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Leptospirosis in northern Tanzania: investigating the role of rodents and ruminant livestock in a neglected public health problem

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Abstract

Leptospirosis is an important but neglected zoonotic disease that is often overlooked in Africa. Although comprehensive data on the incidence of human disease are lacking, robust evidence of infection has been demonstrated in people and animals from all regions of the continent. However, to date, there are few examples of direct epidemiological linkages between human disease and animal infection. In East Africa, awareness of the importance of human leptospirosis as a cause of non-malarial febrile illness is growing. In northern Tanzania, acute leptospirosis has been diagnosed in 9% of patients with severe febrile illness compared to only 2% with malaria. However, little is known about the relative importance of different potential animal hosts as sources of human infection in this area. This project was established to investigate the roles of rodents and ruminant livestock, important hosts of *Leptospira* in other settings, in the epidemiology of leptospirosis in northern Tanzania. A cross-sectional survey of rodents living in and around human settlements was performed alongside an abattoir survey of ruminant livestock. Unusual patterns of animal infection were detected by real-time PCR detection. Renal *Leptospira* infection was absent from rodents but was detected in cattle from several geographic areas. Infection was demonstrated for the first time in small ruminants sub-Saharan Africa. Two major *Leptospira* species and a novel *Leptospira* genotype were detected in livestock. *L. borgpetersenii* was seen only in cattle but *L. kirschneri* infection was detected in multiple livestock species (cattle, sheep and goats), suggesting that at least two distinct patterns of *Leptospira* infection occur in livestock in northern Tanzania. Analysis of samples from acute leptospirosis in febrile human patients could not detect *Leptospira* DNA by real-time PCR but identified social and behavioural factors that may limit the utility of acute-phase diagnostic tests in this community. Analysis of serological data revealed considerable overlap between serogroups detected in cattle and human leptospirosis cases. Human disease was most commonly attributed to the serogroups Mini and Australis, which were also predominant reactive serogroups in cattle. Collectively, the results of this study led to the hypothesis that livestock are an important reservoir of *Leptospira* infection for people in northern Tanzania. These results also challenge our understanding of the relationship between *Leptospira* and common invasive rodent species, which do not appear to maintain infection in this setting. Livestock *Leptospira* infection has substantial potential to affect the well-being of people in East Africa, through direct transmission of infection or through indirect effects on food production and economic security. Further research is needed to quantify the impact of livestock leptospirosis in Africa and to develop effective interventions for the control of human and animal disease.

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Author’s declaration

I declare that this thesis is entirely my own composition and that the research described in this thesis is also my own except where otherwise stated. Some areas of diagnostic testing and analysis were performed in close partnership with study collaborators. Where others have performed or contributed to the work, this has been clearly acknowledged in the text.

Finally, I declare that the work presented in this thesis has not been submitted for any other degree or professional qualification.

Abbreviations

AE	Qiagen® elution buffer
AL	Qiagen® lysis buffer
ATL	Qiagen® tissue lysis buffer
Bp	Base-pair
CI	Confidence interval
COSTECH	Commission for Science and Technology, Tanzania
Ct	Threshold value for qPCR
DALY	Disability-Adjusted Life Year
DF	Degrees of freedom
DPO	Days post onset
DRC	Democratic Republic of Congo
dsDNA	Double stranded DNA
ELISA	Enzyme-linked immunosorbent assay
EMJH-5FU	Ellinghausen-McCullough-Johnson-Harris culture medium with 5'fluorouracil
EPI	Expanded Immunisation Programme (World Health Organisation)
FAO	Food and Agriculture Organisation
gDNA	Genomic DNA
GE	Genomic equivalent
GPS	Global positioning system
HH	Household
HIV	Human immunodeficiency virus
HuLCL	Human lymphoma cell line
KCMC	Kilimanjaro Christian Medical Centre, Moshi
KIT	Royal Tropical Institute, Amsterdam, The Netherlands
LERG	Leptospirosis Epidemiology Reference Group, WHO
LLOD	Lower Limit of Detection
LRT	Likelihood Ratio Test
MAT	Microscopic agglutination test
mAbs	Monoclonal antibodies
MRH	Mawenzi Regional Hospital
MLST	Multilocus Sequence Typing
NAA	Nucleic acid amplification

NIMR	National Institute of Medical Research, Tanzania
NMFI	Non-malarial febrile illness
NTD	Neglected Tropical Diseases
OIE	World Organisation for Animal Health
PCR	Polymerase Chain reaction
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analyses
qPCR	Real-time polymerase chain reaction
RPM	Revolutions per minute
RT	Room temperature
SPP.	Species (plural)
SV	Serovar
TAWIRI	Tanzania Wildlife Research Institute
TE	Tris-EDTA buffer
US CDC	United States Centers for Disease Control
WHO	World Health Organisation

1 Leptospirosis in Tanzania: an introduction to a neglected and complicated disease

1.1 The global impact of leptospirosis

Leptospirosis is often described as the most common and pervasive zoonotic bacterial disease around the world, but it remains overlooked in global infectious disease priorities, (Abela-Ridder et al., 2010). Recent work however has estimated that approximately one million cases of leptospirosis occur each year (Costa et al., 2015a) with a resulting loss around 2.9 million Disability-Adjusted Life Years (DALYs) per annum (Torgerson et al., 2015). Compared to the burden of other neglected tropical diseases, leptospirosis has a similar impact to several diseases included World Health Organisation's list of priority neglected tropical diseases but yet remains relatively under-resourced (Table 1.1) (Murray et al., 2012, Hotez et al., 2007, Hotez, 2009).

Table 1.1: The global burden of leptospirosis measured in Disability Adjusted Life Years (DALYs) per 100,000 population compared to Global Burden of Disease (GBD) estimates for other tropical diseases (Torgerson et al., 2015, Murray et al., 2012)

Disease	DALYs per annum	Confidence Interval
Malaria	1200	921 - 1594
Rabies	52	22 - 145
Leishmaniasis	48	32-71
Schistosomiasis	48	25-91
Leptospirosis	42	18-66
Lymphatic filariasis	40	26-58
Dengue fever	12	15-20

Approximately three-quarters of leptospirosis cases occur in tropical or sub-tropical regions of the world (Costa et al., 2015a, Torgerson et al., 2015). In Southeast Asia, the importance of leptospirosis as a cause of febrile illness is well recognised. *Leptospira* is a leading aetiology of NMFI in the Greater Mekong region (Cambodia, Lao PDR, Viet Nam, Thailand and Yunnan province of China) (Acestor et al., 2012) and a major cause of undifferentiated febrile disease in several other countries including Sri Lanka (Agampodi et al., 2011), Bangladesh (Kendall et al., 2010) and Nepal (Blacksell et al., 2007). Leptospirosis is also a prominent health threat in island communities around the world including the Caribbean (Alleyne, 1987, Everard and Everard, 1993, Everard et al., 1992), a variety of pacific island nations (Lau et al., 2016, Dreyfus et al., 2014a, Berlioz-Arthaud et al., 2007) and the western Indian Ocean islands (Desvars et al., 2013a). However, relatively little is known about the incidence of disease in Africa.

Recent reviews of leptospirosis have highlighted that leptospirosis research in Africa is hampered by a lack of good quality surveillance data (Costa et al., 2015a, de Vries et al., 2014). Seroprevalence studies indicate that human *Leptospira* infection is geographically widespread in sub-Saharan Africa, although serological data are often out-dated particularly for countries in Central Africa (de Vries et al., 2014). Costa et al. (2015a) also highlighted the lack of data from Africa as a whole in their global review of the burden of leptospirosis. However, based on available data, the East African region was predicted to have a relatively high incidence of disease, compared to many other global regions (Costa et al., 2015a). Leptospirosis therefore may be an important, but under-diagnosed cause of undifferentiated fever in East Africa, a region where awareness of the burden of non-malarial febrile illness (NMFI) is growing.

1.2 The problem of non-malarial febrile illness (NMFI) in Africa

Febrile disease is one of the most common reasons for patients to seek health-care in sub-Saharan Africa (Feikin et al., 2011, Crump and Kirk, 2015). Malaria is the major infectious cause of fever in Africa but there is growing evidence that the burden of malaria is decreasing in many parts of the continent (O'Meara et al., 2010, D'Acremont et al., 2010). Declines in malaria incidence are particularly prominent in East Africa over the last decade where marked reductions of up to ~ 80% have been reported in Tanzania (Mmbando et al., 2010, Bhattarai et al., 2007) and Kenya (Okiro et al., 2007, O'Meara et al., 2008).

However, there is no evidence that the reduction in malaria morbidity has been accompanied by an overall reduction in the prevalence of febrile disease in sub-Saharan Africa (D'Acremont et al., 2010). Over diagnosis of malaria is a serious problem in parts of sub-Saharan Africa (D'Acremont et al., 2010, Amexo et al., 2004). Malaria continues to be diagnosed clinically and treated in a high proportion of febrile patients with little recognition or treatment of alternative causes of febrile illness. For example, in one study performed in northern Tanzania, only 46% of more than 4000 patients who received a clinical diagnosis of severe malaria were positive for *Plasmodium* parasitaemia on blood-smear examination (Reyburn et al., 2004). In this population, patient fatality was significantly higher in patients with NMFI (12.1%) than those with laboratory-confirmed malaria (6.9%; $p < 0.001$). Similar scenarios have been described in other parts of the continent including in Kenya (Ye et al., 2009) and Sudan (A-Elgayoum et al., 2009). As yet, relatively little is known about other causes of fever in this part of the world.

1.3 Investigating the causes of NMFI in Tanzania

In response to growing awareness of the problem of NMFI in East Africa, a cohort study of the aetiology of febrile illness was performed in two hospitals in northern Tanzania. In total, 870 patients with non-specific febrile illness were enrolled between 2007 and 2008, and tested for a range of infectious causes of fever known to occur in tropical areas (Crump et al., 2013). On admission, more than 60% of patients received a clinical diagnosis of malaria, but only 2% of study participants received an aetiological diagnosis of malaria after laboratory testing. In contrast, 8.8% of patients met international serological case definitions for acute leptospirosis (Crump et al., 2013, Biggs et al., 2011). This finding demonstrated the relative importance of leptospirosis in this area, but also highlighted many knowledge gaps critical to be able to detect and control the disease. Firstly, all diagnoses of human leptospirosis were made retrospectively with little opportunity to directly inform patient management. In addition, little was known about animal hosts of leptospirosis in the study catchment area limiting the potential to design and target effective disease control interventions. Consequently, work was initiated (including this PhD) to explore the epidemiology of leptospirosis in this area in greater detail.

Leptospirosis, however, is a complicated infection to work with and to control. The disease is difficult to differentiate clinically from other causes of febrile illness and laboratory diagnosis of leptospirosis is challenging even in well-resourced, high-income settings

(Musso and La Scola, 2013, Haake and Levett, 2015). Infection can be caused by one of a wide variety of *Leptospira* organisms, and multiple animal hosts as well as environmental sources may be involved in the epidemiology of infection (Levett, 2001, Ko et al., 2009). However, control strategies rely on thorough understanding the types of circulating leptospires and animal maintenance hosts of infection, as well as thorough surveillance to monitor the outcome of any interventions (Hartskeerl et al., 2011). The next sections of this introductory literature review will briefly outline and discuss important pathogen characteristics, epidemiological features and limitations of available diagnostic tests that have to be taken into consideration when studying this overlooked but important disease.

1.4 *Leptospira* bacteria: the causative agents of leptospirosis

Leptospirosis is caused by infection with pathogenic *Leptospira* bacteria. *Leptospira* are spirochaete bacteria with a characteristic coiled or spiral appearance (Adler and de la Pena Moctezuma, 2010). The *Leptospira* genus is thought to have arisen from an early branch of eubacterial evolution and the taxonomy of this group is complex and continually being up-dated (Levett, 2015). To date, genetic analysis has identified nine pathogenic and six saprophytic (non-pathogenic) *Leptospira* spp., in addition to five *Leptospira* species that exhibit intermediate pathogenic properties (Table 1.2) (Cerqueira and Picardeau, 2009, Levett, 2015).

Table 1.2: Pathogenic, intermediate and saprophytic *Leptospira* species (adapted from Levett (2015)).

Pathogenic *Leptospira*	Intermediate *Leptospira*	Saprophytic *Leptospira*
L. alexanderi	*L. broomii*	*L. biflexa*
L. alstonii	*L. fainei*	*L. meyeri*
L. borgpetersenii	*L. inadai*	*L. terpstrae*
L. interrogans	*L. licerasiae*	*L. vanthielii*
L. kirschneri	*L. wolfii*	*L. wolbachii*
L. kmetyi		*L. yanagawae*
L. noguchii		
L. santarosai		
L. weilii		

Within each species, *Leptospira* are further sub-divided into serovars, which is the smallest taxonomic unit of *Leptospira* bacteria (Levett, 2015). To date, around 282 pathogenic serovars have been described, 88 of which belong to the best-characterised pathogenic species, *L. interrogans* (Ko et al., 2009, Cerqueira and Picardeau, 2009).

The *Leptospira* genome ranges in size from 3.9 mega-bases (MB) for *L. borgpetersenii* and 4.7 MB for *L. noguchii* (Fouts et al., 2016). The genome consists of a large circular chromosome (approximately 3.6 to 4.3 MB) and a smaller replicon roughly 350 kilo-bases (KB) in length (Picardeau, 2015). *L. biflexa,* the prototype saprophytic leptospire, also has a third extra-chromosomal genomic element P74 (74 KB) that has not been detected in any pathogenic *Leptospira* species (Picardeau et al., 2008). Essential housekeeping genes, including the *sec*Y and ribosomal RNA (rRNA) genes are used to classify the bacteria (*rrs, rrl* and *rrf*) and are located mainly on the large circular chromosome (cI). In contrast to most bacteria where the rRNA genes are clustered and co-transcribed, these genes are not linked to one another and are widely scattered along the cI chromosome (Picardeau, 2015). Most of the genes encoding virulence factors, such as *lip*L32 and *lig*B, are also located on chromosome cI (Picardeau et al., 2008). Overall however, there appears to be substantial amount of functional gene redundancy in *Leptospira*, particularly in pathogen-specific genes where up to 78% of genes have no known function (Adler et al., 2011).

Leptospira bacteria also show unusual patterns of genetic organisation and mechanisms of gene regulation (Bulach et al., 2006, Saint Girons et al., 1992). Although a substantial proportion of the genome is shared between pathogenic species, there is relatively little synteny even for species with a short evolutionary distance between them (Picardeau et al., 2008). Pseudogenes and insertion sequences (IS) are common features in the *Leptospira* genome (Picardeau, 2015). IS-mediated sequence disruption and genome reduction is thought to be an important mechanism in the evolution of *Leptospira* and the number of IS-elements varies between species and serovars (Bulach et al., 2006). In general however, the *Leptospira* genome is considered relatively stable as *Leptospira* serovar identity is maintained in *in vitro* culture for more than 80 years in the absence of selective pressure (Picardeau, 2015). Lateral gene transfer and homologous recombination events are considered to make a relatively minor contribution to the overall genetic diversity of *Leptospira* (Picardeau et al., 2008, Picardeau, 2015).

As well as the gene-based taxonomic classification described above, serovars are clustered into 24 serogroups, based on similarities in their serological characteristics (Levett, 2001).

Serogroups do not have any taxonomic value *per se* but are important to understand the epidemiology of infection on a regional level and are used in serological diagnosis of infection (Levett, 2015). Furthermore, serogroups and genetic species show relatively poor concordance. A single serogroup for example may include serovars belonging to several different *Leptospira* species and vice versa (Cerqueira and Picardeau, 2009). However, serogroup classifications remain critical to serological diagnosis of leptospirosis and our understanding of the epidemiology of leptospirosis on a regional scale, and hence this unit continues to be an important part of *Leptospira* classification (Goris and Hartskeerl, 2013, Levett, 2001).

1.5 The epidemiology of leptospirosis

As a zoonotic bacteria (i.e. one that can transmit between animals and people (World Health Organization, 2006)), infection in both people and animals must be considered in the epidemiology of the disease. A large number of animal host species of *Leptospira* have been reported for the 250 or so recognised *Leptospira* serovars (Levett, 2015, Cerqueira and Picardeau, 2009). In a particular ecosystem, the specific role of each susceptible animal host broadly divides into either maintenance or non-maintenance hosts of infection (Ellis, 2015, Levett, 2001).

In the disease ecology literature, a maintenance host is defined as a host that is able to maintain infection without re-introduction from other animal or environment sources of infection (Viana et al., 2014). Maintenance hosts of *Leptospira* generally appear to be well-adapted to infection with their associated serovars and are able to reach a state of equilibrium with the pathogen without major detrimental effects to the host (Bonilla-Santiago and Nally, 2011, Bharti et al., 2003). Persistent renal or genital tract colonisation occurs following *Leptospira* infection in a maintenance host and infectious leptospires may transmitted through sexual contact or shed in the urine of these animals for several years (Levett, 2001, Ellis, 2015). Transmission of infection from maintenance hosts to non-maintenance hosts usually occurs either through direct with infected urine or indirectly as a consequence of environmental contamination (Levett, 2001).

A non-maintenance host of *Leptospira* is usually referred to as an incidental host in the leptospirosis literature, which is defined as a mammalian host that becomes incidentally infected with a *Leptospira* serovar that is not normally maintained by that particular species or population (Ko et al., 2009, Levett, 2001). Virtually any mammal, including

humans, may be an incidental *Leptospira* host (Levett, 2001). Renal colonisation occurs after an initial period of bacteraemia but typically is less well-tolerated than in a maintenance host and may be associated with clinical disease and more severe renal pathology (Zhang et al., 2012, da Silva et al., 2010). Urinary shedding also occurs in incidental hosts but the duration of shedding is generally thought to be short-lived (Levett, 2001). However, the distinctions between maintenance and incidental hosts of *Leptospira* in a particular setting are not mutually exclusive or absolute. A mammal that is a maintenance host for one serovar may be incidentally infected with a different serovar for which it is a non-maintenance host, and the specific factors and mechanisms that define these roles remain poorly characterised (Ellis, 2015, Monahan et al., 2009). However, any infected host that is capable of shedding infectious leptospires in its urine has the potential to act as a source of infection for other people and animals and hence both maintenance and non-maintenance hosts may contribute to the overall reservoir of infection[*] in a particular ecosystem (Haydon et al., 2002).

Leptospira infection has been reported in many different animal species including domestic animals such as cattle, sheep, pigs, horses and dogs. A wide variety of wild animal hosts including numerous rodent species, bats, possums, deer, mongoose and small insectivores also been reported (Ellis, 2015, Bharti et al., 2003) However, two major groups of animal host are considered fundamental to the epidemiology of human infection; rodents and livestock (Figure 1.1) (Haake and Levett, 2015, Mwachui et al., 2015).

[*] A reservoir of infection is defined by Haydon et al as one or more epidemiologically connected populations or environments in which a pathogen can be permanently maintained and from which infection is transmitted to the target population.

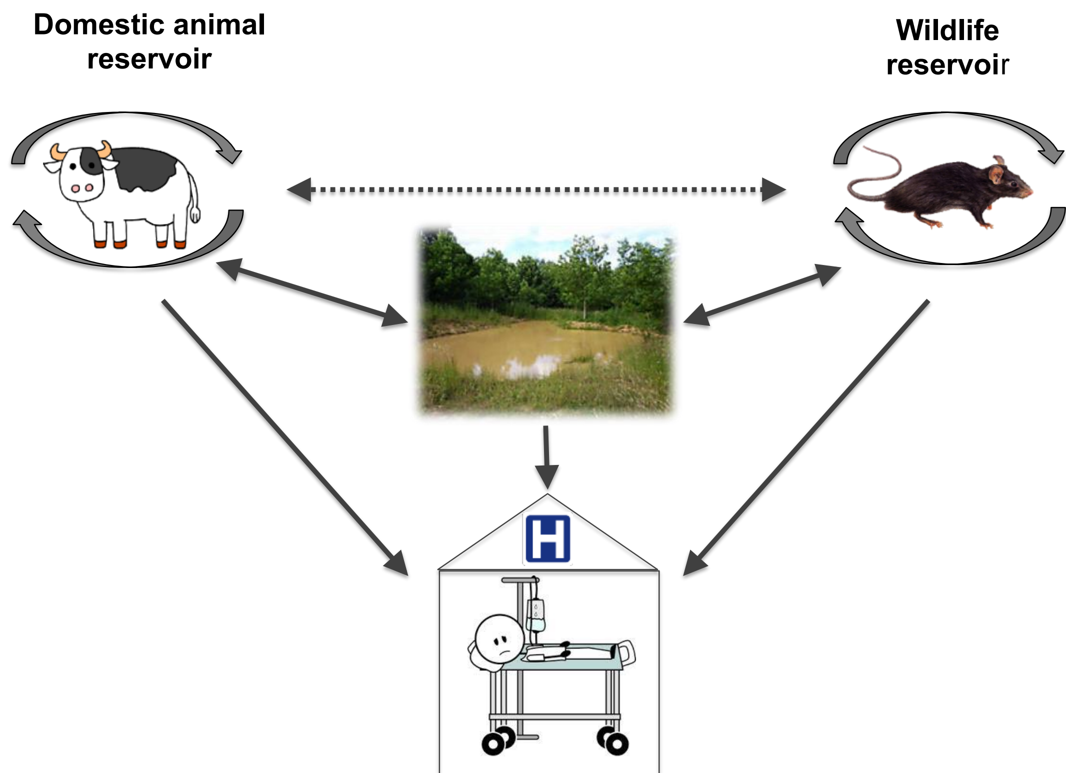

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Figure 1.1: Key features of the epidemiology of leptospirosis.

Domestic and wild animals may maintain infection (curved arrows) and act as sources of infection for people (solid arrows). Transmission of infection occurs through direct contact between animals and people, or indirectly through contaminated soil or water. Transmission may also occur between domestic and wild animal species (dashed arrow).

Rodents are considered by many sources to be the most important animal hosts for human *Leptospira* infection (Haake and Levett, 2015, Levett, 2001), and contact with rodents is a common risk factor for human infection around the world (Mwachui et al., 2015). Rodent-associated leptospirosis is typically seen in environments that are favourable to rodent invasion such as rural areas with abundant food sources (Gratz and Arata, 1975) or urban slum settlements with low-quality housing and poor sanitation and waste management (Masi et al., 2010). As a consequence, rodent-associated leptospirosis is an emerging health problem in urban communities in low-income settings such as slum communities in Brazil (Reis et al., 2008) or rapidly-expanding urban areas of south-east Asia (Kendall et al., 2010, Sakundarno et al., 2014).

Livestock-associated leptospirosis is described as an occupational health risk for professions that bring people into close contact with infected animals, such as dairy farming, veterinary practice or slaughtering or butchering animals for human consumption

(Dreyfus et al., 2014a, Levett, 2001). Livestock production, particularly of cattle and pigs, is a global risk factor for leptospirosis (Mwachui et al., 2015). Livestock contact is a typical part of daily life in subsistence farming communities around the world (Grace et al., 2012b), and hence livestock are potentially important sources of *Leptospira* infection for people living in rural communities, which carry a high burden of infection (Costa et al., 2015a).

Finally, environmentally-acquired leptospirosis can occur in any ecosystem where infected animal hosts live that provides the moist, warm conditions needed for pathogenic *Leptospira* bacteria to survive outside a mammalian host (Lau et al., 2010, Levett, 2001). Tropical areas are particularly well-suited to supporting environmental transmission of leptospirosis (Costa et al., 2015a). Initial contamination of water and soil with *Leptospira* must derive from an infected animal living in that ecosystem. Environmental survival is variable, but may be several weeks for some pathogenic *Leptospira* species (Levett, 2001). Outbreaks of disease may occur after heavy seasonal rainfall or flooding (Lau et al., 2010, Reis et al., 2008). Particular high-risk behaviours associated with environmental transmission including swimming, fishing and walking barefoot have been identified (Mwachui et al., 2015). Recreational exposure to contaminated water sources is also an important and growing source of leptospirosis, particularly in relation to competitive sporting events or adventure travel-related activities (Haake et al., 2002).

1.6 Leptospirosis: the disease

1.6.1 Human disease, presentation and treatment

In people, leptospirosis may range from a mild, flu-like episode to a severe life-threatening disease (Levett, 2001, Haake and Levett, 2015). Asymptomatic infection has also been reported (Bharti et al., 2003). Fever is the most common presenting sign in the acute phase of infection and may be accompanied by other non-specific signs such as headaches, chills and myalgia(Levett, 2001). Conjunctival suffusion is described as a pathognomic sign of leptospirosis but often does not occur (Haake and Levett, 2015). Severe disease manifestations, characterised by multiple organ dysfunction, occur in the secondary phase of infection following tissue localisation of circulating leptospires. The classic disease manifestation is 'Weil's disease', a combination of renal insufficiency and jaundice that is often accompanied by thrombocytopenia and haemorrhage (Cruz et al., 2009, Haake and Levett, 2015). Other life-threatening disease sequelae include meningitis and severe

pulmonary haemorrhagic syndrome (SPHS). SPHS in particular is associated with a high patient mortality rate in excess of 50% (McBride et al., 2005, Ruwanpura et al., 2012, Simpson et al., 1998). For other disease manifestations, the average mortality rate is reported as around 5-10% (Haake and Levett, 2015).

In general, most mild cases of leptospirosis will self-resolve but treatment is indicated when infection is diagnosed to reduce the potential for and impact of secondary sequelae and to shorten the duration of clinical disease (Haake and Levett, 2015, McBride et al., 2005). Pathogenic leptospires shows good *in vitro* susceptibility to a wide range of readily available antimicrobial agents (Wuthiekanun et al., 2015, Bharti et al., 2003) but there continues to be some debate on the most appropriate antibiotic treatment strategy for recognised cases (Suputtamongkol et al., 2004, Vinetz, 2003). Severe cases also require intensive care to support renal function, manage pulmonary complications and reduce mortality (Haake and Levett, 2015).

Chronic health problems have been reported following *Leptospira* infection although data are limited to a single study in the Netherlands (Goris et al., 2013a) and anecdotal reports from New Zealand (Jackie Benschop, personal communication). Sequelae including persistent fatigue, myalgia and headaches have been reported for years following an acute infection. Although recent DALY calculations of leptospirosis gave little weighting to the chronic sequelae of disease in estimates of global morbidity (Torgerson et al., 2015), the issue of long term health problems following an acute episode of leptospirosis is a neglected area of clinical research, which could have consequences for the overall impact of the disease.

1.6.2 Animal disease

In livestock species including cattle, pigs and small ruminants, clinical leptospirosis is well described but varies with the infecting species or serovar (Tibary et al., 2006, Radostits et al., 2000). Acute systematic infection is most common in calves and lambs but is also reported in adult dairy cattle as a cause of acute onset agalactia (‘milk drop syndrome’) (Ellis, 2015). Chronic infections with leptospirosis can result in abortions, stillbirths and reduced fertility in all the major livestock species. Abortion outbreaks are common in leptospirosis infections in pig herds, which can result in large-scale economic losses for commercial units (Gresham, 2003). Reduced weight gain was reported in deer with subclinical *Leptospira* infection (Ayanegui-Alcerreca et al., 2007, Subharat et al., 2012).

Overt clinical disease is frequently reported in dogs and horses. In dogs, four potentially fatal, clinical syndromes (icteric, haemorrhagic, uraemic and reproductive) are described (Faine, 1994, Radostits et al., 2000). Leptospirosis is an important cause of recurrent uveitis in equids (Faber et al., 2000).

In wildlife, little is known about clinical disease. Rodents and other small mammals are common hosts of infection but are not known to suffer from disease following infection with rodent-adapted serovars (Levett, 2001, Reis et al., 2008, Lau et al., 2010, Belmain, 2006). However, in the laboratory, rodent species show differing vulnerability to clinical disease following experimental infection with different serovar. Some species such as hamsters and guinea pigs are susceptible to severe clinical disease and high rates of mortality with typical rodent-associated *Leptospira* serovars (Zhang et al., 2012, Coutinho et al., 2014). Infection has also been demonstrated or induced in other taxonomic classes such as amphibians, reptiles and birds (Faine, 1994, Levett, 2001, Radostits et al., 2000). To date, there is no evidence that these animals shed leptospires or act as sources of infection for people or other animals but it remains a possibility that non-mammalian species may also contribute to the epidemiology of infection in some ecosystems.

1.7 Diagnosis of leptospirosis and *Leptospira* infection

Leptospirosis is notoriously difficult to diagnose due to the non-specific nature of clinical signs in acute cases. Multiple testing strategies are described but each method has limitations and the choice of diagnostic tool is highly dependent upon the goal of testing. Different diagnostic approaches may be taken to diagnose an acutely unwell patient for example than in an epidemiological study investigating evidence of previous exposure in an animal population. The phase of the disease must also be taken into account when selecting an appropriate diagnostic test and sample. Leptospirosis is a biphasic disease consisting of 1) the leptospiraemic phase, when *Leptospira* organisms are circulating in the blood; 2) the leptospiruric or immune phase, when *Leptospira* colonises tissues and antibody production starts shown in Figure 1.2.

Diagnostic methods for *Leptospira* infection may be divided into two complementary groups; firstly those that directly detect the presence of the pathogen through direct visualisation, culture and isolation, or demonstration of the presence of the pathogen’s DNA in normally sterile sites (e.g. blood, kidney, cerebrospinal fluid); or secondly, tests

that rely on demonstration of *Leptospira*-specific antibodies to infer the infection status of a human or animal.

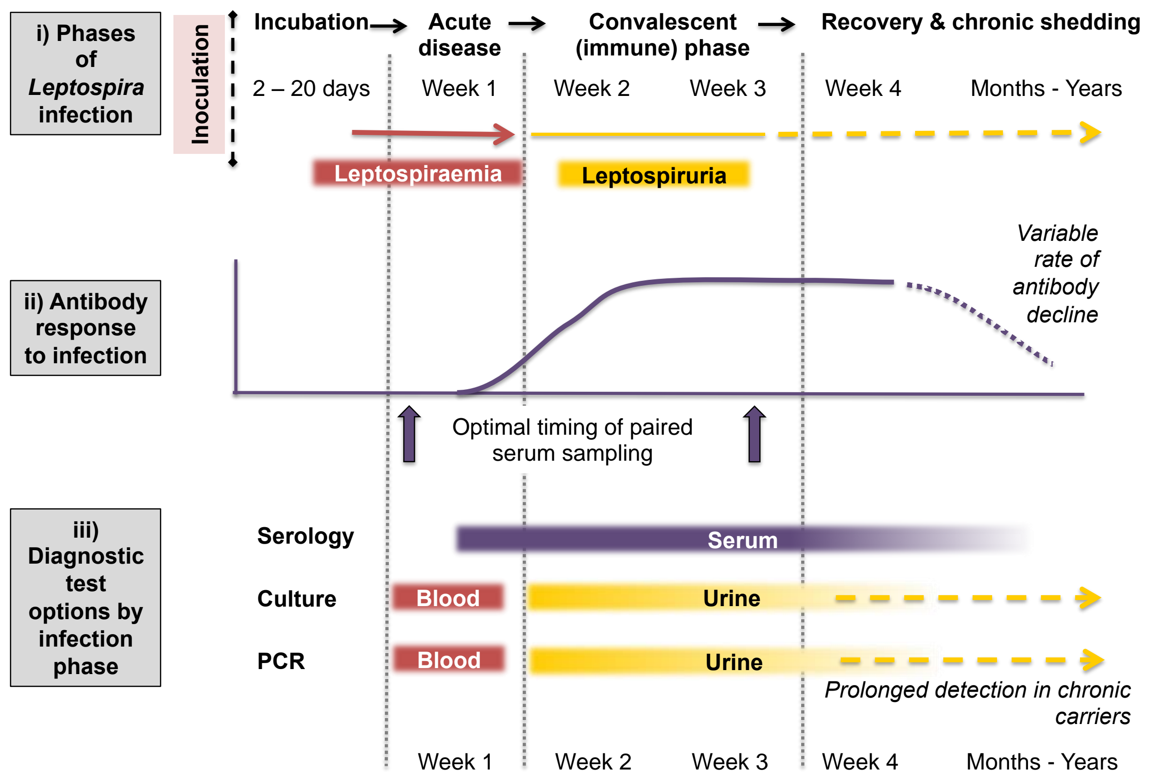


Figure 1.2: Biological phases of leptospirosis and implications for sampling and diagnostic testing (adapted from (Haake and Levett, 2015)).

(i) Leptospirosis is often described as a biphasic disease characterised by a period of leptospiraemia in the acute phase and leptospiruria in the convalescent or immune phase, which coincides with rising antibody titres (ii). Antibody titres may remain elevated for several months to years following infection and decline at a variable rate. (iii) The choice of sample type and diagnostic test is informed by the phase of infection. Serum samples for paired serology should be taken early in the acute leptospiraemic phase and then again 2-4 weeks later in the convalescent phase of infection (shown by arrows). The period where there is a possibility of detection of *Leptospira* by Polymerase Chain Reaction (PCR) or culture in the urine (or kidney) of chronically infected animals is prolonged.

1.7.1 Diagnostic tests for direct detection of *Leptospira* infection

Leptospira culture and isolation is the gold standard test to directly demonstrate *Leptospira* infection and offers the best option for fully characterising the infecting *Leptospira* serovar. This approach therefore is well suited to determining animal hosts of specific serovars, for identifying sources of human infection and for describing population level diversity of *Leptospira.* However, the process is time consuming and laborious as leptospires are slow growing, fastidious bacteria, which require specialist media and long incubation times (up to 6 months according to some sources). As a result, this approach is of limited utility to a clinician concerned with making a rapid diagnosis to inform patient care (Picardeau et al., 2014). Culture is highly specific for *Leptospira* but the sensitivity is relatively low in clinical settings due to issues with timing of sample collection, impacts of prior antibiotic treatment and low bacterial load in clinical samples (i.e. blood and urine) (Faine, 1994, Levett, 2001). Also, no single culture medium is capable of supporting the primary isolation of all known pathogenic leptospires. The potential for serovar selection bias to be introduced through culture and isolation approaches should therefore also be considered (Ellis, 2015).

Direct visualisation of infecting leptospires is possible using histopathology, immunohistochemistry or immunofluorescence techniques to diagnose infection. However, these diagnostic techniques are particularly labour intensive, relatively insensitive and are not well suited to routine surveillance of large numbers of patients or animals.

Over the last decade, the popularity of nucleic acid amplification approaches such as Polymerase Chain Reaction (PCR) to provide rapid diagnosis of human and animal *Leptospira* infection has been growing (Picardeau et al., 2014, Sykes et al., 2011). A positive diagnosis of infection can be made in a matter of hours with PCR rather than the weeks to months required for culture. In human disease, PCR is most useful in the early stages of infection when the diagnostic test can be used to detect bloodstream infection (Figure 1.2). In studies of animal hosts, PCR can be used to demonstrate infection and determine prevalence in different species and geographic settings, as well as to diagnose infection in clinical cases (Desvars et al., 2013c, Sykes et al., 2011). Genetic typing of *Leptospira* species can also be performed directly from PCR products (Morey et al., 2006, Perez and Goarant, 2010). In clinical settings PCR is still subject to some of the same limitations as culture, including issues with timing of sample collection, prior antibiotic use and low bacterial load. PCR assays may also be inhibited by a variety of biological

compounds such as urea in urine samples, which reduce the probability of detecting an infection (Schrader et al., 2012). Overall however, PCR may offer a more practical and sensitive approach to demonstrating infection in the acute phase of illness in people, or in field surveys of animals where conditions often constrain the ability to definitely demonstrate infection by other techniques.

1.7.2 Serological diagnostic tests for *Leptospira* infection

Serology by microscopic agglutination test (MAT) is considered the cornerstone of leptospirosis diagnosis (Goris and Hartskeerl, 2013, World Organisation for Animal Health (OIE), 2014). Definitive serological diagnosis of acute leptospirosis by MAT is reached by demonstrating seroconversion between paired acute and convalescent serum samples. Single sample MAT tests can be used to demonstrate prior *Leptospira* exposure in epidemiological surveillance studies, and a single high titre can also be used to infer current or recent infection. However, a number of important limitations exist for the use of MAT to diagnose acute disease. Optimal sensitivity of the MAT requires prior knowledge of locally prevalent serovars to select a representative test panel (Goris et al., 2012, Goris and Hartskeerl, 2013). Diagnostic sensitivity of a single serum sample is low (~ 6%) during the first 7-10 days of illness due to a delay in antibody response following infection (Figure 1.2) (Limmathurotsakul et al., 2012, Goris et al., 2012). Paired serology improves the sensitivity of testing (~ 82%) but means that infection can only be demonstrated retrospectively and cannot therefore be used to inform patient care (Goris and Hartskeerl, 2013). While the diagnostic specificity of seroconversion in paired MAT titres is good (~ 100%) (Goris and Hartskeerl, 2013), less is known about the specificity of a single acute-phase MAT. For people living in areas where prior exposure may be common, high MAT titres may imply either current or previous infection. Local validation is recommended to test the appropriateness of single titre MAT case definitions (Goris et al., 2012).

In animals, paired serology can also be used to demonstrate seroconversion in an animal, or animal population (Sykes et al., 2011). However, serological approaches have limited utility for identifying animal hosts of *Leptospira* as they cannot be used to detect persistent *Leptospira* infection or shedding. Chronically infected animal hosts may mount only a transient antibody response and hence test negative by serology in the later stages of infection (Bonilla-Santiago and Nally, 2011, Ellis, 2015). Therefore, direct methods of detection are required to definitively identify chronic carriers of *Leptospira* within a population (World Organisation for Animal Health (OIE), 2014).

Another limitation of the MAT is that data can only be used to gain a broad understanding of infecting *Leptospira* serogroups at the population level. The MAT is notoriously unreliable in predicting infecting serogroups early in the course of infection due to a high degree of non-specific or paradoxical reactivity in the early stages of the immune response (Levett, 2003, Smythe et al., 2009). Determining the infecting serovar using MAT data is not possible at any stage as the MAT only shows specificity to the serogroup level (Faine, 1994). Serogroup patterns can be deduced from reaction patterns on convalescent or background population samples. However, cross-reactions are common and should be taken into consideration particularly where multiple serovars are present in a population (Goris et al., 2012, Smythe et al., 2009).

As well as the MAT, a variety of other serology-based rapid tests have been described for the diagnosis of acute leptospirosis in people (Goris et al., 2013b). Most commonly used are enzyme-linked immunoassays (ELISA) targeting either IgM antibodies for acute infection or IgG antibodies for historic infection (Goris and Hartskeerl, 2013, Niloofa et al., 2015). The IgM ELISA for acute leptospirosis is reported shows higher diagnostic sensitivity than the MAT using acute phase serum samples, but typically also has lower diagnostic specificity for acute infections (Bajani et al., 2003, Goris et al., 2013b). Laboratory confirmation of a positive acute-phase ELISA test by either direct pathogen detection (PCR or culture) or evidence of seroconversion by MAT, is required to make a definitive diagnosis of infection or characterise the infecting *Leptospira* type (Picardeau et al., 2014). Finally, unlike the MAT, most ELISA tests are host species-specific (e.g. human or bovine) and therefore cannot be used to generate comparable information between people and animals.

1.8 Control and prevention of leptospirosis

Broadly, strategies to control and prevent *Leptospira* infection in people and animals fall into three categories: 1) directly protecting people or domestic animals from infection or illness; 2) using tactics to reduce transmission from sources of infection; or 3) reducing or eliminating infection from source populations or contaminated environments (Hartskeerl et al., 2011, Haydon et al., 2002). Direct strategies to protect people from disease have been used in some high-risk settings where infection cannot be controlled at source. Prophylactic doxycycline treatment is recommended for travellers or military personnel visiting a highly endemic area (Takafuji et al., 1984, Haake et al., 2002), and human

vaccination has been used as a strategy for some high-risk occupations (Haake and Levett, 2015) or in areas with a high burden of human disease that is attributed to infection with a small number of serovars (e.g. Cuba (Bharti et al., 2003, Martinez Sanchez et al., 2000). However, leptospirosis vaccinations rely on whole-cell preparations that are typically associated with high rates of side effects and a short duration of serovar-specific immunity (Haake and Levett, 2015, Hartskeerl et al., 2011). Hence human vaccinations are not generally practical or an effective method of controlling disease in large populations where multiple serovars are circulating.

More commonly, control measures for human *Leptospira* infection focus on reducing transmission to people by either minimising contact with sources of infection, or reducing the burden of infection in animal source populations (Haake and Levett, 2015). Animal vaccination is one strategy that may reduce infection in animal source populations (Hartskeerl et al., 2011). Vaccination acts to protect the animal from clinical disease and to reduce urinary shedding of infectious bacteria (Ellis, 2015, Bolin and Alt, 2001). As with people, animal vaccination is serovar-specific hence full characterisation of circulating *Leptospira* serovars is important for vaccine selection (Hartskeerl et al., 2011) and clinical disease may still be encountered if animals are exposed to other serovars (Goldstein, 2010, Ellis, 2010). Treatment in infected animal herds has also been described as a method for reducing both urinary shedding and the impact of infection on the animal host (e.g. in cattle in the Netherlands (Hartskeerl et al., 2011)). Both methods of control of infection in animal hosts requires a thorough knowledge of the epidemiology of *Leptospira* infection in local populations and are only really feasible for domestic animal populations (Hartskeerl et al., 2011). Where human disease results from contact with wild animals (e.g. rodents) or environmental sources (or from serovars that cannot be controlled by animal vaccination), the only feasible option for control is to reduce the amount of contact between people and the sources of disease. Examples of the wide variety of control strategies that may need to be considered include improving sanitation in informal urban settlements (Reis et al., 2008), reducing rodent invasion into households (Gratz and Arata, 1975), providing personal protection for people engaged in high-risk occupations (Dreyfus et al., 2014b) or protecting people from risks associated with flooding or other environmental disasters (Lau et al., 2010, Watson et al., 2007). Monitoring the efficacy of leptospirosis control strategies such as these requires a multidisciplinary approach as well as decent public health infrastructure and effective diagnostic testing for accurate surveillance (Hartskeerl et al., 2011). Many of these attributes continue to be lacking from potentially high incidence areas such as sub-Saharan Africa.

1.9 From theory to the field: an overview of study aims, objectives and the outline of this thesis

Leptospirosis is a challenging disease to understand and to work on. The complicated classification system and complex multi-host epidemiology of the pathogen mean that no single approach can be applied to help us understand disease patterns around the world (Hartskeerl et al., 2011). Diagnostic limitations have resulted in limited surveillance, which has contributed to the continued neglect of this disease (Abela-Ridder et al., 2010). Even in the leptospirosis community, Africa still retains a low profile on a global scale (Allan et al., 2015b, Pappas et al., 2008). However, recent evidence demonstrates that leptospirosis is an important cause of NMFI in Tanzania (Crump et al., 2013), and that the incidence of infection is relatively high in East Africa (Costa et al., 2015a). More work is needed to understand the scale of the problem and the epidemiology of infection on this neglected continent.

This study used a multifaceted approach to address some of the gaps in our knowledge of leptospirosis, both on the local level in Tanzania and at the continental level. An outline of the different components of this study is shown in Figure 1.3.

Firstly, a systematic review of available literature on human and animal *Leptospira* infection was performed to assess the geographic distribution and prevalence of *Leptospira* infection in Africa. This study aimed to compile data on human and animal infection from across the continent, taking a ‘One Health’ approach to compile and synthesise the available data on the epidemiology of leptospirosis in Africa in more depth (Chapter 2). Secondly, field studies were established to explore the epidemiology of animal populations in northern Tanzania. Rodents and ruminant livestock have been implicated as important sources of human infection in other settings and therefore were selected as the major animal hosts for investigation in this thesis (Chapter 5 and Chapter 6). qPCR assays were used to diagnose infection in these animal hosts and to diagnose human *Leptospira* infection (Chapter 6.47). The selection of diagnostic assays for use was based on validation data presented in Chapter 4. Infecting *Leptospira* species in people and animal hosts was explored using both serological and genetic typing approaches and serological exposure patterns in people and cattle were also analysed. Finally data from all study elements were assimilated to identify potential sources of *Leptospira* for people in northern Tanzania and to inform evidence-based recommendations for leptospirosis surveillance, control and future research priorities in the region (Chapter 8).

PROBLEM: ACUTE LEPTOSPIROSIS IS AN IMPORTANT CAUSE OF HUMAN FEBRILE ILLNESS IN NORTHERN TANZANIA BUT LITTLE IS KNOWN ABOUT THE EPIDEMIOLOGY OF INFECTION.

Chapter 2: A systematic review of the epidemiology of leptospirosis in Africa

Chapter 3: Core Methodology

Chapter 4: Validation of core lab methods

Chapter 5: **Rodent hosts of *Leptospira***
- Cross-sectional survey of peri-domestic rodents
- Kidney: qPCR, culture

Results:
- Infection prevalence
- *Leptospira* spp. diversity

Chapter 6: Livestock hosts of *Leptospira*
- Abattoir survey of cattle, sheep and goats
- Kidney & urine: qPCR, culture

Results:
- Infection prevalence
- *Leptospira* spp. diversity

Chapter 7: **Human *Leptospira* infection**
- Archived samples from cases of acute leptospirosis (ISAAC study)
- Plasma & urine: qPCR

Results:
- qPCR vs. MAT test performance
- *Leptospira* spp. diversity

Chapter 8: Are rodents and/or ruminant livestock the source of human leptospirosis in Tanzania?
Conclusions, discussion & future perspectives

Figure 1.3: Overview of thesis structure showing chapter outlines, key methodologies and outputs

2 A systematic review of acute human leptospirosis and animal Leptospira infection in Africa

2.1 Introduction

Endemic zoonotic diseases affect impoverished and developing communities around the world but are typically overshadowed in public and clinician awareness by higher profile infections such as malaria and HIV/AIDS (World Health Organization, 2006, Maudlin et al., 2009). In Africa, zoonotic infections are directly affect human health through human morbidity and mortality but may also indirectly impact human well-being as a result of reduced livestock productivity and food security (Schelling et al., 2007, Perry and Grace, 2009, Halliday et al., 2012). However, bacterial zoonoses such as leptospirosis are under-diagnosed and therefore under-reported in Africa. As a result, endemic zoonotic diseases are neglected on the continent (Molyneux et al., 2011, World Health Organization, 2006, Maudlin et al., 2009).

