers in Veterinary Science 9:848664.

Hartskeerl RA, Collares-Pereira M, Ellis WA (2011). Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clinical Microbiology and Infection 17(4):494-501.

Karpagam KB, Ganesh B (2020). Leptospirosis: a neglected tropical zoonotic infection of public health importance-an updated review. European Journal of Clinical Microbiology and Infectious Diseases 39(5):835-846.

Levett PN (2001). Leptospirosis. Clinical Microbiology Reviews 14(2):296-326.

Lilenbaum W, Martins G (2014). Leptospirosis in Cattle: A challenging scenario for the understanding of the epidemiology. Transboundary and Emerging Diseases 61:63-68.

Lucchesi PM, Arroyo GH, Etcheverria AI, Parma AE, Seijo AC (2004). Recommendations for the detection of Leptospira in urine by PCR. Revista de Sociedade Brasileira de Medicina Tropical 37(2):131-134.

Management Entity (2021). Ethiopia's Livestock Systems: Overview and

Areas of Inquiry. Gainesville, USA: Feed the Future Innovation Lab for Livestock Systems.
Marami LM, Gebremedhin EZ, Sarab EJ, Tola GK, Endalew SS, Melkamsew AT, Marco Lo Presti, Vitale M (2021). Seroprevalence and associated risk factors of canine *Leptospira* and *Brucella* species infection in West Shewa Zone, Central Ethiopia. Veterinary Medicine (Auckland, N.Z.) 12:33-42.
Moch RW, Ebner EE, Barsoum LS, Botros BA (1975). Leptospirosis in Ethiopia: a serological survey in domestic and wild animals. The Journal of Tropical Medicine and Hygiene 78:38-42.
Monti G, Montes V, Tortosa P, Tejeda C, Salgado M (2023). Urine shedding patterns of pathogenic *Leptospira* spp. in dairy cows. Veterinary Research 54(1):64.
Musso D, La Scola B (2013). Laboratory diagnosis of leptospirosis: A challenge. Journal of Microbiology, Immunology and Infection 46(4):245-252.
Nally JE, Ahmed AAA, Putz EJ, Palmquist DE, Goris MGA (2020). Comparison of real-time PCR, bacteriologic culture and Fluorescent Antibody Test for the detection of Leptospira borgpetersenii in urine of naturally infected cattle. Veterinary Sciences 7(2):66.
Ngugi JN, Fèvre EM, Mgode GF, Obonyo M, Mhamphi GG, Otieno CA, Cook EAJ (2019). Seroprevalence and associated risk factors of leptospirosis in slaughter pigs; a neglected public health risk, western Kenya. BMC Veterinary Research 15(1):403.
Palma FAG, Costa F, Lustosa R, Mogaji HO, De Oliveira DS, Souza FN, Reis MG, Ko AI, Begon M, Khalil H (2022). Why is leptospirosis hard to avoid for the impoverished? Deconstructing leptospirosis transmission risk and the drivers of knowledge, attitudes, and practices in a disadvantaged community in Salvador, Brazil. PLOS Global Public Health 2(12):e0000408.
Picardeau M (2017). Virulence of the zoonotic agent of leptospirosis: still terra incognita? Nature Reviews Microbiology 15(5):297-307.
Pieracci EG, Hall AJ, Gharpure R, Haile A, Walelign E, Deressa A, Bahiru G, Kibebe M, Walke H, Belay E (2016). Prioritizing zoonotic diseases in Ethiopia using a one health approach. One Health 2:131-135.
Ricardo T, Bergero LC, Bulgarella EP, Previtali MA (2018). Knowledge, attitudes and practices (KAP) regarding leptospirosis among residents of riverside settlements of Santa Fe, Argentina. PLOS Neglected Tropical Diseases 12(5):e0006470.
Rocha BR, Narduche L, Oliveira CS, Martins G, Lilenbaum W (2017). Molecular demonstration of intermittent shedding of *Leptospira* in cattle and sheep and its implications on control. Ciencia Rural 47(8):e20170088.
Qiagen (2020). QIAamp® Viral RNA Mini Handbook. Seventh Edition.
Samir A, Soliman R, El-Hariri M, Abdel-Moein K, Hatem ME (2015). Leptospirosis in animals and human contacts in Egypt: broad range surveillance. Revista de Sociedade Brasileira Medicina Tropical 48(3):272-277.
Schoonman L, Swai ES (2010). Herd- and animal-level risk factors for bovine leptospirosis in Tanga region of Tanzania. Tropical Animal Health and Production 42(7):1565-1572.
Shapiro BI, Gebru G, Desta S, Negassa A, Negussie K, Aboset G, Mechal H (2015). Ethiopia livestock master plan: Roadmaps for growth and transformation. Kenya, Nairobi: International Livestock Research Institute (ILRI).
Shapiro BI, Gebru G, Desta S, Negassa A, Nigussie K, Aboset G, Mechal H (2017). Ethiopia livestock sector analysis. ILRI Project Report. Kenya, Nairobi: International Livestock Research Institute (ILRI).
Thrusfield M (2005). Veterinary Epidemiology. 3rd edition. London: Blackwell Science Ltd. pp. 227-247.
Tsegay K, Potts AD, Aklilu N, Lötter C, Gummow B (2016). Circulating serovars of *Leptospira* in cart horses of central and southern Ethiopia and associated risk factors. Preventive Veterinary Medicine 125:106-15.
Tulu D (2020). Epidemiology and zoonotic implication of leptospirosis in domestic animals in Ethiopia. Academic Journal of Animal Diseases 9(1):19-32.
Vijayachari P, Sugunan AP, Umapathi,T, Sehgal SC (2001). Evaluation of darkground microscopy as a rapid diagnostic procedure in leptospirosis. Indian Journal of Medical Research 114:54-58.
Vincent AT, Schiettekatte O, Goarant C, Neela VK, Bernet E, Thibeaux R, Ismail N, Khalid MKNM, Amran F, Masuzawa T,Nakao R, Korba AA, Bourhy P, Veyrier FJ, Picardeau M (2019). Revisiting the taxonomy and evolution of pathogenicity of the genus Leptospira through the prism of genomics. PLOS Neglected Tropical Diseases 13(5):e0007270.
De Vries SG, Visser BJ, Nagel M, Goris MGA, Hartskeerl RA, Grobusch MP (2014). Leptospirosis in Sub-Saharan Africa: A Systematic Review. International Journal of Infectious Diseases 28:47-64.
World Health Organization (WHO) (2003). Human Leptospirosis: Guidance for Diagnosis, Surveillance and Control. Geneva, Switzerland: World Health Organization.
WHO. Leptospirosis (2009). Fact sheet. WHO Regional Office for South-East Asia: World Health Organization.
Yadeta W, G. Michael B, Abdela N (2016). Leptospirosis in animal and its public health implications: a review. World Applied Sciences Journal 34(6):845-853.
Yimer E, Koopman S, Messele T, Wolday D, Newayeselassie B, Gessese N, Degefe B, Sabders EJ (2004). Human leptospirosis, in Ethiopia: a pilot study in Wonji Hospital. Ethiopian Journal of Health Development 18:48-51.
Yupiana Y, Vallée E, Wilson P, Weston JF, Benschop J, Collins-Emerson J, Heuer C (2020). On-farm risk factors associated with Leptospira shedding in New Zealand dairy cattle. Epidemiology and Infection 148:e219.