Leptospirosis is thought to be one of the most common and widespread zoonotic infections worldwide. Disease is caused by infection with a pathogenic serovar of *Leptospira* spp. bacteria (Hartskeerl et al., 2011, Adler and de la Pena Moctezuma, 2010). More than 250 pathogenic *Leptospira* serovars are known to exist that are classified into 24 serogroups based on their serological phenotype (Chapter 1.4) (Cerqueira and Picardeau, 2009, Levett, 2001). Nine pathogenic species have been described (Cerqueira and Picardeau, 2009, Evangelista and Coburn, 2010), which may be carried by a wide range of mammals that act as a source of infection for people and other animals (Levett, 2001, Hartskeerl et al., 2011). Mammalian hosts of *Leptospira* may be asymptomatic carriers of infection or develop clinical disease following infection (Levett, 2001, Faine, 1994). Infectious leptospires are shed in the urine of infected animals for months to years following infection. *Leptospira* serovars often demonstrate a degree of animal host preference and some common relationships between serovars and their hosts are reported (Bharti et al., 2003). Knowledge of the serovars circulating in local animal populations is necessary to determine sources and transmission routes for human infection (Hartskeerl et al., 2011).

In people, infection with *Leptospira* occurs through direct or indirect contact with infected urine from an animal host (Adler and de la Pena Moctezuma, 2010, Hartskeerl et al., 2011,

Bharti et al., 2003). However, recognising leptospirosis in the early stages of disease is challenging. Most commonly, human leptospirosis presents as a non-specific ‘flu-like disease that is difficult to distinguish from other causes of febrile illness in tropical areas (Levett, 2001, Cruz et al., 2009, McBride et al., 2005). However, infection can lead to severe secondary sequelae including renal failure, jaundice, meningitis and severe pulmonary haemorrhagic syndrome (SPHS). A mortality rate of 50% has been reported in complicated cases (Bharti et al., 2003, McBride et al., 2005).

Leptospirosis is thought to be widespread in tropical areas where people and animals live in close contact, and warm and humid conditions favour environmental survival of the pathogen (Hartskeerl et al., 2011, Adler and de la Pena Moctezuma, 2010). In other tropical and sub-tropical regions such as South-East Asia and South America, leptospirosis is recognised as an important cause of renal failure and febrile disease (Cruz et al., 2009, McBride et al., 2005, Crump et al., 2013, Acestor et al., 2012). However, despite its global importance, substantial gaps persist in our understanding of the burden and epidemiology of leptospirosis in Africa. Reports and a recent review from the WHO Leptospirosis Epidemiology Reference Group (LERG) indicate that leptospirosis incidence may be high in Africa, but also highlight the lack of available data (World Health Organization, 2011, Abela-Ridder et al., 2010, Costa et al., 2015a). Seroprevalence studies demonstrate that *Leptospira* exposure is widespread in people and animals in Africa (de Vries et al., 2014, Benkirane et al., 2014). Yet, little is known about the distribution or prevalence of human disease or the epidemiology of infection in different animal hosts in Africa.

2.1.1 Aims and objectives

This chapter describes a systematic literature review performed to tackle gaps in our understanding and awareness of the epidemiology of acute human leptospirosis and animal *Leptospira* infection in Africa. The objectives for this study component were:

Objective 1: Summarise current knowledge of the geographic distribution, prevalence and incidence of acute human leptospirosis in Africa;

Objective 2: Summarise the geographic distribution, host range and prevalence of *Leptospira* infection in animal hosts in Africa;

Objective 3: Describe the species and serogroups of infecting *Leptospira* serovars involved in acute human leptospirosis and animal infection in Africa.

A version of the work presented in this chapter has been previously published and is freely available at http://dx.doi.org/10.1371/journal.pntd.0003899 (Allan et al., 2015a)

2.2 Systematic review methodology

2.2.1 Search strategy

This systematic review following the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Moher et al., 2009). References for this review were identified through searches of eight international and regional databases. Database searches were performed in PubMed, Web of Science, Biosis, CABI abstracts, Zoological Record, Africa-Wide NiPAD, and Africa Index Medicus for resources published between 1st January 1930 – 31st May 2013, and Embase (Ovid) for resourced published between 1947 -2013. An additional search was performed on 24th November 2014 to identify additional articles published in print or online between 1st June 2013 and 31st October 2014. Search terms for each database were developed with guidance from two library scientists (Megan Von Isenberg and Alastair Allan) (Table 2.1). Additional articles for inclusion were identified by bibliography hand searches of relevant articles (Hopewell et al., 2007). Results were exported into Microsoft Excel (Microsoft Corporation, Redmond, WA, USA). The number of unique references from each database was determined after removing duplicates.

Table 2.1: Full search terms used for each study databases

Database	Publication Date Limits	Search Strategies
- Africa-Index Medicus (World Health Organization Global Health Library)	January 1930-October 2014	(Leptospirosis OR *Leptospira*)
- Africa-Wide:NiPAD (now EBSCO Host Africa Wide Information)	January 1930-October 2014	(SU (leptospirosis OR leptospira) OR TX (leptospirosis OR leptospira) AND (AB Africa* OR GE Africa OR SU Africa OR TI Africa* OR KW Africa)
- BIOSIS Previews; **- CABI Abstracts;** **- Web of Science Core Collection;** **- Zoological Record**	January 1930-October 2014	Search 1: Topic=(leptospirosis) OR Topic=(leptospira) OR title=(leptospirosis) OR title=(leptospira); Search 2: topic=(Africa*) OR title=(Africa*); Search 3: Combine Search 1 AND Search 2
- Embase (Ovid; including Embase Classic and Embase)	January 1947-October 2014	(leptospirosis [sh] OR leptospira [sh] OR leptospirosis [tw] OR leptospira [tw]) AND (africa*[sh] OR africa*[tw])
- Pubmed	January 1930-October 2014	(leptospirosis[mesh] OR leptospirosis[Text Word] OR leptospira[Text Word] OR leptospira[mesh) AND (africa[mesh] OR africa*[Text Word])

2.2.2 Study selection and criteria for exclusion by abstract review

Abstracts and titles of references identified by database search were compiled in EndNote (Thomson Reuters, Philadelphia, PA, USA). Each reference was independently reviewed by two researchers (KA and Holly Biggs (HMB)) to determine whether each article met pre-determined abstract inclusion and exclusion criteria. A third researcher (Jo Halliday (JEBH) served as a tiebreaker for any discordant decisions. References were included if they presented data on human or animal *Leptospira* spp. infection from any country within the United Nations (UN) definition of Africa (United Nations Statistics Division, 2012). We excluded any abstract that did not include original human or animal leptospirosis research data, including reviews, textbooks, letters to the editor, policy papers, and lay press and media stories. Additionally, we excluded any abstract in which did not investigate for the presence of naturally occurring cases of leptospirosis in human or animal populations including studies which described in vitro or in vivo experiments, laboratory methods descriptions or abstracts that described only environmental data. We excluded reports of returned travellers because of potential uncertainty around the specific location where infection was acquired. A full list of abstract exclusion criteria with coding hierarchy is given in Table 2.2.

Table 2.2: Title and abstract review exclusion criteria.

Numbers and letters refer to hierarchy used to determine the decision for exclusion

Category		Exclusion criteria
1. Article Type	a.	Review or summary article without original data
	b.	Editorial, letter to the editor opinion, commentary or policy article without original data
	c.	Textbook or handbook rather than publication of new data
	d.	Lay media publications or broadcasts
	e.	Abstracts with corresponding full manuscripts with the same data
2. Language		Exclude if title/abstract are NOT: ENGLISH, FRENCH, PORTUGESE, SPANISH, DUTCH, GERMAN, ITALIAN, AFRIKAANS
3. Geographic focus		Exclude countries not included in the UN macro-geographical definition of Africa
4. Topic focus	a.	Wrong agent
	b.	Experimental data (in vitro or in vivo cellular, molecular, biochemical or other studies that do not include naturally occurring cases of leptospirosis in humans or animals)
	c.	Laboratory methods descriptions
	d.	Leptospirosis included in the diagnostic evaluation or as a differential diagnosis, but diagnosis of leptospirosis infection or exposure was not reached
	e.	Social science or environmental or climate modeling data only
	f.	Case reports of returned travelers

2.2.3 Study selection and criteria for inclusion by full text review

Articles eligible for inclusion after abstract review, as well as references for which no abstract was available, were retrieved in full text form. Non-English language articles identified for full text review (n=97) included 83 French, seven German and four Italian language articles

translated with the assistance of native language speakers (Ferdinand von Göetzen and Erika Abbondati). In addition, two Afrikaans (n=2) articles and one Dutch article were also identified and were translated using online translation software (Google Translate, Mountain View, California, USA) with support from a Dutch language speaker (Julie Woodfield).

Case definitions for human acute leptospirosis and carrier animal status were pre-defined based on WHO and international reference laboratory guidelines (Table 2.3)(World Health Organization, 2011, World Organisation for Animal Health (OIE), 2014). Serological diagnostics were not included in the case definition for carrier animals because of incapacity to differentiate between prior exposure and current infection. Articles describing studies that used laboratory animal inoculations as a diagnostic test for leptospirosis were not considered eligible for inclusion in the review due to concerns over diagnostic sensitivity and the potential for false positive results in contaminated laboratory animal colonies (Faine, 1994).

Full text articles were reviewed by two investigators (KA, HMB) and excluded if they failed to meet case definitions or if insufficient information was provided in the study methodology to determine whether the case definitions were met. Articles were also excluded if they were found to meet any of the abstract exclusion criteria during full-text review.

Table 2.3: Case definitions for acute human leptospirosis and animal *Leptospira* infection

Human acute leptospirosis definition – confirmed cases
Compatible acute illness, plus ≥1 of the following:
≥ 4 fold rise in Microscopic Agglutination Test (MAT) titre between acute and convalescent serum
Culture* and isolation of pathogenic *Leptospira* spp. from blood, urine, CSF or tissues
Pathogenic *Leptospira* spp. DNA detected by Polymerase Chain Reaction (PCR) from blood/blood derivatives, urine, cerebrospinal fluid, or tissues
Detection of *Leptospira* spp. in tissue by immunohistochemical techniques
Human acute leptospirosis definition – probable cases
Compatible acute illness, plus ≥1 of the following:
MAT titre ≥1:400 in single or paired serum samples
Presence of IgM antibodies by enzyme-linked immunosorbent assay (ELISA) or dipstick
Presence of IgM or a fourfold increase in IFA antibody titre in acute and convalescent serum samples
Animal *Leptospira* infection definition - confirmed
Clinical signs present <u>or</u> absent, plus ≥ 1 of the following:
Culture* and isolation of pathogenic *Leptospira* spp. from a normally sterile site
Pathogenic *Leptospira* spp. DNA detected by PCR or real-time PCR (qPCR) from a normally sterile site
Typing of previously isolated serovar
Detection of *Leptospira* spp. in clinical specimens by immunohistochemistry

* Culture in any of the following media: Ellinghausen-McCullough-Johnson-Harris (+/- 5'Fluorouracil), Fletcher, Korthoff, Stuart, Vervoot or Noguchi culture media.

2.2.4 Data extraction and synthesis

Two reviewers (KA, HMB) independently extracted pre-determined qualitative and quantitative data from each article eligible for inclusion. Extracted data included: geographical location (country, locality); study year and duration; study setting (e.g. hospital, community, abattoir) and type (e.g. cohort study, surveillance study, case report); study inclusion criteria and diagnostic methodology; sample size and species tested; number of positive cases and prevalence estimate; incidence (human population-based studies only); and results of serological and genetic typing on infecting *Leptospira* spp.

Data on infection prevalence and incidence were compiled for studies that used comparable inclusion criteria and diagnostic methodologies, Prevalence and incidence ranges were summarised by study type (human studies), location or host species (animal studies) if three or more references reporting comparable data were identified. Data on serological and genetic typing of *Leptospira* isolates or *Leptospira* DNA detected in human and animal infections were compiled and summarised by country and species. Additional serogroup and species data of reported serovars was obtained from the Leptospirosis Library, maintained by the Leptospirosis Reference Centre, Royal Tropical Institute (KIT), Netherlands (Royal Tropical Institute (KIT), 2014).

2.2.5 Critical assessment of methodological quality and bias

The risk of bias in included studies such as selection or reporting bias was assessed following an adaptation of the Cochrane guidelines for systematic reviews of medical interventions (Higgins and Green, 2008). Methodological quality and detection bias was assessed by comparison to pre-determined case definition criteria to control for heterogeneity in study design and diagnostic methodology (Table 2.3). Other types of bias including selection bias based on study type and design, and attrition bias were also evaluated for individual studies. Studies classified as high-risk for bias were not included in quantitative analysis of leptospirosis prevalence and incidence. In studies with incomplete reporting of case definitions and diagnostic criteria, data was only included where a valid assessment of methodological quality could be performed. For references where only a subset of reported positives met our study criteria, prevalence was re-calculated after data adjustment.

2.3 Results

Systematic database searches yielded 681 unique articles from a total of 1201 references. After abstract and full text review, 95 references were considered eligible for inclusion. Hand searches identified two additional articles that met inclusion criteria but were not identified in the original database searches. Reasons for full-text exclusion are detailed in Figure 2.1. In total, 97 articles describing acute human leptospirosis or animal *Leptospira* infection in 26 (44.8%) of 58 African countries were included in analysis (Figure 2.2). Major potential sources of bias identified in eligible studies were selection bias, attrition bias in studies that relied on paired serology (MAT) for confirmatory diagnosis, and reporting bias through incomplete reporting of diagnostic methodology and results.

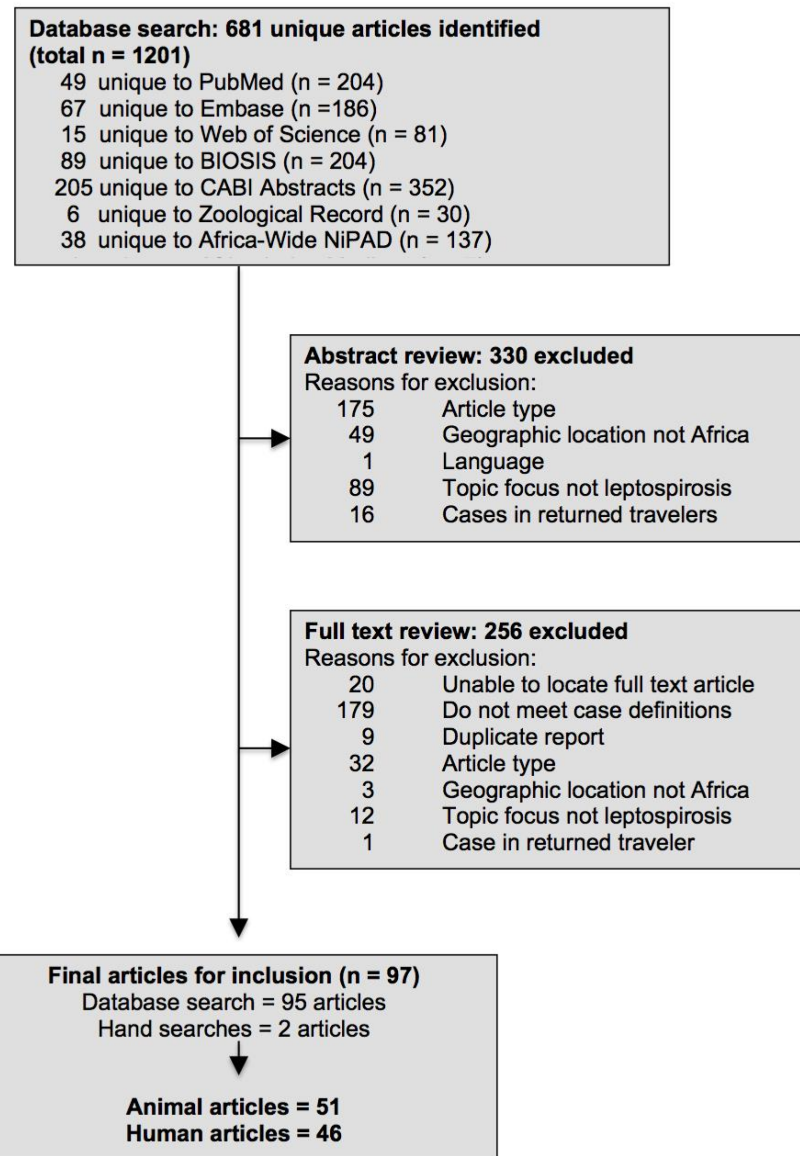


Figure 2.1: Flow chart showing the selection of eligible articles for study inclusion following criteria defined in Table 2.2 and Table 2.3, based on PRISMA guidelines (Moher et al., 2009)

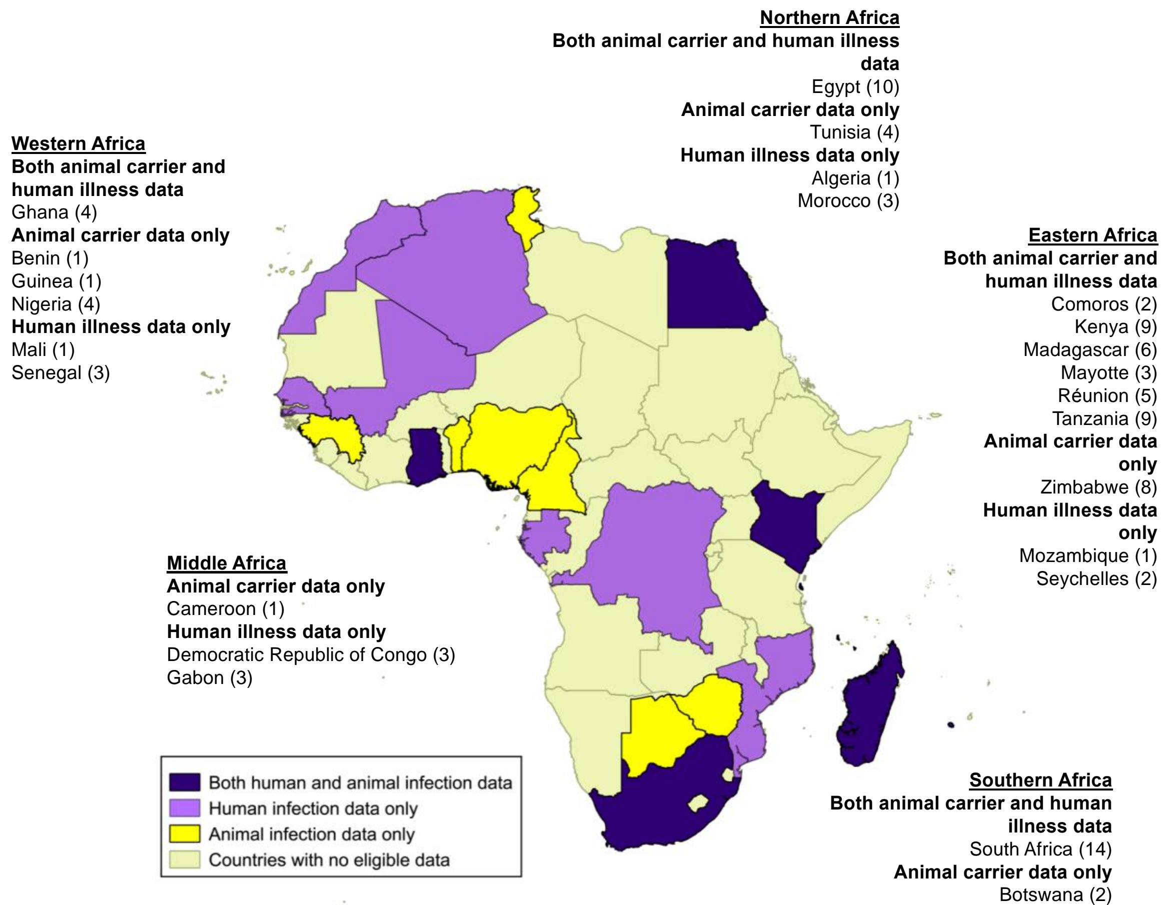


Figure 2.2: Geographic distribution of eligible reports (number per country) of human leptospirosis and animal *Leptospira* infection in Africa

2.3.1 Acute human leptospirosis studies

Full citations and study details for eligible articles (excluding case reports) reporting human leptospirosis in Africa can be found in Appendix 1, where they are also cited in full.

Acute human leptospirosis was reported in 46 articles from 18 African countries (Figure 2.2) (Appendix 1). Most articles came from South Africa (n = 6), Egypt (n = 5) and Kenya (n = 5). Leptospirosis cases were reported in 21 articles describing hospital or health centre-based cohort studies. Five articles reported cases detected by passive population-based surveillance, and two articles described an active case-finding approach used in the event of a febrile disease outbreak. Non-specific febrile illness was the most frequently described inclusion criterion for cohort or surveillance studies. However, in

three hospital-based cohort studies, jaundice was the primary inclusion criterion. In one study conducted in the Democratic Republic of the Congo (DRC), leptospirosis testing was performed on a cohort of patients with haemoglobinuria.

2.3.2 Diagnostic methodologies for human studies

The Microscopic agglutination test (MAT) was the primary method of case diagnosis in the majority of human studies (89.1%; n = 46) (Appendix 1). IgM enzyme linked immunosorbent assay (ELISA) was used in a total of nine studies, most commonly as part of a multi-faceted diagnostic approach. *Leptospira* culture from blood in combination with serological diagnostics was used to diagnose infection in fifteen (32.6%) studies. Nine (19.5%) studies also used PCR detection as well as culture and serology in a three-tonged approach. Gene targets for diagnostic PCR assays included: *lbf1*(Bourhy et al., 2010, Bourhy et al., 2012), *lip*L32, 16S (*rrs*) and *ligA*.

2.3.3 Human leptospirosis prevalence

Reported leptospirosis prevalence was highly variable and differed with study design and inclusion criteria. In 11 hospital-based prospective cohort studies that recruited patients with non-specific febrile illness and used MAT with or without adjunct diagnostics tests to diagnose cases, prevalence ranged from 2.3% to 19.8%. The number of patient tested in these studies ranged from 39 to 2441 patients (median = 166) (Biggs et al., 2011, Collares-Pereira et al., 1997, de Geus et al., 1969, de Geus et al., 1977b, de Geus et al., 1977a, Forrester et al., 1969, Hogerzeil et al., 1986, Ismail et al., 2006, Murray et al., 2011, Parker et al., 2006, Tagoe et al., 2010). A hospital-based prospective cohort study of febrile patients (n = 2523) that diagnosed leptospirosis by direct pathogen detection only (PCR and culture) reported 13.7% prevalence in Mayotte (Bourhy et al., 2010). Where jaundice was the main study enrolment criterion (three studies), prevalence of acute leptospirosis ranged from 2.0% to 16.1% (n = 99 – 392 patients) (Kinebuchi and Afoakwa, 1973, Hogerzeil et al., 1986, Ismail et al., 2006). Acute leptospirosis was also reported in eight (9.8%) of 82 patients affected by an outbreak of acute pneumonia in a mining camp in DRC (Bertherat et al., 2014) and three (25.0%) of 12 patients from an outbreak of acute febrile disease in a pastoralist community in northern Kenya (Ari et al., 2011). In the cohort of patients with haemoglobinuria, only one patient (2.3%; n = 38) met leptospirosis case definitions (Delacollette et al., 1995).

2.3.4 Human leptospirosis incidence

Incidence estimates were calculated from five population-based surveillance studies (Bourhy et al., 2012, Desvars et al., 2011, Pages et al., 2014, Renault et al., 2011, Yersin et al., 1998) and two hospital-based prospective cohort studies (Biggs et al., 2013b, Pinn, 1992). A regional incidence of 75 to 102 cases per 100,000 people per year was reported for northern Tanzania (Biggs et al., 2013b). This was the only estimate of leptospirosis incidence from mainland Africa identified by systematic review. Incidence was calculated using multipliers derived from a population-based health-care utilisation survey to generate population level estimates from hospital prevalence data. More estimates were available for island countries included in the macro-geographical definition of Africa. In the Seychelles, average annual incidence was estimated as 60 to101 cases per 100,000 (Pinn, 1992, Yersin et al., 1998). In Réunion, the reported average annual incidence ranged from 3.1 to 12.0 cases per 100,000 based on a variety of different data sources (Renault et al., 2011, Desvars et al., 2011, Pages et al., 2014). In Mayotte, the reported average annual incidence was 25 cases per 100,000, calculated from data generated by four years of active hospital-based surveillance (Bourhy et al., 2012).

2.3.5 Human case reports

Sixteen case reports describing leptospirosis in 34 patients were eligible for study inclusion. Reported clinical manifestations included febrile illness, jaundice, meningitis, and acute respiratory distress. Case reports describe leptospirosis in patients from South Africa (n=6) (Gear et al., 1958, Klopper, 1969, Maze and Kirsch, 1981, Newman and Cohen, 1962, Samson and Pillay, 1966, Zaltzman et al., 1981), Gabon (n=3) (Koko et al., 2001, Perret et al., 1994, Magne et al., 2013), Morocco (n=3) (Lahsen et al., 2010, Mailloux, 1967, Mailloux, 1971), Algeria (n=1) (Aubry et al., 1975), Mali (n=1) (Mailloux et al., 1974), Réunion (n=1) (Legris et al., 2014), and Senegal (n=1) (Payet et al., 1965). With the exception of Réunion and Senegal, case reports were the only eligible articles on acute human leptospirosis in these countries.

2.3.6 Animal *Leptospira* infection studies:

Full citations and study details for eligible articles reporting animal *Leptospira* infection in Africa can be found in Appendix 2, where they are also cited in full.

Leptospira spp. infection in animals was reported by 51 eligible references describing studies performed in 17 African countries (Figure 2.2). Wild animal surveys were the most common study types (41.2%; n = 51) followed by serovar typing of *Leptospira* spp. previously isolated from naturally infected animal hosts (25.5%), livestock disease outbreaks (13.7%) and abattoir surveys (13.7%). Four references stated that human leptospirosis outbreaks were the inciting cause for animal investigations.

2.3.7 Carrier animal species:

Leptospira spp. infection was demonstrated in a large number of different animal hosts (Appendix 2) including cattle (*Bos* spp.); pigs (*Sus scrofa domestica*); goats (*Capra aegagrus hircus*); Rusa deer (*Rusa timorensis*); dogs (*Canis lupis familiaris*); cats (*Felis catus*); rodents including the African grass rat (*Arvicanthus niloticus*), African giant pouched rat (*Cricetomys gambianus*), lesser tufted-tailed rat (*Eliurus minor*), fringe-tailed Gerbil (*Gerbilliscus robustus*), rusty-bellied brush-furred rat (*Lophuromus sikapusi*), multimammate mouse (*Mastomys* sp.), house mouse (*Mus musculus*), Norway rat (*Rattus norvegicus*), black rat (*Rattus rattus*), South African pouched mouse (*Saccostomys campestris*); and a variety of other free-living mammal species including shrews (*Crocidura* spp. and *Suncus murinus*); mongoose (*Herpestes ichneumon*, *Mungo mungo* and *Paracynictis selousi*); tenrecs (see Appendix 2 for species details); and numerous bat species (see Appendix 2 for species details). Studies demonstrating infection in cattle were most common (n=20 of 51) followed by pigs (n = 8), black rats (n = 8), Norway rats (n = 7) and house mice (n = 7).

2.3.8 Diagnostic methodologies for animal studies

Culture was the most common method of diagnosis for *Leptospira* infection in animal studies (43 (84.3%) of 51 studies). PCR assays were used to demonstrate *Leptospira* spp. infection in 13 (25.5%) studies, mainly of rodents and other small mammals. Culture and PCR were used in combination to determine infection status in three studies. As with human studies, a variety of genetic targets were used to detect *Leptospira* DNA by PCR,

including *lip*L32/*hap1, sec*Y, *rrl*, and *rrs*. Only one study used PCR assays to demonstrate infection in domestic animals (Desvars et al., 2013c).

2.3.9 Prevalence in animal populations

Leptospira infection prevalence varied widely between studies. Studies that used PCR diagnosis reported higher infection prevalence than studies that relied on *Leptospira* culture and isolation. For example, *Leptospira* infection prevalence reported in black rats ranged from 11.0% to 65.8% in six studies that used PCR to detect infection (n = 33-141; median = 79) (Desvars et al., 2013c, Desvars et al., 2012, Felt et al., 2011, Houemenou et al., 2013, Rahelinirina et al., 2010, Halliday et al., 2013). Culture was also used in two of these studies (Felt et al., 2011, Rahelinirina et al., 2010). Where results could be directly compared, prevalence estimates were substantially higher by PCR (11.0%, n=100; and 28.7%, n=94) than by culture (4.0% and 3.2%). Across six unique studies of Norway rats, higher prevalence was also detected by PCR than by culture (culture: 2.7% - 8.5% (n = 130-919, median = 256) (Chadli and Bakoss, 1965, Lazuga and Bonnefous, 1962, Rademan et al., 1964)) versus PCR: 10.0% - 41.7% (n = 10-96, median = 11) (Rahelinirina et al., 2010, Houemenou et al., 2013, Halliday et al., 2013)), although the sample size was relatively smaller in studies using a PCR-based approach. In cattle however, the majority of reports used culture alone to diagnose infection. Four abattoir-based surveillance studies of cattle from Egypt, Nigeria and Zimbabwe (Feresu, 1992, Ezeh et al., 1989a, Diallo and Dennis, 1982, Hatem et al., 2014) detected renal *Leptospira* infection by culture in 1.1% to 10.4% of sampled animals (n = 74 – 625: median = 480). In a single PCR-based study, prevalence was estimated as 18.2% (n = 77) in Mayotte (Desvars et al., 2013c).

2.3.10 Animal disease

Although the study was not designed to evaluate clinical disease in animals, several studies reported overt clinical illness associated with *Leptospira* infection in animals. Clinical leptospirosis was reported in cattle and pigs in South Africa, Botswana and Kenya (de Lange et al., 1987, Gummow et al., 1999, Herr et al., 1982, Herr and Winnen, 1983, Tabel and Losos, 1979, Te Brugge and Dreyer, 1985, Van Rensburg, 1973). Abortions and poor reproductive success were the most common reported clinical signs (de Lange et al., 1987, Gummow et al., 1999, Herr et al., 1982, Herr and Winnen, 1983, Te Brugge and Dreyer, 1985, Van Rensburg, 1973). Acute non-reproductive disease of cattle (anaemia, icterus, haemoglobinuria, death) was also reported in two articles (Gummow et al., 1999, Tabel

and Losos, 1979). In three reports, chronic interstitial nephritis was reported in kidney samples collected from abattoirs (de Lange et al., 1987, Gummow et al., 1999, Hunter et al., 1987). Similar histological lesions were observed in the kidneys of two culture-positive dogs that sampled as part of a surveillance study in Egypt (Maronpot et al., 1971).

2.3.11 Serological typing of *Leptospira* from human and animal infections

Isolates belonging to 15 serogroups were reported in cohort studies conducted in the DRC (Van Riel et al., 1956), Egypt (Murray et al., 2008, Murray et al., 2011), Ghana (Hogerzeil et al., 1986), Kenya (de Geus et al., 1969, de Geus et al., 1977b, de Geus et al., 1977a) and Mayotte (Bourhy et al., 2010, Bourhy et al., 2012), and in a case report from South Africa (Maze and Kirsch, 1981) (Table 2.4). Mini and Icterohaemorrhagiae were the most commonly reported serogroups in human infections. In animal studies, isolates belonging to 12 serogroups were reported and at least one animal host was identified within Africa for 11 (73.3%) of the 15 reported human-infecting serogroups (Table 2.4). However, only six serogroups were detected in human and animal populations from the same country. These were: serogroups Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona and Pyrogenes in Egypt and serogroup Autumnalis in Kenya. Cattle were identified as carrier hosts for the largest number of *Leptospira* serogroups (n=9) but several other animal species, such as African grass rats and black rats were also identified as carrier hosts for multiple serogroups. Frequently, serogroups associated with human febrile illness were reported in several animal species. For example: serogroup Icterohaemorrhagiae, one of the most commonly reported serogroups in leptospirosis cases in Africa, was isolated from cattle, Norway rats, Egyptian mongoose and an Egyptian fox.

Table 2.4: Serogroups of *Leptospira* isolated from human and animal infections by country

	Human Studies	Animal Studies	
Serogroup	Country	Host species	Country
Australis	Kenya (de Geus et al., 1977a)	African grass rat (*Arvicanthus niloticus*)	Nigeria (Diallo and Dennis, 1982)
		Cattle (*Bos* spp.)	Zimbabwe (Feresu, 1992)
Autumnalis	Kenya (de Geus et al., 1977a)	African grass rat (*Arvicanthus niloticus*)	Kenya (Dikken et al., 1981)
Ballum	Not reported	African giant pouched rat (*Cricetomys gambianus*)	Tanzania (Machang'u et al., 2002, Machang'u et al., 2004)
		African grass rat (*Arvicanthus niloticus*)	Nigeria (Diallo and Dennis, 1982)
		South African pouched mouse (*Saccostomys campestris*)	Kenya (Dikken et al., 1981)
Bataviae	Egypt (Murray et al., 2011, Murray et al., 2008)	Cattle (*Bos* spp.)	Zimbabwe (Feresu, 1992, Feresu et al., 1999a)
		Rusty-bellied brush-furred rat (*Lophuromys sikapusi*)	Cameroon (Le Bras et al., 1977)
Canicola	Egypt (Murray et al., 2008)	Black rat (*Rattus rattus*)	Egypt (Felt et al., 2011), Madagascar (Rahelinirina et al., 2010)

	Kenya (de Geus et al., 1977a)	Brown rat (*Rattus norvegicus*)	Madagascar (Rahelinirina et al., 2010)
	South Africa (Maze and Kirsch, 1981)	Dogs (*Canis lupus familiaris*)	Egypt (Maronpot et al., 1971)
		Pigs (*Sus scrofa domesticus*)	South Africa (Van Rensburg, 1973)
Djasiman	Ghana (Hogerzeil et al., 1986)	Not reported	
Grippo-typhosa	DRC (Van Riel et al., 1956)	Black rat (*Rattus rattus*)	Egypt (Felt et al., 2011)
	Egypt (Murray et al., 2008)	Cattle (*Bos* spp.)	Kenya (Tabel and Losos, 1979), Zimbabwe (Feresu, 1992, Feresu et al., 1995)
	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)	House mouse (*Mus musculus*)	Egypt (Barsoum et al., 1973, Brownlow and Dedeaux, 1964)
Hebdomadis	DRC (Van Riel et al., 1956)	Cattle (*Bos* spp.)	Zimbabwe (Feresu, 1992, Feresu et al., 1996)
	Kenya (de Geus et al., 1969, de Geus et al., 1977b, de Geus et al., 1977a)		

Icterohaem-orrhagiae	Egypt (Murray et al., 2011, Murray et al., 2008)	Brown rat (*Rattus norvegicus*)	South Africa (Rademan et al., 1964) Tunisia (Lazuga and Bonnefous, 1962)
	Ghana (Hogerzeil et al., 1986)	Cattle (*Bos* spp.)	Egypt (Hatem et al., 2014), Tanzania (Mgode et al., 2006), Zimbabwe (Feresu, 1992, Feresu et al., 1993)
	Kenya (de Geus et al., 1969, de Geus et al., 1977b)	Egyptian fox (*Vulpes vulpes niloticus*)	Egypt (Barsoum et al., 1973)
		Egyptian mongoose (*Herpestes ichneumon*)	Egypt (Barsoum et al., 1973)
Mini	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)	Not reported	
Pomona	Egypt (Murray et al., 2011, Murray et al., 2008)	Cattle (*Bos* spp.)	Botswana (Herr and Winnen, 1983), Egypt (Hatem et al., 2014), South Africa (Gummow et al., 1999, Herr et al., 1982), Zimbabwe (Feresu, 1992, Feresu et al., 1995)
	Mayotte (Bourhy et al., 2012)	Pigs (*Sus scrofa domesticus*)	South Africa (de Lange et al., 1987, Gummow et al., 1999, Hunter et al., 1987)
Pyrogenes	Egypt (Murray et al., 2008)	Black rat (*Rattus rattus*)	Egypt (Felt et al., 2011)
	Kenya (de Geus et al., 1977a)	Cattle (*Bos* spp.)	Nigeria (Diallo and Dennis, 1982, Ezeh et al., 1989a, Ezeh et al., 1989b, Ezeh et al., 1990),

	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)		Zimbabwe (Feresu, 1992, Feresu et al., 1994)
Sejroe	Not reported	Black rat (*Rattus rattus*)	Egypt (Felt et al., 2011)
		Cattle (*Bos* spp.)	Nigeria (Ezeh et al., 1989a, Ezeh et al., 1989b) South Africa (Te Brugge and Dreyer, 1985) Zimbabwe (Feresu, 1992)
Tarassovi	DRC (Van Riel et al., 1956)	Cattle (*Bos* spp.)	Zimbabwe (Feresu, 1992, Feresu et al., 1998)
		Fringe-tailed gerbil (*Gerbilliscus robustus*)	Kenya (Dikken et al., 1981)
		Pigs (*Sus scrofa domesticus*)	Tunisia (Bakoss, 1969, Bakoss and Chadli, 1965)
Wolfii	Egypt (Murray et al., 2008)	Not reported	
***Mini/ Hebdomadis**	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)	Not reported	
***Pyrogenes/ Ballum**	Mayotte (Bourhy et al., 2012)	Not reported	

Footnotes:

* Cross-reactive isolates

2.3.12 Genetic typing of *Leptospira* from human and animal infections:

Five pathogenic *Leptospira* species were isolated from human patients with acute disease (Table 2.5). *L. interrogans* was the most commonly reported species in both human and animal studies, followed by *L. borgpetersenii* and *L. kirschneri*. Multiple animal hosts were identified for these *Leptospira* species from a variety of countries.

By country, the largest number of different *Leptospira* species was reported in Kenya, where two studies isolated leptospires belonging to five species – *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. noguchii* and *L. santarosai* (de Geus et al., 1977b, de Geus et al., 1977a). However two of these, *L. noguchii* and *L. santarosai,* were not detected in any other study in Africa. In Mayotte, four *Leptospira* species –*L. borgpetersenii*, *L. borgpetersenii*-like, *L. interrogans* and *L. kirschneri* – were identified in people, as well as in concurrent study of black rats performed during the same period (Bourhy et al., 2010, Bourhy et al., 2012, Desvars et al., 2012). Sequencing and alignment of *Leptospira* isolates from rat kidneys (Desvars et al., 2012) showed perfect identity with isolates derived from people (Bourhy et al., 2012). Divergent *Leptospira* spp. (described as *L. borgpetersenii*-like or *L. borgpetersenii* Group B) detected in human cases and small mammals in Mayotte and Madagascar (Bourhy et al., 2012, Dietrich et al., 2014, Bourhy et al., 2010, Desvars et al., 2012) have subsequently been reclassified as a new species *L. mayottensis* (Bourhy et al., 2014).

Table 2.5: Leptospira species[a] reported in human and animal infections by country

	Human Studies	Animal Studies	
Species	**Country**	**Host species**	**Country**
L. borgpetersenii	Kenya (de Geus et al., 1977b, de Geus et al., 1977a)	African grass rat (*Arvicanthus niloticus*)	Nigeria (Diallo and Dennis, 1982)
	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)	Black rat (*Rattus rattus*)	Benin (Houemenou et al., 2013) Egypt (Felt et al., 2011) Mayotte (Desvars et al., 2012)
		Cattle (*Bos* spp.)	Nigeria (Diallo and Dennis, 1982, Ezeh et al., 1989a, Ezeh et al., 1989b, Ezeh et al., 1990) South Africa (Te Brugge and Dreyer, 1985) Zimbabwe (Feresu et al., 1994)
		Comoro rousette (*Rousettus oblivious*)	Comoros (Lagadec et al., 2012)
		Fringe-tailed gerbil (*Gerbilliscus robusta*)	Kenya (Dikken et al., 1981)
		Giant African pouched rat (*Cricetomys gambianus*)	Tanzania (Machang'u et al., 2004)
		Lesser tufted-tailed rat (*Eliurus minor*)	Madagascar (Dietrich et al., 2014)
		Long-winged bats (*Miniopterus* spp)[b]	Madagascar (Dietrich et al., 2014)

		Madagascar free-tailed bat (*Otomops madagascariensis*)	Madagascar (Lagadec et al., 2012)
		Multimammate mouse (*Mastomys* sp.)	Benin (Houemenou et al., 2013)
		Pigs (*Sus scrofa domesticus*)	Tunisia (Bakoss, 1969)
		Shrew tenrecs (*Microgale* spp.)[c]	Madagascar (Dietrich et al., 2014)
		South African pouched mouse (*Saccostomys campestris*)	Kenya (Dikken et al., 1981)
***L. borgpetersenii* -like**[d]	Mayotte (Bourhy et al., 2012)	Black rat (*Rattus rattus*)	Mayotte (Desvars et al., 2012)
		Shrew tenrec (*Microgale cowani, Microgale dobsoni*)	Madagascar (Dietrich et al., 2014)
L. interrogans	Egypt (Murray et al., 2008)	African giant shrew (*Crocidura oliveri*)	Benin (Houemenou et al., 2013)
	Ghana (Hogerzeil et al., 1986)	African grass rat (*Arvicanthus niloticus*)	Nigeria (Diallo and Dennis, 1982)
	Kenya (de Geus et al., 1977b, de Geus et al., 1977a)	Asian house shrew (*Suncus murinus*)	Madagascar (Rahelinirina et al., 2010)
	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)	Banded mongoose (*Mungo mungo*)	Botswana (Jobbins et al., 2013)
		Black rat (*Rattus rattus*)	Egypt (Felt et al., 2011), Mayotte (Desvars et al., 2012), Madagascar (Rahelinirina et al., 2010)

		Brown rat (*Rattus norvegicus*)	Benin (Houemenou et al., 2013) Madagascar (Rahelinirina et al., 2010)
		Cattle (*Bos* spp.)	Botswana (Herr and Winnen, 1983) Nigeria (Diallo and Dennis, 1982) South Africa (Herr et al., 1982), Zimbabwe (Feresu et al., 1999a)
		Comoro rousette bat (*Rousettus oblivious*)	Comoros (Lagadec et al., 2012)
		House mouse (*Mus musculus*)	Kenya (Halliday et al., 2013) Madagascar (Rahelinirina et al., 2010)
		Pigs (*Sus scrofa domesticus*)	South Africa (Hunter et al., 1987, Van Rensburg, 1973)
		Rusty-bellied brush-furred rat (*Lophuromys sikapusi*)	Cameroon (Le Bras et al., 1977)
L. kirschneri	Egypt (Murray et al., 2008)	African grass rat (*Arvicanthus niloticus*)	Kenya (Dikken et al., 1981)
	Kenya (de Geus et al., 1977a)	Black rat (*Rattus rattus*)	Mayotte (Desvars et al., 2012)
	Mayotte (Bourhy et al., 2010, Bourhy et al., 2012)	Cattle (*Bos* spp.)	Kenya (Tabel and Losos, 1979), Tanzania (Mgode et al., 2006) Zimbabwe (Feresu et al., 1995)

		House mouse (*Mus musculus*)	Kenya (Halliday et al., 2013)
		Shrew (*Crocidura* spp.)	Benin (Houemenou et al., 2013)
		Streaked tenrec (*Hemicentetes nigriceps, H. semispinosus*)	Madagascar (Dietrich et al., 2014)

Footnotes:

[a]Methodology includes genetic typing of isolates, DNA sequencing following PCR detection, extrapolation of serovar data with species determined by reference to KIT *Leptospira* library.

[b] *Miniopterus* spp. include *Miniopterus gleni*, *Miniopterus goudoti, Miniopterus griffithsi, Miniopterus mahafaliensis*, *Miniopterus majori*, *Miniopterus soroculus*

[c] *Microgale* spp. include *Microgale longicaudata*, *Microgale majori*, *Microgale principula*

[d]Described as *L. borgpetersenii*-like,(Bourhy et al., 2012) *L. borgpetersenii* Group B (Desvars et al., 2012) and recently re-classified as *L. mayottensis*(Bourhy et al., 2014)

2.4 Discussion

This systematic review synthesizes and compiles an abundance of data on the epidemiology of human leptospirosis and *Leptospira* infection in animals, despite the fact that the disease is often overlooked in public health priorities in Africa. There is substantial evidence that acute leptospirosis is an important cause of febrile illness in people in Africa. As yet, few studies evaluate the population-level incidence of disease but where available, estimates indicate that incidence is high in both island and mainland populations. Three *Leptospira* species - *Leptospira borgpetersenii, L. interrogans* and *L. kirschneri* - predominate reports and a wide variety of *Leptospira* serogroups have been recognised in human and animal infections. Infection has been recognised in a wide range of domestic and wild animal species from across Africa but studies linking animal infections with acute human disease are rare.

Acute leptospirosis was diagnosed in up to 19.8% of hospital inpatients with non-specific febrile illness in studies identified by this review. In sub-Saharan Africa, recent reports have highlighted that clinical over-diagnosis of malaria may obscure other causes of febrile illness (Crump et al., 2013, Reyburn et al., 2004). The evidence synthesised here demonstrates that acute leptospirosis infection is geographically widespread across the continent. Consistent with recommendations in other tropical settings such as South America (Manock et al., 2009, Bharti et al., 2003) and South-East Asia (Suttinont et al., 2006, Gasem et al., 2009, Kendall et al., 2010), leptospirosis should be considered as an important differential diagnosis for non-specific febrile illness in Africa.

Few estimates of leptospirosis incidence in Africa could be identified by our review, revealing a major gap in research and surveillance outputs to date. The majority of incidence estimates identified came from the western Indian Ocean islands where annual incidence reports ranged from 3.1 to 101 cases per 100,000 people. In contrast, only one report of annual leptospirosis incidence was identified from mainland Africa but this estimate from Tanzania (75 to 102 cases per 100,000 people (Biggs et al., 2013b)) is consistent with both the Indian Ocean island data and the predicted figure of median African incidence (95.5 cases per 100,000) from the WHO leptospirosis burden epidemiology reference group (LERG) (World Health Organization, 2011). At present, given the lack of population level data for Africa, continental estimates of incidence should be interpreted with caution. However, all data that are available thus far indicate that the overall leptospirosis burden in Africa is likely to be high relative to other global regions. If incidence estimates identified by this review are representative of the true burden of

disease, up to 750,000 people in Africa will contract clinical leptospirosis each year, representing a substantial burden of febrile disease (Abela-Ridder et al., 2010).

Literature review has revealed three predominant *Leptospira* species and a large number of pathogenic *Leptospira* serogroups involved in human and animal infection across the continent. Animal hosts including livestock and rodents were reported for the majority of human-infecting *Leptospira* species and serogroups. The findings of this review indicate that both livestock and rodents are important in human disease transmission but that the major hosts of human-infecting serovars may vary across Africa. However, there was little geographical overlap in serogroup reports between human and animal studies. Few articles were identified that described *Leptospira* serovar diversity in human cases and animal populations from the same country. Investigations that attempted to link data on acute human leptospirosis with evidence of *Leptospira* infection in local animal populations were scarce. Studies on the island of Mayotte were the exception to this. The same genetic type of *Leptospira* (*L. borgpetersenii* Group B, now classified as *L. mayottensis* (Bourhy et al., 2014)), were detected from both human and black rat infections, implicating the black rat as the animal source of these infections (Desvars et al., 2012, Bourhy et al., 2012). This result demonstrates the value of integrating human and animal research in a so-called 'One Health approach' to identify sources and transmission routes of human leptospirosis. Considering the epidemiology of infection in both human and animal populations can provide evidence to design targeted, evidence-based intervention strategies.

A number of limitations and potential sources of bias were identified over the course of this literature review. Whilst underreporting is a substantial concern for the continent as a whole, some regions e.g. western Indian Ocean islands may be relatively over-represented in this review due to reporting or publication bias. Factors such as the level of research investment, logistical connections or disease education should be taken into consideration when assessing the relative geographic distribution of disease reports. Patient selection bias was also observed in some human studies that limited the utility of prevalence data from these sources. Methodological inclusion criteria, designed to control for the quality of diagnostic data, may have biased the selection of eligible studies towards the later decades of our review period, when technologies such as PCR became available.

Marked heterogeneity in methods and reporting criteria for serological diagnostic data was a major limiting factor. Differences in selected MAT panels prevented the meaningful synthesis and comparison of serogroup reactivity data between different human studies. Incomplete reporting was also a common reason for article exclusion during full-text

review and hence valuable disease data may have been missed as a result. Other methodological limitations include the use of the broad geographical database search term (‘Africa’) rather than individual country names and the large number of non-English language articles that were identified. The inclusion of non-English language articles allowed data published in local language journals (e.g. in Afrikaans in South Africa and French in western Africa) or during the European colonial era to be evaluated. Wherever possible, articles were translated by a study author in close partnership with a proficient language speaker. However, it is possible that some eligible studies may have been overlooked for inclusion due to translation limitations. Finally, the nature of the systematic review methodology means that only the most relevant and rigorous studies were included in this review. Additional information on infecting *Leptospira* serogroups and species may also exist in references that were not identified by our database searches or did not meet the study case definitions for acute human leptospirosis for example.

Therefore, the data on human leptospirosis included in this review is probably only the tip of the proverbial iceberg of the burden of disease in Africa. Generating good quality, representative data on the incidence and burden of leptospirosis on the continent will be a major challenge for future research. Systematic review studies such as this one can help to raise awareness of the human health threat among researchers and policy makers. However, surveillance remains limited due to lack of awareness of the disease amongst clinicians and poor accessibility to diagnostic tests (Soors et al., 2013, Rutherford et al., 2010, Petti et al., 2006). For medical clinicians, the non-specific nature of the typical symptoms of patients with acute leptospirosis poses a substantial diagnostic challenge in developing countries where laboratory capacity rarely exists to diagnose the infection (Crump et al., 2013, Cruz et al., 2009, McBride et al., 2005). Hence, increasing clinician awareness of the diagnosis and treatment of alternative causes of non-malarial fever should be a priority in resource-limited settings (Crump et al., 2011a). Identifying risk factors for human disease could also help to identifying patient groups at high risk of *Leptospira* infection, and hence target surveillance and control strategies towards these groups.

Linkages between *Leptospira* infections in people and animals are rarely addressed in the existing literature in Africa yet human and animal *Leptospira* infections are inextricably linked. Knowledge of animal hosts of human-infecting *Leptospira* types is critical to identify sources of infection and understanding the epidemiology, transmission and control of leptospirosis in each setting (Adler and de la Pena Moctezuma, 2010, Levett, 2001). In

the future, greater emphasis should be placed on performing multidisciplinary human and animal leptospirosis studies where a human disease problem in recognised. Linking investigations of animal reservoir populations with confirmed human cases may improve our understanding of the role of different animal species in the transmission of pathogenic *Leptospira* serovars in diverse environmental settings (Hartskeerl et al., 2011, Cleaveland et al.). Using an integrated 'One Health' may also provide an invaluable opportunity to explore the direct and indirect impacts of animal *Leptospira* infection on human health in Africa (Mazet et al., 2009, Zinsstag et al., 2011).

Finally, this review reveals that livestock are important hosts of *Leptospira* infection in Africa, and may play a more substantial role in human disease transmission than is widely recognised. Furthermore, the clinical and sub-clinical productivity impacts of *Leptospira* infection in domestic animal populations in Africa are poorly understood. Clinical disease associated with *Leptospira* infection was identified by this review (Chapter 2.3.10). Various *Leptospira* serovars of economic importance are described around the world and infection can be associated with production losses in a variety of livestock species (O'Doherty et al., 2015, Cortizo et al., 2015, Gummow et al., 1999, Ellis, 1994). More than 300 million of the world's poorest people live in Africa, and at least 60% of this population are at least partially dependent on livestock for their livelihood (Grace et al., 2012b). Evaluating the impact of *Leptospira* infection on livestock health and productivity as well as human health is therefore an important priority for prospective leptospirosis research in Africa. Control of *Leptospira* infection in livestock species has considerable potential to directly and indirectly improve human health and well-being in Africa, through reduced zoonotic disease transmission and increased productivity in livestock that subsistence farming communities depend upon (Halliday et al., 2015, O'Doherty et al., 2015, Cortizo et al., 2015, Hartskeerl et al., 2011).

3 Core Methods

3.1 Research questions, objectives and study components

The following components of this thesis aim to explore animal sources for human *Leptospira* infection in the Kilimanjaro Region of northern Tanzania. Focusing on two groups of animal host known to be important in other settings, the following research questions were addressed:

1) What are the patterns of *Leptospira* infection in rodents and ruminant livestock (cattle, sheep and goats) in this area?

2) Which *Leptospira* species and subtypes infect rodents and ruminant livestock in the Kilimanjaro Region?

3) Which *Leptospira* species and subtypes are responsible for acute leptospirosis in people in the Kilimanjaro Region?

4) Can molecular and serological information about infecting *Leptospira* types be used to infer the source of human infection in this area?

3.2 Study site

The United Republic of Tanzania, comprising Tanganyika (Tanzania Mainland) and Zanzibar has a total population of 44.9 million (Tanzania National Bureau of Statistics, 2012) and is projected to become the second most populous country in sub-Saharan Africa by 2050 based on current growth rates. The country is organised into 30 geographical regions, with 25 of these on the Tanzanian mainland. This study was based in the Kilimanjaro Region of northern Tanzania (Figure 3.1), which was selected on the basis of previous studies demonstrating that a high proportion of severe febrile illness in people can be attributed to acute leptospirosis (Crump et al., 2013).

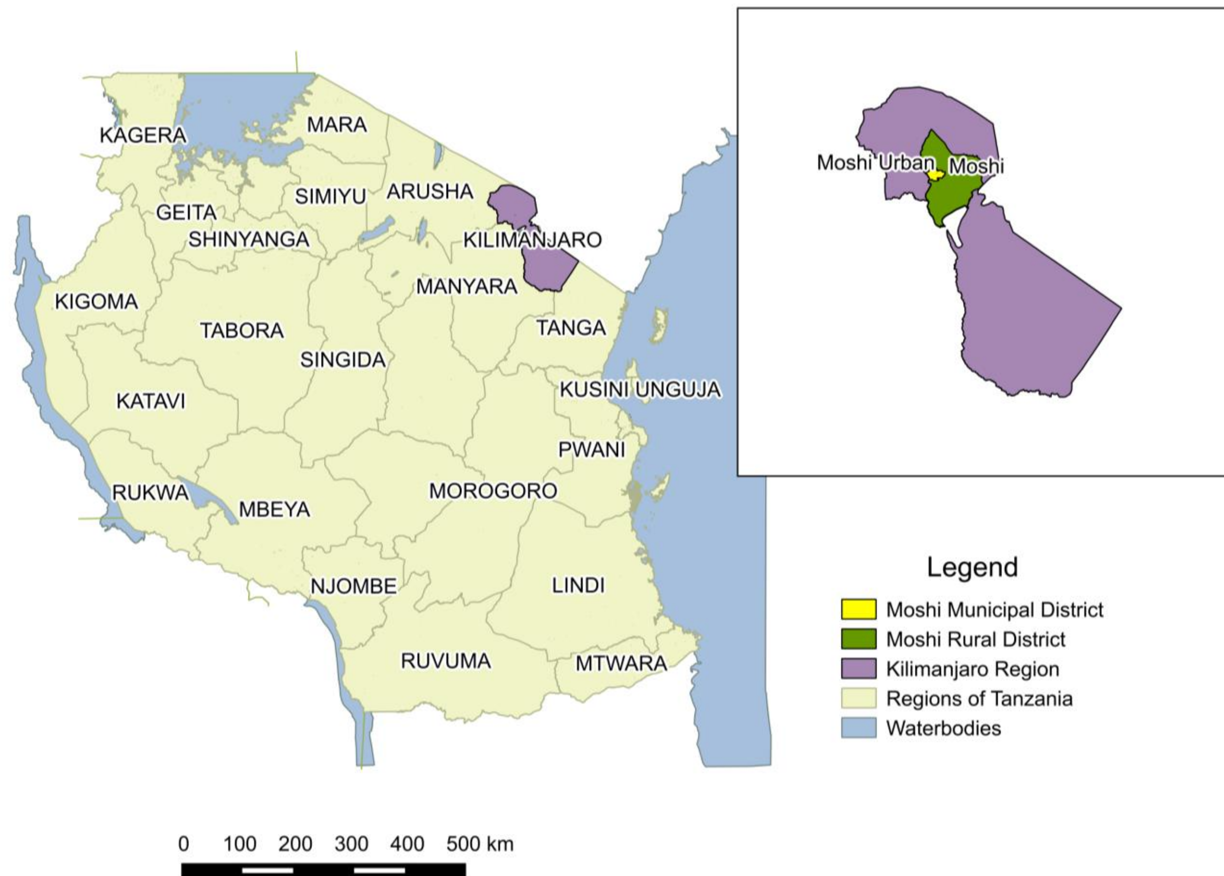


Figure 3.1: Map of Tanzania showing the administrative regions of Tanzania (main map) and the location of the Moshi Municipal and Rural Districts within the Kilimanjaro Region (insert)

The Kilimanjaro region has a population of 1.64 million with an average intercensal annual population growth rate of 1.8% (national average: 2.7%; 2002-2012). The population density of the region is estimated as 124 people per km^2 (national average: 51 per km^2) and 20.9% of the population are classified as urban (Tanzania National Bureau of Statistics, 2012, Tanzania National Bureau of Statistics, 2002). The climate in the region typically follows a pattern of long rains from March to May and short rains from October to December with the coolest months coinciding with the long dry season from June to September.

The Kilimanjaro Region is further sub-divided in seven administrative districts (Figure 3.2). Two study districts; Moshi Municipal and Moshi Rural Districts were chosen as the core study site for their proximity to hospitals (Mawenzi Regional Hospital (MRH) and Kilimanjaro Christian Medical Centre (KCMC)) involved in previous and on-going febrile disease surveillance studies and to cover both urban and rural populations.

Moshi Municipal District is the administrative centre of the Kilimanjaro region. The district has a population of 184,292 across living in 21 wards (an organisational unit of consisting of approximately 10 villages – see Figure 3.2 for more details). The Tanzania National Census (Tanzania National Bureau of Statistics, 2012) classifies all wards in this district as urban, although many people grow crops and keep small numbers of livestock, particularly poultry for personal use. The average household size in this district is 4.0 people (national average = 4.8 people per household) (Tanzania National Bureau of Statistics, 2012).

Moshi Rural District has a population of 466,737 people (Tanzania National Bureau of Statistics, 2012) across 31 wards (average household size: 4.2 people). The wards in this district are predominantly classified as rural (Tanzania National Bureau of Statistics, 2012) and characterised by small scale farming systems including a mix of agriculture and smallholder livestock farming. The environment ranges from lush high-altitude mountainous regions, where coffee, bananas and avocados dominate cash crop production, to drier low-altitude pasture land and plains where mainly maize and beans are cultivated. In both environments, livestock is typically kept in small numbers and usually confined to zero-grazing units or tethered.

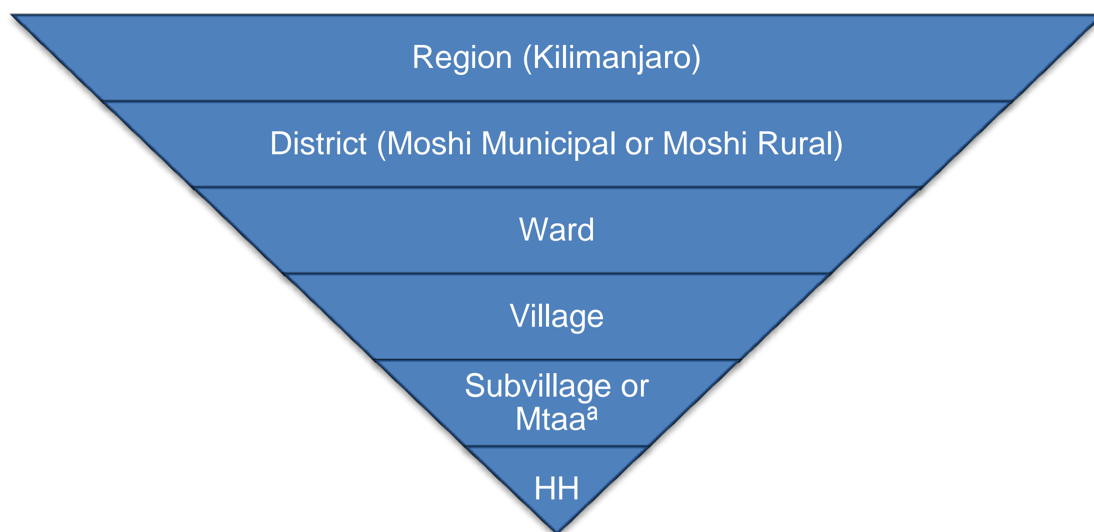


Figure 3.2: Hierarchy of Tanzanian administrative units used in this study

[a]Subvillage or Mtaa are the names used in rural or urban areas respectively; HH = household.

Both study hospitals are located within the Moshi Municipal District and are the main hospitals for the provision of health-care to the population of the Kilimanjaro Region. Mawenzi Regional Hospital (MRH) is 300-bed hospital that provides primary hospital care, whereas Kilimanjaro Christian Medical Centre (KCMC) is a larger, 458-bed referral hospital that provides tertiary care to several regions in the north of the country. The catchment area for these hospitals covers a diverse range of agro-ecological settings including small to moderate sized urban communities (e.g. Moshi), agro-pastoral communities living on the slopes of Kilimanjaro and the surrounding plains (e.g. villages within the districts of Moshi Rural) and pastoral, nomadic communities (e.g. from the region of Arusha).

Aetiological febrile disease surveillance has been performed at these hospitals on two occasions. The first study was performed on a cohort of 870 febrile patients between 2007 and 2008 (Crump et al., 2013). The results of this study were the inciting cause for this project and have been outlined in Chapter 1.3 of the general introduction. This study was also the source of data and samples analysed in Chapter 7. A second period of febrile disease surveillance was performed from February 2012 to May 2014. A total of 1,115 patients were recruited into this study. In parallel, a cross-sectional field study of human and linked livestock populations was also implemented in the Kilimanjaro and Arusha Regions (Figure 3.1) to explore risk factors for human and animal exposure to leptospirosis and other bacterial zoonoses (BacZoo Study; 2013 to 2014). At the time of analysis,

laboratory testing of these data were not available for inclusion in this study. However, preliminary results are included for context in individual chapter discussions.

3.3 Studies to explore *Leptospira* infection in northern Tanzania

Studies undertaken by this PhD are divided into three complementary strands of work, exploring *Leptospira* infection in three linked populations in the core study area:

i. Cross-sectional study of peri-domestic rodents (Chapter 5);
ii. Abattoir surveillance of ruminant livestock (Chapter 6);
iii. Febrile patients with serologically confirmed acute leptospirosis (Chapter 7).

The following methods are considered core methodology for the following study components and are compiled for reference. Evaluation and validation of study diagnostic methodology can be found in Chapter 4.

3.4 Ethical clearance

The field protocol was reviewed and approved in Tanzania by the Commission for Science and Technology (COSTECH) Certificate No. 2012-471-ER-2005-141; National Institute of Medical Research (NIMR), Tanzania (protocol number: NIMR/HQ/R.8a/Vol.IX/1499) and the Tanzanian Wildlife Research Institute (TAWIRI). Ethical approval for human sampling and testing was granted from the Kilimanjaro Christian Medical Centre (KCMC) Research Ethics Committee, NIMR and the Institutional Review Boards of Duke University Medical Center and the United States for the Centers for Disease Control. In the UK, the University of Glasgow College of Medicine, Veterinary Medicine and Life Sciences Ethics Committee (protocol number: 200120020) and University of Glasgow Faculty of Veterinary Medicine Ethics and Welfare Committee (Ref. 01a/13 & 02a/13) also approved the study protocol.

3.5 Sample collecting and processing

This section describes the processing and testing procedures common to multiple sample types or study components. Details of the design of each study component and methods for sample collection can be found in Chapters 5, 6 and 7.

3.5.1 Kidney sample collection and pre-processing

Kidney samples were collected from a cross-sectional survey of rodents (Chapter 5) and a slaughterhouse survey of cattle, sheep and goats (Chapter 6). For rodents, one whole kidney was placed into 70-96% ethanol immediately after sterile harvesting and stored at ambient temperature for up to one year prior to processing. For ruminants, a 1 x 2 cm piece of kidney tissue was dissected across the cortico-medullary junction using a sterile blade following surface sterilisation with a flamed blade. Kidney tissue was placed into 70-96% ethanol immediately after harvesting and stored at ambient temperature for up to one year prior to processing.

3.5.2 Urine sample collection and pre-processing

Urine samples were collected by cystocentesis directly from the urinary bladder following slaughter of ruminant livestock in the abattoir. Urine samples were stored at -80°C. Samples were thawed at room temperature and then heat-inactivated at 67°C for one hour prior to DNA extraction.

3.5.3 Human sample collection and pre-processing

Archived plasma and urine samples were also available from patients with acute leptospirosis (Chapter 7). These were not heat treated prior to DNA extraction.

3.6 DNA Extraction from samples for detection of *Leptospira* infection

DNA extraction was performed using the QIAamp DNA Blood Mini Kit spin-column protocol (Qiagen, Maryland, USA). Modifications (described in full below) were made to the standard protocols to improve DNA extraction efficacy following recommendations of expert laboratories (WHO/FAO/OIE Collaborating Leptospirosis Laboratory, Royal Tropical Institute, Amsterdam (KIT); and Moredun Research Institute, Edinburgh).

3.6.1 Extraction of DNA from pure *Leptospira* isolates

After a minimum of ten passages, 1ml *Leptospira* culture (2.5-5 x 10^8 cells/ml) was centrifuged at 13,000 rpm for 20 minutes and the supernatant discarded. The pellet was suspended in to a mix of 180µl Qiagen® lysis buffer (AL) and 20µl proteinase K and incubated at 56°C for one to two hours and mixed regularly by pulse vortex. Then 200µl AL buffer (200µl) was added to each sample and mixed. Finally, 200µl absolute ethanol was added before proceeding to the standard spin column extraction protocol (Chapter 3.6.5).

3.6.2 Extraction from kidney tissue

Approximately 25 milligrams (mg) kidney tissue was dissected across the cortico-medullary junction from each kidney sample and diced finely using a sterile scalpel blade and petri-dish that was changed for each sample. The dissected tissue was added to a mix of 180µl Qiagen® tissue lysis buffer (ATL) and 20µl proteinase K, mixed vigorously by pulse vortex and incubated for three hours at 56°C until the tissue was completely lysed. For ruminant tissues, 4µl Rnase A (100mg/ml; Qiagen, Maryland, USA) was added to each sample and incubated at room temperature (RT) for two minutes before proceeding with DNA extraction. Then 200µl AL buffer was added to each sample, which was mixed again and incubated at 70°C for ten minutes to dissolve any residual precipitate. Finally, 200µl ethanol was added to each sample before proceeding with the standard spin column procedure (Chapter 3.6.5).

3.6.3 Extraction from urine

DNA was extracted from urine using an extraction protocol optimised to maximise the yield of bacterial DNA from urine samples (Ruth Zadoks and Ian Heron, personal communication). Briefly, up to 1000µl of urine was centrifuged at 10,000xG for ten minutes. The supernatant was discarded and the remaining pellet was re-suspended in 200µl Tris-EDTA (TE) buffer (Biotechnology grade, pH 8.0; VWR International Ltd, Magna Park, Lutterworth UK). The centrifuge step was repeated to wash the pellet and the supernatant discarded. The washed pellet was then re-suspended in an enzyme mix containing 50µl lysozyme (10mg/ml), 50µl mutanolysin (1mg/ml) and 4µl lysostaphin (1mg/ml) made up to a final volume of 200µl with TE buffer, and incubated at 37°C for one hour. Subsequently, a solution containing 180µl AL buffer and 20µl proteinase K were added to each sample and incubated at 56°C for 1 hour to complete digestion. Finally, 200µl absolute ethanol was added before proceeding to the standard spin column extraction protocol (Chapter 3.6.5).

3.6.4 DNA extraction from human plasma

Frozen plasma samples were thawed in a Class 2 Biological Safety Cabinet (BSC) for one hour at room temperature. For DNA extraction, 400µl plasma was added to a mix of 360µl AL buffer and 40µl proteinase K and incubated at 56°C for ten minutes. Then 200µl absolute ethanol was added before proceeding to the standard spin column extraction protocol (Chapter 3.6.5).

3.6.5 Standard spin column procedure

The following steps were used for all extraction protocols and sample types.

Following the addition of ethanol, the resulting mixture was then applied to a QIAamp® DNA Mini spin column and processed according to manufacturer's recommendations (Qiagen, 2012). Each mixture/column was incubated for three minutes at room temperature before centrifugation at 8000 rpm for one minute. The filtrate was discarded. Each spin column was washed with 500µl Qiagen® wash buffer 1 (AW1) and incubated for two minutes at room temperature before centrifugation at 8000 rpm for one minute. The filtrate was discarded. Then 500µl Qiagen® wash buffer 2 (AW2) was added to each spin column, which was incubated for three minutes at room temperature before centrifugation at 13,000

rpm for one minute. The filtrate was discarded and the spin column was centrifuged at 13,000 rpm for four minutes and allowed to dry at ambient temperature.

Final elution was performed in two stages with 50% final volume of Qiagen® elution buffer (AE) added to each spin column before incubation at room temperature for three minutes before centrifugation at 13,000 rpm for three minutes. This step was repeated with a reduced incubation time of one minute. Final elution volumes were 200µl for tissue samples and *Leptospira* isolates; 60µl for urine samples, and 100µl for plasma samples.

DNA was quantified using a NanoDrop spectrophotometer (ThermoScientific, Waltham, MA) for kidney samples and the Qubit® Fluorometer (ThermoScientific) using the Qubit® dsDNA High Sensitivity assay kit. DNA extracts were stored at -20°C prior to qPCR testing.

3.7 qPCR protocols for the detection of pathogenic *Leptospira* samples

3.7.1 *sec*Y SYBR® Green qPCR assay

The *sec*Y SYBR® Green assay was run at WHO/FAO/OIE Collaborating Leptospirosis Reference Laboratory, at the Royal Tropical Institute, Amsterdam (KIT) (Ahmed Ahmed (AA). The protocol was run as published on Biorad CFX96 qPCR platform, using the primer set *sec*YIVF/*sec*YIV to amplify a 202 base-pair (bp) fragment (Table 3.1)(Ahmed et al., 2009). Test wells with a threshold cycle (Ct) value of ≤ 35 and a melt temperature TaqMan® 79-84°C were considered positive (Ahmed et al., 2012).

3.7.2 *lip*L32 TaqMan® qPCR assay

The *lip*L32 TaqMan® qPCR assay was run on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) following published protocols (Stoddard et al., 2009, Stoddard, 2013). Amplification of a 245 bp product was performed using the primer set lipL32-45F and lipL32-286R (Table 3.1), and a 19bp 5'FAM®-labelled probe with a 3'BHQ quencher dye.

Table 3.1: Primer and probe sequences used for the amplification and detection of *Leptospira* DNA by qPCR

Reference	Primer	Sequence
Ahmed et al. (2009)	*sec*YIVF	5'-GCG ATT CAG TTT AAT CCT GC-3
	*sec*IV	5'-GAG TTA GAG CTC AAA TCT AAG-3'
Stoddard et al. (2009)	*lip*L32-45F	5'-AAG CAT TAC CGC TTG TGG TG-3'
	*lip*L32-286R	5'-GAA CTC CCA TTT CAG CGA TT-3'
	Probe: *lip*L32-189P	FAM-5'-AA AGC CAG GAC AAG CGC CG-'3-BHQ1
Smythe et al. (2002)	16S (*rrs*) Lepto F	5'-CCC GCG TCC GAT TAG-3'
	16S (*rrs*) Lepto R	5'-TCC ATT GTG GCC GR$^{A/G}$A CAC-3'
	Probe: 16S Lepto P	FAM-5'-CTC ACC AAG GCG ACG ATC GGT AGC-3'TAMRA

The following reaction conditions were used: 1x Platinum® qPCR Supermix-UDG (Invitrogen, Carlsbad, CA), 500nmol/L forward (lipL32-45F) and 500nmol/L reverse (lipL32-286R) primers; 100nmol/L probe (lipL32-189P) and 5µl DNA extract for each sample in a total reaction volume of 25µl. The amplification protocol consisted of: a pre-incubation step at 50°C for two minutes; denaturation at 95°C for ten minutes; followed by 45 amplification cycles (95°C for 15 seconds, 60°C for 60 seconds) with fluorescence acquisition performed at the end of each cycle. The reaction was run with the addition of low concentration ROX (50nmol/L) to normalise fluorescent reporter signal from December 2014 onwards, following re-calibration of the ABI 7500 qPCR platform.

Reactions were run using MicroAmp Optical 96-well reaction plates (Invitrogen, Carlsbad, CA). On each plate, PCR grade water was used as a negative control and DNA extracted from a pure culture of *L. interrogans* serovar Copenhagenii Strain Wijnberg (KIT Biomedical Research, Amsterdam, NL) was used as a positive control. Reaction profiles were analysed using Applied Biosystems 7500 System Sequence Detection (SDS) Software Version 1.2.4 (Applied Biosystems, Carlsbad, CA 2001-2004). Ct values were calculated using the automated baseline and threshold cycle (Ct) parameters, set by the

SDS software and manually adjusted as necessary. Test wells were considered positive where a Ct value of < 40 was obtained.

3.7.3 Human endogenous process control for the *lip*L32 TaqMan® qPCR assay

The published *lip*L32 TaqMan® qPCR assay protocol also includes methodology for a control qPCR reaction against an endogenous human *rnaseP* gene, designed to control for DNA extraction efficiency and PCR inhibition in individual samples (Stoddard, 2013, Stoddard et al., 2009). For human plasma and urine specimens tested in this thesis, a single replicate of the *rnaseP* was run for each sample on the same reaction plate as the two *lip*L32 test reactions.

rnaseP TaqMan® qPCR assay was performed on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using the primer set *rnase*P-F and *rnase*P-R as published (Stoddard, 2013, Stoddard et al., 2009). Amplification was detected through the use of a 24-bp 5'FAM®-labelled probe with a 3'BHQ quencher dye. The qPCR assay was run using: 1x Platinum® qPCR Supermix-UDG (Invitrogen, Carlsbad, CA), 400nmol/L forward (*rnase*P-F) and 400nmol/L reverse (*rnase*P-R) primers; 120nmol/L probe (*rnase*P) and 5µl DNA extract for each sample in a total reaction volume of 25µl. Low concentration ROX (50nmol/L) was added to normalise background fluorescence signal as per manufacturer's recommendations. The amplification was run under the same reaction conditions as the *lip*L32 qPCR reaction (Chapter 3.7.2) with fluorescence acquisition performed at the end of each cycle. Test wells were considered positive where a Ct value of < 40 was obtained.

3.7.4 16S (*rrs*) TaqMan® qPCR assay

The 16S (*rrs*) TaqMan® qPCR assay was performed on the ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) following published methodology (Smythe et al., 2002). Amplification of an 87-bp product was performed using the primers 16S LeptoF and 16S LeptoR, and detected with a 24-bp 5'FAM®-labelled probe with a 3'TAMRA quencher dye (Table 3.1).

The qPCR assay was run using the following conditions: 1x TaqMan® Universal PCR Mastermix (containing ROX^{TM} Passive Reference dye) (Invitrogen, Carlsbad, CA), 600nmol/L forward (LeptoF) and 600nmol/L reverse (LeptoR) primers; 400nmol/L probe

(16S LeptoP) and 5µl DNA extract for each sample in a total reaction volume of 25µl. The amplification protocol consisted of: pre-incubation at 50°C for two minutes; denaturation at 95°C for ten minutes; followed by 40 amplification cycles (95°C for 15 seconds, 60°C for 60 seconds) with fluorescence acquisition performed at the end of each cycle.

Reactions were run using MicroAmp Optical 96-well reaction plates as described for *lip*L32 assay. Reaction profiles and Ct values were analysed as described above. Test wells were considered positive where a Ct value of < 40 was obtained.

3.8 Culture and isolation of *Leptospira* from animal kidneys

Culture for *Leptospira* bacteria was performed from a subset of rodents, cattle and goats in collaboration with Marga Goris (MG) and Rudy Hartskeerl (RH) at KIT. Selection of animals for culture is described in Chapters 5 and 6.

For each sample, approximately 25 mg of kidney tissue was dissected across the cortico-medullary junction using a sterile scalpel blade following surface sterilisation with a flamed blade. On a sterile surface the capsular surface was removed and the remaining sample was finely diced and then added to 1ml Ellinghausen-McCullough-Johnson-Harris (EMJH) media supplemented with 5-Fluorouracil (5-FU). The kidney-EMJH solution was agitated using a sterile 1ml syringe to form a fine homogenate. A serial ten-fold dilution series (1:10, 1:100, 1:1000) was then made in three 5ml tubes of EMJH-5FU (1:10 dilution) and mixed by gently inverting the vial three times. Each aliquot was sealed and labelled with the animal identification number, dilution and date. Aliquots were stored at room temperature (18-22°C) for up to four weeks prior to shipment to KIT.

Upon arrival, aliquots of culture media were incubated at 30°C and examined for *Leptospira* growth by dark-field microscopy every four weeks for three months and then again after six months of incubation (MG and KA). The presence of non-*Leptospira* organisms, such as fungi, yeast and other bacteria was recorded. Samples positive for *Leptospira* were sub-cultured in EMJH media prior to serological and genetic typing (see Chapter 3.10).

3.9 Serological typing of *Leptospira* isolates

Serological characterisation of pathogenic *Leptospira* isolates was performed at KIT (MG and KA) following internationally agreed guidelines. Isolates were typed after ten *in vitro* passages in EMJH media. The agglutination characteristics for each isolate were determined in two stages: 1) using the new isolate as an antigen, a microscopic agglutination test (MAT) was performed using a panel of rabbit serum representative of all known pathogenic serogroups; and 2) typing using a panel of monoclonal antibodies (mAbs) (raised in BALB/c mice) to define a serovar-specific antigenic profile for each isolate (Hartskeerl et al., 2006, Faine, 1994).

3.10 Sequence-based typing of *Leptospira* isolates and non-isolate samples

3.10.1 Single locus sequence typing of *Leptospira*

For isolates: single locus sequence typing of a ~600bp fragment of the *sec*Y gene was performed at KIT (AA and KA) based on the protocol described by Victoria et al. (2008). Briefly, amplifications were performed using 10µl genomic DNA (gDNA) template in a 25µl reaction containing 160nmol/L *sec*YII and *sec*YIV primers (Table 3.2). All PCR assays included a non-template control (PCR grade water). PCR products were visualised by gel electrophoresis on a 1.5% agarose gel and purified using the QIAquick® PCR Purification Kit following to manufacturer's instructions (Qiagen, Maryland, USA). PCR products were sequenced at local facilities and compared to a database for reference *Leptospira* serovars (Victoria et al., 2008) for species determination.

Table 3.2: Primers and annealing temperatures used for *Leptospira* species typing PCR assays

Gene target	Primer name	Primer sequence*	Annealing Temp.
***sec*Y** (Victoria et al., 2008)	secYII	5'-GAA TTT CTC TTT TGA TCT TCG-3'	54°C
	secYIV	5'-GAG TTA GAG CTC AAA TCT AAG-3'	
***sec*Y** (Dietrich et al., 2014)	secYFd	5'-ATG CCG ATC ATY TTY GCT TC-3'	52°C
	secYR3	5'-TTC ATG AAG CCT TCA TAA TTT CTC A-3'	

*Mixed or 'Wobble' base definitions: Y = C or T

For qPCR positive samples, sequence analysis of the infecting *Leptospira* species was performed using single locus amplification and sequencing of a ~ 470 bp segment of the *sec*Y gene using primers modified for use with non-isolate samples from the East African region (Madagascar and Mayotte) (Table 3.2)(Dietrich et al., 2014). Assays were run at the University of Aberdeen (Mark Moseley (MM) and KA). PCR conditions were run following published protocols optimized by PCR platform (Moseley et al., *in preparation*). Amplifications were performed using 5µl genomic DNA (gDNA) template in a 25µl reaction containing 500nmol/L secYFd and secYR3 primers (Table 3.2). All PCR assays included a non-template control (PCR grade water) and a positive control of DNA extracted from a pure isolate of *L. interrogans* or *L. borgpetersenii*. PCR products were visualised by gel electrophoresis on a 1.5% agarose gel and purified using the QIAquick® PCR Purification Kit following to manufacturer's instructions (Qiagen, Maryland, USA). Purified product was quantified using a Nanodrop ND1000 spectrophotometer (ThermoScientific, Massachusetts, USA) and sequenced by Eurofins Genomics GmbH (Ebersburg, Germany).

3.10.2 Multi-locus sequence typing

Multi-locus sequence typing (MLST) was performed at KIT (AA) following a seven loci typing scheme (Boonsilp et al., 2013). DNA was extracted from pure cultures of each isolate after ten *in vitro* passages. Sequences from the PCR amplicons were compared to references sequences available through an online MLST database (previously available at http://leptospira.mlst.net, accessed 19th June 2015; now available at http://pubmlst.org/leptospira/)(Jolley and Maiden, 2010, Jolley and Maiden, 2016). *Leptospira* species and serovar were determined by allelic profile generation.

3.11 Summary

A summary flow chart of diagnostic testing performed on different sample types is shown in Figure 3.3. The results of sampling, diagnostic testing and *Leptospira* sequence typing are presented and discussed in the following chapters.

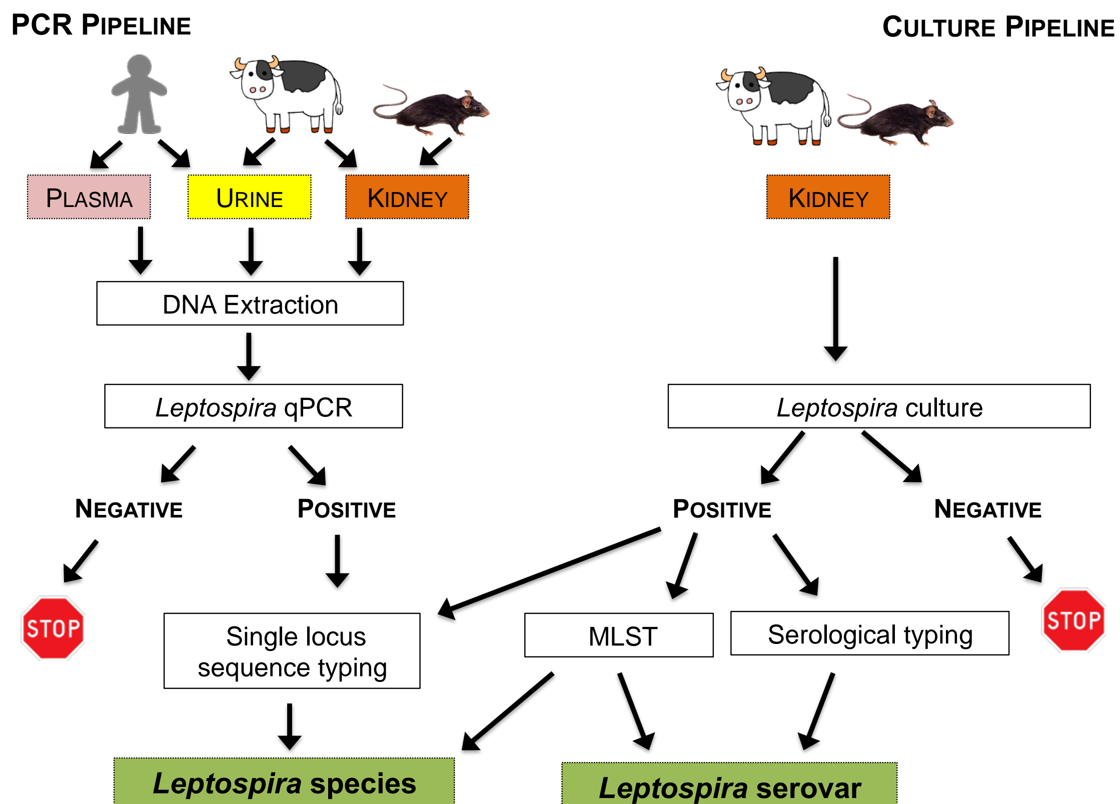


Figure 3.3: Flow chart of diagnostic testing approaches used for different species and sample types in this thesis

4 Validation of core study methods

4.1 Introduction to molecular approaches for the diagnosis of *Leptospira* infection

Molecular methods, such as nucleic acid amplification (NAA) are increasingly being used in the diagnosis of *Leptospira* infection. Assays mostly rely on one of two NAA-based approaches - traditional (end-point) polymerase chain reaction (PCR) or real-time polymerase chain reaction (qPCR) – both of which are designed to specifically amplify a specific gene fragment of target pathogen (Biassoni and Raso, 2014). PCR-based diagnosis of *Leptospira* infection has a number of advantages of more traditional methods of *Leptospira* detection. PCR is fast and typically more sensitive than culture, which may take several months to yield positive results (Faine, 1994, Levett, 2001, Picardeau et al., 2014). qPCR can be used to quantify the bacterial load in an infection (Agampodi et al., 2012), and sequence analysis of PCR or qPCR products also offers the opportunity to obtain pathogen genetic information from (Agampodi et al., 2013, Boonsilp et al., 2011). Whilst PCR is also subject to a number of limitations (Chapter 1.7.1), these approaches are have proved useful in both clinical and research settings and hence were selected as the major diagnostic test in this study. This chapter describes the selection of a PCR-based diagnostic assay for the diagnosis of *Leptospira* infection in rodent, ruminant livestock and people in this study, based on evaluation of the literature and validation of the analytical performance of each test in controlled conditions.

4.1.1 Assessing the performance of PCR-based diagnostic assays

The goal for a PCR-based test for *Leptospira* infection is to maximise the number of true positive infections diagnosed by the test (i.e. diagnostic sensitivity), whilst minimising the number of false positives detected (i.e. diagnostic specificity). In general, PCR-based diagnostic assays are evaluated by comparison of their analytical and diagnostic sensitivity and specificity.

Analytical sensitivity is used in the initial assessment and validation of a diagnostic test. It is defined as the dilution of a known quantity of pathogen DNA below which the diagnostic assay fails to detect $\geq$ 95% of replicates (World Organisation for Animal Health (OIE), 2008). It is assessed *in vitro* using serial dilutions of purified pathogen DNA to

determine the lowest detectable number of genome copies or organisms within a reaction. This is termed the lowest limit of detection (LLOD).

Diagnostic sensitivity is used to assess the performance of a test under "real-world" conditions. It is defined as the proportion of truly infected individuals detected from a population of people or animals (Thrusfield, 1995). Evaluation of diagnostic sensitivity uses clinical or field samples from a population of known infection status (evaluated previously using a gold standard or reference test) to calculate the proportion of 'true' positives that particular diagnostic assay can detect. As the calculation of diagnostic sensitivity relies on patient or animal samples it may be more sensitive to bias introduced by sample characteristics or study design than analytical sensitivity.

Analytical specificity is also used in the initial assessment and validation of a diagnostic test. It is defined as the ability of a diagnostic assay to detect a particular target organism when present in a sample (World Organisation for Animal Health (OIE), 2008). For the diagnosis of *Leptospira* infection, analytical specificity is required both to the genus, to differentiate *Leptospira* from other bacterial organisms, and within the genus to detect only a subset of *Leptospira* species with pathogenic properties (Picardeau et al., 2014). In laboratory evaluations of analytic specificity, validation studies usually test a wide range of *Leptospira* types as well as a range of other microorganisms.

Diagnostic specificity is also used to evaluate the performance of a test in clinical or field samples from a human or animal population. It is defined as the proportion of true negative patients or animals that are detected from a test population and is particularly important in a clinical setting where false positive test results may give rise to inappropriate treatment or over-estimation of a disease burden (Saah and Hoover, 1997, Thrusfield, 1995).

Together the sensitivity and specificity of a test can be used to select a robust diagnostic approach for disease surveillance. For *Leptospira* detection, the analytical and diagnostic performance of a variety of different qPCR assays has been described in the literature.

4.1.2 PCR-based assays for diagnosis of *Leptospira* infection in people and animals

A variety of PCR assays are available for use in the diagnosis of *Leptospira* infection in people and animals. Available assays target either conserved *Leptospira* housekeeping genes where sequence-specific primers are used to differentiate between pathogenic and non-pathogenic species, or genes encoding specific *Leptospira* virulence factors, which are

found only in the pathogenic species. Traditional PCR protocols for the diagnosis of *Leptospira* infection were first described in the early 1990s (Gravekamp et al., 1993, Merien et al., 1992). These have subsequently been superseded by qPCR assays, which offer automated detection and quantification of the amplification reaction through the use of a fluorescent reporter dye that accumulates exponentially alongside the target PCR product (Biassoni and Raso, 2014). Automated detection of amplification increases the analytical sensitivity of qPCR assays over traditional assays often down to the level of a single genomic equivalent (GE) (Ahmed et al., 2009). Two main technologies are used to facilitate automatic detection of DNA amplification in qPCR assays, either SYBR® Green dye, a fluorescent dye that binds double-stranded DNA (dsDNA) PCR products or sequence-specific TaqMan® fluorescent-labelled probes, which generate detectable fluorescence after binding to a complementary PCR product (Applied Biosystems, 2014, Biassoni and Raso, 2014). TaqMan® assays generally offer greater analytical specificity than SYBR® Green assays, which are prone to false positives due to the non-specific nature of their dsDNA binding properties.

4.1.3 Review of qPCR protocols for the detection of pathogenic *Leptospira* infection in people and animals

A number of qPCR assays have been described for the detection of pathogenic *Leptospira* DNA in clinical samples (Stoddard et al., 2009, Picardeau et al., 2014). Three qPCR assays developed and validated at international leptospirosis reference laboratories were selected for detailed analytical evaluation.

i) *sec*Y SYBR® Green qPCR assay

The *sec*Y SYBR® Green qPCR assay was developed at the WHO/OIE/FAO Leptospirosis Reference Laboratory, Royal Tropical Institute (KIT), Amsterdam (Ahmed et al., 2009). The assay targets the *Leptospira sec*Y gene, a housekeeping gene located on the large leptospiral chromosome (cI) that encodes a pre-protein translocase important for the export of protein across the cytoplasmic membrane (Haake and Levett, 2015, Durack et al., 2015). The analytical sensitivity of the *sec*Y SYBR® Green qPCR assay is reported as 1.0 genomic equivalents (GE) for *L. interrogans,* 1.2 GE for *L. santarosai* and 1.5 GE for *L. weilli.*

Table 4.1: Comparison of analytical specificity of diagnostic qPCR assays for pathogenic *Leptospira* spp.