SUPPORTING INFORMATION
S1 Appendix. Questionnaire

QUESTIONNAIRE - Assessment of Knowledge, Attitudes and Practices

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 ð Male head of household ð Other adult (>18)

Questions related to demographic characteristics of the household

⌋ Male ð Female

1. Sex:

2. Marital status:

⌋ Single ð Married ð 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. What is your occupation?

⌋ Employed: A) Government B) Private C) NGO ⌋ Builder/carpenter
⌋ Farmer ⌋ Unemployed/retired/housewife

7. Does your household have:

⌋ Electricity ⌋ Television / Radio
⌋ Refrigerator ⌋ Telephone/Mobile phone

8. 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)

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

	Piped water	Surface water	Ground/b ore-hole	Rain water	River water	Bottled water
Drinking water for household						
Drinking water for animals						
Water for food preparation						
Water for cleaning house and utensils						
Water for hand washing and laundry						

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

Yes: (please specify when) No

11. Which of the following animal species do you have?

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

Questions related to the knowledge, attitude and practices of households regarding leptospirosis, risk factors for leptospirosis and diseases in general

12. o you think that animals can be a source of human diseases?
Yes No

If yes, how can humans get a disease from animals?

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

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

⌋ Yes ð No

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

⌋ Yes ð No

15. Have you heard of diseases transmitted by urine of cattle to humans?

⌋ Yes ð No

16. Did you hear about a disease called leptospirosis?

⌋ Yes ð No

If yes, how did you hear about it? ……………………………….

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

Seek veterinary assistance | Slaughter the animal

| Treat the animal self | Do nothing

| Sell the animal | Others (please, specify)

18. 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)

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

Yes No

20. The following symptoms can be found when someone has leptospirosis, which you can get from contact with water or urine of cattle or rats. 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 diseases			
Jaundice			
Bleeding			

21. Indicate if you agree with the following statements:

	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 or my family walks through the water with the animals			
Rats come inside the house			
I use rat traps or rat poison around my house			

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.

S2 Appendix. Real-time PCR using *Leptospira* specific lipL32 and lfb1 PCR detection assays

Real-time PCR using *Leptospira* specific lipL32 and lfb1 PCR detection assays

Developed by MRC-Holland and AMC, Department of Medical Microbiology and Infectio n Prevention, Expertise Centre for Reference and Research on Leptospirosis / OIE Reference Laboratory for Leptospirosis in the Netherlands.

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 species identification.
2. LipL32 PCR detection involving a TaqMan probe hydrolysis assay that specificall y

detects the Real-Time formation of a lipL32 PCR product. No species 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, reverse primers and probes:

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 Expertise Centre for Reference and Research on Leptospirosis / OIE Reference Laboratory for Leptospirosis in the Netherlands.

	µl
10x SALSA PCR buffer	2.5
LFB1-F (100 µM)	0.1
LFB1-R (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 PCR Reaction with TAQ-Man probe and degenerate primers

	µl
10x biolabs buffer	2.5
LipL32-47Fd (100 µM)	0.175
LipL32-301Rd (100 µM)	0.175
LipL32 Probe (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 3:00 min

Step 7 40°C for 0:05 min

Step 8 95°C = END