Intermediate *Leptospira* species are also shown for comparison of test specificity to the pathogenic species. Key: (1) Detected by qPCR assay in validation studies; (0) not detected by qPCR assay in validation studies; (-) not tested in validation studies

qPCR Assay	*sec*Y SYBR® Green qPCR		*lip*L32 TaqMan® qPCR			16S (*rrs*) TaqMan® qPCR	
Reference	(Ahmed et al., 2009)	(Bourhy et al., 2011)	(Stoddard et al., 2009)	(Bourhy et al., 2011)	(Thaipadungpanit et al., 2011)	(Smythe et al., 2002)	(Thaipadungpanit et al., 2011)
Pathogenic spp.							
L. alstonii[*]	1	1	-	1	1	-	1
L. alexanderi	1	1	1	1	1	-	1
L. borgpetersenii	1	1	1	1	1	1	1
L. interrogans	1	1	1	1	1	1	1
L. kmetyi	-	0	-	1	-	-	-
L. kirschneri	1	1	1	1	1	1	1
L. noguchii	1	1	1	1	1	1	1
L. santarosai	1	1	1	1	1	1	1
L. weilii	1	1	1	1	1	1	1
L. mayottensis[†]	-	0	-	1	-	-	-

Intermediate spp.							
L. broomii	0	0	0	0	-	-	-
L. fainei	0	0	0	0	-	1	-
L. inadai[‡]	0/1	0	0	0	0	0	1
L. licerasiae	0	0	-	0	-	-	-
L. meyeri[§]	1	0	0	0	0	1	0
L. wolfii	-	0	-	0	0	-	1

[*] Also called Genomospecies 1
[†] Recently reclassified from a divergent *L. borgpetersenii* type to a new species (Bourhy et al. (2014)
[‡] For *L. inadai;* 0/1 denotes varying qPCR results are obtained across validation studies using different strains and serovars
[§] Classified as saprophytic by some sources; different serovars used across validation studies.

The assay shows a high degree of specificity to the *Leptospira* genus but also amplifies some intermediate *Leptospira* species (*L. inadai* and *L. meyeri*) reducing the overall test specificity for detection of pathogenic *Leptospira* (Table 4.1). Independent validation trials have highlighted that this approach may fail to detect some of the more recently described pathogenic species, specifically *L. kmetyi* and *L. mayottensis* (Bourhy et al., 2014, Bourhy et al., 2011). Melt curve analysis is needed to determine whether an observed amplification is specific to pathogenic *Leptospira* species (Ahmed et al., 2009).

Despite some limitations, the *sec*Y SYBR® Green qPCR assay is used routinely in the diagnosis of acute human leptospirosis in the Netherlands. Compared to culture alone, the diagnostic sensitivity and specificity are reported as 100% and 93% in this population (Ahmed et al., 2009). The assay has been used to demonstrate *Leptospira* infection in epidemiological studies of rodents and ruminant populations in a variety of countries including Kenya, Morocco and Benin (Halliday et al., 2013, Houemenou et al., 2013, Benkirane et al., 2014).

ii) *lip*L32 TaqMan® qPCR Assay

The *lip*L32 gene, which is also located on chromosome cI, encodes an outer membrane lipoprotein specific to pathogenic *Leptospira* species that is thought to play an important role in virulence (Haake et al., 2000, Murray, 2013). A TaqMan® qPCR assay targeting this gene was developed and validated at the United States Centers for Disease Control, Georgia (Stoddard et al., 2009, Galloway and Hoffmaster, 2015). A human-specific internal control assay using the human *rnaseP* housekeeping gene has also been described for use with this assay (Stoddard et al., 2009).

All published validation studies for the *lip*L32 TaqMan® qPCR assay have demonstrated a high degree of analytical specificity to pathogenic *Leptospira* serovars (Table 4.1), but figures for the analytical sensitivity vary by study. From the original CDC validation study, the reported 95% LLOD ranged from 20 to 50 GE/µl *L. interrogans* depending on the qPCR platform used (Stoddard et al., 2009). A ten-fold improvement was reported following additional optimisation of the protocol (Galloway and Hoffmaster, 2015). However, an independent validation study reported the repeatable detection limit of one leptospire (1.0 GE) per reaction (Bourhy et al., 2011), and in direct comparisons, the *lip*L32 TaqMan® qPCR assay was more sensitive than either the *sec*Y SYBR® Green assay (Ahmed et al., 2009) or a third qPCR assay targeting the *lfb*1 gene (Merien et al., 2005). This variation in reported analytical sensitivity may be a consequence of

heterogeneity in reporting methodology, but the sensitivity of this assay is also influenced by differences in the choice of PCR platform (Stoddard et al., 2009) or the presence of a passive reference dye to normalise background fluorescence (Galloway and Hoffmaster, 2015). These factors should therefore also be considered when validating the use of this assay in other settings.

The *lip*L32 TaqMan® qPCR assay is commonly used in both clinical and research applications. The assay has been used to provide early diagnosis for acute human leptospirosis in a variety of tropical settings including Mayotte (Bourhy et al., 2010), Thailand (Bourhy et al., 2010, Thaipadungpanit et al., 2011) and Uruguay, where the assay was able to detect infection in the acute phase of illness in 30% (n = 85) of leptospirosis cases confirmed by demonstration of seroconversion by MAT (Gonzalez et al., 2013). The assay has also been used for epidemiological surveillance of a range of animal species including rodents in Canada and South-East Asia (Himsworth et al., 2013, Cosson et al., 2014), dogs in Ireland (Rojas et al., 2010) and livestock and wildlife on the island of La Réunion (Desvars et al., 2013c).

iii) 16S (*rrs*) TaqMan qPCR assay

The 16S (*rrs*) TaqMan® qPCR assay developed at the WHO/OIE/FAO Leptospirosis Reference Laboratory, Queensland Health Scientific Services, Brisbane, was the first diagnostic *Leptospira* qPCR assay to be described in the literature (Smythe et al., 2002). The assay targets the *Leptospira* 16S rRNA (*rrs*) gene, which is a housekeeping gene is common to all bacteria and used to define the taxonomy of different bacterial genera (Levett, 2015). Two copies of the *rrs* gene are located in chromosome cI in pathogenic and saprophytic *Leptospira* species (Picardeau et al., 2008). The 16S diagnostic assay is therefore designed based on regions of the *rrs* gene that are conserved between pathogenic species but not in non-pathogenic or intermediate species (Smythe et al., 2002). The analytical sensitivity is reported as approximately 2 GE for the two most common *Leptospira* species, *L. interrogans* and *L. borgpetersenii* (Smythe et al., 2002). However, the 16S (*rrs*) assay is not entirely specific to pathogenic *Leptospira* species and amplification of intermediate *Leptospira* species, including *L. fainei, L. inadai, L. meyeri* and *L. wolfii,* has been demonstrated in some validation studies (Table 4.1) (Thaipadungpanit et al., 2011, Smythe et al., 2002).

The 16S (*rrs*) assay has been used in epidemiological surveillance of *Leptospira* in wild animal populations including flying foxes in Australia (Cox et al., 2005) and bats and rodents in the Western Indian Ocean islands (Lagadec et al., 2012, Dietrich et al., 2014). As a human diagnostic test, the 16S (*rrs*) TaqMan® qPCR assay has been used widely in tropical settings including Australia (Smythe et al., 2002), Mayotte (Bourhy et al., 2012), Peru (Ganoza et al., 2010) and Thailand (Thaipadungpanit et al., 2011). In Sri Lanka, the 16S (*rrs*) assay was able to detect infection in the acute phase of illness in 51% (n = 105) of leptospirosis cases confirmed by demonstration of seroconversion by MAT (Agampodi et al., 2012). In a case-control study of leptospirosis cases diagnosed by culture and/or a positive MAT results (based on seroconversion or a single titre ≥ 1:400) performed in Thailand, the 16S (*rrs*) assay showed higher diagnostic sensitivity than the *lip*L32 assay (56% vs. 43%, p<0.001) but lower test specificity (90% vs. 93%, p = 0.06) (Thaipadungpanit et al., 2011).

Whilst all three diagnostic tests are used widely in human and animal studies, data from validation studies indicates that some differences in test sensitivity may exist, which may also be influenced by the testing conditions or sample type. Therefore, independent evaluation indicated to provide a better understanding of the sensitivity and performance of selected diagnostic tests for our purposes.

4.1.4 Objectives

The overall goal of this study component was to evaluate several qPCR approaches to select an appropriate diagnostic test for the detection of *Leptospira* infection in people and animals from Tanzania. Assays were performed at the University of Glasgow. The following specific study objectives were addressed:

Objective 1: Measure and compare the analytical sensitivity for:

i) *lip*L32 TaqMan® qPCR assay under different reaction conditions

ii) 16S (*rrs*) TaqMan® qPCR assay under standard reaction conditions;

Objective 2: Evaluate the effect of sample inhibition on the *lip*L32 TaqMan® qPCR assay;

Objective 3: Compare the diagnostic performance of the *lip*L32 TaqMan® qPCR assay (run at the University of Glasgow) with the *sec*Y SYBR® Green qPCR assay run at the WHO/OIE/FAO Leptospirosis Reference Laboratory, KIT.

4.2 Methods

4.2.1 Preparing *Leptospira* DNA titration series

Validation studies were performed using genomic DNA (gDNA) extracted from:

i) pure culture of *L. interrogans* serovar Copenhagenii Strain Wijnberg obtained from the WHO/FAO/OIE Leptospirosis Reference Laboratory at the Royal Tropical Institute, Amsterdam (KIT).

ii) pure culture of *L. borgpetersenii* serovar Hardjo isolated during this project from cattle in Tanzania (Chapter 6).

The starting genomic DNA (gDNA) concentration was determined using the Qubit® dsDNA High sensitivity assay kit. A ten-fold dilution series was prepared using PCR grade water to a minimum concentration of 10^0 genome copy per microlitre.

4.2.2 Calculating genomic equivalence (GE)

The number of genomic equivalents in a sample was calculated using the following formula (URI Genomics & Sequencing Center, 2004):

$$GE = \frac{\text{(amount of DNA (ng) * Avogadro's number)}}{\text{length of the template (base-pairs) x } (1\text{x}10^9) \text{ x average weight of 1 mol (bp)}}$$

Where:

- Avogadro's number: 6.022×10^{23} molecules/mole
- The molecular weight of any double-stranded DNA template is estimated by taking the product of its length (in bp) and 650g (the average weight of 1 mol bp).
- The genomic length of *L. interrogans* and *L. borgpetersenii* is estimated as 4,627 Kilobases (Kb) and 3,391 Kb respectively (Adler and de la Pena Moctezuma, 2010, Nascimento et al., 2004).

4.2.3 Diagnostic cut-offs and case definitions

Positive case definitions or cut-offs for positive test results were predefined as:

***sec*Y:** A minimum of two of three replicates amplified with Ct ≤ 35, and a melt temperature (Tm) between 79°C and 84°C (Ahmed et al., 2012)

***lip*L32:** A minimum of one or two replicates amplified with Ct ≤ 40 (Stoddard et al., 2009)

16S (*rrs*): A minimum of one of two replicates amplifies with Ct ≤ 40 (Smythe et al., 2002)

4.2.4 Effect of ROX on analytical sensitivity of the *lip*L32 TaqMan® qPCR assay

To measure the effect of adding ROX on the performance of the *lip*L32 TaqMan® qPCR assay, a titration series of DNA extracted from *L. interrogans* serovar Copenhagenii was made from a starting concentration of 0.133 ng/µl, equivalent to ~ 10^5 GE of *L. interrogans*. Each dilution step was run in triplicate a) without ROX (Stoddard et al., 2009) and b) with the addition of ROX to normalise baseline fluorescence (Galloway and Hoffmaster, 2015). Assays were run on a single plate to minimise the introduction of between-run variability.

4.2.5 Analytical sensitivity of *lip*L32 TaqMan® qPCR assay

Starting DNA concentrations for assessment of *lip*L32 TaqMan® qPCR performance were 1.71 x 10^{-2} ng/µl for *L. interrogans* and 2.32 x 10^{-2} ng/µl *L. borgpetersenii*, equivalent to ~ 10^4 GE. The qPCR assay was performed as described in Chapter 3.7.2. Nine replicates of each DNA dilution step were tested, divided equally across three different plates and reaction runs, each with three replicates of a particular titration step. Three negative control replicates using PCR grade water were also used on each test plate.

4.2.6 Analytical sensitivity of the 16S (*rrs*) TaqMan® qPCR assay

Starting DNA concentrations for assessment of 16S (*rrs*) TaqMan® qPCR performance were curves were 1.71 x 10^{-2} ng/µl for *L. interrogans* and 2.32 x 10^{-2} ng/µl *L. borgpetersenii*, equivalent to ~ 10^{4} GE. The qPCR assay was performed as described in Chapter 3.7.4. Eight replicates of each DNA dilution step were tested on three different plates and reaction runs (Plate 1 and 2 = 3 replicates each; Plate 3 = 2 replicates only). Three negative control replicates using PCR grade water were also used on each test plate.

4.2.7 Assessing sample inhibition on the *lip*L32 TaqMan® qPCR assay

The effect of sample inhibition on the *lip*L32 TaqMan® PCR assay was evaluated by spiking DNA extracted from rodent kidneys with known concentrations of DNA extracted from *Leptospira interrogans* serovar Copenhagenii. Samples were selected to cover the typical range of DNA concentrations obtained following DNA extraction from 25 milligrams of rodent kidney (Chapter 3.6.2). A ten-fold titration series was made as described above (Chapter 4.2.1) using the DNA extracts in place of PCR grade water.

Table 4.2: Details of rodent kidney DNA extracts used to test for PCR inhibition

Sample ID	Rodent species	DNA concentration
R0355	*Rattus rattus*	367 ng/µl
R0379	*Rattus rattus*	244 ng/µl
R0387	*Mus* sp.	120 ng/µl

qPCR tests were run in triplicate alongside a standard curve using the same titration series but diluted in PCR-grade water. Negative controls were also run in triplicate for each DNA extract and for water. All PCR tests were run in the same plate to avoid between-batch variations.

4.2.8 Comparison of diagnostic performance of *sec*Y SYBR® Green qPCR assay and *lip*L32 TaqMan® qPCR assay in rodent tissue samples

A double-blinded trial was performed to compare the diagnostic sensitivity and specificity of the *sec*Y SYBR® Green assay run at WHO/FAO/OIE Collaborating Leptospirosis Reference Laboratory, Royal Tropical Institute, Amsterdam (KIT) (AA) and the *lip*L32 TaqMan® qPCR assay run at the University of Glasgow (KA). Ethanol-fixed rodent kidney samples were available from 26 *Rattus norvegicus* of known *Leptospira* infection status trapped in the Netherlands between 2011 and 2013. DNA was extracted at the University of Glasgow using the protocol described in Chapter 3.6.2. Each sample was tested in duplicate using the *lip*L32 TaqMan® qPCR assay as described in Chapter 3.7.2, and in triplicate using the *sec*Y SYBR® Green assay (Chapter 3.7.1)(Ahmed et al., 2009).

4.2.9 Statistical analysis

Statistical analyses and plots were performed in R (R Core Team, 2015). A standard curve for each qPCR assay and test *Leptospira* species was plotted using the log of the number of GE in each reaction as the explanatory variable. The analytic sensitivity for each *Leptospira* spp. was defined as the lowest dilution detectable in 100% of qPCR replicates (100% LLOD).

The Ct value was considered the primary outcome variable for all analyses of diagnostic test performance. Multivariable linear models were performed to explore the statistical significance of candidate explanatory variables of the Ct value. Variables considered included the $\log_{10}$ transformed GE, ROX status of the test (ROX included or not), *Leptospira* species, test batch (either test plate or dilution series in the case of inhibition studies) and qPCR assay (*lip*L32 or 16S). A forward step-wise method for model building was used and models were compared using likelihood ratio tests (LRT) with a significance cut-off of $p \leq 0.05$. Interaction terms were included in the models and retained if significant.

For the double-blinded control study, the results of the *lip*L32 TaqMan® qPCR assay was compared to *sec*Y SYBR® Green qPCR assay run at the reference laboratory. Test agreement was assessed using the Cohen's *kappa* statistic.

4.3 Results

4.3.1 Effect of ROX on analytical sensitivity of the *lip*L32 TaqMan® qPCR assay

The analytical sensitivity (LLOD) for the *lip*L32 assay with and without ROX at which 100% of replicates were positive was 1.33 GE (Table 4.3). Under both conditions, a single replicate (n = 1/3) was positive at the 1.33 x 10^{-1} GE concentration (data not shown).

Table 4.3: Comparison of the *lip*L32 assay run with and without ROX using DNA extracted from *L. interrogans*

Starting DNA concentration ng/µl	Number of GE per reaction	Mean Ct ROX (n = 3)	Ct SD ROX	Mean Ct No ROX (n=3)	Ct SD NoROX
1.33 x 10^{-1}	1.33 x 10^{5}	17.1	0.227	17.9	0.137
	1.33 x 10^{4}	20.4	0.0834	21.1	0.080
	1.33 x 10^{3}	23.7	0.321	24.2	0.335
	1.33 x 10^{2}	26.9	0.165	27.4	0.550
	1.33 x 10^{1}	30.3	0.206	30.6	0.628
	1.33 x 10^{0}	33.5	1.38	35.3	0.372

A linear relationship was demonstrated between the number of GE and measured Ct value within the tested range. By linear regression, both the $\log_{10}$-transformed GE and presence of ROX were statistically significant explanatory variables for Ct values observed (Table 4.4). Every ten-fold increase in the test $\log_{10}$ GE was associated with a reduction in the Ct value of 3.23 cycles (95% CI: -3.31 – -3.15). The addition of ROX into the reaction was estimated to reduce the Ct value by 0.518 (95% CI: -0.74 – -0.29). There was an indication of a weak interaction effect between ROX status but the addition of this interaction did not significant improve the model fit ($p = 0.086$).

Table 4.4: Logistic regression model of the effect of GE and ROX on the *lip*L32 TaqMan® qPCR assay

Variable	Level	Coefficient	95% CI	t-value	p-value	n
Intercept		34.3	34.0 – 34.6	240	< 0.001	
Log10 GE		-3.23	-3.31 – -3.15	-83.8	< 0.001	18
ROX Status:	NoROX	ref				9
	ROX	-0.52	-0.74 – -0.29	-4.75	< 0.001	9

Adjusted R^2 = 0.996; LRT statistic = 2.01, df = 1; p < 0.001

4.3.2 Analytical sensitivity of *lip*L32 TaqMan® qPCR assay

The analytical sensitivity (100% LLOD) of the *lip*L32 TaqMan® qPCR assay with ROX was measured as 17 genome copies of *L. interrogans* and 32 genome copies of *L. borgpetersenii* per reaction (Table 4.5). At a further ten-fold dilution step, six (66.7%) out of nine replicates were positive for *L. interrogans* (1.7 genome copies per reaction), and seven (77.8%) out of nine replicates were positive for *L. borgpetersenii* (3.2 genome copies per reaction).

A linear relationship was demonstrated between $\log_{10}$GE and observed Ct value up to the 100% LLOD (Figure 4.1). By linear regression, both the $\log_{10}$GE and the test batch were significant explanatory variables and included in the final model (Table 4.6). Every ten-fold increase in the test $\log_{10}$ GE was associated with a reduction in the Ct value of -3.44 (95% CI: -3.45 – -3.39). A significant difference was observed in Ct values between batches but the magnitude of the effect was small (< 1 cycle).

Table 4.5: Mean Threshold Cycle (Ct) for a) *lip*L32 TaqMan® qPCR assay and b) 16S (*rrs*) TaqMan® qPCR assay run with a standard ten-fold titration series with DNA extracted from pure isolates of i) *L. interrogans* and ii) *L. borgpetersenii*

			a) *lip*L32 qPCR		b) 16S qPCR	
***Leptospira* species**	**Starting DNA concentration (ng/µl)**	**GE per reaction**	**Mean Ct (n = 9)**	**SD (Ct)**	**Mean Ct (n = 8)**	**SD (Ct)**
i) *L. interrogans*	1.71×10^{-2}	1.71×10^{4}	22.8	0.355	23.9	1.03
		1.71×10^{3}	26.2	0.458	28.7	1.07
		1.71×10^{2}	29.9	0.524	33.3	1.22
		1.71×10^{1}	33.0	0.627	38.1	1.22
		1.71×10^{0}	36.3*	0.729	NA	NA
		1.71×10^{-1}	NA	NA	NA	NA
ii) *L. borgpetersenii*	2.32×10^{-2}	3.16×10^{4}	21.9	0.356	23.2	1.34
		3.16×10^{3}	25.4	0.307	27.8	1.35
		3.16×10^{2}	28.9	0.363	32.8	1.28
		3.16×10^{1}	32.2	0.395	37.5	1.51
		3.16×10^{0}	35.5**	1.03	39.8***	NA
		3.16×10^{-1}	NA	NA	NA	NA

*n= 6 of 9 replicates amplified; ** n = 7 of 9 replicates amplified; *** n = 1 of 8 replicates amplified

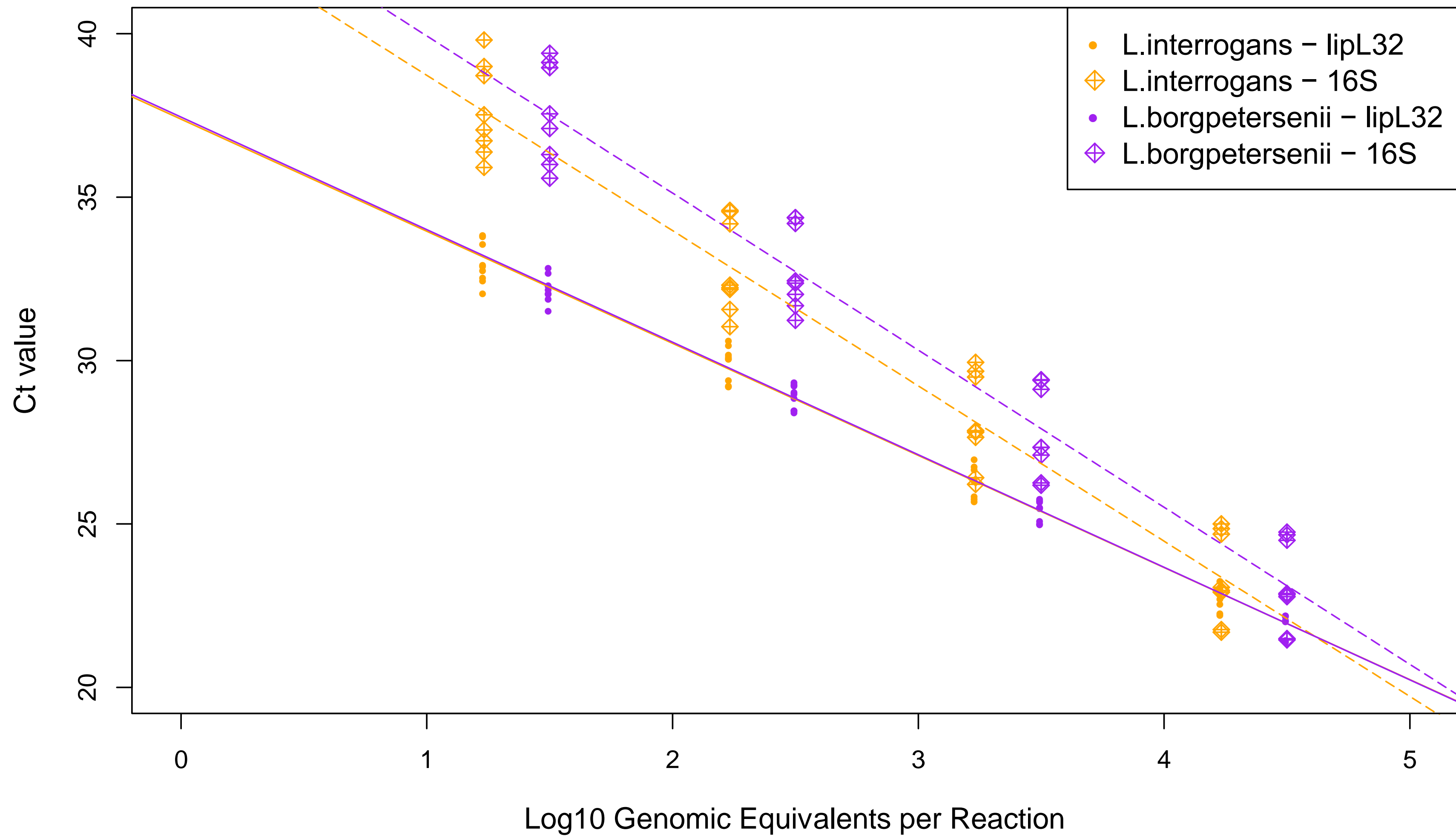


Figure 4.1: Standard curve for a) *lip*L32 TaqMan® qPCR assay and b) 16S (*rrs*) TaqMan® qPCR assay based on a ten-fold titration series of DNA extracted from pure isolates of (i) *L. interrogans* and (ii) *L. borgpetersenii*

Table 4.6: Logistic regression model for *lip*L32 TaqMan® qPCR assay

Variable	Level	Coefficient	95% CI	t-value	p-value	n
Intercept		37.5	37.3 – 37.6	513	< 0.001	
Log10 GE		-3.44	-3.45 – -3.39	-163	< 0.001	72
Batch	1	ref				24
	2	-0.53	-0.64 – -0.41	-9.12	< 0.001	24
	3	0.39	0.27 – 0.51	6.74	< 0.001	24

Adjusted R^2 = 0.997; LRT statistic = 10.2, df = 1; p < 0.001

4.3.3 Analytical sensitivity of 16S (*rrs*) TaqMan® qPCR assay

The analytical sensitivity (100% LLOD) of the 16S (*rrs*) TaqMan® assay was measured as 17 genome copies of *L. interrogans* and 32 genome copies of *L. borgpetersenii* per reaction (Table 4.5). Below 10^1 GE, amplification was only observed for one (12.5%) out of eight *L. borgpetersenii* replicates, and none of the *L. interrogans* replicates.

A linear relationship was demonstrated between $\log_{10}$GE and observed Ct value up to the 100% LLOD (Figure 4.1). By linear regression the $\log_{10}$GE, *Leptospira* species and the test batch were significant explanatory variables and included in the final model (Table 4.7). Every ten-fold increase in the test $\log_{10}$ GE was associated with a reduction in the Ct value of 4.78 cycles (95% CI: -4.97 – -4.71). The Ct values for *L. interrogans* were significantly lower with the 16S (*rrs*) assay than Ct values for *L. borgpetersenii*. A significant difference was also observed in Ct values between batches.

Table 4.7: Logistic regression model for 16S (*rrs*) TaqMan® qPCR assay

Variable	Level	Coefficient	95% CI	t-value	p-value	n
Intercept		44.1	43.9 – 44.4	333	< 0.001	
Log10 GE		-4.77	-4.85 – -4.71	-133	< 0.001	64
***Leptospira* species**	*borgpetersenii*	ref				32
	interrogans	-1.10	-1.26 – -0.94	-13.6	< 0.001	32
Batch	1	ref				24
	2	2.06	1.89 – 2.25	22.2	< 0.001	24
	3	-1.02	-1.22 – -0.81	-9.80	< 0.001	16

Adjusted R^2 = 0.997; LRT statistic = 19.1, df = 1; p < 0.001

4.3.4 Comparison of analytical sensitivity and reliability between *lip*L32 and 16S qPCR assays

Finally, data from both qPCR assays were compiled into a final model to explore the effect of test on Ct value (Table 4.8), and compare the analytic sensitivity between the two assays. Although the 100% LLOD of detection was equivalent for the two assays, at concentrations < 10 GE per reaction, the *lip*L32 assay was able to detect 13 (72.2%) of 18 reactions compared to the 16S (*rrs*) assay, which detected only 1 (6.25%) of 16 reactions replicates. By linear regression the log_{10}GE, *Leptospira* species and *lip*L32 assay were significant explanatory variables and were included in the final model. Statistically significant interaction effects were observed between the qPCR test type and the log_{10}GE, and the qPCR test type and the *Leptospira* species. The overall effect of batch was variable in the final model.

Table 4.8: Final multivariable linear model for Ct value (both qPCR assays)

Variable	Level	Coefficient	95% CI	t-value	p-value	n
Intercept		44.4	43.7 – 45.1	127	< 0.001	
Log_{10} GE		-4.78	-4.97 – -4.58	-48.5	< 0.001	136
Test	16S (*rrs*)	ref				64
	*lip*L32	-7.19	-8.09 – -6.28	-15.7	< 0.001	72
***Leptospira* species**	*borgpetersenii*	ref				68
	interrogans	-1.10	-1.54 – -0.66	-4.97	< 0.001	68
Batch	1	ref				48
	2	0.77	0.41 – 1.12	4.29	< 0.001	48
	3	-0.12	-0.49 – -0.25	-0.64	0.53	40
Log_{10}GE*Test[*lip*L32]		1.34	1.07 – 1.61	9.92	< 0.001	-
Species[Int]*Test[*lip*L32]		1.08	0.47 – 1.68	3.53	< 0.001	-

Adjusted $R^2 = 0.965$; LRT statistic = 9.69, df = 1; $p < 0.001$

The final multivariable linear model was used to predict Ct values to compare the relative sensitivity of the qPCR assays within the range of the experimental data (i.e. between 1.5 and 4.0 log_{10}GE). Predicted Ct values were consistently lower for the *lip*L32 assay than those obtained from the 16S (*rrs*) assay used as the reference test for both *L. interrogans* and *L. borgpetersenii*. The greatest magnitude of effect was observed for reactions with low numbers of genomic copies (Table 4.9).

Table 4.9: Predicted Ct values for the *lip*L32 and 16S (*rrs*) TaqMan® qPCR assays using the final multivariable linear model (Table 4.8)

Leptospira spp.	Test	Ct values	
		1.5 Log_{10}GE	4.0 Log_{10}GE
L. interrogans	*lip*L32	32.2	23.7
	16S (*rrs*)	36.4	24.4
L. borgpetersenii	*lip*L32	32.3	23.7
	16S (*rrs*)	37.5	25.5

4.3.5 Assessment of qPCR inhibition in rodent samples spiked with *L. interrogans* DNA

A significant linear relationship was demonstrated with the *lip*L32 TaqMan® qPCR assay between log_{10} GE and measured Ct value in all three rodent kidney DNA preparations as well as the water control titration series (Figure 4.2). Recorded Ct values for two samples, R0355 and R0379 were significantly different from the water control (Table 4.10). In both cases, spiked rodent samples showed statistically significantly lower Ct values than the standard curve. However, the effect size was in the region of 0.5 to one Ct in both cases.

Table 4.10: Logistic regression model to assess PCR inhibition in the *lip*L32 TaqMan® qPCR assay

Variable	Level	Coefficient	95% CI	t-value	p-value	n
Intercept		11.4	10.8 – 12.0	38.0	< 0.001	57
Log10 GE		-3.03	-3.12 – -2.94	-64.4	< 0.001	57
Sample	H_2O	ref				13
	R0355	-0.93	-1.30 – -0.55	-4.94	< 0.001	14
	R0379	-0.44	-0.81 – -0.06	-2.36	< 0.05	15
	R0387	-0.16	-0.53 – -0.21	-0.878	0.38	15

Adjusted $R^2 = 0.987$; LRT statistic = 6.79; $p < 0.001$

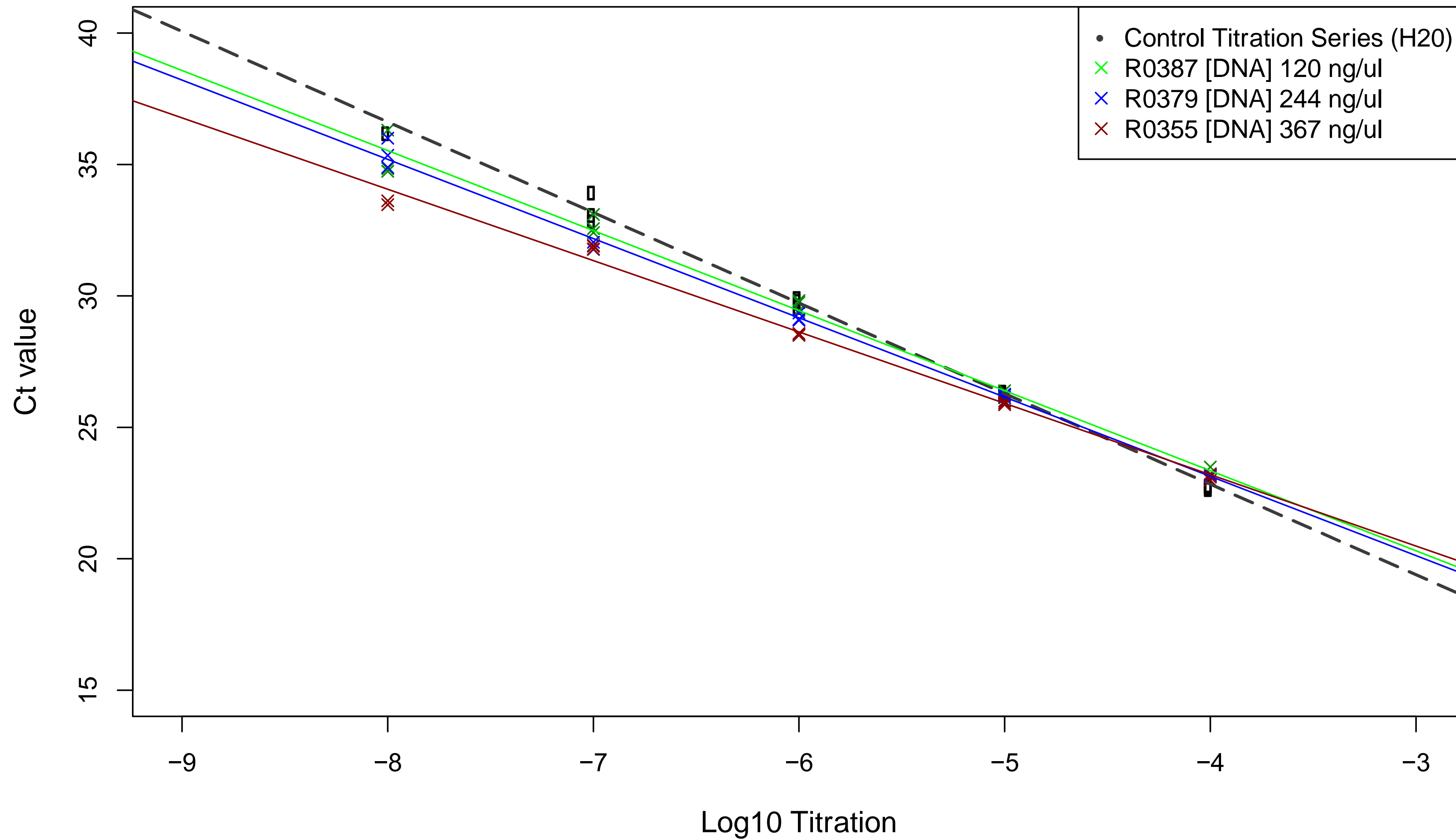


Figure 4.2: Observed Ct values for rodent kidney DNA extracts spiked with *L. interrogans* DNA compared with a standard control titration series (PCR-grade water)

4.3.6 Comparison of diagnostic performance of *sec*Y SYBR® Green qPCR assay and *lip*L32 TaqMan® qPCR assay in Dutch rodent kidneys

Comparing results from *sec*Y SYBR® Green qPCR assay and *lip*L32 TaqMan® qPCR assay in Dutch rodent kidneys, the two tests showed almost perfect agreement (Cohen's kappa statistic: 0.917 (95% CI: 0.758 – 1.08) (Thrusfield, 1995). *Leptospira* infection was detected in nine (34.6%; n = 26) samples by the *sec*Y assay and 10 (38.5%) samples by *lip*L32 assay (Table 4.11). The observed Ct values ranged from 15 to 30 for the *sec*Y assay (n = 9, median = 21) and 17 to 33 for the *lip*L32 assay (n = 10, median = 22). For the one discrepant sample, amplification with the *lip*L32 assay was only observed in one of two test replicates, with a Ct value of 33.

Table 4.11: Comparison of the diagnostic performance of the *lip*L32 TaqMan® and *sec*Y SYBR® Green qPCR in detecting *Leptospira* infection in rodent tissues

	*sec*Y qPCR positive	*sec*Y qPCR negative	Total
***lip*L32 qPCR positive**	9	1	**10**
***lip*L32 qPCR negative**	0	16	**16**
Total	**9**	**17**	**26**

4.4 Discussion and Conclusions

The validation data summarised in this chapter provides a quantitative assessment of two qPCR assays, the *lip*L32 TaqMan® qPCR assay and the 16S (*rrs*) qPCR assay, used for the diagnosis of *Leptospira* infection in people and animals from Tanzania described in subsequent chapters. Although both tests are widely used in both clinical and epidemiological settings, these analyses revealed some significant differences in analytical sensitivity between the two assays. Both tests demonstrated the same 100% LLOD but observed Ct values are significantly lower for the *lip*L32 assay. The *lip*L32 was able to detect more replicates at very low concentrations of *Leptospira* DNA than the 16S (*rrs*) assay. No significant inhibitory effects were observed when qPCR reactions were run in the presence of highly concentrated DNA extracts from rodent kidney samples. Furthermore, the diagnostic sensitivity of the *lip*L32 assay to detect *Leptospira* infection in naturally infected Dutch rodent kidneys was equivalent to the *sec*Y SYBR® Green assay carried out at an international reference laboratory. Overall, validation test results indicate that, under our laboratory conditions, the *lip*L32 assay is a highly sensitive test for the detection of *Leptospira interrogans* and *L. borgpetersenii*.

The addition of ROX improved the analytic sensitivity of the *lip*L32 TaqMan® qPCR assay in line with recent published data (Galloway and Hoffmaster, 2015). ROX is a passive reference dye added to qPCR reactions to normalise background fluorescence. In this study, the addition of ROX to the *lip*L32 TaqMan® qPCR reaction mix was associated with a small but significant increase in the analytical sensitivity of the assay. Ct values for reactions containing ROX were detected around half a cycle earlier (i.e. 0.5 Ct lower) than those without. Based on the original assay protocol (Stoddard et al., 2009), ROX was not included in the reaction mix when testing field samples from the first year of my field study (Chapter 5 and 6), but was later introduced following repeat assay optimisation (see Chapter 3.7.2 for methodology). Given the small size of the effect, it is unlikely the addition of ROX will have substantially altered the ability of the assay to detect positive infections in field samples. However, the influence of ROX should be taken into account if the Ct values from the assay were used to quantify the leptospiral load in test samples for example. ROX was included in the reaction mix for all other validation studies on *lip*L32 assay in this chapter.

In direct comparison of the analytical sensitivity of the assays, the optimised *lip*L32 assay demonstrated substantially greater analytical sensitivity than the 16S (*rrs*) assay. Both assays demonstrated a strongly significant linear relationship between the observed Ct value and the number of GE in each reaction. Whilst the 100% LLOD was the same for both tests, observed and predicted Ct values were consistently and substantially lower for the *lip*L32 assay than the 16S (*rrs*) assay (Figure 4.1 and Table 4.9), particularly at low concentrations of *Leptospira* per test reaction. The ability to detect low leptospiral loads is an important characteristic of a diagnostic test. Estimates from the literature indicate that the bacterial load in the blood of a bacteraemic patients ranges from 10^2 to 10^6 *Leptospira* organisms per millilitre (Agampodi et al., 2012), which translates roughly to 2×10^0 to 2×10^4 GE per reaction for human blood samples using the methods for DNA extraction (Chapters 3.6.4) and *lip*L32 qPCR testing (Chapter 3.7.2) described in Chapter 3. The low end of this range is equivalent to the final detectable dilution step used in the validation study (Table 4.5; 1.7×10^0 for *L. interrogans* and 3.2×10^0 for *L. borgpetersenii*). In the titration step containing less than ten GE per reaction, the *lip*L32 assay was able to detect *Leptospira* DNA in 72% of replicates (n = 18) compared to only ~ 6% (n = 16) with the 16S (*rrs*) assay run with the same test DNA concentrations. These results indicate that the *lip*L32 assay is more likely than the 16S (*rrs*) assay to detect infection in patients with low leptospiral loads, assuming that the sensitivity of the test is not influenced by any other sample or study factors. However, testing multiple replicates is also recommended to increase the sensitivity of the approach in this scenario.

The mean leptospiral load in naturally infected rodents is predicted to fall within the linear dynamic range of the *lip*L32 assay demonstrated in this study. The median observed Ct value for naturally infected Dutch rodents (*Rattus norvegicus)* was 23 cycles, which is approximately equivalent to 10^4 GE of *L. interrogans* per reaction (Table 4.9). This estimate of bacterial load corresponds with figures for *Leptospira* infection reported in the literature. In a study of black rats in Mayotte, the mean infection load in rodent kidneys has reported as 3.9×10^4 leptospires per mg of kidney tissue (Desvars et al., 2013b). In this experimental set up, this leptospiral load is equivalent to 2.2×10^2 GE per qPCR reaction and is predicted to result in a Ct value of 29.5 based on the final multivariable linear model. Based on the validation study of Dutch rodents and the literature on leptospiral load, the sensitivity range of the *lip*L32 appears to be appropriate for detecting *Leptospira* infection in naturally infected rodent populations.

For the purpose of GE calculations in this study, *Leptospira* organisms were considered to contain a single copy of each of the target genes. However, some sources suggest that a

single leptospire may contain up to five copies of the *lip*L32 gene based on observations from other spirochaetes (e.g. *Borrelia*) when actively replicating during *in vitro* propagation for example (Bourhy et al., 2011), and that two copies of the *rrs* gene are present in some *Leptospira* species (Picardeau et al., 2008). An increased number of copies of the target gene per leptospire is one possible explanation for the observed difference in analytical sensitivity between the *lip*L32 and 16S (*rrs*) qPCR tests. More pathogen-specific data may become available on the number of copies of each of the target genes (or their homologs) as whole-genome sequencing data for *Leptospira* becomes available (Fouts et al., 2016, Xu et al., 2016).

Inter-batch variability was observed in both assays, although the effect size was greater for the 16S (*rrs*) assay. Variation between batches is acknowledged as an unavoidable limitation of qPCR testing with particular consequences for studies attempting to quantify leptospiral load (Applied Biosystems, 2014, Desvars et al., 2013b). Universal fluctuations, which affect the precision of all replicates within a reaction run, results from temporal differences in fluorescence emission (often resolved by the addition of a passive reference such as ROX) or the effects of temperature and air dissolved in the samples, which tend to be standard within plates but can lead to variation between plates (Applied Biosystems, 2014). Atypical optical fluctuations, such as optical warping or large air bubbles in samples, or errors in the reaction preparation e.g. pipetting or mixing errors, can also affect the results of single replicates and are harder to account for between plates. In this study, testing in triplicate across multiple plates reduces the influence of atypical fluctuations on the calculation of the analytical sensitivity of an assay.

In the individual assay models, no difference in analytical sensitivity for the *lip*L32 assay was observed between *L. interrogans* and *L. borgpetersenii* indicating that the assay performance is equivalent for the two test serovars. In contrast, the *Leptospira* species was a statistically significant explanatory variable for Ct value for the 16S (*rrs*) assay, which was slightly more sensitive for *L. interrogans* than *L. borgpetersenii* (95% CI: -0.941 to -1.26). Coupled with conclusions from the available literature on analytical specificity (summarised in Table 4.1), this data supports the conclusion that the *lip*L32 TaqMan® qPCR assay is a robust choice for the detection of pathogenic *Leptospira* species involved in human and animal infections.

Evaluation of the analytical performance of the *lip*L32 assay in rodent samples found no evidence for PCR inhibition in rodent kidney DNA samples. The observed 100% LLOD was the same for all tested samples, although the assay appeared slightly more sensitive at

detecting *Leptospira* DNA diluted in rodent kidney DNA extracts than in the water control. The effect size, which ranged from 0.16 to 0.9 cycles, was largest in the most concentrated DNA samples (R0355: DNA concentration = 367 ng/µl; beta coefficient = -0.93; 95% CI -0.55 to -1.30) but was of the same order of magnitude as the average effect of batch on the *lip*L32 assay validation study (Chapter 4.3.2; Table 4.6). High concentrations of DNA, along with a variety of other biological compounds are known to inhibit PCR reactions and reduce reaction efficiency (Burkardt, 2000). Kidney samples are particularly at risk of PCR inhibitors due to the high concentrations of urea that may be found in crudely processed samples (Schrader et al., 2012). Although this study used a small number of field samples to evaluate evidence for systemic inhibition, these results indicate that PCR inhibition is not a systemic problem within this testing pipeline. However steps to reduce the effect of qPCR inhibitors, such as diluting sample DNA prior to testing or the use of an internal control reaction (Schrader et al., 2012), should still be considered for future testing.

Further evidence that the *lip*L32 qPCR assay is performing well in 'real-world' samples comes from direct comparison of diagnostic test results for to a population of Dutch rats of known infection status to the *sec*Y SYBR® Green qPCR reference test. A high degree of agreement was observed between the test results. However, one sample tested positive by *lip*L32 testing in one out of two test replicates but failed to amplify in the *sec*Y assay. Assuming perfect diagnostic sensitivity and specificity for the *sec*Y assay, this observation could be interpreted as a false positive *lip*L32 result. An alternative explanation is that this is actually a false negative result for the *sec*Y assay. This interpretation is perhaps more likely given data on analytical sensitivity and specificity presented in other assay validation studies (Bourhy et al., 2011, Stoddard et al., 2009, Ahmed et al., 2009). More robust conclusions cannot be made without further analysis (e.g. repeat testing and/or sequencing of the product to check for reaction specificity), but this discrepancy does highlight the problem of selecting 'gold standard' reference tests for *Leptospira* diagnostic assays (Limmathurotsakul et al., 2012).

Overall, this chapter brings together a number of independent validation studies that explore the strengths and limitations of two *Leptospira* diagnostic qPCR assays. Each study component was designed and performed independently with the exception of the comparison of validation studies of the *lip*L32 TaqMan® qPCR assay and the 16S (*rrs*) TaqMan® qPCR described in sections 4.2.5 and 4.2.6, which were performed in parallel. Therefore, other direct comparisons between the different components of this chapter should be made with caution.

The studies were performed using the same stock control DNA (*L. interrogans* serovar Copenhagenii) over the period of approximately one year, over which time evidence of DNA degradation is apparent. An example of this can be seen by comparing the starting concentration of *L. interrogans* DNA for the ROX study (Table 4.3), which was performed in January 2015, with starting DNA concentration for the *lip*L32 and 16S (*rrs*) assay validation studies (Table 4.5), which were performed in February 2016. Prolonged storage or repeat freeze-thaw cycles may have contributed to the observed reduction in gDNA concentration between the tests, and concurrent loss of DNA integrity may also have affected the observed analytical sensitivity of each of the assays. Repeat testing with a new stock of control DNA could evaluate this possibility in the future.

Efforts were made to minimise variation within each study component. However, during the laboratory set up of these studies, standardisation focused on the titration steps rather than the absolute DNA concentration or GE (e.g. difference in the starting concentrations of *L. borgpetersenii* and *L. interrogans*) used in each assay. This design limits direct comparison of the data presented in this chapter with other published validation studies, but does not limit the interpretation of data presented in this chapter or its relevance to the rest of this thesis.

In conclusion: the primary goal of this chapter was to justify the selection of a qPCR assay for the detection of *Leptospira* infection in human and animal samples from Tanzania. The *lip*L32 assay consistently outperformed the 16S (*rrs*) assay and is therefore considered a robust choice for a primary diagnostic tool to meet the study objectives. The high analytical and diagnostic sensitivity demonstrated by the *lip*L32 assay is predicted to support more accurate measurement of infection prevalence in target populations, as well as offering a greater probability of detecting infection in patients with low leptospiral loads. The high degree of analytical specificity reported for this assay also supports this choice. The two other assays discussed - *sec*Y SYBR® Green qPCR assay and 16S (*rrs*) TaqMan® qPCR assay - were subsequently used as secondary, confirmatory tests to corroborate results in specific scenarios described later in this thesis (Chapter 5 and 7).

5 *Leptospira* infection in rodents in the Kilimanjaro Region

5.1 Introduction

Worldwide, rodents are considered to be ubiquitous hosts of *Leptospira* and the most important source of infection for people (Haake and Levett, 2015, Levett, 2001). The order Rodentia is extremely diverse (Wilson and Reeder, 2005) and *Leptospira* infection has been reported in a large number of different rodent species and on most continents of the world (reviewed in (Meerburg et al., 2009, Kosoy et al., 2015)). The tremendous variety of rodent hosts and settings in which these rodents are found makes it difficult to draw universal conclusions about the epidemiology of leptospirosis in rodent populations (Ellis, 2015, Levett, 2001). However, for some commensal rodent species that live in close proximity to people, patterns of infection have been relatively well-characterised through more intensive laboratory or field studies. These species offer some important insights into the different patterns of *Leptospira* infection in the rodent host, which have implications for the maintenance and transmission of infection in different environmental settings.

5.1.1 Patterns of *Leptospira* infection in rodents

Rattus norvegicus (also known as the Norway or brown rat); *Rattus rattus* (also known as the black rat, ship rat or roof rat) and *Mus musculus* (also known as the house or laboratory mouse) are among the best studied and most widespread commensal rodent species. Since the first isolation of *Leptospira* from *R. norvegicus* and *R. rattus* in Japan 1915, these two *Rattus* species have been considered as the archetypal hosts of *Leptospira* (Adler, 2015, Ido et al., 1917). *R. norvegicus* is the main laboratory model for chronic *Leptospira* infection and infection has been shown to persist in the kidneys of these hosts for several months following infection (Bonilla-Santiago and Nally, 2011, Athanazio et al., 2008). Although no laboratory models have been reported for *R. rattus*, it is generally assumed that the dynamics and susceptibility to *Leptospira* infection are similar to those observed in *R. norvegicus* (Kosoy et al., 2015, Levett, 2001). However, marked variability in the outcome of *Leptospira* infection is reported in other laboratory rodent species. Some species, such as hamsters and guinea-pigs, are extremely sensitive to some infections and succumb to severe acute clinical disease that is usually fatal even with *Leptospira* serovars that are well tolerated by other rodent species (da Silva et al., 2010, Faine, 1994). Outside

the laboratory, there is virtually no information on clinical disease in rodent species in their natural habitat (Ellis, 2015) but the outcome of infection in rodents in a natural setting is likely to influence the ability of a particular rodent host species to maintain *Leptospira* infection for prolonged periods of time.

5.1.2 Relationships between rodents and *Leptospira* serovars

Another factor that determines the outcome of infection in rodent hosts is particular type of *Leptospira* involved. Some specific associations are described between common rodent hosts and particular *Leptospira* serovars. *R. norvegicus* appears particularly well-adapted carry *L. interrogans* serovar Copenhagenii and *L. interrogans* serovar Icterohaemorrhagiae (Levett, 2001, Bharti et al., 2003). Evidence for this association comes from laboratory models of infection and epidemiological data from infections in natural settings where extremely high loads of the pathogen appear to be tolerated and maintained (Costa et al., 2015b, Bonilla-Santiago and Nally, 2011). A similar relationship is described between *Mus musculus* and *L. borgpetersenii* serovar Ballum (Adler and Faine, 1977, Matthias and Levett, 2002). In other species, less is known about associated *Leptospira* types although the recent application of molecular tools to investigate this question in field settings is also revealing trends of rodent host specificity for some *Leptospira* genotypes (Dietrich et al., 2014). On the whole however, the factors that determine relationships between host species and *Leptospira* serovars are poorly understood and may be variable between populations and environmental settings (Bharti et al., 2003).

5.1.3 Factors that influence rodent *Leptospira* infection prevalence

A wide variety of risk factors for rodent *Leptospira* infection have been reported and prevalence in rodent populations appears to vary considerably between geographic locations. Spatial differences in infection prevalence have been reported on macro-geographical scales, such as between countries in South-East Asia (Cosson et al., 2014), and on a much finer scale between city blocks or trapping grids in Vancouver and Nairobi for example(Halliday et al., 2013, Himsworth et al., 2013). Patterns of rodent infection have also been reported to vary by season and climate, with higher prevalence of rodent infection associated with increased rainfall at some sites (Munoz-Zanzi et al., 2014a, Theuerkauf et al., 2013). Fluctuations in rodent abundance are thought to influence the temporal dynamics of infection in a particular host (Holt et al., 2006). Infection prevalence also often varies by species at the same sites (e.g. between *Mus musuculus* and *Rattus*

rattus in Nairobi (Halliday et al., 2013) or between different *Rattus* species in Western Polynesia (Theuerkauf et al., 2013)). However a consistent relationship between prevalence and age and bodyweight in reported in *Rattus* species (Himsworth et al., 2013, Desvars et al., 2013b, Costa et al., 2014). Overall, risk factors for rodent infection appear to be complex and highly variable and site-specific investigations are warranted to identify locally important factors that may influence the prevalence and dynamics of infection at a particular site.

5.1.4 *Leptospira* infection in rodents in Tanzania

Substantial data exists to support the role of the rodents in the epidemiology of leptospirosis in Tanzania. Seroprevalence surveys of rodents indicate that rodent exposure to infection is common in southern and western Tanzania (Mgode et al., 2014, Assenga et al., 2015, Machang'u et al., 1997). Definitive evidence for rodents as hosts of *Leptospira* infection in Tanzania also exists. Four different *Leptospira* serovars have been isolated from the African giant pouched rat, *Cricetomys gambianus,* in the Morogoro Region in the central Tanzania (Mgode et al., 2015, Machang'u et al., 2004)(Table 5.1). *C. gambianus* is a widespread adaptable rodent species in Tanzania that lives in a diverse range of habitats from forests to farmland and rural villages (Kingdon, 1997). A captive population of this species has also been established in Tanzania, which has supported better characterisation of the pathogens associated with this rodent host (Machang'u et al., 2002, Machang'u et al., 2004). In a natural setting, *Leptospira* infection has also been demonstrated in multimammate mice (*Mastomys natalensis*). This is another widespread indigenous rodent species that demonstrates dramatic population 'outbreaks' associated with major crop and food losses for people in rural areas in Tanzania (Gratz and Arata, 1975, Leirs et al., 1996). *Leptospira* infection has been demonstrated in these species by PCR (6.3%; n = 18) and by culture (Table 5.1). Two *Leptospira* serovars have been isolated from this species, one of which is also associated with infection in *C. gambianus.*

Table 5.1: Previous reports of *Leptospira* isolated from rodents in Tanzania

Serogroup	*Leptospira* species	Serovar	Rodent species
Australis	*L. interrogans*	Lora	*Mastomys natalensis* (Multimammate mice) (Mgode et al., 2015)
Ballum	*L. borgpetersenii*	Kenya	*Cricetomys gambianus* (African giant pouched rat) (Machang'u et al., 2004) *Mastomys natalensis* (Mgode et al., 2015)
Canicola	*L. interrogans*	Canicola	*Cricetomys gambianus* (Mgode et al., 2015)
Ictero-haemorrhagiae	*L. kirschneri*	Mwogolo	*Cricetomys gambianus* (Mgode et al., 2015)
	L. kirschneri	Sokoine	*Cricetomys gambianus* (Mgode et al., 2015, Machang'u et al., 2004)

Both *C. gambianus* and *Mastomys natalensis* are known to be present in the Kilimanjaro Region alongside a number of other rodent species. However, to date there are no studies that have investigated *Leptospira* infection in rodent hosts in this region despite a high incidence of human disease (Biggs et al., 2011, Biggs et al., 2013b, Crump et al., 2013). Understanding the epidemiology of *Leptospira* infection in rodents in this area may offer important insights into the sources of infection for people and be used to inform potential control strategies for human infection.

5.1.5 Study aims and objectives

This study component aimed to explore the role of rodents in the epidemiology of leptospirosis in the Moshi Municipal and Moshi Rural Districts where the majority of human leptospirosis cases (described in more detail in Chapter 7) occur. This chapter describes a cross-sectional surveillance study of rodents establish to address the following research objectives:

Objective 1: Determine the prevalence and patterns of *Leptospira* infection in rodents living in the peri-domestic environment in the Moshi Municipal and Moshi Rural Districts of northern Tanzania.

Objective 2: Evaluate the potential role of rodents as a source of *Leptospira* infection for people.

5.2 Methods

To explore *Leptospira* infection in rodents in the Kilimanjaro Region, a cross-sectional study of rodents was performed in randomly selected villages within the Moshi Municipal and Moshi Rural Districts. The majority of human leptospirosis cases identified by the hospital-based febrile disease surveillance, described in Chapter 3 and 7, originated from villages within these two districts. Rodent trapping was performed in eight-week blocks conducted in: 1) wet season of 2013 (8th May to 27th June 2013); 2) wet season of 2014 (6th May to 24th June 2014); and 3) dry season of 2014 (5th August and 23rd September 2014). Rodents were trapped in and around households in the study villages and kidney samples were collected for direct determination of *Leptospira* infection prevalence by qPCR. Questionnaires were also conducted in the study households to explore potential risk factors for rodent infection. Detailed methodology for this study is described below.

5.2.1 Village sampling frame

The geographical sampling frame was composed of villages within Moshi Municipal and Moshi Rural Districts from which people have sought health care at Kilimanjaro Christian Medical Centre (KCMC) or Mawenzi Regional Hospital (MRH) for febrile illness and been enrolled in the on-going fever surveillance studies between 1st March 2012 and 28th February 2014 (Chapter 3). Village data were extracted from the Febrile Surveillance Clinical Review Form (CRF) database (curated by KCMC-Duke University Medical Center Research Collaboration) on 5th April 2013 and 22nd April 2014. Data were cleaned and de-duplicated, and village names were matched to those listed in the National Census of Tanzania, 2002 (Tanzania National Bureau of Statistics, 2002). Excel (Microsoft® Excel® for Mac 2011, Version 14.6.1) was used to randomly select 15 study villages without replacement. One additional village was selected by convenience as a pilot village for rodent trapping. Village sampling was performed in order of random selection. Where sampling access to a village was not possible, the next randomly selected village was taken as a replacement.

5.2.2 Subvillage selection

Consent for study participation was obtained from the Village Chairperson of each study village, who also provided a list of subvillages (also known as Mtaa in urban areas; see Figure 3.2) within each village. A single subvillage was selected as the representative sampling location within each study village using random number allocation.

5.2.3 Household selection

Household selection was performed using a modification of the World Health Organization's (WHO) Expanded Program for Immunization (EPI) random walk method recommended for cluster surveys in geographic areas that lack a robust, data-based framework for household or population sampling (Milligan and Bennett, 2004, Bostoen and Chalabi, 2006). Recruitment was performed along two transects each of approximately 500 metres in length on average. The administrative centre of the sub-village centre was defined as the starting point for each transect. The direction of the first sampling transect was determined at random by spinning a pen to select a compass bearing. In villages with nucleated structure (e.g. in urban areas), a second transect was established using the same central starting point as the first, but taking a compass bearing perpendicular (90°) to the first. In villages with a linear structure, for example in mountainous areas where settlements typically run along natural valleys and ridges, a second transect was established using the same central starting point but at a compass bearing of 180°C from the first transect. A GPS device (Garmin eTrex®10, Garmin Ltd., Olathe, Kansas, USA) was used to follow existing pathways and roads that best matched the selected compass bearing. The route of each transect was recorded using sketch mapping and GPS waypoint marking. The exact GPS co-ordinates for each recruited household were also recorded.

Households[†] were enrolled alternatively from either side of the path in a zigzag pattern. A minimum of ten metres was required between the compound borders of each study household. A total of ten households were recruited along each transect. Adaptations to standard methods were made where necessary based on village layout. For example: in one village, houses were arranged and numbered in a regular grid layout within a worker's 'camp' on a sugar cane plantation. At this site, households were selected through random number selection (without replacement) generated using Microsoft® Excel. In several

[†] A household is defined as a permanent structure where one or more inhabitants who share the same cooking facilities sleep over night for the purposes of this study.

villages, multi-household compounds were encountered, where up to ten or so households occupied a single compound. In these situations, only one household within the compound was selected for study participation. Manual random number generation was used to select a single household within the compound. Briefly, consecutive numbers were sequentially assigned to each household defined as a working in a clockwise direction from the compound entrance or gateway. Numbers were written on folded papers that were then mixed, with the number hidden from view. A local village representative (e.g. subvillage chairperson or Livestock Field Officer), who was blinded to the number allocation process, picked one paper and the household corresponding to the chosen number was selected for participation.

5.2.4 Household recruitment

The head of the household or other nominated adult household representative was informed of the study objectives and requirements and written consent for study participation was obtained (see Appendix 3 for copies of the study consent forms and participant information sheets). Where consent was refused or could not be obtained, the nearest consenting neighbour was recruited as a replacement.

5.2.5 Household data collection

Household questionnaires were performed to characterise study households and explore risk factors for rodent *Leptospira* infection. Questionnaires were also designed to complement questionnaires carried out in a parallel cross-sectional seroprevalence survey for bacterial zoonoses (brucellosis, leptospirosis and Q fever) in human and livestock populations within the Kilimanjaro Region (Chapter 3).

Questionnaires included simple questions about household demography (e.g. household size, number of children), followed questions focusing on factors known to influence the presence of invasive rodents in a household (Gratz and Arata, 1975, Bonner et al., 2007) or general environmental risk factors for *Leptospira* transmission (Lau et al., 2010, Levett, 2001, Sarkar et al., 2002). Risk factor question topics included the physical properties of the compound or household (e.g. building materials of house, presence of electricity and piped water and type of toilet system), environmental properties (e.g. distance to an open water source, flooding within the previous 12 months), household livestock ownership and agricultural characteristics (e.g. types and numbers of animals kept at the compound, types

of crops grown in the compound) and questions specifically relating to perceived rodent abundance (e.g. frequency of rodent sightings inside the house, food stores and animal housing, evidence of rodent damage to stored food, rodent control practices and perception of rodent abundance in different seasons of the year). Where rodent control was reported, the method and frequency of use was also recorded. Where possible, questions were also included to measure the internal validity of questioning.

Household questionnaires were translated into Kiswahili by professional translators, and back translated by KA with assistance from native Kiswahili speakers. Informal piloting and training was performed with field staff. Questionnaires were then piloted under supervision at the pilot village. Where necessary, additional training was provided and minor amendments were made to the questionnaire. A full version of the final study questionnaire in English and Kiswahili can be found in Appendix 4.

At study households, Tanzanian field assistants conducted questionnaires in Kiswahili. Direct observation was performed for quality control where possible. Household responses were recorded on paper questionnaires by study field assistants and independently reviewed for consistency (KA). Missed questions or ambiguities in responses were clarified at the household and validated by direct observation where possible. Data were entered manually into a Microsoft® Excel database for subsequent analysis. The study area (Moshi Municipal and Moshi Rural Districts) and locations for each patient was mapped using QGIS (Version 2.4.0-Chugiak, 2014).

5.2.6 Rodent trapping

A standard set of five rodent traps was placed within each of the study households. Where possible, traps were placed in standard locations including kitchens, food storage areas and animal housing areas. In some households trap placement was also adapted according to strong preferences of study participants. In the first year of trapping (2013), four large Sherman® traps (dimensions: 3 x 3.5 x 9 inches) and one small Sherman® trap (dimensions: 2 x 2.5 x 9 inches) were set (HB Sherman Traps, Tallahassee, USA) (Figure 5.1). Sherman traps were baited with a stiff mixture of peanut butter and oats and chopped carrots. Tomahawk traps were baited with a couple of pieces of locally available dried fish.

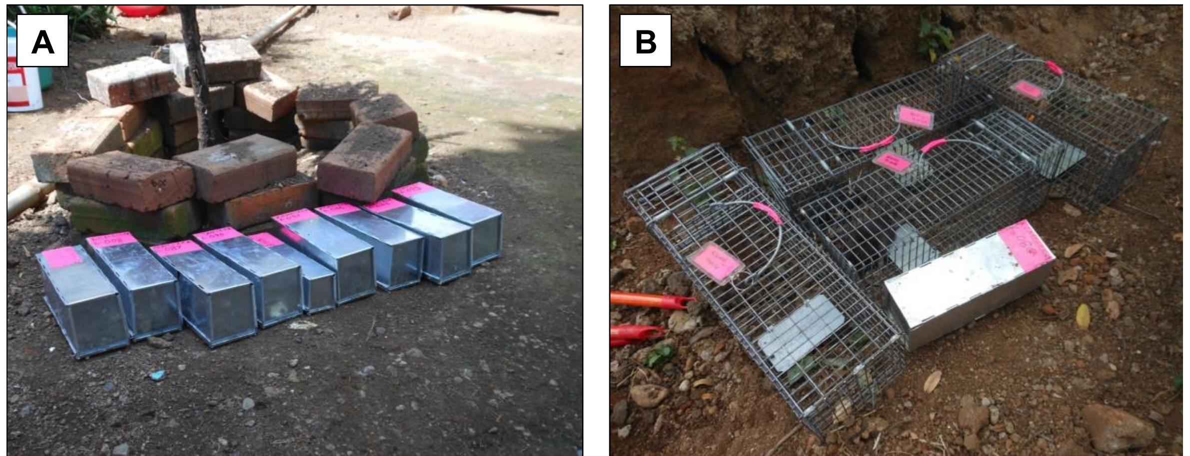


Figure 5.1: Examples of rodent traps used in Tanzania: a) two sizes of Sherman traps (note the small Sherman trap in the centre of the image; b) Tomahawk wire traps with a large Sherman for size comparison.

A pilot study was carried out to determine the number of trap nights needed. Traps were set for three nights in the pilot village and four nights in the first study village, after which the number of nights spent in each village was extended due to concerns over the low trap success. For subsequent villages, the traps were set for an average of eight nights (range: 7-10 nights) and checked every morning. Trap status for each individual trap was recorded as: i) open; ii) closed (empty); iii) closed (full); and iv) damaged, faulty or absent (i.e. missing, stolen or unable to check). Full traps were removed and replaced. Soiled or insect-infested food was replaced. If non-target species were trapped (e.g. frogs, lizards, birds), traps were recorded as closed (empty) and the non-target species animal was released. In some villages, additional traps were also placed in non-study household sites at the request of local village officials and householders (e.g. maize milling sheds, bars). Rodents trapped in these traps were tested for *Leptospira* but were not included other analyses. At the end of each trapping period, study traps were removed and study participants were gifted a locally procured rodent snap trap as gesture of appreciation for taking part in the study.

5.2.7 Calculating trap success as a proxy for rodent abundance

Corrected trap success was calculated and used as an index of rodent abundance for each village (Table 5.2) (Cunningham and Moors, 1996, Nelson and Clark, 1973). Firstly, the number of **traps nights** per village was calculated as the total number of traps set

multiplied by the number of nights spent at each location. Next, a correction factor was applied to account for three sources of 'lost trap nights': 1) disturbed traps (i.e. closed/ empty traps); 2) full traps that were no longer available to trap more rodents; or 3) damage to the trap that prevented it from functioning normally. **Lost trap nights** was calculated by subtracting half a night from the total number of trap nights for each closed, damaged trap or lost traps assuming that in each case, the trap would have only been available to trap a rodent for half the night. Traps lost to follow up were also included in this correction for the first night, and then subtracted from total traps set for subsequent nights. Therefore, the **corrected number of trap nights** was calculated as the total number of trap nights minus the total number of trap nights lost in each location. Finally, **adjusted trap success** was calculated as an index of rodent abundance in each village by dividing total number of rodents caught by the total number of corrected trap nights and expressed as a percentage.

Table 5.2: Summary of definitions used to calculate adjusted trap success as an index of rodent abundance for each village

	Definition (Cunningham and Moors, 1996)
Trap nights	Traps (n) x Nights (n)
Lost trap nights	Closed (n) + damaged traps (n) / 2
Corrected trap nights	Traps nights – Lost trap nights
Adjusted trap success	Total rodents caught per session (n) / corrected trap nights

n = number

5.2.8 Rodent necropsy and sampling

Traps containing rodents were removed to a central, well-ventilated processing point for euthanasia and tissue sampling. Euthanasia was performed following UK and international guidelines for humane euthanasia (Home Office, 2014, Leary et al., 2014). First, a cotton wool swab soaked in liquid halothane inhalant anaesthetic was placed inside a tough, Ziplok® bag, used as an anaesthetic chamber. Rodents were placed inside this chamber and monitored until deep anaesthesia was induced (judged by lack of response to external stimuli and a slow or agonal respiratory pattern). Euthanasia was performed by cervical dislocation, followed by immediate blood sampling by cardiocentesis.

Body weight, body length, tail length, foot and ear lengths, presence of wounds and presence of ectoparasites were recorded for each individual. Phenotypic characteristics and morphometric measurements including body length, tail length, foot and ear lengths were used to determine the rodent species by comparison with published data (The Field Museum, 2011, Cunningham and Moors, 1996). An identifying photograph of each individual was also taken for species validation.

Rodent gender and age class (mature or immature) was determined using external sexual features using the following definitions (Cunningham and Moors, 1996):

Immature female	A sheet of translucent skin covers the vagina.
Immature male	Testes are not fully descended into the scrotum.
Mature female	The vagina is open (no sheet of skin). Nipples may be prominent.
Mature male	The testes are descended into the scrotum fully, which hides the anus.

Full necropsy and comprehensive tissue sampling was then performed. When observed, external parasites were collected and stored in 70-96% ethanol. Pregnancy status of female animals was recorded during necropsy. For diagnosis of *Leptospira* infection by qPCR, one kidney from each rodent was collected and preserved in 70-96% ethanol. In a subset of rodents (n = 100) a portion of kidney tissue was use to inoculate EMJH-5FU culture media for *Leptospira* spp. culture (Chapter 3.8). Selection of individual rodents for *Leptospira* culture was largely governed by logistical constraints (i.e. availability of culture media and shipping constraints). When culture media was available, vials were inoculated for the first five individuals sampled each day and continued on consecutive trapping days until a maximum of 50 rodents had been sampled each year. Following inoculation samples were shipped to the WHO/FAO/OIE Collaborating Leptospirosis Reference Laboratory, Royal Tropical Institute, Amsterdam (KIT) for propagation, isolation and typing of positive samples.

5.2.9 DNA extraction from rodent kidney tissues

DNA was extracted from ethanol-fixed kidneys as described in Chapter 3.6.2 and quantified using a NanoDrop spectrophotometer (ThermoScientific, Waltham, MA). Prior to qPCR testing, total genomic DNA (gDNA) was diluted 1:10 in PCR-grade water (or 1:5

in samples with a final DNA concentration < 100ng/µl) to mitigate the effect any residual qPCR inhibitors.

5.2.10 *lip*L32 TaqMan® qPCR assay for *Leptospira* infection

Samples were run on MicroAmp® 96 well plates and tested in duplicate with the *lip*L32 assay as described in Chapter 3.6.2 (Stoddard, 2013, Stoddard et al., 2009). The final test concentration of gDNA ranged from approximately 50ng to 150ng per 25µl qPCR reaction. Each plate included: two replicates of a *Leptospira* positive control; *L. interrogans* serovar Copenhagenii Strain Wijnberg at ~10^2 genome copies numbers; two replicates of a non-template extraction control, and two replicates of a negative control (PCR-grade water). Each reaction run was considered valid when both negative controls were negative and at least one replicate of the *Leptospira* positive controls amplified with Ct value < 40. Samples were considered positive when at least one test well amplified the *lip*L32 target with a Ct value < 40.

5.2.11 *sec*Y SYBR® Green qPCR assay for *Leptospira* infection

For validation of test results, a randomly selected subset of 60 samples (30 per sampling year) were sent for confirmatory testing using the *sec*Y SYBR® Green qPCR assay at KIT (Ahmed Ahmed). Samples were tested in triplicate as described in Chapter 3.6.2. Samples were considered positive when at least two test wells amplified the *sec*Y target with a Ct value < 35 and a melt temperature (Tm) within the specific *Leptospira* reference range of 79 to 84°C.

5.2.12 Statistical analysis

To allow an expected prevalence of infection of 10% to be estimated with a confidence level of 0.95 and a precision level of 0.95, the target sample size was calculated as 139 rodents per trapping season (Sergeant, 2016).

Statistical analysis was performed in R (R Core Team, 2015). Two-sample and paired t-tests were run to compare adjusted trap success between seasons and district types (urban vs. rural). Binomial confidence intervals were calculated for prevalence estimates. Pie charts and bar plots were plotted in R.

5.3 Results

5.3.1 Village and household recruitment

Rodent trapping was performed in a total of 11 randomly selected villages and one pilot village shown in Figure 5.2. One village (Village F) were sampled in both wet and dry seasons of 2014. In total, six study villages and pilot villages were located in the Moshi Rural District and five villages were located in wards in the Moshi Municipal District.

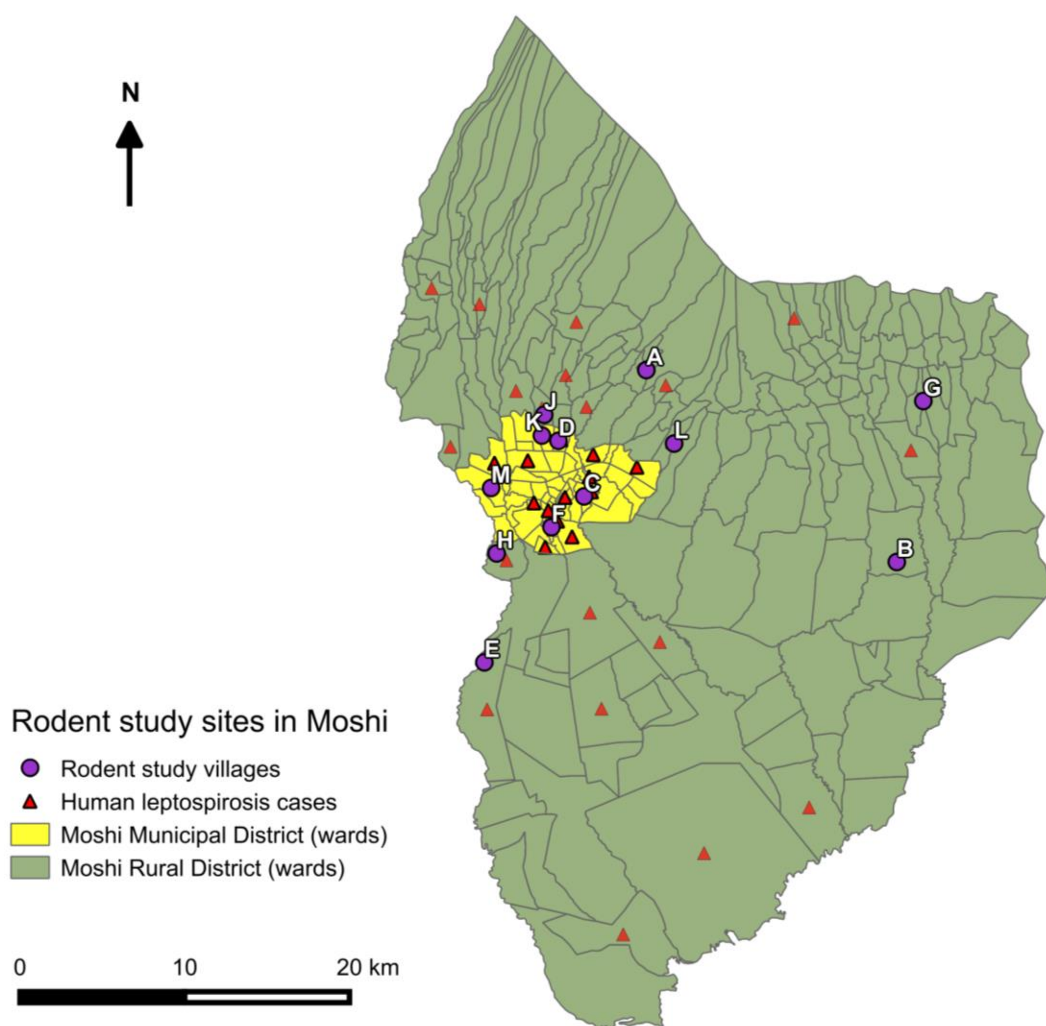


Figure 5.2: Map of rodent study villages labelled according to study ID (Table 5.3) and location of villages with human leptospirosis cases from febrile disease surveillance study (Figure 7.4)

In total, 230 households were recruited into the study. Study retention was high. Over both years, only four households (1.7%, n = 230) withdrew from participation following the start of the study and were subsequently replaced with neighbouring households. Illness in the family or travel was cited as the reason for withdrawal.

5.3.2 Characterisation of the study villages by questionnaire

Questionnaire data were available for analysis for 227 of 230 study households. Responses were used to provide summary information about each village (Table 5.3). Study villages covered a range of environments (rural and urban) and altitudes (748 to 1307 metres above sea-level). The population density for study villages, calculated at ward level by the Tanzanian 2012 Census ranged from 80 to 14,961 people per km^2 with the highest densities observed in urban settings. Household population size ranged from 3.8 people per household in the Village G up to 13.4 people in Village F (national average = 4.8 people per household; (Tanzania National Bureau of Statistics, 2012)).

Infrastructure and housing quality also varied by village. The proportion of households with grid electricity provision ranged from 10% to 80%. In all villages, up to 50% of houses were made out of mud and manure as opposed to bricks or cement, which was the other common building material. Pit latrines were common in all but one village. In this village (Village E), all questionnaire respondents had access to a communal block of squat toilets provided by the owners of the sugar cane plantation where this village was located.

Overall, the majority of respondents cultivated some kind of crop at the household. Ruminant livestock ownership was significantly lower in urban areas compared to rural areas ($\chi^2 = 31.5$, $p < 0.001$). Pigs were also kept at small number of households from villages in both urban and rural districts.

Table 5.3: Village characteristics and summary of questionnaire data

Village ID	Pilot (A)	B	C	D	E	F	G	H	J	K	L	M
Ward	Mbokomu	Kilema Kusini	Mji Mpya	Rau	Arusha Chini	Boma Mbuzi	Mamba Kusini	Kindi	Uru Kusini	Longuo	Kimochi	Karanga
District type	Rural	Rural	Urban	Urban	Rural	Urban	Rural	Rural	Rural	Urban	Rural	Urban
Elevation[a] (m)	1307	883	798	890	748	796	1263	792	967[i]	940	1051	898
Ward pop. Density (people/km^2)	555	433	10,813	2,386	80	14,691	808	678	962	1,420	438	933
Responses[b] (n = HH)	10	20	20	19	20	20	20	20	18	20	20	20
Median time in village (years)	51.0	16.0	14.5	19.0	10.0	32.0	41.0	34.0	32.5	25.0	44.0	10.0
Average size of HH	4.2	5.9	7.7	6.6	4.5	13.4	3.8	4.7	5.5	4.4	4.5	5.0
% HH made of mud	30.0	0	5.0	0	0	30.0	45.0 (n = 19)	40.0	11.1	5.0	25.0	5.0

% HH with electricity	20.0	30.0	65.0	78.9	20.0	60.0	45.0	10.0	61.1	80.0	50.0	60.0
% HH with pit latrine	80.0	75.0	47.4 (n = 19)	82.4 (n = 18)	0	55.0	75.0	70.0	77.8	60.0	100.0	60.0
% HH with flooding in last year	0	20.0	35.0	21.1	0	10.0	0	30.0	22.2	10.0	0 (n = 19)	10.0
% HH with crops	NA	NA	65.0	100	65.0	45.0	100	100	100	60.0	100	80.0
Cattle per HH[d]	0.4	0.6	0	0.1	0.1	0	0.25	0.3	0.25	0	0.9	0.1
Goat/Sheep per HH[d]	1.8	4.6	1.0	0.9	2.2	0	2.0	2.0	3.5	0.3	3.8	0.75
Pigs per HH[d]	3.5	0.2	0	0.5	0	0	0.6	0	0.22	0	1.25	0.65
Dogs/cats per HH[d]	0.2	1.9	0.5	1.1	0.1	0.5	0.5	0.8	0.4	0.7	0.6	0.3

[a]Elevation of subvillage taken from altitude of transect starting point; [b]Number of households with complete responses i.e. the denominator for subsequent calculations unless otherwise indicated; [d]Mean number of animals per household (HH) by village.

5.3.3 Rodent sightings, control and perceptions of abundance

Rodent sightings were commonly reported by study respondents. In total, 111 (48.9%) of 227 respondents reported seeing rodents or evidence of rodents in their house every day in the month prior to the sampling visit. A further 41 (18.1%) respondents reported seeing rodents or evidence of rodents in their house less than every day but more than once a week. Of 179 respondents with fields around their households, 61 (34.1%) reported seeing rodents or evidence of rodents in those fields more than once per week.

The majority of respondents (89.9%) used some form of rodent control in their household. Chemical control (zinc phosphide poison) was the most frequently reported method of controlling rodents used in 86.6% of households. Domestic carnivores (cats or dogs) were also used as a means of rodent control in 30.8% of households.

Questionnaire respondents reported that the dry season was the peak season for rodent abundance. The short rains were considered the season with the fewest rodents although many respondents also were unsure of rodent abundance at that time of year (Table 5.4).

Table 5.4: Perception of rodent abundance by season (all villages; n = 227 respondents)

	Many	Few	None	Don't know	NA
Long Rains	88	109	4	23	3
Short Rains	26	144	15	39	3
Dry season	170	34	1	20	2

5.3.4 Rodent trapping results and trap success

A summary of trapping effort and trap success by village is shown in Table 5.5. In total, 384 (98.2%) of rodents were trapped in randomly selected houses over 9427 trap nights (8730 after adjustment for lost nights), with an overall adjusted trap success of 4.40% (binomial confidence interval: 3.99 – 4.85%). By village, adjusted trap success ranged from 1.94% to 10.4%. Rodents were trapped in 60% of study households, with averaging at 1.54 rodents per household. An additional seven rodents were trapped through targeted trap placement in extra houses at the request of local villagers.

No significant difference was observed between adjusted trap success (two-sample t-test: $p = 0.716$) or the number of households with rodents (two-sample t-test: $p = 0.124$) between urban and rural villages. In general, no significant difference was observed in adjusted trap success between wet and dry seasons (two sample t-test; $p = 0.282$). However, in Village F where the same households were visited in both the wet (F) and dry (N(F)) season of 2014, the adjusted trap success was significantly higher in the wet season than the dry season (paired t-test: $t(8.9) = 2.65$, $p < 0.05$).

Table 5.5: Summary of rodent trapping results and testing by study village

Village ID	Pilot (A)	B	C	D	E	F	G	H	J	K	L	M	N (F)[i]	Total
District type	Rural	Rural	Urban	Urban	Rural	Urban	Rural	Rural	Rural	Urban	Rural	Urban	Urban	
Season, year	Wet 2013	Wet 2013	Wet 2013	Wet 2013	Wet 2013	Wet 2014	Wet 2014	Wet 2014	Wet 2014	Dry 2014	Dry 2014	Dry 2014	Dry 2014	
Households (n)	10	20	20	20	20	20	20	20	20	20	20	20	20	**250**
Trap nights per village (n)	3	4	7[ii]	10	8[iii]	8	8	8	8	8	8	8	8	**96**
Trap nights (n)	150	350[iv]	700	1000	800	826	800	801	800	800	800	800	800	**9427**
Adjusted trap nights (n)	143	304	650	932	738	731	773	748	742	722	751	751	747	**8730**
Rodents trapped (n)	14	13	31	25	39	76	15	35	20	23	22	38	33	**384**
Adjusted trap success (%)	9.79	4.28	4.77	2.68	5.28	10.4	1.94	4.68	2.70	3.19	2.93	5.06	4.42	**4.40**

% HH with rodents	60.0	45.0	60.0	50.0	50.0	90.0	40.0	60.0	65.0	0.55	60.0	65.0	80.0	**60.0**
Rodents per HH[v]	1.40	0.65	1.55	1.25	1.95	3.80	0.75	1.75	1.00	1.15	1.10	1.90	1.65	**1.54**
Extra rodents	-	-	-	-	-	-	-	1	-	2	1	3	-	**7**
N tested by *lip*L32 qPCR	14	13	29	25	39	75	15	36	19	25	22	41	32	**385**
N tested by culture	0	0	13	22	15	0	0	0	0	0	0	25	25	**100**

[i]Village N(F) is the same village as F but trapped in the dry season;
[ii] Traps removed for three nights over the weekend hence trap nights were broken up into two consecutive trapping periods of three and four nights respectively;
[iii]Traps removed for three nights over the weekend hence trap nights were broken up into two consecutive trapping periods of four nights each;
[iv]In this village, only ten households were recruited for the first trap night;
[v]Mean number of animals per household (HH) by village
Abbrev: HH = household, n = number

5.3.5 Rodent species

In total, the black rat (*Rattus rattus*) was the most common species trapped (326 (83.4%) of 391). Other species trapped included two mouse species: house mice (*Mus musculus*), (n = 44; 11.3%) and African pygmy mice (*Mus minutoides*) (n = 3; 0.77%); and a small number of other endemic rodents species including multimammate mice (*Mastomys natalensis*)(n = 8; 2.05%), spiny mice (*Acomys* sp.)(n = 7; 1.79%), the striped bush squirrel (*Paraxerus flavovittis*) (n = 3; 0.77%) (Figure 5.3). *Rattus rattus* was the most common species trapped in 11 (91.7%) of 12 study villages in both rural and urban locations. *Mus musculus* however was only trapped in two urban villages (Village C: n = 25 (80.6%) of 31 rodents; and Village F: Wet season 7 (9.21%) of 76 rodents; Dry season (N(F)) 12 (36.4%) of 33 rodents).

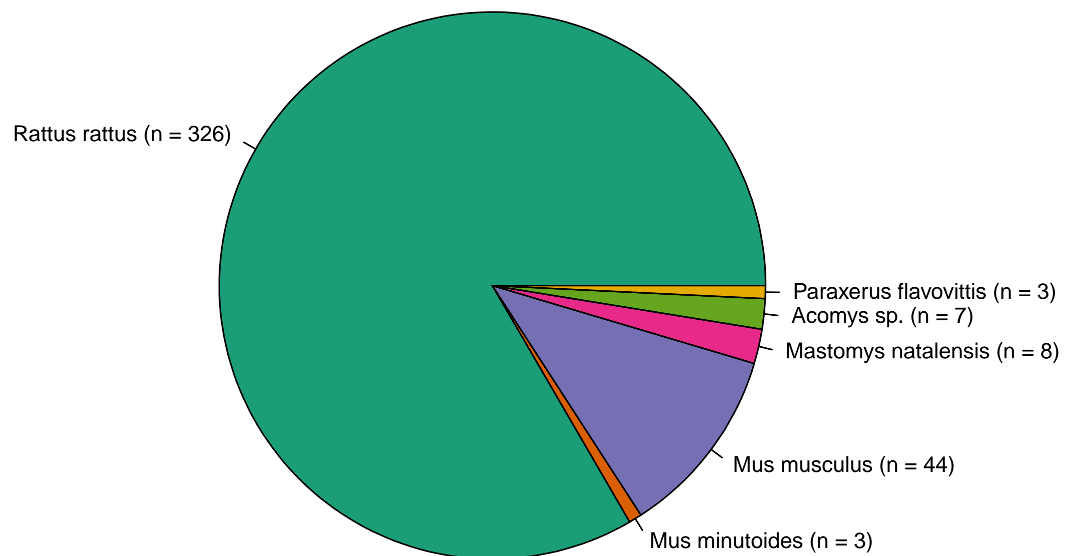


Figure 5.3: Pie chart of rodent species trapped (with sample sizes)

5.3.6 Rodent gender and age class

Overall, female rodents accounted for 57.4% (n = 391) of the trapped population. The majority of animal trapped were sexually mature (58.9%) but the proportion varied by village (Figure 5.4). For the most common species: significantly more immature *R. rattus* were trapped in during wet season sampling than in the dry season ($\chi^2 = 20.5$, $p < 0.001$). For *Mus musculus,* no difference was observed between seasons (χ^2; $p = 0.696$). Pregnancy data collection was incomplete and could not be analysed.

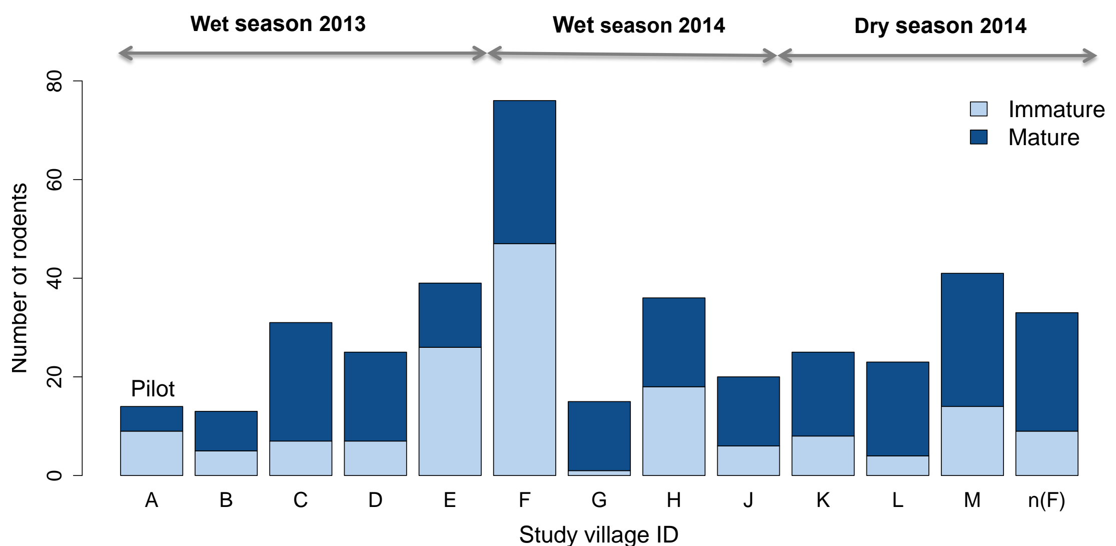


Figure 5.4: Age class of trapped rodents (all species) by study village and season

5.3.7 *Leptospira* culture results

Culture media was inoculated from 100 rodents between 2013 and 2014 (Table 5.5). Kidney sampled for culture were taken from *R. rattus* (n = 76), *M. musculus* (n = 18), *Mastomys natalensis* (n = 4), *M minutoides* (n = 1) and *Mastomys natalensis* (n = 1). *Leptospira* organisms were not isolated from any sample (Table 5.6).

5.3.8 Results from *lip*L32 TaqMan® qPCR testing for *Leptospira* infection

Six rodent kidney samples (three *R. rattus* and three *M. musculus*) were excluded from qPCR testing due to DNA extraction failure, indicated by a final DNA concentration < 10 ng/µl. In total, 0 of 385 rodent kidney samples tested by *lip*L32 qPCR TaqMan® assay were positive for *Leptospira* infection (Table 5.6).

Table 5.6: Results for *Leptospira* testing on rodent kidneys (qPCR and culture)

Leptospira diagnostic test	Number of rodents tested per test	Number of positive rodents	Prevalence (Binomial CI)
***lip*L32 TaqMan® qPCR**	385	0	0% (0.00 - 0.01%)
***Leptospira* culture (EMJH-5FU)**	100	0	0% 0.00 – 0.04%
***sec*Y SYBR® Green qPCR**	60	0	0% 0.00 – 0.06%

5.3.9 Results from *sec*Y SYBR® Green qPCR testing for *Leptospira* infection

The *sec*Y SYBR® Green qPCR assay (KIT) was run as a confirmatory test on DNA extracts from a randomly selected subset of rodent kidneys. In total, 0 of 60 rodent kidney samples tested by *sec*Y SYBR® Green qPCR were positive for *Leptospira* infection (Table 5.6).

5.4 Discussion

The goal of this study component was to explore the patterns of *Leptospira* infection in rodents that live in close proximity with people in two districts of northern Tanzania where the incidence of human leptospirosis is high. Contrary to expectations, all rodents trapped and tested in this study (n = 385) were negative for *Leptospira* infection (Table 5.6). This was a surprising finding but is believed to be a true reflection of the infection status of commensal rodents living in this area. Considerable efforts were made in the design of this study to ensure that a representative sample of rodents was trapped and tested in this study. Sampling was performed over two years (and two seasons in 2014) to account for any short-term fluctuations in rodent infection prevalence and 12 different sites. Sites were randomly selected (with the exception of the pilot village) and covered a variety of environments from densely populated urban areas to mountainous rural villages (Table 5.3). A variety of trap types and sizes were used to ensure representative sampling of different ages, sizes and species of rodent, which are all factors known to influence the prevalence of infection in other settings (Desvars et al., 2013b, Halliday et al., 2013, Costa et al., 2015b). Therefore the animals trapped in this study are believed to be a reliable representation of rodents living in the peri-domestic environments in this geographical area.

Substantial work went into the selection appropriate diagnostic tests and to validate test results in this study. Two complementary testing approaches (*lip*L32 qPCR and culture) were used to diagnose *Leptospira* infection in kidney samples. The *lip*L32 qPCR assay was selected based on evidence of high analytical sensitivity (Chapter 4.3.2) and excellent specificity for pathogenic *Leptospira* species (Stoddard et al., 2009, Bourhy et al., 2011), as well as extensive field validation in studies of *Leptospira* infection in rodents in other settings (Himsworth et al., 2013, Cosson et al., 2014, Desvars et al., 2013c, Munoz-Zanzi et al., 2014a). Furthermore, the validity of the entire qPCR testing pipeline from sample preservation through DNA extraction to qPCR was demonstrated by: i) the high level of agreement in test results between the *lip*L32 and *sec*Y qPCR assays in a batch of Dutch rodent samples of known infection status (Chapter 4.2.8) and in a randomly selected subset of the Tanzanian rodent samples (Chapter 5.3.9); ii) the utility of the same testing pipeline in demonstrating *Leptospira* infection in livestock samples (Chapter 6.2.6); and iii) the consistency between qPCR and culture results for a subset of 100 rodents (n = 391).

All final DNA elutions were diluted in PCR-grade water (1:10 dilution) prior to testing to mitigate the risk of qPCR inhibition in these samples. PCR inhibition, which can reduce the diagnostic sensitivity of a test in clinical samples, is a particular risk in kidney samples because of high concentrations of urea in the unprocessed tissue (Schrader et al., 2012). Systematic evaluation for qPCR inhibition was not carried out in this study although no evidence could be detected during a small scale validation study (Chapter 4.2.7) or when testing other batches of samples (e.g. the Dutch rodent kidney samples used to validate the *lip*L32 qPCR assay against the *sec*Y qPCR assay (Chapter 4.2.8) and the *lip*L32 qPCR testing of livestock samples (Chapter 6.2.6)). Overall, the final sample size of rodents tested by qPCR was considered sufficient at the 95% confidence level to demonstrate freedom from disease in this population even allowing for a low prevalence of infection (1.0%) and an imperfect test (80% sensitivity) (Sample size for freedom testing with imperfect tests: n = 368; population sensitivity 0.95 (Sergeant, 2016, Cameron and Baldock, 1998)).

As no obvious study design or diagnostic testing limitations could be identified in the study to account for the absence of *Leptospira* infection in this rodent population, the results of this cross-sectional rodent study are considered to be a robust representation of the infection status of the rodent population at the time of sampling. Therefore on the basis of these study results, we can conclude that the peri-domestic rodent population was not a source of *Leptospira* infection for people living in the Moshi study area over the period of this study. This conclusion raises some intriguing questions about patterns and drivers of *Leptospira* infection in rodent populations in northern Tanzania. It is important to note that the vast majority of rodents trapped in this study have been shown to be susceptible hosts of infection in other nearby settings (e.g. *R. rattus* and *M. musculus* in Nairobi (Halliday et al., 2013), *Mastomys natalensis* in Morogoro (Mgode et al., 2015, Mgode et al., 2005)). Furthermore, this is not an isolated population of rodents that may have been protected from the introduction of infection by some kind of geographical barrier. Moshi is located on the major highway connecting the cities of Nairobi (where rodent *Leptospira* infection has been demonstrated (Halliday et al., 2013)) and Dar es Salaam, a major sea-port. Commensal rodents are known to exploit human transport networks to disperse and invade new areas (Brouat et al., 2014, Aplin et al., 2011). Therefore geographic isolation does not seem like a feasible explanation for the lack of *Leptospira* infection in the Moshi rodents.

However, geographic and environmental factors may play a role in the absence of detectable infection at this site. Fine-scale and regional variation in *Leptospira* prevalence is reported in studies around the world (Cosson et al., 2014, Himsworth et al., 2013). In the

African region, marked variation in *Leptospira* infection prevalence was reported between the same rodent species sampled in different study sites in Madagascar (Rahelinirina et al., 2010) and a study in Niger demonstrated *Leptospira* infection in rodents from irrigated agricultural areas but not in rodents living in densely population urban areas (Dobigny et al., 2015). The specific environmental factors that influence rodent *Leptospira* infection are not well understand and are likely to vary in different geographic regions and with different host and *Leptospira* species. In the future, multi-site studies in Tanzania could offer insights into whether the patterns of infection detected in this study are a widespread phenomenon or the consequence of some as yet unidentified site-specific factors.

A variety of host population factors may influence the ability of a rodent population to maintain *Leptospira* infection in a particular setting. The presence of susceptible rodent species within a community is important, and these species must be present in sufficient abundance or density for the pathogen to be maintained within the population. In the disease ecology literature, this concept is referred to as the critical community size, defined as size of a host population below which a disease cannot persist in the long term (Lloyd-Smith et al., 2005, Haydon et al., 2002). In general, fluctuations in the abundance of rodent hosts are thought to influence the dynamics of *Leptospira* infection in rodent communities (Holt et al., 2006, Davis and Calvet, 2005). Factors relating to the abundance of susceptible hosts are one possible explanation for the absence of *Leptospira* infection in peri-domestic rodents in Moshi in the trapping seasons of 2013-14. However, whilst factors relating to host abundance could be plausible for the less commonly trapped species in this study (e.g. *Mastomys natalensis* (Figure 5.3)), this seems improbable for *R. rattus,* which was trapped in large numbers in some villages and houses. Up to 21 *R. rattus* individuals were caught in a single house of an eight-day trapping period in one village (village F). *Leptospira* infection has also been demonstrated in similarly-abundant *R. rattus* populations in other settings (Munoz-Zanzi et al., 2014a). Therefore, if infection was absent from an apparently abundant population of hosts, it seems plausible that this host species (*R. rattus*) is in fact not well-adapted to maintaining the specific types of *Leptospira* circulating in Moshi, and hence is not a maintenance host in this setting.

With regards to the composition of the rodent host community in Moshi, it was notable that *Rattus norvegicus* was absent from this site. *R. norvegicus* is well-described as a maintenance host of *Leptospira* both in natural settings and in the laboratory (Chapter 5.1.1). *R. rattus* is generally assumed to fill the same ecological niche as *R. norvegicus* in the maintenance of the common rat-associated leptospires but to date, there is little to no experimental evidence to support this assumption. In natural settings, studies

of *R. norvegicus* appear well-adapted to maintain infection with common rodent-associated serovars in the absence of other hosts (e.g. (Costa et al., 2014, Himsworth et al., 2013). However, in other sites in Africa, *Leptospira* infection has also been demonstrated as absent from an abundant population of *R. rattus* where no *R. norvegicus* was trapped (Dobigny et al., 2015). In contrast, where *Leptospira* has been demonstrated in *R. rattus* in the East African region, infected *R. norvegicus* individuals have also been identified in living in the same environment (e.g. Kenya (Halliday et al., 2013) and Madagascar (Rahelinirina et al., 2010)). It is difficult to know how representative the available literature is as negative results often go unpublished and representative trapping of all rodent species in a particular environment may not be performed. However, it is possible that *R. rattus* has a very different role in the maintenance of *Leptospira* infection that its better studied cousin, *R. norvegicus*. Although this hypothesis remains largely speculative at present, sub-Saharan Africa may offer a unique opportunity to better characterise the true role of *R. rattus* in the maintenance of *Leptospira* infection as this area still remains a stronghold of *R. rattus,* with *R. norvegicus* incursions currently limited to the coast or major cities (Aplin et al., 2011, Brouat et al., 2014, The Field Museum, 2011).

As well as posing some fascinating questions about the factors that support *Leptospira* maintenance in a commensal rodent population, the findings of this study also have some important implications for our understanding of the sources of infection for people. Although rodents are often assumed to be the source of infection for people (Haake and Levett, 2015), the lack of infection in rodents living in close proximity to people in Moshi implies that other animal hosts are more important in the epidemiology of leptospirosis this setting. Investigations into the other potential hosts of *Leptospira* infection are recommended to further explore the epidemiology of leptospirosis in northern Tanzania and identify potential sources of infection for people. One such investigation, into the epidemiology of leptospirosis in ruminant livestock species in northern Tanzania is described in the next chapter of this thesis (Chapter 6).

6 *Leptospira* infection in ruminant livestock in the Kilimanjaro Region

6.1 Introduction

Ruminant livestock species are important hosts in the epidemiology of *Leptospira* infection. All major ruminant livestock species are susceptible, and once infected may shed leptospires in their urine for several years following infection (Ellis, 2015). Infected livestock therefore may pose a considerable zoonotic disease risk for people who live and work in close proximity with these animals (Haake and Levett, 2015, Mwachui et al., 2015). Cattle are the best known ruminant livestock host of *Leptospira*, but infection is also reported in sheep, goats (Martins and Lilenbaum, 2014), water buffalo (Marianelli et al., 2007), camelids (Ellis, 2015) and even deer where they are farmed commercially (Ayanegui-Alcerreca et al., 2007, Desvars et al., 2013c). *Leptospira* infection also has detrimental effects on the health of livestock and can lead to production losses resulting from reproductive failure, for example abortions or infertility, or reduced milk yield (Ellis, 1994, Ellis, 2015). As a consequence, livestock leptospirosis results in financial losses for livestock keepers. Although these economic impacts of infection have not yet been systematically quantified, in a low-income setting such as Tanzania where livestock plays an important role in food security, household income and the national economy (Food and Agriculture Organization of the United Nations, 2005), livestock leptospirosis has the potential to be a pervasive threat to both human and animal wellbeing.

6.1.1 The epidemiology of *Leptospira* infection in ruminant livestock

The epidemiology of *Leptospira* infection in ruminant livestock is classically described in two broad patterns, reviewed in more detail in the general introduction of this thesis (Chapter 1.5). A livestock host may become infected with a particular *Leptospira* serovar that is typically maintained within the population of that particular livestock species (Blackmore and Hathaway, 1979). Alternatively, an animal may become 'accidentally' infected with a *Leptospira* serovar that spills over from another, conspecific animal population, for example a wildlife host of infection (Lilenbaum and Martins, 2014). Direct transmission through urinary contact or indirect transmission through contact with a contaminated environmental reservoir may occur in either epidemiological scenario. However, sexual transmission is also implicated in infection maintenance in livestock

populations as leptospires frequently colonise the genital tract of infected hosts belonging to several major ruminant livestock species (Lilenbaum et al., 2008, Ellis, 1994).

Clinical disease may occur in ruminant livestock following *Leptospira* infection. Acute systemic illness is relatively rare but can present in the initial leptospiraemic phase of infection resulting in pyrexia, anaemia, jaundice and can be fatal in young animals (Ellis, 2015). In dairy cattle, an acute drop in milk production is also reported in the early stage of infection (Dhaliwal et al., 1996a). In the second phase of infection, leptospires colonise the kidney of infected hosts and are shed in the urine. The bacteria may also localise to the uterus of pregnant females leading to vertical transmission of infection, abortion of infected foetuses or poor neonatal viability (Ellis et al., 1986, Ellis, 1994, Cortizo et al., 2015). Chronic infection is typically subclinical although fertility may be persistently suppressed and infected animals may continue to shed *Leptospira* for years following the initial infection (Leonard et al., 1992).

A wide variety of *Leptospira* serovars and serogroups have been reported in ruminant livestock. However, a particularly close relationship is reported between certain livestock species and specific *Leptospira* serovars (Bharti et al., 2003). The main example is the relationship between cattle and the Hardjo serovars. Bovine infection is frequently associated with one of the two Hardjo serovars, either *L. borgpetersenii* serovar Hardjo (Hardjobovis) or *L. interrogans* serovar Hardjo (Hardjoprajitno) (Ellis, 2015). Cattle appear to be well adapted to sustaining prolonged infections with these serovars (Leonard et al., 1992), which they are able to maintain independently of environmental factors or the presence other animal hosts (Ellis, 1984). Bovine Hardjo infection has been reported in most continents including Europe (Dhaliwal et al., 1996a, van Schaik et al., 2002), North and South America (Van De Weyer et al., 2011, Salgado et al., 2014), Australasia (Fang et al., 2014a, Elder and Ward, 1978), Asia (Odontsetseg et al., 2005, Bahaman et al., 1988) and Africa (Feresu et al., 1999b, Te Brugge and Dreyer, 1985). Sheep have also been shown to maintain *L. borgpetersenii* serovar Hardjo in isolation from cattle, and thus are also implicated as adapted maintenance hosts of this particular serovar in some settings (Arent et al., 2013, Vallee et al., 2015, Cousins et al., 1989). Other serovar associations have been reported, (e.g. cattle infections with *L. interrogans* serovar Kennewicki) but these relationships are less well-characterised and more geographically variable than the Hardjo-cattle association (Ellis, 2015, Lilenbaum and Martins, 2014).

6.1.2 Risk factors for livestock *Leptospira* infection

Environmental and farm management aspects are known to be risk factors for livestock *Leptospira* infection. Environmental factors such as the presence of other infected animal hosts, warm wet conditions that promote the *ex vivo* survival of *Leptospira* and heavy seasonal rainfall leading to standing water and flooding are risk factors for *Leptospira* infection that apply equally to any animal species including people (Lau et al., 2010, Mwachui et al., 2015, Ellis, 1984). However, specific farm management factors are also important for livestock infection. Firstly, biosecurity is important as infection may be introduced into a herd when infected animals are bought as replacement stock (Williams and Winden, 2014). Herd size and mixed management and grazing of multiple livestock species have also been shown to be risk factors for infection (Oliveira et al., 2010, Lilenbaum and Souza, 2003, Ryan et al., 2012, Subharat et al., 2012). In addition, certain strategies for reproductive management such as the use of natural service may predispose to infection due to the localisation of leptospires to the genital tract in the major livestock species, (Ellis, 2015, Lilenbaum et al., 2008). In tropical areas of the world where many livestock management risk factors and environmental risk factors for livestock leptospirosis coincide, a large proportion of the cattle population may be at risk of infection (Ellis, 1984).

6.1.3 Livestock leptospirosis in Africa

Although surveillance is somewhat fragmented in Africa, livestock leptospirosis appears to be prevalent in many parts of the continent. Serological surveys in sub-Saharan Africa have demonstrated that *Leptospira* exposure is widespread in cattle in the region (de Vries et al., 2014). In the systematic review described in Chapter 2, cattle were the most commonly reported animal host species of *Leptospira* infection in Africa and were associated with a wide range of serovars and serogroups (Table 2.4). In small ruminants reports of infection are fewer but serological exposure has been identified in both sheep and goats in sub-Saharan Africa (de Vries et al., 2014), and infection was demonstrated by PCR in sheep in Morocco (Benkirane et al., 2014) and goats on the island of Mayotte (Desvars et al., 2013c). Therefore all three of these major ruminant production animal species may play a role in the epidemiology of leptospirosis in Africa.

6.1.4 Leptospirosis in Tanzanian livestock

In Tanzania, both serological and microbiological evidence exists for *Leptospira* infection in cattle. In the Morogoro Region of central Tanzania, two serovars – *L. kirschneri* serovar Sokoine (serogroup Icterohaemorrhagiae) and *L. kirschneri* serovar Grippotyphosa (serogroup Grippotyphosa) – were isolated from the urine of cattle sampled in the abattoir (Mgode et al., 2015, Mgode et al., 2006). In addition, seroprevalence surveys performed across the country have demonstrated widespread seroreactivity in cattle (Table 6.1). All studies used the microscopic agglutination test (MAT) for serological diagnosis of *Leptospira* exposure. In general, heterogeneity in MAT test serovars and serogroups limited the scope for comparisons between regions and studies. However, serogroup Sejroe was used in all studies and this serogroup was demonstrated as the most common reactive serogroup in nearly all the seroprevalence studies of cattle conducted in Tanzania (Karimuribo et al., 2008, Swai and Schoonman, 2012, Assenga et al., 2015, Schoonman and Swai, 2010, Machang'u et al., 1997).

In goats, evidence of *Leptospira* exposure is limited to a single study in the Katavi Region, which tested cattle, goats and people from the same study households. Considerable seroreactivity was demonstrated in goats in this study with 17.7% of animals demonstrating reactivity to at least one of the six test serovars (Assenga et al., 2015). In goats, reactivity to Icterohaemorrhagiae using *L. kirschneri* serovar Sokoine, previously isolated from cattle in Tanzania, was most common, but reactivity to serogroup Sejroe was also reported (Table 7.1).

As a group, data from these seroprevalence studies indicate that *Leptospira* exposure is common ruminant livestock across Tanzania. Demonstration of pathogenic leptospires in the urine of cattle in Morogoro indicates that this species has the potential to be source of human *Leptospira* infection in Tanzania, particularly for abattoir workers and other people coming into close contact with cattle. However, to date, there are no available data from livestock infection in the Kilimanjaro Region, where the burden of acute human leptospirosis is high.

Table 6.1: Predominant reactive serogroups from seroprevalence studies for ruminant livestock species (single MAT titre ≥ 1:160)

	Goats	Cattle					
Study region (number of animals tested)	Katavi (n=248) (Assenga et al., 2015)	Katavi (n=1103) (Assenga et al., 2015)	Tanga (n=51) (Swai and Schoonman, 2012)	Tanga (n=654) (Schoonman and Swai, 2010)	Usambara (n=80) (Karimuribo et al., 2008)	Tanga (n=230) (Swai et al., 2005)	Various (n=374) (Machang'u et al., 1997)
Study type	Cross-sectional	Cross-sectional	Abattoir surveillance	Cross-sectional	Case-control	Cross-sectional	Abattoir surveillance
Serogroup: Australis	0.4%	0.82%	-	-	-	-	-
Ballum	0%	0%	-	-	-	-	-
Bataviae	-	-	3.9%	1.8%	-	0.9%	-
Canicola	0%	0%	-	-	0%	-	-
Grippotyphosa	1.6%	4.8%	-	-	2.5%	0.4%	-
Hebdomadis	0.8%	7.7%	-	-	-	-	-
Icterohaemorrhagiae	3.2%	4.7%	-	-	3.8%	-	-
Pomona	-	-	0%	1.2%	2.5%	1.3%	-
Pyrogenes	-	-	-	-	-	-	1.9% (n=360)
Sejroe	2.8%	17.6%	29.4%	15.0%	12.5%	3.5%	5.6%
Tarassovi	-	-	17.6%	12.2%	-	4.8%	-

6.1.5 Goals and objectives of this chapter

This study component aimed to explore patterns of *Leptospira* infection in cattle, sheep and goats, which are the three major ruminant livestock species kept for food production in northern Tanzania. Livestock are considered an important source of *Leptospira* for people in other settings, but little is known about the role of livestock in the epidemiology of human infection in Tanzania. This chapter describes an abattoir surveillance study of ruminant livestock established to address the following research objectives:

Objective 1: Determine the prevalence and patterns of *Leptospira* infection in three different ruminant livestock hosts in the Kilimanjaro Region;

Objective 2: Identify and described common types of *Leptospira* bacteria found in livestock infections;

Objective 3: Explore serological reactivity patterns in cattle to:

i) compare serological data on common reactive serogroups with genetic data derived from livestock samples;
ii) generate serological data comparable with data available from human leptospirosis cases in northern Tanzania.

Objective 4: Compare the performance of different diagnostic tests for *Leptospira* in the Tanzanian cattle population.

6.2 Methods

To explore *Leptospira* infection in livestock the Kilimanjaro Region, an abattoir surveillance study was established within the Moshi Municipal District. This district is the administrative centre for the region and also the location of the hospital-based febrile disease surveillance, described in Chapter 3.2 and Chapter 7. Kidney samples were collected from cattle, sheep and goats and tested for *Leptospira* infection by qPCR and culture. Urine and serum samples were also collected from a subset of animals (mostly cattle) and the performance of different testing approaches was compared in this setting. *Leptospira* from ruminant infections were typed to characterise the pathogen in qPCR or culture positive cases. Detailed methodology for this study is described below.

6.2.1 Slaughterhouse sampling frame

Slaughterhouses for ruminant livestock were identified in liaison with the District Veterinary Officer for the Moshi Municipal District. In total, one abattoir and 17 smaller slaughter slabs were identified within the District (Figure 6.1). Data on the average number of cattle slaughtered at each site per week was collected from Livestock Field Officers (LFOs) responsible for meat inspection at each of the District slaughterhouses. The weekly throughput ranged from an average of 210 cattle per week at the main Moshi abattoir down to one animal per week at some smaller slaughter slabs (shown in labels on Figure 6.1). The main Moshi abattoir and four small slaughter slabs were selected for convenience of livestock sampling on the basis of moderate-to-high throughput of cattle, convenient locations and cooperative LFOs.

6.2.2 Sample collection

Slaughterhouse sampling was performed opportunistically in two sampling sessions that were performed between 1st May and 18th July 2013 and 5th December 2013 to 11th September 2014. Sampling at the main Moshi abattoir was only performed during 2013 due to closure for refurbishment in 2014.

A maximum of ten animals per species were sampled each day. In liaison with LFOs, animal details were recorded including source of animal (market vs. household), region and district of origin, approximate age (adult vs. juvenile), sex and breed.

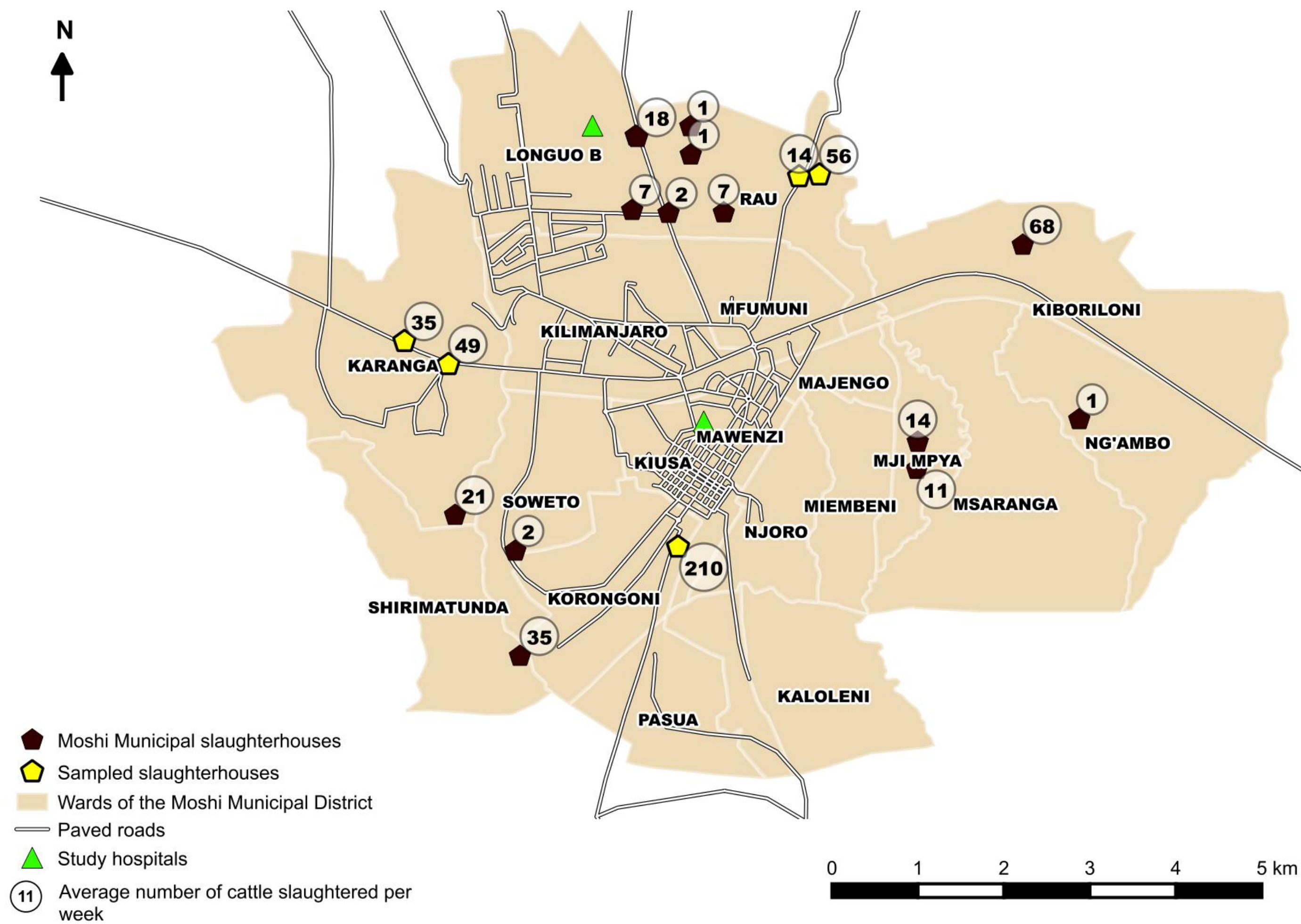


Figure 6.1: Map showing the location and average weekly throughput of cattle in the Moshi Municipal slaughterhouses

For each animal slaughtered, one kidney was collected into a clean, labelled, single-use Ziplok® bag. Samples of kidney tissue (3 x 1 x 1 cm) were taken across the cortico-medullary junction within three hours of slaughter and placed directly into 70-96% ethanol. From a subset of animals, urine samples were taken by urinary bladder cystocentesis during meat inspection, and stored without preservatives at minus 80°C. Blood samples were also collected from a different subset of cattle prior to slaughter. Serum was separated by centrifugation and stored without preservatives at minus 80°C.

6.2.3 *Leptospira* culture from cattle and goat kidneys

In a subset of cattle (n=100) and goats (n=49), a portion of kidney tissue was used to inoculate EMJH-5FU culture media for *Leptospira* spp. culture (Chapter 3.8). Selection of individual carcasses for *Leptospira* culture was largely governed by logistical constraints (i.e. availability of culture media and shipping constraints). When culture media was available, vials were inoculated for up to five individuals per day and continued on consecutive sampling days until a maximum of 50 cattle had been sampled each year. Culture from goats was only attempted in 2014. Following inoculation samples were shipped to the WHO/FAO/OIE Leptospirosis Reference Laboratory, Royal Tropical Institute (KIT), Amsterdam for propagation, isolation and typing of positive samples.

6.2.4 Typing of isolated *Leptospira* bacteria

Serological typing of isolates (MG) was performed at KIT (MG) following standard *Leptospira* typing methods outlined in Chapter 3.9. DNA was extracted from pure cultures of each isolate (Chapter 3.6.1). Genotyping of isolates (AA) was performed by PCR-based amplification and sequencing of the *sec*Y gene based on the protocol described by Victoria *et al.* (2008) to identify *Leptospira* species (Chapter 3.10).

Multi-locus sequence typing (MLST) was also performed to identify the *Leptospira* sequence type (ST) as described by Boonsilp *et al.* (2013) (Chapter 3.10). A set of seven different primer pairs targeting different loci within *Leptospira* housekeeping genes (*glmU*, *pntA*, *sucA*, *tpiA*, *pfkB, mreA* and *caiB)* were used to generate products for sequencing. PCR reactions, product purification and sequencing were performed at KIT (AA). Sequence analysis was performed by KA using sequence analysis software (Sequencher® version 5.3, Gene Codes Corporation MI). Trimmed sequences were aligned with reference sequences (n = 1046) curated by an online MLST database

(http://pubmlst.org/leptospira/(Jolley and Maiden, 2010, Jolley and Maiden, 2016) to generate a unique allelic profile for each isolate. Finally each allelic profile was compared to a database of 223 profiles to determine the ST and the most similar *Leptospira* serovar (Boonsilp et al., 2013).

6.2.5 DNA extraction from livestock kidney and urine samples

DNA was extracted from ethanol-fixed kidneys as described in Chapter 3.6.2 and quantified using a NanoDrop® spectrophotometer (ThermoScientific, Waltham, MA). Prior to PCR or qPCR testing, total genomic DNA (gDNA) was diluted 1:10 in PCR-grade water (or 1:5 in samples with a final DNA concentration < 100ng/µl) to mitigate the effect any residual qPCR inhibitors. The test concentration of gDNA ranged from approximately 50ng to 150ng per 25µl qPCR reaction.

DNA was extracted from urine samples as described in Chapter 3.6.3 and quantified by Qubit® Fluorometer (ThermoScientific) using the Qubit® dsDNA High Sensitivity assay kit. Final DNA concentrations ranged from <0.05 ng/µl to >60 ng/µl. DNA extracted from urine samples were tested undiluted due to low final concentrations.

6.2.6 Nucleic acid amplification by *lipL32* TaqMan® qPCR assay for *Leptospira* infection

Samples were run on MicroAmp® 96 well plates and tested in duplicate with the *lip*L32 assay as described in Chapter 3.7.2 (Stoddard, 2013, Stoddard et al., 2009). Each plate included: two replicates of a *Leptospira* positive control; *L. interrogans* serovar Copenhagenii Strain Wijnberg at ~10^2 genome copies numbers; two replicates of a non-template extraction control, and two replicates of a negative control (PCR-grade water). Each reaction run was considered valid when both negative controls were negative and at least one replicate of the both the *Leptospira* positive controls amplified with Ct value < 40. Samples were considered positive when at least one test well amplified the *lip*L32 target with a Ct value < 40.

6.2.7 Identification of infecting *Leptospira* species from PCR-positive samples

Infecting *Leptospira* species in *lip*L32 qPCR-positive kidney samples were identified by PCR-based amplification and sequencing of the *sec*Y gene used a protocol modified for use with non-isolate samples from the East African region (Dietrich et al., 2014). A 470-bp fragment of the *sec*Y gene was amplified and sequenced as described in Chapter 3.10 at the University of Aberdeen (MM). DNA from *Leptospira* isolates obtained from cattle in Tanzania (Chapter 6.2.4) was also sequenced using the Dietrich *et al.* protocol to allow *Leptospira* sequence from qPCR-positive kidney DNA samples and kidney isolates from the same animal to be directly compared.

6.2.8 Sequence analysis

Analysis of *sec*Y sequences was performed using MEGA7.0 (Kumar et al., 2015). Electropherograms for forward sequences from *sec*Y PCR products were compiled and were checked by eye. Sequences were trimmed to exclude sequence of low quality at either end of the reads and to exclude any ambiguous base calls. Then, the most appropriate evolutionary model for the sequence nucleotide substitution rate was selected using the MEGA7.0 model test function. Finally, a phylogenetic tree of *sec*Y sequences from livestock *Leptospira* sequences was constructed using the maximum likelihood method. The stability of internal nodes within the tree was evaluated using 1000 bootstrap iterations. Sequences from each cluster as identified in the phylogenetic tree were compared to published sequences in GenBank® (Benson et al., 2011). Sequences from *Leptospira* serovars with a high proportion of sequence similarity (≥ 98%) were also included for reference in the final tree.

6.2.9 Serological diagnosis of Leptospira exposure in cattle by Microscopic Agglutination Test (MAT)

For a subset of cattle, sera were tested for evidence of *Leptospira* by MAT at KIT (performed by KA under supervision from MG) following standard protocols (Goris and Hartskeerl, 2013, Hartskeerl et al., 2006). Sera were tested against a panel of 26 *Leptospira* serovars from 17 serogroups (Table 6.2). Serogroups and representative serovars for inclusion on the panel were chosen using the following rationale:

1. Reference *Leptospira* serovars recommended for representative MAT testing by WHO/FAO/OIE Collaborating Reference Laboratories (Goris and Hartskeerl, 2013)
2. Serovars used in MAT panels in previous studies of human disease in Tanzania (Biggs et al., 2011)
3. Serovars previously isolated from livestock and rodents in Tanzania (Mgode et al., 2015)
4. Serovars previously isolated from people and animals in other parts of Africa (Royal Tropical Institute (KIT), 2014).

For each sample and test serovar, the MAT titre was defined as the highest dilution at which ≥ 50% of leptospires were still agglutinated (Goris and Hartskeerl, 2013). Samples were considered positive for seroreactivity with a MAT titre ≥1:40 against at least one serovar. Predominant reactive serogroups were defined for each animal as the serogroup of the test serovar with the highest observed titre. Where equivalent titres were observed to more than one serogroup, all serogroups were recorded.

6.2.10 Analysis of serological reactivity profiles

Serovar and serogroup serological reactivity was assessed within the dataset of cattle MAT titres. Heat maps plotted using in R (R Core Team, 2015) using the gplots package (Warnes et al., 2015) were used to identify distinct serological patterns in cattle titres. All MAT titres for cattle were included. A negative result (MAT < 1:20) was given the value of 10 for the purpose of analysis. Data were arranged in a matrix where each row represented the $\log_{10}$-transformed reciprocal MAT titres for a given animal and each column represented the MAT test serovar. Column order was fixed alphabetically by serovar. Initially, row ordering was generated at random to check that the results of subsequent clustering analyses were not influenced by the order of data entry. Next, Euclidean distances (i.e. square root of the sum of squared differences between the elements of a pair of rows) were calculated for pairwise sample comparisons between each row of the matrix (Everitt, 2005). A hierarchical clustering algorithm based on a complete linkage cluster method was performed using the core stats package in R (R Core Team, 2015) to reorder rows. Data were plotted using the clustered arrangement of cattle samples, and colour coded according to the magnitude of the MAT titre.

Table 6.2: Serovar panel for MAT testing of cattle sera

Serogroup	Serovar	Abbrv[‡]	1.[§] Reference serovar	2. Human disease	3. Tanzania serovar	4. African serovar
Australis	Australis	AusAus	✓	✓		
	Bratislava	AusBra	✓	✓		
	Lora	AusLor			✓	
Autumnalis	Lambwe	AusLam		✓		✓
Ballum	Ballum[**]	BalBal	✓			
Bataviae	Bataviae	BatBat	✓			
Canicola	Canicola	CanCan	✓	✓	✓	
Celledoni	Celledoni	CelCel	✓	✓		
Cynopteri	Cynopteri	CynCyn	✓			
Djasiman	Djasiman	DjaDja	✓	✓		
Grippotyphosa	Grippotyphosa	GriGri	✓	✓	✓	
Hebdomadis	Hebdomadis	HebHeb	✓	✓		
Icterohaem-orrhagiae	Copenhagenii	IctCop	✓			
	Icterohaem-orrhagiae	IctIct	✓	✓		
	Sokoine	IctSok			✓	
Mini	Mini[††]	MinMin	✓	✓		
Pomona	Pomona	PomPom	✓			
Pyrogenes	Kwale	PyrKwa				✓
	Pyrogenes	PyrPyr	✓	✓		
	Nigeria	PyrNig				✓
Sejroe	Hardjobovis	SejHbo	✓			
	Hardjo	SejHar	✓			
	Sejroe	SejSej	✓			
Semaranga	Patoc	SemPat	✓			
Tarassovi	Kanana	TarKan				✓
	Tarassovi	TarTar	✓	✓		

[‡] Abbreviations are used in Figures 6.5 and 6.6 in the results section of this chapter
[§] Number refers to rationale used for selection, referred to in the text
[**] In place of serovar Kenya (included by Assenga et al. 2015)
[††] In place of serovar Georgia (included by Biggs et al. 2011), which was not available for use

Spearman rank correlation coefficients were calculated to quantify pairwise associations in MAT titres between serovars in cattle serum samples. Correlation coefficients for each pairwise comparison were displayed in a correlation matrix generated in R using the package corrgram (Wright, 2015). Significance levels of each coefficient were set and displayed at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

6.2.11 Statistical analysis

Statistical analysis was performed in R (R Core Team, 2015). Binomial confidence for point prevalence estimates intervals (Wilson method) were calculated using the Hmisc package (Harrell et al., 2016). The Fisher's exact test was performed to compare infection prevalence in male and female animals. The McNemar's Chi-squared test was performed to test the significance of diagnostic test differences between kidney and urine qPCR results from the same animals. The degree of agreement between different diagnostic test approaches (e.g. kidney qPCR vs. urine qPCR; kidney qPCR vs. kidney culture; kidney qPCR vs. MAT) was assessed by calculating the Cohen's *kappa* statistic for inter-rater agreement (Thrusfield, 1995).

6.3 Results

6.3.1 Summary of ruminant livestock sampling

In total, samples were collected from 453 cattle, 167 goats and 90 sheep. Sampling was opportunistically divided across the five slaughterhouses as detailed in Table 6.3. Cattle were sampled at all study slaughterhouses. Goats were only sampled at the two Karanga slaughter slabs due to low availability at other sites.

Table 6.3: Sampling summary by slaughterhouse and livestock species

Slaughterhouse ID	Ward	Cattle	Sheep	Goats
SS01	Rau	92	-	-
SS02	Rau	273	-	-
SS03	Boma Mbuzi	70	40	-
SS04	Karanga	6	2	12
SS05	Karanga	12	48	141
NA	-	-	-	14*
Total	-	**453**	**90**	**167**

* Sampled at Karanga slabs but individual slab ID not recorded.

6.3.2 Livestock origins: the journey to slaughter

All animals sampled in this study were sourced from primary and secondary markets, mainly in the Arusha and Kilimanjaro Regions prior to slaughter. However, the majority of animals originated from regions much further afield. Of 453 cattle sampled, 384 (84.8%) originated from the Manyara Region, mainly from the districts of Mbulu (n= 296) and Babati Districts (n= 65) (Figure 6.2). Other regions of cattle origin included Arusha, Dodoma, Kilimanjaro, Mwanza, Singida, Tabora and Tanga. Manyara Region was also the region of origin of 115 (68.9%) goats and 48 (53.3%) sheep (Figure 6.3). District information was not available for goats but sheep also commonly came from the Babati District (n = 32). The rest of the small ruminants sampled came from the Arusha Region.

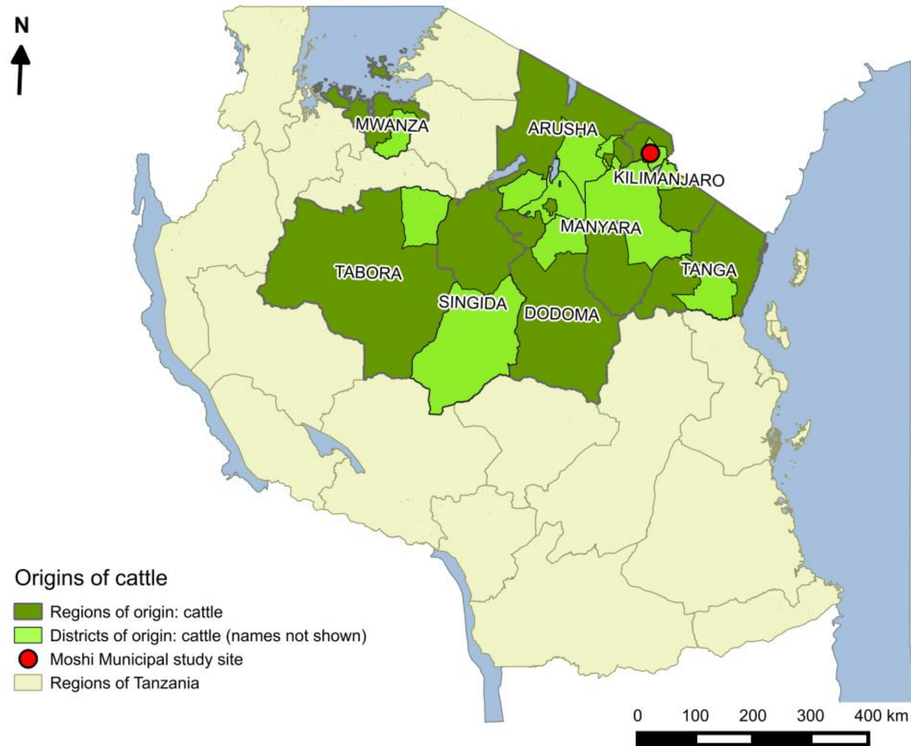


Figure 6.2: Regions and districts of origin of cattle sampled at Moshi slaughterhouses

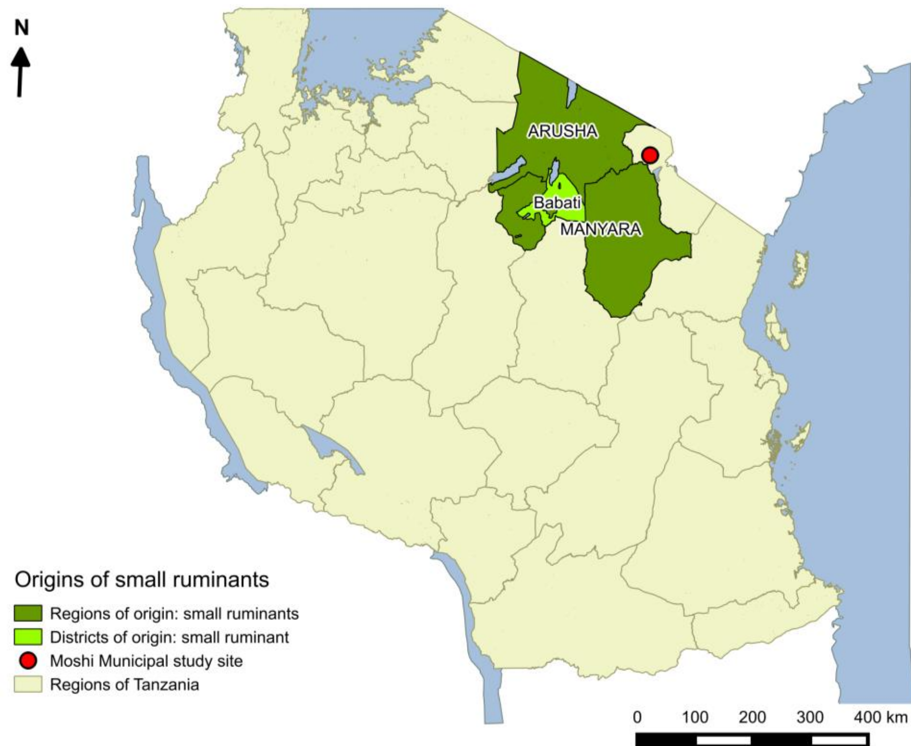


Figure 6.3: Regions and districts of origin of small ruminants sampled at Moshi slaughterhouses

6.3.3 Livestock demographics

Virtually all animals sampled at the abattoir were indigenous breeds. For cattle, the vast majority of animals of animals sampled (99.6%; n = 453) were indigenous humped Zebu breeds (*Bos indicus*). For small ruminants, only three animals (1.17%; n = 257) were classed as non-indigenous breeds. The majority of animals sampled were male (81.7% (n = 453) of cattle, 70.1% of goats (n = 167) and 53.3% of sheep (n=90)) and most animals were adult. Only a small number of juvenile animals were sampled (30 cattle, 11 sheep and 4 goats).

6.3.4 Summary of samples available for diagnostic testing

In total, qPCR was performed on kidney samples from 453 cattle, 167 goats and 90 sheep. Urine qPCR was also performed on a subset of 73 cattle, 29 goats and 40 sheep. Serum samples were available for MAT serology from 56 cattle (Table 6.4)

Table 6.4: Summary of ruminant livestock sampling by species, sample type and diagnostic test

Species:	Cattle	Goats	Sheep
Diagnostic test:			
Kidney qPCR only	190	88	50
Kidney qPCR and culture	100	50	0
Kidney qPCR and urine qPCR	73	29	40
Kidney qPCR and serology	56	0	0
Total number of animals sampled	**453**	**167**	**90**

6.3.5 Results of the *lip*L32 TaqMan® qPCR assay – kidney samples

Pathogenic *Leptospira* infection was detected by *lip*L32 qPCR in 32 (7.06%) of 453 cattle with kidneys available for testing. In small ruminants, *Leptospira* infection was detected in 2 (1.20%) of 167 goats and 1 (1.11%) sheep of 90 tested (Table 6.5).

Table 6.5: *Leptospira* qPCR results (*lip*L32 qPCR) for kidney and urine samples from livestock species

	Cattle		Goats		Sheep	
	Kidney	**Urine**	**Kidney**	**Urine**	**Kidney**	**Urine**
Total tested	**453**	**73**	**167**	**29**	**90**	**40**
qPCR negative	420	61	165	28	89	40
qPCR positive	32	12	2	1	1	0
Prevalence (%)	**7.06**	**16.4**	**1.12**	**3.45**	**1.12**	**0**
Binomial confidence interval (%)	5.05–9.80	9.66–26.6	0.33–4.26	0.18–17.2	0.06–6.03	0–8.76

All positive small ruminants (n = 3) originated from the Manyara Region. In cattle, positive cases were detected in cattle from Manyara, Arusha, Singida, Dodoma and Tanga regions. No significant difference was observed in infection prevalence between male and female cattle or adult or juvenile animals (Fisher's exact tests; $p > 0.05$).

6.3.6 Results of the *lip*L32 TaqMan® qPCR assay – urine samples

Leptospira infection was detected in 12 (16.4%) out of 73 cattle urine samples, one (3.45%) of 29 goat urine samples tested and no sheep urine samples (n = 40) (Table 6.5). All kidney qPCR positive cattle and sheep were also positive by urine qPCR; however urine qPCR testing failed to detect infection in one kidney-qPCR positive sheep. In cattle, urine qPCR identified significantly more *Leptospira* positive animals than kidney qPCR (Table 6.6; McNemar's $\chi^2 = 5.14$; df = 1, $p = 0.023$). Overall, moderate agreement was observed between the two tests (Cohen's *kappa* statistic 0.544; 95% CI 0.223 – 0.865; p = 0.008) (Thrusfield, 1995).

Table 6.6: Comparison of results from kidney qPCR versus urine qPCR for cattle

	Urine positive	Urine negative	Total
Kidney positive	5	0	5
Kidney negative	7	61	68
Total	**12**	**61**	**73**

6.3.7 *Leptospira* culture from cattle and goat kidneys

Leptospira isolates were obtained for 4 out of 100 cattle kidneys tested by culture. All culture-positive cattle were also qPCR-positive, but culture failed to demonstrate *Leptospira* infection in two qPCR-positive cattle (Table 6.7). However, in general, substantial agreement was observed between the two tests for cattle (Cohen's *kappa* statistic 0.790; 95% CI 0.502 – 1.08; p = 0.005) (Thrusfield, 1995). For goats, *Leptospira* infection was not detected by either qPCR or culture in any kidneys tested by both methods.

Table 6.7: Comparison of results from kidney qPCR versus kidney culture for cattle

	Kidney qPCR positive	Kidney qPCR negative	Total
Culture positive	4	0	4
Culture negative	2	94	96
Total	**6**	**94**	**100**

6.3.8 Typing of *Leptospira* isolates

All *Leptospira* isolates (n = 4) derived from culture of cattle kidneys were serologically typed as *L. borgpetersenii* serovar Hardjo (Hardjobovis), serogroup Sejroe. By MLST, an identical sequence profile was generated for all four isolates (ST 152), which corresponded to strains of *L. borgpetersenii* serovar Hardjo (Hardjobovis) in the reference database.

6.3.9 Results of *sec*Y typing of *Leptospira* from PCR-positive samples

After trimming and alignment, a 435-bp fragment of the *sec*Y gene was available for sequence analysis from 20 (60.6%) of 33 qPCR-positive kidney samples. By host species, this corresponded to 18 sequences derived from cattle infections, one sequence from an infected goat (C0417) and one sequence from an infected sheep (C0481). The majority of *Leptospira* sequences were derived from animals that originated in the region of Manyara, which was the most common region of origin for this study (section 6.3.2).

The phylogenetic relationships between livestock-derived *sec*Y sequences were inferred using a Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). From BLAST searches, *sec*Y sequences published for fully characterised *Leptospira* serovars with a high degree of similarity to livestock sequences were also included in the final sequence alignment of reference (Bulach et al., 2006, Victoria et al., 2008). The resulting phylogenetic tree is shown in Figure 6.4. *sec*Y sequences corresponding to *Leptospira borgpetersenii* were identified in 13 (72.2%) cattle samples. Two distinct clusters of *L. borgpetersenii* sequence types, which shared 97.7% sequence similarity, were identified in 100% of bootstrap replicates.

Sequences from eight (44.4%) cattle were grouped into a cluster (Figure 6.4: **Lb1**) that shared 100% similarity with sequences derived from reference strains of *L. borgpetersenii* serovar Hardjo (Hardjobovis strains L550 and JB197; accession number CP000348.1 (Bulach et al., 2006)). Sequences derived from cattle originating from Manyara, Arusha and Tanga Regions aligned perfectly in this group. *sec*Y sequences from cattle isolates obtained by this study described in Chapter 6.3.8 also aligned perfectly (100% similarity) with the Lb1 cluster.

Sequences from five cattle (27.8%) originating from Manyara and Singida were grouped into a cluster of *L. borgpetersenii* sequences (Figure 6.4: **Lb2**) that was clearly distinct from the serovar Hardjo cluster (Lb1). The only GenBank sequences with 100% similarity to this Lb2 group were reported from cattle infections in Brazil (Accession number KP862647.1 (Hamond et al., 2016)). However, *sec*Y sequences from several *L. borgpetersenii* reference serovars (*L. borgpetersenii* serovars Balcanica, Moldaviae, Nyanza, Tarassovi and Tunis (Victoria et al., 2008)) demonstrated 98% similarity with this cluster.

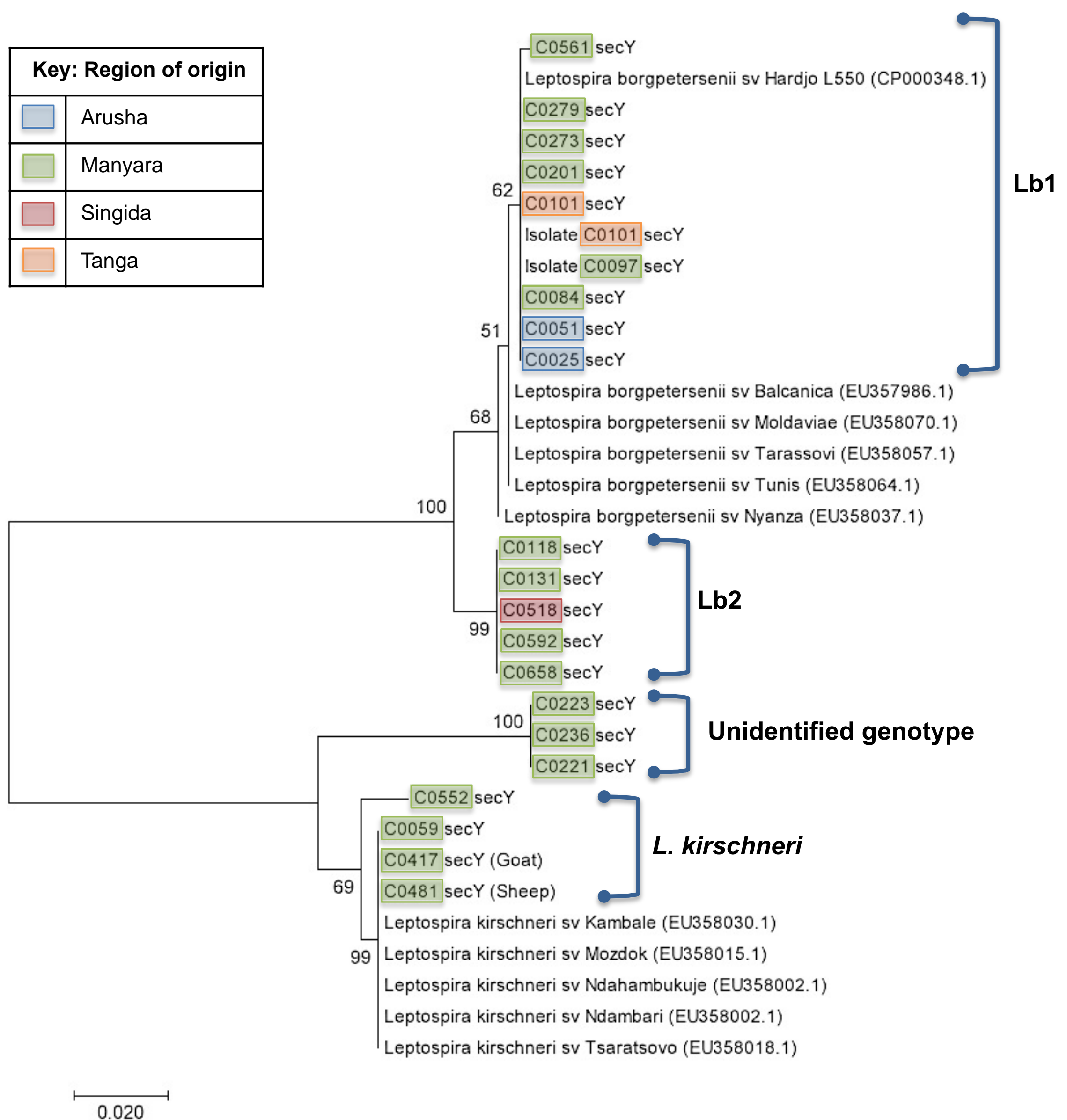


Figure 6.4: Molecular phylogenetic analysis of the *Leptospira sec*Y gene (435-bp fragment) by Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992).

Sequence analysis was performed on DNA extracted from PCR-positive livestock kidney samples. Selected reference serovars (including GenBank accession numbers) and isolates from Tanzanian cattle (this study) are shown for comparison. The tree with the highest log likelihood (-1036.7115) is shown and drawn to scale, with branch lengths measured in the number of substitutions per site. Nodal bootstrap support values are shown. Labels (Lb1, Lb2 etc.) refer to text descriptions. Livestock host species is cattle unless otherwise specified. Colour coding of identification (ID) numbers refers to the region of origin of each animal (Key). Unique ID numbers were sampled sequentially. ID numbers < 500 were sampled from May to July 2013. ID numbers > 500 were sampled from January to September 2014. Abbreviations: Lb = *L. borgpetersenii;* sv = serovar.

*sec*Y sequences corresponding to *L. kirschneri* were identified in samples from two (11.1%) cattle, one sheep and one goat (shown as ***L. kirschneri*** in Figure 6.4) all originating from the Manyara Region. Within this grouping, a subdivision of one cattle sample (C0552) was observed in 69% of bootstrap replicates. This sequence (C0552) showed lower overall similarity (98.9%) compared to the rest of the livestock-derived *L. kirschneri* sequence types. Livestock-derived sequences in the main cluster showed 100% similarity with *sec*Y sequences from several *L. kirschneri* reference serovars and including three serovars (Kambale, Ndahambukuje and Ndamnari) previously isolated from patients in the Democratic Republic of Congo (formerly Zaire) (Victoria et al., 2008, Royal Tropical Institute (KIT), 2014).

Finally, a cluster of three cattle-derived *Leptospira sec*Y sequences that showed relatively little similarity to previously reported *Leptospira sec*Y sequences was observed (labelled as **Unidentified genotype** in (Figure 6.4All cattle in this group originated from the Manyara Region (Mbulu District) and were sampled at two neighbouring slaughter slabs over a two-day period. Genetic sequences in this group showed only 95% similarity to the *L. kirschneri* group (Figure 6.4) and the most analogous published sequence (e.g. *L. kirschneri* serovar Grippotyphosa: accession number EU358028.1 (Victoria et al., 2008).

6.3.10 MAT results

MAT serological testing was performed on 56 cattle also tested for *Leptospira* infection by kidney qPCR. Using a cut-off MAT titre ≥ 1:40, serological reactivity was demonstrated against eight *Leptospira* serogroups in 27 (48.2%) of 56 animals (Table 6.8). Reactivity against at least one of the two Hardjo serovars was also common (14.3%; n = 56).

Overall, the pattern of predominant reactive serogroups followed the overall trend in seroprevalence, with the exception of serogroup Hebdomadis (Table 6.8). A single predominant serogroup could not be determined in four animals, which demonstrated equivalent titres to more two serogroups. These were serogroups Australis and Tarassovi (n=1); serogroups Icterohaemorrhagiae and Mini (n=1); serogroups Mini and Sejroe (n=1); and serogroups Mini and Tarassovi (n = 1).

Within predominant serogroups, a degree of serovar-specificity was observed. For serogroup Australis, high titres were only observed against serovar Lora. For serogroup Icterohaemorrhagiae, high titres were only observed against serovar Sokoine.

Table 6.8: Overall serogroup prevalence (MAT titres ≥1:40) and predominant serogroup prevalence in cattle by MAT

Serogroup	N positive (Titres ≥1:40)	Predominant serogroups (prevalence)
Mini	12 (21.4%)	8 (29.6%)
Sejroe	9 (16.1%)	5 (18.5%)
Tarassovi	8 (14.3%)	3 (11.1%)
Australis	3* (5.36%)	2* (7.41%)
Grippotyphosa	3 (5.36%)	3 (11.1%)
Semarang	3 (5.36%)	1 (3.70%)
Hebdomadis	2 (3.57%)	0
Icterohaemorrhagiae	2∞ (3.57%)	1∞ (3.70%)
Any serogroup	**27 (48.2%)**	**-**

* For serogroup Australis, titres ≥ 1:40 were observed to serovar Lora only;

∞ For serogroup Icterohaemorrhagiae; titres ≥ 1:40 were observed to serovar Sokoine only.

6.3.11 Assessing serological trends in cattle by heat map

Log_{10} transformed MAT titres (MAT ≥ 1:20) from 54 cattle were plotted as heat maps shown in Figure 6.5. *Leptospira* MAT serovars (see Table 6.2 for full serovar details) were plotted on the x-axis with cattle clustered by similarity shown by the dendrogram on the y-axis. Clustering algorithms reveal four main patterns (labelled on Figure 6.5) in MAT titres in this cattle serology dataset:

A: Cattle with **no observed MAT titres** (< 1:20) against any test serovar (n = 12).

B: Cattle with moderate to high MAT titres (yellow) against a **single serogroup**, most commonly serogroups Grippotyphosa, Sejroe or Tarassovi (n = 8).

C: Cattle with MAT titres to **at least two of the serogroups Mini, Sejroe and Hebdomadis**. Most commonly, high titres (yellow) are observed to both serogroups Mini and Sejroe. Hebdomadis titres are typically lower (dark blue) (n = 12).

D: Cattle with low titres (dark blue) against a range of other serogroups including Australis, Autumnalis, Icterohaemorrhagiae (serovar Sokoine) and Tarassovi (n = 22). This pattern is seen mostly in the group labelled D but also in a small group of samples between groups B and A.

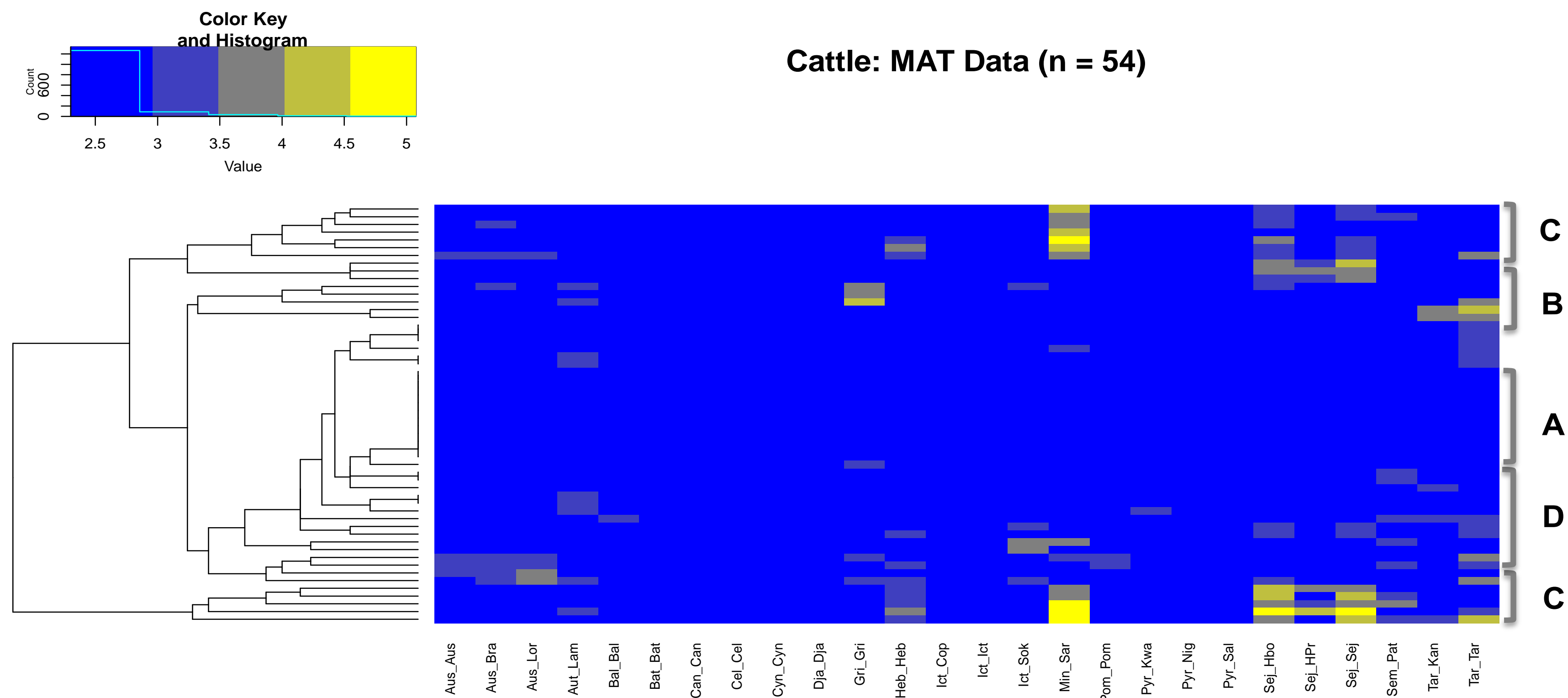


Figure 6.5: Heat map showing $\log_{10}$ transformed MAT titres by *Leptospira* MAT serovars for cattle.

The dendrogram on the y-axis illustrates the clustering of cattle (rows) based on serological similarity. Colours indicate the magnitude of MAT titres (Colour Key). Test serovars are shown on the x-axis (see Table **6.2** for full serovar details). Patterns in animal clustering are shown on the right: A: Cattle with no observed MAT titres to any serovar (n = 12); B: Cattle with titres to one serogroup (n = 8); C: Cattle with titres to serogroups Mini, Sejroe and Hebdomadis (n = 12); D: Cattle with low titres against a range of serogroups (n = 22). NB: The hierarchical clustering algorithm of the dendrogram is continuous; hence sections C at the top and bottom of the axis are part of the same cluster.

6.3.12 Assessing correlation in MAT titres between serovars

Spearman's rank correlation coefficients (r) for pairwise comparisons between MAT titres are shown in matrix form in Figure 6.6. In this figure, serovars with no evidence of reactivity (MAT titre < 1:20) have been excluded for ease of interpretation.

A high degree of correlation in MAT titres was observed between test serovars of the same serogroup. For example, within the Tarassovi serogroup, MAT titres against serovars Tarassovi (TarTar) and Kanana (TarKan) were highly correlated ($r = 0.64$, $p < 0.001$). Significant correlations in MAT titre were observed between all of the three test serovars belonging to the serogroup Australis (serovars Australis (AusAus), Bratislava (AusBra) and Lora (AusLor); $p < 0.001$– see Figure 6.6 for pairwise correlation coefficients). Significant correlations were also observed between all serovars from the Sejroe serogroup (Hardjobovis (SejHbo), Hardjoprajitno (SejHPr) and Sejroe (SejSej); $p < 0.001$– see Figure 6.6 for pairwise correlation coefficients).

In addition, significant correlation was observed between MAT titres for serovars across the Mini-Hebdomadis-Sejroe serogroup complex. For example, statistically significant correlation was demonstrated between MAT titres against the representative serovars for serogroups Hebdomadis (HebHeb) and Mini (MinSar) ($r = 0.68$, $p < 0.001$), and between both of these serogroups and all of the test Sejroe serovars ($p < 0.001$– see Figure 6.6 for pairwise correlation coefficients).

	Aus_Aus	Aus_Bra	Aus_Lor	Aut_Lam	Bal_Bal	Gri_Gri	Heb_Heb	Ict_Sok	Min_Sar	Pom_Pom	Pyr_Kwa	Sej_Hbo	Sej_HPr	Sej_Sej	Sem_Pat	Tar_Kan
Aus_Bra	0.58 ***															
Aus_Lor	0.79 ***	0.62 ***														
Aut_Lam	−0.13	0.29 *	0.05													
Bal_Bal	−0.04	−0.05	−0.04	−0.06												
Gri_Gri	−0.08	0.17	0.03	0.37 **	−0.04											
Heb_Heb	0.14	0.17	0.15	0.06	−0.07	−0.07										
Ict_Sok	−0.08	0.20	0.08	0.10	−0.04	0.11	−0.06									
Min_Sar	−0.07	−0.11	−0.11	−0.04	−0.06	−0.12	0.68 ***	−0.06								
Pom_Pom	0.49 ***	0.39 **	0.24	−0.06	−0.02	−0.04	0.20	−0.04	−0.06							
Pyr_Kwa	−0.04	−0.04	−0.04	0.31 *	−0.02	−0.04	−0.07	−0.04	−0.06	−0.02						
Sej_Hbo	−0.08	−0.04	−0.06	0.13	−0.06	−0.07	0.70 ***	−0.05	0.63 ***	−0.06	−0.06					
Sej_HPr	−0.08	−0.10	−0.09	0.14	−0.04	−0.08	0.43 ***	−0.09	0.39 **	−0.04	−0.04	0.83 ***				
Sej_Sej	−0.08	−0.12	−0.11	0.05	−0.06	−0.13	0.61 ***	−0.11	0.62 ***	−0.06	−0.06	0.92 ***	0.77 ***			
Sem_Pat	−0.01	0.04	−0.08	−0.13	0.18	−0.13	0.21	0.04	0.32 *	0.18	−0.06	0.08	−0.05	0.16		
Tar_Kan	−0.09	0.02	−0.10	−0.05	0.26	−0.09	−0.07	−0.09	−0.00	−0.04	−0.04	−0.09	−0.10	−0.04	0.09	
Tar_Tar	0.14	0.24	0.19	0.18	0.07	0.13	0.17	−0.03	0.12	0.07	−0.08	0.04	−0.05	0.11	−0.00	0.64 ***

Figure 6.6: Matrix of Spearman's rank correlation coefficients (correlogram) for MAT titres from cattle by test serovar

See Table 6.2 for full serovar details Statistical significance of pairwise correlation coefficients (label) is shown by stars ($p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Shading corresponds to the numeric magnitude of the correlation coefficient ranging from -0.13 (pale grey) to 0.92 (dark blue).

6.3.13 Comparison of serology and qPCR results for determining *Leptospira* prevalence and diversity in livestock

Of 56 cattle tested by MAT, four were positive for *Leptospira* infection by kidney qPCR (Table 6.9). Of these, only two were positive by MAT using a cut-off of MAT titre ≥ 1:40. Overall, agreement was very low between the two testing approaches (Cohen's kappa statistic; 0.005; 95% CI -0.265 – 0.276; p = 0.485)(Thrusfield, 1995).

Table 6.9: Comparison of kidney qPCR versus MAT results for any serogroup (MAT titre ≥ 1:40)

	qPCR positive	qPCR negative	Total
MAT positive	2	25	**27**
MAT negative	2	27	**29**
Total	**4**	**52**	**56**

Table 6.10: Summary of PCR and MAT positive cattle with serological profiles and genotype data (no observed MAT titres ≥ 1:20)

Cattle ID	*Leptospira sec*Y genotype	MAT reactive serogroup (serovar*)				
		Autumnalis	Mini	Pyrogenes (Kwale)	Sejroe (Hbo)	Semarang
C0518	*L. borgpetersenii* (Lb2)	1:20	-	1:20	-	-
C0552	*L. kirschneri*	-	1:80	-	1:20	-
C0592	*L. borgpetersenii* (Lb2)	-	1:80	-	-	-
C0603	NA	-	-	-	-	1:20

*Serovar given where multiple test serovars were used for a single serogroup by MAT; Hbo: Hardjobovis

Where available, data on reactive MAT titres against *Leptospira* serogroups was compared to *Leptospira* species information for qPCR positive cattle. For qPCR-positive cattle, serological reactivity was often below the pre-defined MAT cut-off titre (≥ 1:40) used to determine serogroup prevalence in this study (Table 6.10). Data on infecting *Leptospira* species based on *sec*Y sequence analysis was available for three animals. Two cattle were infected with *L. borgpetersenii* from the non-Hardjo cluster (Lb2). Of these, one animal showed serological reactivity against serogroup Mini at a titre of 1:80, and the second showed low levels of reactivity to serogroups Autumnalis and Pyrogenes. In one cow with *L. kirschneri* infection, serological reactivity was demonstrated to serogroups Mini and Sejroe. Overall, no relationship between the infecting *Leptospira* sequence type and MAT reactive serogroups could be deduced.

6.4 Discussion

The results presented in this chapter indicate that *Leptospira* infection is common in ruminant livestock in this area. Using molecular methods, a high prevalence of *Leptospira* infection was detected in cattle from across northern Tanzania. Infection was demonstrated for the first time in small ruminants from the East African region. This study also represents the first reported isolation of *L. borgpetersenii* serovar Hardjo from cattle in East Africa. Serological data suggests that *L. borgpetersenii* exposure to this serovar is common in cattle, but also identified a number of other dominant sero-reactive serogroups in this livestock species. From the results presented in this chapter, *L. borgpetersenii* appears to be the predominant *Leptospira* species involved in cattle infection and was only detected in this host species. In contrast, *L. kirschneri* infection was detected in multiple livestock species (cattle, sheep and goats). In addition, the use of a sequence-based approach directly on clinical samples identified an unusual *Leptospira* genotype that has not been previously described in international databases.

Overall, this study provided some novel and intriguing insights into the epidemiology of leptospirosis in livestock in Tanzania. The use of an abattoir sampling platform proved to be a productive method to investigate *Leptospira* infection in livestock in a wide geographic area. The majority of animals sampled in this study originated from distant regions and travelled long distances before arriving at slaughterslabs in Moshi (Figure 6.2). Infection was detected in animals that originated from various regions across the northern half of Tanzania, indicating that *Leptospira* infection is widespread in Tanzanian livestock. However, direct extrapolation of prevalence estimates from this study should be performed with caution. Infection prevalence data generated from animals sold for slaughter may not be entirely representative of the prevalence of disease in the general population due to the potential for selection bias (Cleaveland et al., 2007, McKenna et al., 2004). Animals may be culled from a herd on the basis of particular characteristics that affect the likelihood of infection in an endemic setting, such as older age or poor reproductive performance for example. In this setting where animals travel long distances to slaughter, it is also possible that the animals may have become infected after leaving their home regions through mixing with other herds in cattle-holding areas, at watering holes or markets or even the abattoir lairage where they may be held for several days (Juvenile Urio and Francis William, personal communication). Further work may be needed to understand the prevalence of *Leptospira* infection in the home grazing areas of these cattle. However, as a preliminary study of livestock leptospirosis in Tanzania, this work offers some important insights that will have real and practical benefits to future surveillance of the disease.

Firstly, slaughterhouse sampling enabled kidney and urine samples to be collected from a large number of animals for the direct detection of *Leptospira* organisms by qPCR. In Africa, molecular approaches for the diagnosis of *Leptospira* infection in livestock have previously only been used in a small number of studies (Chapter 2). However, where they have been applied, relatively high estimates of prevalence have been obtained (Desvars et al., 2013c). In this study, qPCR testing proved more sensitive than culture for detecting *Leptospira* infection in livestock. qPCR detected an additional 50% of infections over culture where kidney samples were tested by both methods, demonstrating its utility in this setting. The use of urine samples rather than kidney samples for qPCR testing further improved the probability of detecting infection in cattle. The reason for this discrepancy is uncertain. A number of limitations for demonstrating infection by urine testing have been reported including intermittent *Leptospira* shedding, low bacterial load and the risk of PCR inhibition from the high level of urea in urine samples (World Organisation for Animal Health (OIE), 2008, Levett, 2001, Schrader et al., 2012). However, a previous study of cattle and sheep performed in New Zealand demonstrated comparable results between the kidney and urine samples (Fang et al., 2014b). Most likely, the difference in detection sensitivity in this study is a consequence of the sampling approach. A single, relatively small volume of kidney tissue was taken from each animal for DNA extraction and subsequent qPCR testing, which may have missed more localised infections that are common in chronic *Leptospira* infection in cattle (Bill Ellis, personal communication). Urine samples may be more representative of the true infection status of the animal, as urine from different parts of the kidney will be pooled and mixed together, removing the problem of trying to target a focal area of infection in the kidney tissue. Therefore, in this study, figure for infection prevalence based on kidney qPCR for cattle are likely to be underestimates of the true prevalence of bovine infection in this setting. Urinary qPCR is recommended for future studies of *Leptospira* infection in this setting, which has clear advantages for surveillance of live animals as well as those destined for slaughter.

The use of an abattoir-based study also allowed better characterisation of circulating *Leptospira* types. *Leptospira* culture resulted in the definitive demonstration of *L. borgpetersenii* serovar Hardjo (serogroup Sejroe) infection in Tanzanian cattle, which expands the known global distribution of this cattle-associated serovar. Prior to this study, Hardjobovis had only been reported in cattle from Nigeria, South Africa and Zimbabwe on the African continent (Ezeh et al., 1989a, Te Brugge and Dreyer, 1985, Feresu et al., 1999b), although the high prevalence of serogroup Sejroe in seroprevalence studies had indicated its presence in East Africa for some time (see references in Table 6.1). The

almost ubiquitous nature of Hardjobovis in cattle populations worldwide confers some functional benefits for surveillance and control of this particular *Leptospira* serovar in Tanzanian cattle. Serovar-specific diagnostic tests for Hardjo already exist to aid the diagnosis of infection in cattle (Yan et al., 1999), as do vaccinations, which could be evaluated for their feasibility in the Tanzanian context (Dhaliwal et al., 1996b, Bolin and Alt, 2001).

However, further analysis suggests that a much wider variety of serological and genetic types of *Leptospira* are circulating in Tanzanian livestock than is implicated by culture alone. Four distinct clusters of *Leptospira* genotype were identified by *sec*Y sequence analysis of qPCR-positive samples including two groups of *L. borgpetersenii* sequence type, a cluster of *L. kirschneri* and a fourth cluster of sequences from an unidentified *Leptospira* species. To date, relatively little is currently known about the genetic diversity of *Leptospira* in mainland Africa (Allan et al., 2015a), and African serovars are likely to be under-represented in reference collections used to develop typing schemes (Boonsilp et al., 2013, Thaipadungpanit et al., 2007). However, where sequence-based typing approaches have been used in the region, a remarkable array of *Leptospira* genotypes has been reported (Dietrich et al., 2014, Gomard et al., 2016). The use of a relatively short fragment of a single gene to perform phylogenetic analysis in this study limits the robustness of distinguishing between genotypes within a *Leptospira* species (e.g. the two clusters within *L. borgpetersenii* sequences). However, the *sec*Y is well characterised for many known pathogenic *Leptospira* species and serovars. Based on reported proportional similarities for serovars of the same *Leptospira* species (Victoria et al., 2008), a similarity of only 95%, as observed between the unidentified cluster of *Leptospira* genotypes identified from cattle in this study and its nearest known neighbour (*L. kirschneri*), is on the threshold of similarities for species level distinctions for this gene locus. Analysis of additional gene targets for this *Leptospira* genotype could help to determine whether this cluster represents an unreported divergent form of *L. kirschneri* or distinct, unreported *Leptospira* genotype.

Serological analysis revealed that cattle are exposed to several different serogroups in addition to serogroup Sejroe, including Australis, Grippotyphosa, Icterohaemorrhagiae and Tarassovi. Statistically significant correlations were observed in serological reactivity (MAT titres) for the serogroups Mini, Hebdomadis and Sejroe (Figure 6.6) indicating that titres to these serogroups could be cross-reactive or co-occur. However, for other serogroups, reactivity was demonstrated without any evidence of cross or correlated reactivity. On the basis of this analysis, at least five serological types of *Leptospira* are presumed to circulating in these cattle populations. Reactive serogroups from this study

will be used to in this study inform the selection of a serovar panel for future MAT testing in livestock in this area.

The observed patterns of *Leptospira* infection in livestock, supported by background information about the biology and typical transmission routes of *L. borgpetersenii* and *L. kirschneri*, led to the hypothesis that two distinct transmission cycles may be occurring in Tanzanian livestock (Figure 6.7). *L. borgpetersenii* infection was seen only in cattle indicating a degree of host specificity in this *Leptospira* species. Published genomic analysis of *L. borgpetersenii* has revealed considerable loss of genes involved in environmental survival, indicating that *L. borgpetersenii* is evolving towards strict dependence on direct host-to-host transmission (Bulach et al., 2006, Picardeau et al., 2008). In northern Tanzania, we hypothesize that *L. borgpetersenii* relies on direct cow-to-cow transmission for the maintenance and propagation of infection.

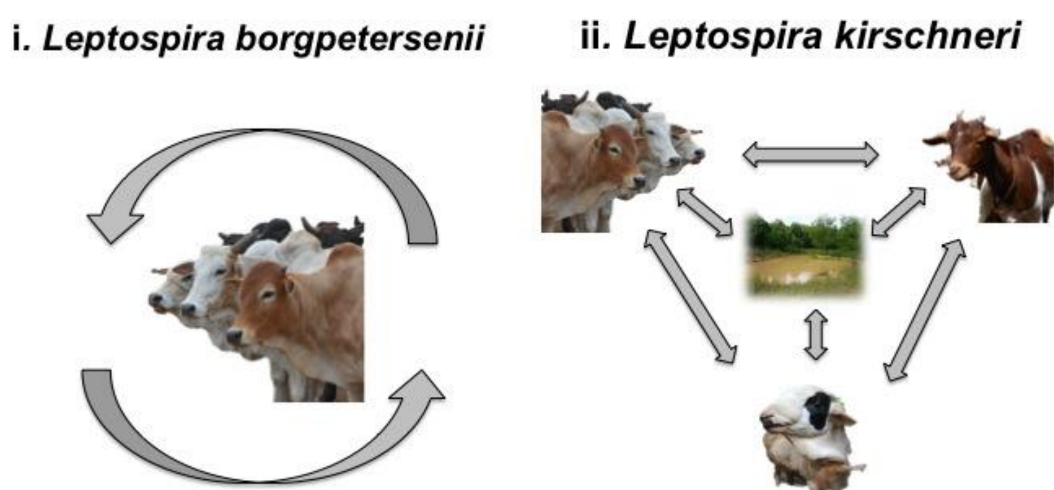


Figure 6.7: Proposed transmission cycles for major *Leptospira* species detected in Tanzanian livestock

In contrast, *L. kirschneri* was detected in cattle, sheep and goats sampled in this study. Sequence-based analysis showed perfect alignment of the *sec*Y locus in sequence derived from all three host species (Figure 6.4). This infection pattern indicates that a more complex, multi-host epidemiology may be important for this *Leptospira* species. Genetically *L. kirschneri* is similar to *L. interrogans*, and is thought to be able to survive in

the environment for several weeks enabling indirect routes of infection to play a more prominent role in its transmission (Fouts et al., 2016, Andre-Fontaine et al., 2015). Indirect or environmental transmission is hypothesised to be more important in for *L. kirschneri* than *L. borgpetersenii* and would better explain the multi-host infection patterns observed in this study. In pastoral and agropastoral systems in Tanzania, cattle and small ruminants are often kept in mixed flocks and share grazing areas, watering holes and overnight housing (Figure 6.8). Mixed herds are known to be an important risk factor for cattle *Leptospira* infection in other settings (Ryan et al., 2012, Lilenbaum and Souza, 2003). Small ruminants may be responsible for direct or indirect transmission of *L. kirschneri* to cattle in settings where they come into close contact, for example in the abattoir lairage. Alternatively, all three species may be infected from another animal host or environmental reservoir. In either scenario, small ruminants should not be overlooked when considering potential hosts for *Leptospira* infection in Tanzania.

(i) Mixed herd management in pastoral farming systems

(ii) Mixed species housing in the abattoir lairage

Figure 6.8: Tanzania livestock are often managed in mixed herds both in pastoral farming systems (i) and in the abattoir lairage (ii)

The epidemiology, maintenance and transmission of the two main livestock-infecting *Leptospira* types may also have implications for transmission of *Leptospira* infection from livestock to people. Reduced environmental survival of *L. borgpetersenii* dictates that close contact with the infected tissues or urine of animal is necessary for human infection. This may occur through milking, assisting with parturition or slaughter. In contrast, a propensity for greater environmental survival and cross-species transmission in *L.*

kirschneri means that there are more potential routes for people to come into contact with this pathogen. As livestock travel long distances before arriving at the slaughterhouses they have the potential to transmit infection to people (and other animals) anywhere on their journeys from their home grazing areas. In Moshi, the high prevalence of infection and urinary shedding of *Leptospira* suggests that infected livestock may pose a considerable health risk to slaughterhouse workers and butchers. Kidneys have commercial value in Tanzania where they are sold for consumption and hence, the handling and preparation of *Leptospira*-infected offal may also pose a zoonotic disease risk to the consumer.

Finally, the high prevalence of infection may also pose health problem to the animals themselves. Very little is known about the impact of *Leptospira* infection on cattle in Africa, but the potential for production losses are considerable given how widespread the infection appears to be. Many questions remain unanswered and there remains a fair degree of uncertainty regarding transmission routes and the role that livestock play in the epidemiology of human disease in northern Tanzania. Exploring the similarities between *Leptospira* types detected in cattle and people is an important priority in this setting and a focus of the final data chapter of this thesis (Chapter 7).

7 Human leptospirosis in northern Tanzania

7.1 Introduction

Leptospirosis is an important but overlooked cause of human febrile illness on the African continent. Over the past five years, a mounting body of evidence has demonstrated that acute leptospirosis contributes substantially to the burden of non-malarial febrile illness in Africa (reviewed and discussed in Chapter 2). Although human disease surveillance on the continent is limited, available figures indicate that eastern sub-Saharan Africa is one of the most severely affected regions in the world (Costa et al., 2015a). Estimates of annual disease incidence range from 26 cases per 100,000 population in the region as a whole (Costa et al., 2015a) to more than 100 cases per 100,000 population in Tanzania (Biggs et al., 2013b). However, despite demonstration of a high burden of human disease, relatively little is known about the types and sources of *Leptospira* bacteria responsible for human disease in Tanzania, where the disease remains overlooked in public health priorities.

7.1.1 Human *Leptospira* exposure in Tanzania

To date, the majority of data on *Leptospira* infection in people in Tanzania comes from serological surveillance studies, which reveal widespread exposure in the general population. The first serological evidence for human exposure came from an opportunistic study of agricultural workers from a variety of Tanzanian regions (Machang'u et al., 1997). This pioneering investigation was limited in scope as only a very small number of *Leptospira* serovars were used for serological testing by microscopic agglutination test (MAT) (Table 7.1. However, this study laid the foundations for leptospirosis research in Tanzania. Subsequent surveys have since used broader serological panels for MAT testing to generate more representative estimates of prevalence.

Seroprevalence data generated from cross-sectional surveys or hospital-based cohort studies are available for four different regions of Tanzania (Table 7.1). In the Tanga Region (north-east Tanzania), a cross-sectional survey of 199 city inhabitants demonstrated a Seroprevalence of 15.1% by MAT (Schoonman and Swai, 2009). Study participants were considered positive for *Leptospira* exposure with at MAT titre ≥ 1:160 to at least one *Leptospira* serovar from a panel encompassing six different serogroups. In the Katavi Region (west Tanzania), a cross-

sectional study targeting livestock-owning households estimated human seroprevalence as 30.0% (n= 267) (Assenga et al., 2015). In this study, sera were also tested by MAT against a panel of six *Leptospira* serogroups but only two serogroups were directly comparable between the Tanga and Katavi studies).

In a hospital-based study of 370 children presenting with febrile illness at Kilosa District hospital in the Morogoro Region of central Tanzania, ELISA (IgM and IgG combined) was used to generate an estimate of 15.9% seroprevalence (Chipwaza et al., 2015). Secondary MAT testing was subsequently performed on 200 ELISA-positive patients using the same serovar panel as described for the Katavi cross-sectional study (Table 7.1). Reactivity to at least one *Leptospira* serovar was demonstrated by a MAT titre ≥ 1:160 in 26 (13.0%) ELISA-positive patients. This result highlights discrepancies between the two test methodologies but confirms considerable exposure in this patient cohort. Finally, seroprevalence estimates are also available from a hospital-based study exploring the aetiology of febrile disease performed at two hospitals in the Kilimanjaro Region of northern Tanzania (Biggs et al., 2011, Crump et al., 2013). This study performed the most comprehensive MAT testing to date, using a panel of 20 different *Leptospira* serovars representing a total of 17 serogroups. Of 831 adult and paediatric patients tested by MAT in the Kilimanjaro study, 346 (41.6%) showed at MAT titre ≥ 1:100 against at least one *Leptospira* serogroup (Table 7.1).

Together, these studies demonstrate substantial *Leptospira* exposure in the Tanzanian population. Seroprevalence estimates also indicate that *Leptospira* exposure is common in patients with febrile illness who seek health-care in the Morogoro and Kilimanjaro Regions. However, diagnosing leptospirosis as the cause of an acute episode of febrile illness requires a more rigorous approach than simply demonstrating serological exposure. Definitive diagnosis of acute leptospirosis by MAT requires demonstration of seroconversion between acute and convalescent samples taken two to four weeks apart in the presence of compatible clinical signs (World Health Organization, 2011, Costa et al., 2015a). A single high MAT titre is also accepted as evidence of probable infection in the presence of compatible clinical signs. These case definitions were used by the Kilimanjaro febrile disease study to provide the most robust evidence of *Leptospira* infection as a cause of acute febrile illness in Tanzania.

Table 7.1: ***Leptospira*** **exposure in people in Tanzania reported by study, Tanzanian region and** ***Leptospira*** **serogroup**

Study location (citation)	**Morogoro (Machang'u et al., 1997)**	**Tanga (Schoonman and Swai, 2009)**	**Kilimanjaro (Biggs et al., 2011)**	**Katavi (Assenga et al., 2015)**	**Morogoro[**] (Chipwaza et al., 2015)**
Study type (n = participants)	Opportunistic study (n = 375)	Cross-sectional survey (n = 199)	Febrile patients (n = 831)	Livestock owners (n = 267)	Febrile children (n = 200)[††]
MAT Cut-off titre	≥1:160	≥1:160	≥1:100	≥1:160	≥1:160
Overall seroprevalence[‡‡]	0.3%	15.1%	41.6%	30.0%	13.0%
MAT Serogroup[§§]					
Australis	-	-	12.1%	1.5%	1.0%
Autumnalis	-	-	14.5%	-	-
Ballum	-	-	0.2%	1.1%	3.0%
Bataviae	-	4.5%	0.6%	-	-
Canicola	-	-	0.8%	-	-
Celledoni	-	-	2.4%	-	-
Cynopteri	-	-	0.5%	-	-
Djasiman	-	-	2.0%	-	-
Grippotyphosa	0.3%	-	0.6%	4.9%	3.0%
Hebdomadis	-	-	0.6%	3.4%	3.0%
Icterohaemorrhagiae	Not detected	5.5%	8.3%	9.0%	4.5%

Javanica	-	-	0.2%	-	-
Mini	-	-	22.8%	-	-
Pomona	-	0.5%	0.1%	-	-
Pyrogenes	-	-	0.7%	-	-
Sejroe	-	3.0%	0.5%	15.7%	0.0%
Tarassovi	-	1.0%	0.6%	-	-

[**] Some patients are positive for more than one serogroup
[††] Selected from larger population of febrile children on the basis of positive Total IgELISA results
[‡‡] Overall seroprevalence is the number of patients with a MAT titre ≥ threshold for any tested serogroup
[§§] For representative serovars for each serogroup, see original references

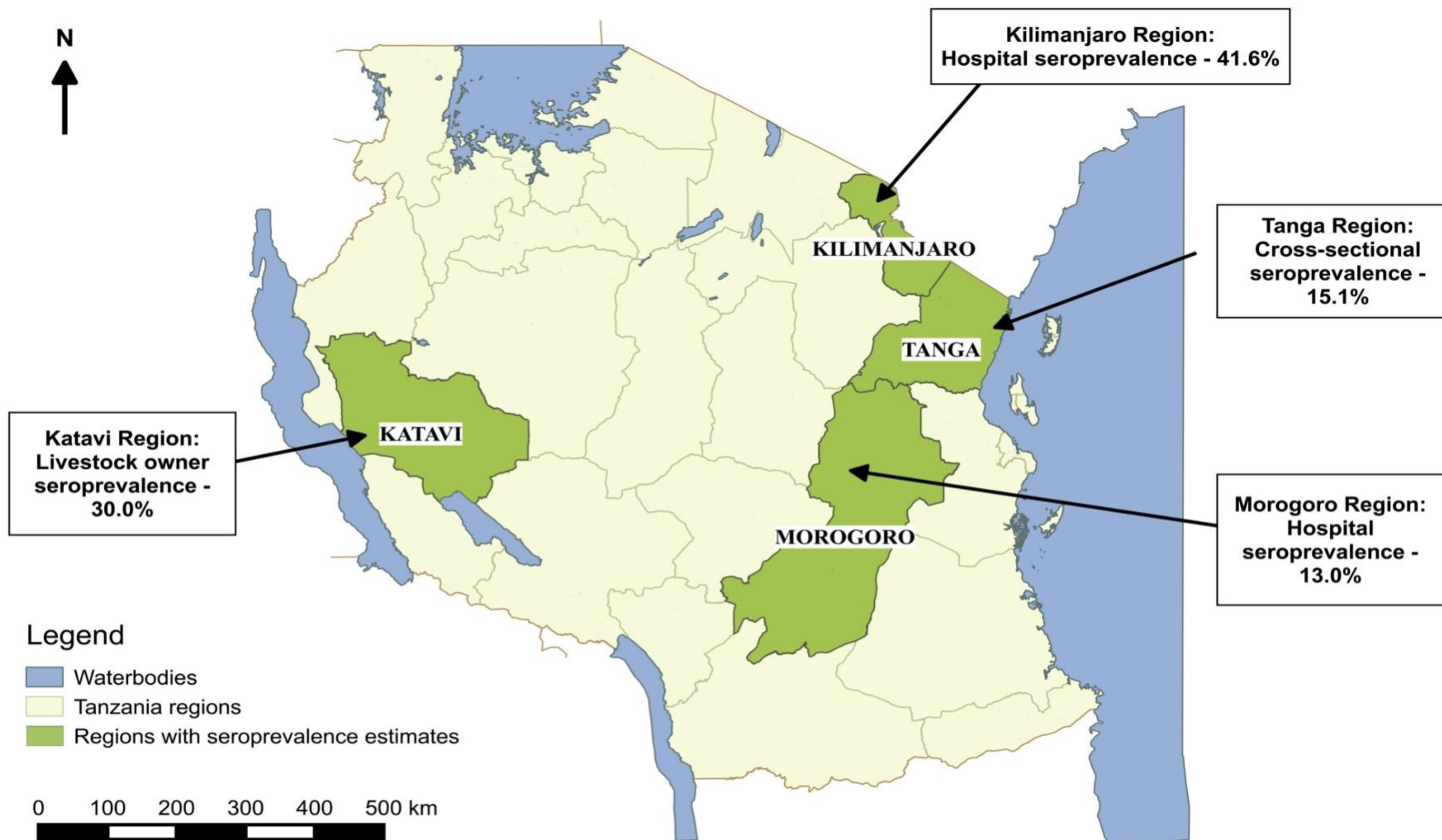


Figure 7.1: Map of Tanzania showing regions and *Leptospira* seroprevalence estimates from published surveillance studies (Biggs et al., 2011, Schoonman and Swai, 2009, Chipwaza et al., 2015, Assenga et al., 2015).

Arrowheads of labels indicate the approximate location of studies within each region.

7.1.2 Acute leptospirosis as a cause of human febrile illness in Tanzania

Acute leptospirosis has been demonstrated as an important cause of febrile illness in in the Kilimanjaro Region of northern Tanzania (Crump et al., 2013, Biggs et al., 2011). A prospective cohort study was performed in two hospital facilities - Kilimanjaro Christian Medical Centre (KCMC) and Mawenzi Regional Hospital (MRH) - both located in the Moshi Municipal District, which is the administrative centre of the Kilimanjaro Region (Chapter 3.2). Between 17 September 2007 and 25 August 2008, 870 paediatric and adult patients with fever were enrolled into the study (Crump et al., 2013). Standard diagnostic tests including malaria screening by blood smear, bacterial and fungal blood culture and HIV serology were carried out on admission to the hospital. Retrospective serological diagnostic testing was performed for a range of tropical and zoonotic infections including leptospirosis at international reference laboratories. Cases of acute leptospirosis were identified using predefined case definitions based on WHO guidelines (World Health Organization, 2011). Confirmed acute leptospirosis was defined by a four-fold increase in MAT titre between acute and convalescent serum samples. Probable leptospirosis was defined as a MAT titre ≥ 1:800[7] in a single serum sample.

Of 831 patients with at least one serum sample available, 70 (8.4%) patients met the case definitions for confirmed or probable acute leptospirosis. Of 453 febrile patients with paired serum samples available, a total of 40 (8.8%) demonstrated a four-fold rise in antibody titre to at least one *Leptospira* serovar. However, attempts to isolate *Leptospira* bacteria from these patients were unsuccessful (Crump & Galloway, personal communication) and questions remain about the types of *Leptospira* involved in human disease in this region.

[7] This is a more stringent definition than suggested in WHO guidelines, which recommends a probable case should be defined as a single MAT titre ≥ 1:400.

7.1.3 *Leptospira* species and serovars associated with human leptospirosis in Tanzania

Overall, little is known about the *Leptospira* serovars and species responsible for human leptospirosis in Tanzania. In the Kilimanjaro Region, serological data was used to identify common reactive serogroups in leptospirosis cases, which included serogroups Mini, Australis, Autumnalis and Icterohaemorrhagiae (Biggs et al., 2011). However, data from other studies that performed serology and *Leptospira* isolation in parallel indicate that the identity of an infecting *Leptospira* serovar cannot be accurately predicted with MAT, which is unable to reliably discriminate to the serovar level (Haake and Levett, 2015, Levett, 2003, Murray et al., 2009). Whilst MAT reaction profiles can be useful to identify broad patterns of *Leptospira* serogroups circulating at the population level, cross reactions between serogroups are common in the acute phase, and may confound interpretation of MAT data (Levett, 2003, Goris and Hartskeerl, 2013). Therefore, definitive determination of the infecting *Leptospira* species, serovar and serogroup for individual patients requires direct serological or molecular typing of *Leptospira* isolates (Levett, 2001, Faine, 1994). Understanding and characterising *Leptospira* types responsible for human disease is vital to design locally appropriate MAT serovar panels for diagnosis as well as to identify sources and transmission routes for human infection.

7.1.4 Limitations for diagnosis and surveillance of human leptospirosis in Tanzania

The lack of robust information on human-infecting leptospires in animal hosts is a major limiting factor in our ability to identify sources for human infection in Tanzania. Leptospirosis is a complex multi-host disease and many different serovars associated with numerous animal hosts may be found in a single area (Bharti et al., 2003). Understanding which serovars and animal hosts are important for human disease is vital to target infection control strategies (Hartskeerl et al., 2011, Desvars et al., 2013a). A good working knowledge of *Leptospira* types in both humans and animals in a community is critical to design appropriate control programmes for human disease.

For human disease surveillance and clinical management in Tanzania, providing an accurate and timely diagnosis of leptospirosis to patients at the point of care is an important limitation of the current health-care system. Laboratory diagnosis of leptospirosis remains challenging even in well-resourced high-income laboratory settings, which often use a complex, multi-faceted approach to diagnose infection (Musso and La Scola, 2013). At present, little laboratory capacity exists to support the diagnosis of leptospirosis in most primary health-care facilities in Tanzania. Clinician awareness of the disease is poor (Chipwaza et al., 2014, Zhang et al., 2016) and leptospirosis is frequently misdiagnosed in tropical settings, as clinical signs in the acute phase are virtually impossible to differentiate from other causes of febrile illness, including malaria (Haake and Levett, 2015, Hartskeerl et al., 2011). Point-of-care diagnostic tests are needed to help improve case recognition in a clinical setting.

Currently, all available diagnostic tests for *Leptospira* infection are associated with challenges or limitations that restrict their utility to provide an early diagnosis in resource-limited settings. Serological diagnostic tests such as the MAT can only provide a definitive diagnosis of leptospirosis in the convalescent stage of illness following seroconversion (Goris and Hartskeerl, 2013, Goris et al., 2012). Other serological approaches such as the IgM ELISA may be more sensitive than the MAT in the early phase of infection but lack diagnostic specificity in endemic settings (World Health Organization, 2011, Goris et al., 2011). The utility of culture and isolation to provide an acute phase diagnosis for leptospirosis is restricted by the long growth period of the organism (Levett, 2001). However other direct detection approaches such as PCR and real-time PCR (qPCR) may be of greater value to the clinician as they provide a rapid and more timely test result (Picardeau et al., 2014). *Leptospira* DNA can be detected in the blood of an infected patient during the first week of illness, and in urine in the subsequent phase weeks (Figure 1.2), although factors such as the timing of sampling, the leptospiral load and prior antibacterial use may reduce the diagnostic sensitivity of PCR assays in a clinical setting (Haake and Levett, 2015, Stoddard et al., 2009). However, an additional benefit of PCR detection is that sequence-based typing can be performed on PCR products to determine the infecting *Leptospira* species in positive cases, therefore generating additional epidemiological data on human infections (Boonsilp et al., 2011). PCR-based approaches have not yet been described for *Leptospira* infection in a Tanzanian health-care setting but the infrastructure to support PCR testing already exists in some of the larger Tanzanian hospitals. Hence PCR testing may offer a feasible tool to improve diagnosis of acute leptospirosis in Tanzania.

7.1.5 Goals and objectives of this study

Although leptospirosis has been demonstrated as an important cause of febrile illness in northern Tanzania, little is known about the types of *Leptospira* that are infecting people in Tanzania. This lack of knowledge is limiting our ability to understand the sources and transmission routes for human infection and to design effective control programmes for the region. Furthermore, all diagnoses of acute leptospirosis in the Kilimanjaro Region have so far been made retrospectively at remote reference laboratories outside the country. Evaluating acute phase diagnostic tests for use at the point-of-care in Tanzania is an important next step in providing diagnostic data to inform patient care, and in establishing sustainable capacity for in-country disease surveillance.

To address these limitations and knowledge gaps, this study component aims to address the following objectives:

Objective 1: To pilot and evaluate the use of qPCR as a diagnostic assay to detect *Leptospira* infection in febrile patients in northern Tanzania;

Objective 2: To explore patterns of *Leptospira* infection in pre-defined human cases to:

i) identify and describe common human-infecting *Leptospira* types;

ii) explore serological reactivity profiles within the cohort of cases.

Objective 3: To infer possible sources of *Leptospira* infection by comparing available serological and molecular data from human patients with data from linked animal populations.

7.2 Methods

This study uses data and samples collected as part of the previously described hospital-based febrile disease cohort study performed in the Kilimanjaro Region of Tanzania. Full study details can be found in the following references: (Biggs et al., 2011, Crump et al., 2013).

7.2.1 Selection of human samples for analysis

Archived plasma (n = 372) and urine (n = 301) samples were available for patients enrolled in the febrile disease cohort study between September 2007 and August 2008. Samples and data from patients with acute leptospirosis were selected for further analysis on the basis of serological test results previously generated by the United States Centers for Disease Control and Prevention (US CDC) (Biggs et al., 2011). Leptospirosis testing was performed by MAT using a panel of 17 *Leptospira* serogroups (Table 7.2) (Biggs et al., 2011). Serological data available from the previous study were re-classified for this analysis based on new, more inclusive case definitions for acute leptospirosis. In line with current WHO recommendations, leptospirosis cases were defined as:

1. Demonstration of seroconversion (four-fold rise antibody titre) by MAT on paired acute and convalescent serum samples;
2. Single MAT titre of ≥ 1:400 on either acute or convalescent samples (World Health Organization, 2011).

Plasma and urine samples from patients that met one or both of the case definitions were selected for molecular analysis. Plasma samples were also selected from five randomly selected febrile patients with no evidence of exposure to leptospirosis (MAT titres < 1:100 in paired acute and convalescent serum samples against any of the test serovars) as negative controls for the qPCR study.

Table 7.2: Serovars used for the diagnosis of human acute leptospirosis by Microscopic Agglutination Test (MAT) (Biggs et al., 2011)

Serogroup	Species and Serovar(s)	Abbreviation*
Australis	*L. interrogans* serovar Australis	AusAus
	L. interrogans serovar Bratislava	AusBra
Autumnalis	*L. interrogans* serovar Autumnalis	AutAut
Ballum	*L. borgpetersenii* serovar Ballum	BalBal
Bataviae	*L. interrogans* serovar Bataviae	BatBat
Canicola	*L. interrogans* serovar Canicola	CanCan
Celledoni	*L. weilii* serovar Celledoni	CelCel
Cynopteri	*L. kirschneri* serovar Cynopteri	CynCyn
Djasiman	*L. interrogans* serovar Djasiman	DjaDja
Grippotyphosa	*L. kirschneri* serovar Grippotyphosa	GriGri
Hebdomadis	*L. santarosai* serovar Borincana	HebHeb
Icterohaemorrhagiae	*L. interrogans* serovar Mankarso	IctMan
	L. interrogans serovar Icterohaemorragiae	IctIct
Javanica	*L. borgpetersenii* serovar Javanica	JavJav
Mini	*L. santarosai* serovar Georgia	MinGeo
Pomona	*L. interrogans* serovar Pomona	PomPom
Pyrogenes	*L. interrogans* serovar Pyrogenes	PyrPyr
	L. santarosai serovar Alexi	PyrAle
Sejroe	*L. interrogans* serovar Wolfii	SejWol
Tarassovi	*L. borgpetersenii* serovar Tarassovi	TarTar

*Serovar abbreviations are used in the results of this chapter and included for reference here

7.2.2 DNA extraction from human plasma and urine samples

DNA was extracted from archived human plasma and urine samples from the selected patients. DNA extraction from human plasma was performed as described in Chapter 3.6.4. DNA was extracted from human urine samples as described in Chapter 3.6.3.

7.2.3 qPCR for the diagnosis of *Leptospira* infection

***lip*L32 TaqMan® qPCR assay:**

All samples were run on MicroAmp® 96 well plates and tested in duplicate with the *lip*L32 TaqMan® qPCR assay (Stoddard, 2013, Stoddard et al., 2009) and singularly with an internal control *rnaseP* qPCR assay as described in Chapter 3.7.2. Each plate included: two replicates of a *Leptospira* positive control; DNA extracted from *L. interrogans* serovar Copenhagenii Strain Wijnberg at ~10^2 genome copies numbers; two replicates of DNA extracted from a human lymphoma cell line (HuLCL) as a positive control for the *rnaseP* reaction; two replicates of a non-template extraction control; and two replicates of a negative control (PCR-grade water). Reaction runs were considered valid when at least one replicate of both positive controls (*L. interrogans* and *rnaseP*) amplified with Ct values < 40, and all replicates of the negative controls showed no evidence of amplification. Test reactions were considered positive with a Ct value < 40.

16S (*rrs*) TaqMan® qPCR assay

All samples were run on MicroAmp® 96 well plates and tested in duplicate with the 16S (*rrs*) TaqMan® qPCR assay (Smythe et al., 2002) as described in Chapter 3.7.4. Each plate included: two replicates of a *Leptospira* positive control; *L. interrogans* serovar Copenhagenii Strain Wijnberg at ~10^2 copies numbers; two replicates of a non-template extraction control; and two replicates of a negative control (PCR-grade water). Reaction runs were considered valid when at least one replicate of the *L. interrogans* control amplified with Ct values < 40 and when all replicates of the negative controls showed no evidence of amplification. Test reactions were considered positive with a Ct value < 40.

The outcome of each qPCR test was compared in contingency tables. The result of the *rnaseP* qPCR assay was used to monitor for inhibition in the samples. Samples negative for *rnaseP* amplification were excluded from further analysis.

7.2.4 Analysis of patient data for leptospirosis cases

Patient data collected by questionnaire at the time of study enrolment was extracted from the study database. Variables for analysis included factors that may influence the outcome of diagnostic testing, such as sample timing (days post onset (DPO) of illness) or patient reported prior antibiotic treatment; and spatial factors associated with patterns of *Leptospira* infection (home location defined by region, district and village). Region, district and village shapefiles were sourced from the Tanzania National Bureau of Statistics (NBS) Housing and Population Census 2012 (Tanzania National Bureau of Statistics, 2012). The core study area (Kilimanjaro Region) and home village for each patient was mapped using QGIS (Version 2.4.0-Chugiak, 2014).

Patients were grouped by DPO of clinical illness to assess the likely phase of their infection at the time of sample collection. 'Week 1' defined as 1-6 DPO corresponds to the typical leptospiraemic phase when leptospires are most likely to be detectable in the blood (Figure 1.2). 'Week 2' and 'Week 3' defined as 7-13 DPO and 14-20 DPO respectively correspond to the typical leptospiruric phase.

Data on urinary antibacterial activity (indicating recent antibiotic treatment) from samples collected at the time of study enrolment in the original febrile disease surveillance were also extracted from the study database. Urinary antibacterial activity was measured using a modified bioassay approach described for the epidemiological surveillance of antimicrobial use (Crump et al., 2011b, Liu et al., 1999). Briefly, filter paper discs soaked in patient urine were placed on plates of solid culture media pre-streaked with pure cultures of *Bacillus subtilis, Escherichia coli*, and *Streptococcus pyogenes* respectively. After overnight incubation, the diameter of the growth inhibition zone around each disc was recorded. For the purposes of this study, a patient sample with a zone of inhibition greater than the diameter of the test disc (8mm) for at least one of the three test bacteria was considered positive for urinary antibacterial activity.

7.2.5 Analysis of serological reactivity profiles in leptospirosis cases

Serological profiles, previously generated at the CDC as described by Biggs *et al.* (2011), were also extracted from the study database for further analysis. Firstly, the predominant reactive serogroup, defined as either the serogroup with a four-fold rise in MAT titre for paired serum samples or the serogroup with the highest MAT titre for single serum samples (acute or convalescent) was determined for each case. The prevalence of each predominant reactive serogroup was calculated for the cohort of leptospirosis cases. Where equivalent titres were observed to more than one serogroup, all serogroups were recorded. Where patients met both definitions (i.e. a four-fold rise and a titre > 1:400) the predominant serogroup was defined as the serogroup with evidence of a four-fold rise.

Serovar and serogroup serological reactivity was assessed within the cohort of cases. Heat maps plotted using in R (R Core Team, 2015) using the gplots package (Warnes et al., 2015) were used to identify distinct serological patterns and serogroup-specificity in acute and convalescent titres of leptospirosis cases. Briefly, all MAT titres for patients that met study case definitions were included. A negative result (MAT < 1:100) was given the value of 10 for the purpose of analysis. Data were arranged in a matrix where each row represented the $\log_{10}$-transformed reciprocal MAT titres for a given patient and each column represented the MAT test serovar. Column order was fixed alphabetically by serovar. Initially, row ordering was generated at random to check that the results of subsequent clustering analyses were not influenced by the order of data entry. Next, Euclidean distances (i.e. square root of the sum of squared differences between the elements of a pair of rows) were calculated for pairwise sample comparisons between each row of the matrix (Everitt, 2005). A hierarchical clustering algorithm based on a complete linkage cluster methods was then used to reorder rows into groups of patients with similar reactivity profiles. Data were plotted using the clustered arrangement of patient samples, and colour coded according to the magnitude of the MAT titre.

Spearman rank correlation coefficients were calculated to quantify pairwise associations in MAT titres between serovars in acute and convalescent serum samples respectively. Correlation coefficients for each pairwise comparison were displayed in a correlation matrix generated in R using the package corrgram (Wright, 2015). Significance levels of each pairwise correlation coefficient were set and displayed at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

7.2.6 Using serological data to explore sources of human *Leptospira* infection

Finally, data on human *Leptospira* infection was compared with data generated through linked animal studies, described in Chapter 6. Predominant serogroups from human cases were compared to those detected in cattle in the abattoir study and plotted in R using the VennDiagram package (Chen, 2015).

7.3 Results

7.3.1 Samples for qPCR testing for *Leptospira* infection

From the total study cohort, 73 patients met the case definitions for acute leptospirosis. Archived samples were available for qPCR testing from 62 (84.9%) cases. Both plasma and urine samples were available from 33 cases. Plasma samples only were available from 25 patients, and urine samples only were available from four patients.

7.3.2 Timing of presentation and prior antibiotic treatment

The median time between onset of illness and study enrolment for all leptospirosis cases was 7 days (range: 1 to 366 days) (n = 73). In total, 30 (41.1%) of 73 leptospirosis cases presented at hospital within the first seven days of clinical illness. Antibiotic treatment prior to study enrollment was reported in 21 (28.8%) cases. For cases with plasma samples available for testing (n = 58), 22 (37.9%) were enrolled within the first week of febrile illness i.e. the leptospiraemic phase (Figure 7.2). Antibiotic treatment prior to study enrolment was reported by questionnaire in 19 cases (32.8%).

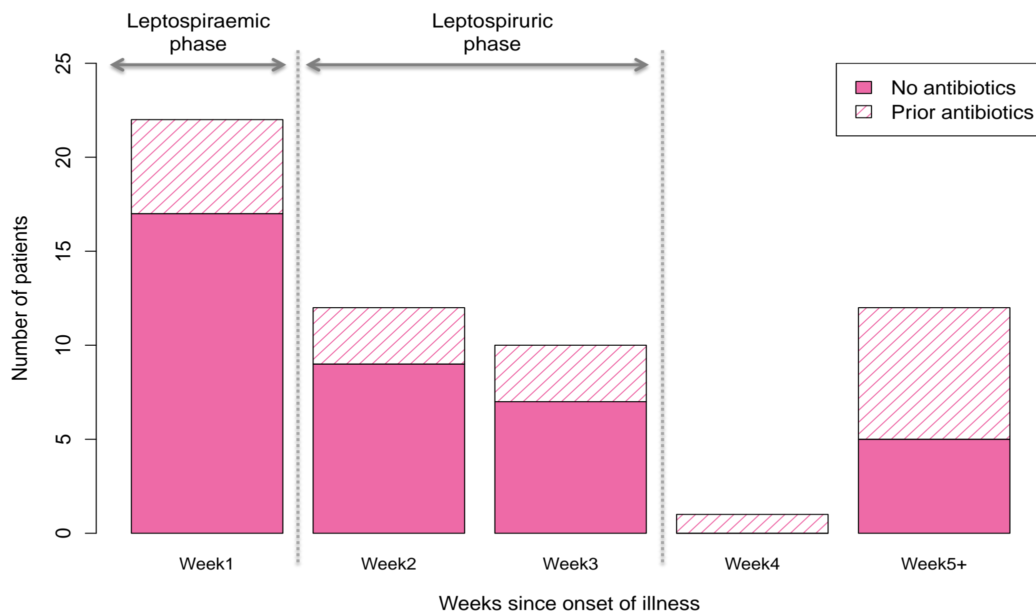


Figure 7.2: Timing of presentation and reported prior antibiotic treatment for leptospirosis cases with plasma samples available for qPCR testing.

For cases with urine samples available for testing (n = 37), 16 (43.2%) were enrolled within the hypothetical leptospiruric phase (7 to 21 days from the onset of febrile illness) (Figure 7.3). A further eight cases were enrolled after at least 21 days of clinical illness, when leptospiruria may persist intermittently. Antibiotic treatment prior to study enrolment was reported in 11 cases (29.7%).

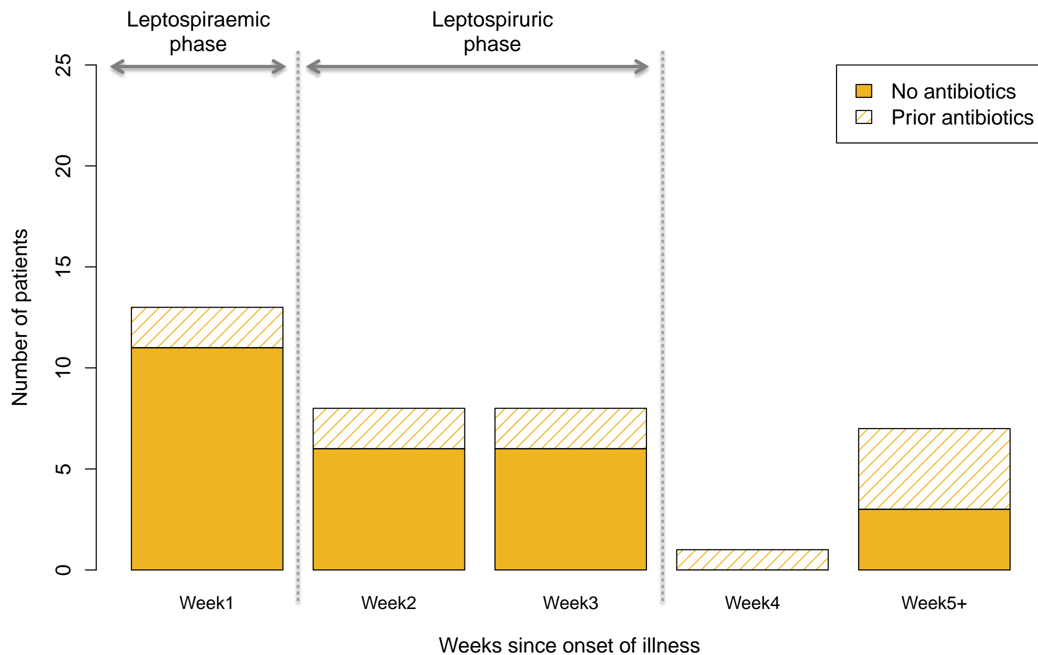


Figure 7.3: Timing of presentation and reported prior antibiotic treatment for leptospirosis cases with urine samples available for qPCR testing.

7.3.3 Comparing reported antibiotic treatment with urine antibacterial activity

Data on urinary antibacterial activity was available for 48 cases. In total, 31 (64.6%; n = 48) cases had evidence of urinary antibacterial activity by bioassay but only ten (20.8%; n = 48) of these cases had reported prior antibiotic treatment by questionnaire. Two (4.17%) patients that reported taking antibiotics prior to admission were negative for urinary antibacterial activity. Under-reporting of antibiotic use was estimated as 67.5% in this patient cohort (Table 7.3).

Table 7.3: Reported antibiotic treatment versus measured urinary antibacterial activity for: i) all leptospirosis cases; ii) cases with plasma available for qPCR testing; iii) cases with urine available for qPCR testing

		Reported antibacterial treatment										
		i) All cases				ii) Plasma cases				iii) Urine cases		
		0	1	NA	(n)	0	1	NA	(n)	0	1	(n)
Urine anti-microbial activity	0	15	2	0	**17**	11	2	0	**13**	13	2	**15**
	1	21	10	0	**31**	15	9	0	**24**	12	9	**21**
	NA	15	9	1	**25**	12	8	1	**21**	1	0	**1**
Total (n)		**51**	**21**	**1**	**73**	**38**	**19**	**1**	**58**	**26**	**11**	**37**

Key: Positive (1); negative (0), not available (NA), total number (n). Discrepant results are highlighted in red.

By week of illness, the greatest proportion of cases with evidence of urine antibacterial activity was observed in week 4 (100%, n = 2) and week 5 (87.5%, n = 8) although sample sizes were small. However, urine antibacterial activity was detected in 63.6% (n = 22) of cases enrolled in the first week of illness, suggesting that early antibiotic treatment prior to seeking hospital health-care is commonplace for patients with acute leptospirosis in the Kilimanjaro Region.

7.3.4 qPCR for the detection of *Leptospira* infection

With the exception of two urine samples, *rnaseP* was amplified for all tested samples (n =58 plasma samples; 37 urine samples) indicating good DNA extraction efficiency and minimal PCR inhibition. No qPCR amplification was detected by either the *lip*L32 or 16S (*rrs*) TaqMan®® qPCR assays in DNA extracted from plasma and urine samples from leptospirosis cases or controls (Table 7.4).

Table 7.4: Summary of qPCR results (*lip*L32, 16S (*rrs*) and *rnaseP*) for plasma and urine samples from leptospirosis cases

Sample type	No. of seropositive samples tested	No. of samples positive by qPCR assay		
		rnaseP	*lip*L32	16S (*rrs*)
Plasma	58	58	0	0
Urine	37	35	0	0
Total:	**95**	**93**	**0**	**0**

7.3.5 Summary of serological results from leptospirosis cases

Paired acute and convalescent MAT titres were available for 39 (53.4%) of 73 leptospirosis cases that met one or both of the study case definitions. MAT titres from single serum samples were available for the remaining 34 patients, of which 32 were sampled in the acute phase of illness and two were sampled in the convalescent phase only.

7.3.6 Geographic origin of leptospirosis cases

Most of the leptospirosis cases (64.8%; n = 73) came from villages within the Kilimanjaro Region (Figure 7.4). In this region, the majority of cases came from villages within the Moshi districts (Moshi Rural (n = 24 patients from 20 villages) and Moshi Municipal (n = 17 patients from 14 villages). The remaining cases came from Hai District (14 patients from nine villages), two villages in Same District (n = 2) and one village in Mwanga District (n = 1). Cases were also detected from other Tanzanian regions including Manyara (n = 6), Arusha (n = 4), Tanga (n = 2) and Dodoma (n =1). Data on home location were not available for three cases.

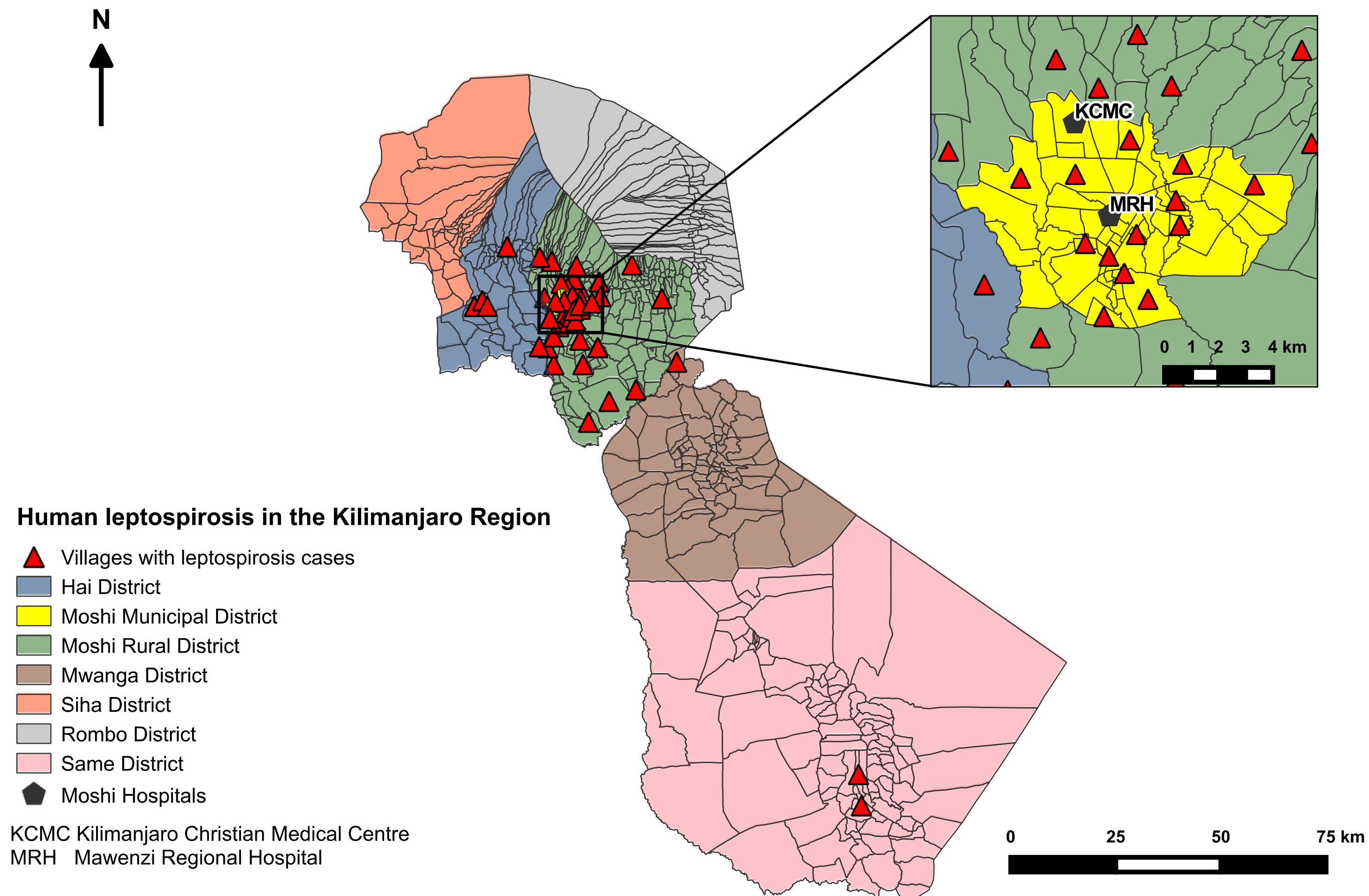


Figure 7.4: Map of the Kilimanjaro Region showing home village locations of leptospirosis cases.

7.3.7 Predominant serogroups for leptospirosis cases

In total, 11 predominant serogroups were recorded (Table 7.5). Serogroups Mini (30.1%), Australis (26.0%) and Autumnalis (13.7%) were the most prevalent predominant reactive serogroup in the cohort of leptospirosis cases. A single predominant serogroups could not be determined in six patients that demonstrated equivalent MAT titres to more than one serogroup. Of this six, five patients demonstrated equivalent reactivity to two serogroups by MAT (Autumnalis and Djasiman (n=2); Australis and Grippotyphosa (n=1); Australis and Mini (n=1); and Autumnalis and Mini (n=1)). In addition, one patient demonstrated equal titres to four serogroups (Australis, Autumnalis, Djasiman and Icterohaemorrhagiae).

Table 7.5: Number and proportion (prevalence) of leptospirosis cases (n = 73) with each predominant serogroup

Predominant serogroup	Number of cases	Prevalence (%)
Mini	22	30.1
Australis	19	26.0
Autumnalis	10	13.7
Celledoni	6	8.22
Icterohaemorrhagiae	3	4.11
Djasiman	2	2.74
Canicola	1	1.37
Grippotyphosa	1	1.37
Hebdomadis	1	1.37
Pyrogenes	1	1.37
Tarassovi	1	1.37
Not determined*	6	8.22

*Due to multiple serogroups with equivalent titres

7.3.8 Assessing serological patterns by heat map

Acute and convalescent log_{10} transformed MAT titres were plotted as heat maps shown in Figure 7.5 and Figure 7.6. *Leptospira* MAT serovars (see Table 7.2 for full serovar details) were plotted on the x-axis with patients clustered by similarity shown by the dendrogram on the y-axis. The clustering algorithms reveal four main patterns (labelled on heat maps) in MAT titres that were observed in both acute and convalescent samples:

A & A_C: Patients with **no observed MAT titres** (< 1:100) against any test serovar. For acute samples **(A)** this cluster comprises patients that were negative in their acute sample and demonstrated seroconversion between acute and convalescent samples (n = 9). For convalescent samples **(A_C)**, this cluster represents a group of nine patients of 39 patients with paired serology that met the case definition for a single elevated titre (MAT titre ≥ 1:400) on acute serology and did not demonstrate seroreactivity (MAT titre < 1:100) to any serovar on convalescent serology.

B & B_C: Patients with MAT titres against a **single** serogroup (B = 27; B_C = 15). In both the acute and convalescent sample groups, several patients demonstrate high to moderate titres against serogroup Mini only. In the heat map showing convalescent serology data (**B_C**), a second cluster of patients that show moderate to high moderate titres against Celledoni only can be seen.

C & C_C: Patients with titres to **two to three serogroups** (C = 20; C_C = 11). In the acute serology sample set **(C)**, patients in this group show evidence of seroreactivity mainly against serogroups Mini and Autumnalis, with a smaller number also demonstrating reactivity against serogroup Icterohaemorrhagiae. In the heat map showing convalescent serology data (**C_C**), patients in this group show seroreactivity Australis, either alone or in combination with another serogroup (most commonly Mini but also Canicola, Djasiman or Icterohaemorrhagiae.

D & D_C: Patients with titres against **more than three serogroups** (D = 17; D_C = 4). The heat map illustrating data from acute serology shows that nearly all the patients in this cluster **(D)** react to at least one Australis serovar and up to ten different *Leptospira* serovars. In general, convalescent serology data (**D_C**) demonstrated greater serogroup specificity than acute serology and co-occurring reactivity between serogroups was more variable.

In the convalescent serology dataset, two patients show high titres (bright yellow) to multiple serogroups indicating a high degree of co-reactivity (**E_C**). These hyper-reactive patients were excluded from further analysis of serogroup patterns for convalescent serology.

Overall, acute MAT results from leptospirosis cases showed evidence of serological reactivity to 15 of 17 tested serogroups. Only Ballum and Pyrogenes (Figure 7.5) were not represented in reactive serogroups. In the convalescent serology dataset, titres were observed to fewer serogroups (n = 11) when the two hyper-reactive patients were excluded (**Ec**). The convalescent serology data of the remaining patients (n = 39) shows that titres were not observed to serogroups Bataviae, Cynopteri, Javanica, Pomona and Sejroe. Notably, titres to serogroup Autumnalis were seen less frequently in convalescent phase samples than in acute phase samples. Where observed, convalescent titres to Autumnalis were never seen without co-reactivity to at least one other serogroup.

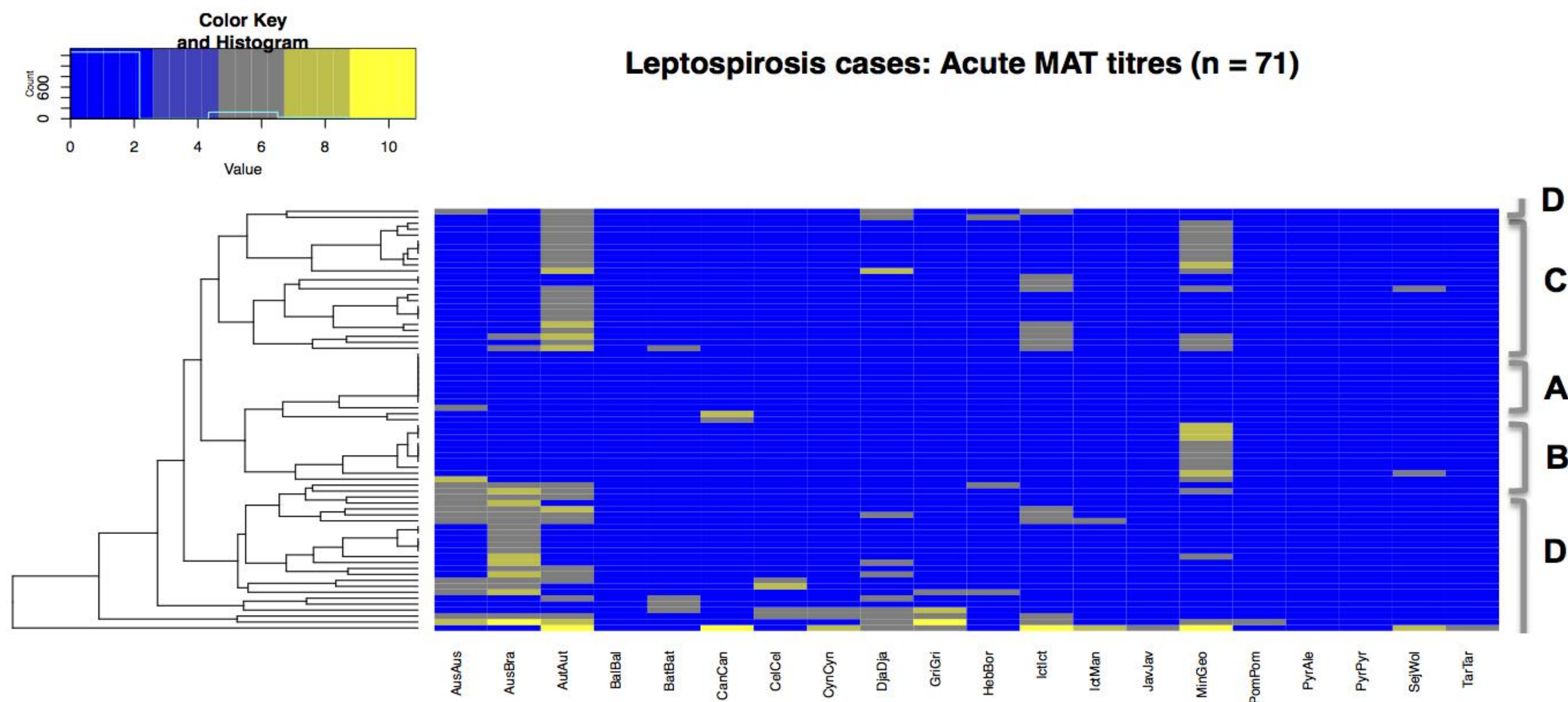


Figure 7.5: Heat map showing log_{10} transformed MAT titres by *Leptospira* serovar for acute phase sera from human leptospirosis cases.

The dendrogram on the y-axis illustrates the clustering of patients (rows) based on serological similarity. Colours indicate the magnitude MAT titres (Colour Key). Test serovars are shown on the x-axis (see Table **7.2** for full serovar details). Patterns in patient clustering are shown on the right: A: Patients with no observed MAT titres to any serovar (n = 9); B: Patients with titres to one serogroup (n = 27); C: Patients with titres to 2-3 serogroups (n = 20); D: Patients with titres against > 3 serogroups (n = 17). The hierarchical clustering algorithm used to make the dendrogram is continuous; hence sections D at the top and bottom of the axis are part of the same cluster.

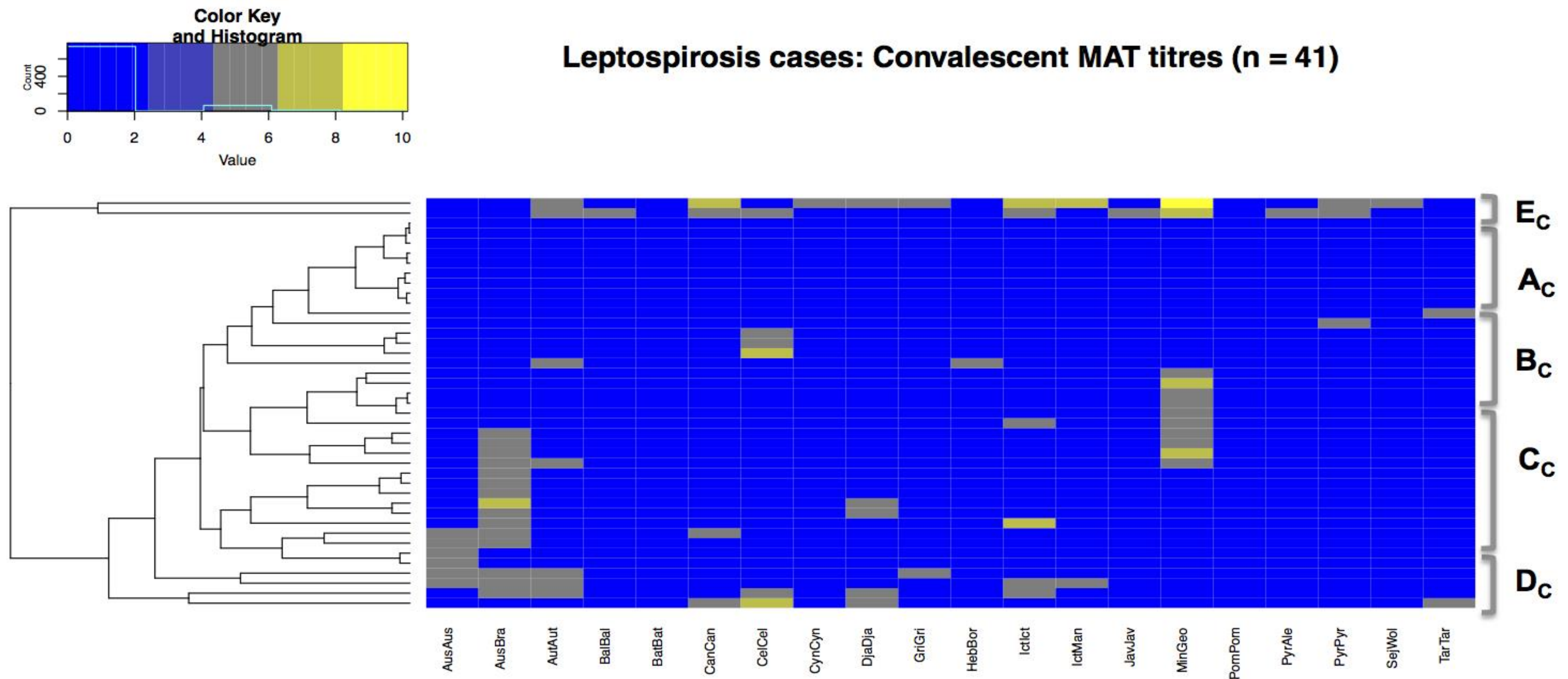


Figure 7.6: Heat map showing $\log_{10}$ transformed MAT titres by *Leptospira* serovar for convalescent phase sera from human leptospirosis cases.

The dendrogram on the y-axis illustrates the clustering of patients (rows) based on serological similarity. Colours indicate the magnitude MAT titres (Colour Key). Test serovars are shown on the x-axis (see **Table 7.2** for full serovar details). Patterns in patient clustering are shown by on right: A_C: Patients with no observed MAT titres to any serovar (n = 9); B_C: Patients with titres to one serogroup (n = 15); C_C: Patients with titres to 2-3 serogroups (n = 11); D_C: Patients with titres against > 3 serogroups (n = 4); E_C: Hyper-reactive patients not used for analysis of population patterns (n = 2).

7.3.9 Assessing correlation in MAT titres between serovars

Spearman's rank correlation coefficients (r) for pairwise comparisons between MAT titres are shown in matrix form in Figure 7.7 (acute serology data) and Figure 7.8 (convalescent serology data).

A high degree of correlation in MAT titres was observed between multiple test serovars in the acute serology data set (Figure 7.7). As expected, highly significant correlations were observed between serovars of the same serogroup (for example; between serovar Australis (AusAus) and serovar Bratislava (AusBra) in the Australis serogroup ($r = 0.84$; $p < 0.001$), and between serovar Icterohaemorrhagiae and serovar Mankarso in the Icterohaemorrhagiae serogroup ($r = 0.99$, $p < 0.001$). However, strong correlations were also observed between test serogroups (for example, titres against serogroup Mini was significantly correlated with titres to the serogroups Canicola, Cynopteri, Icterohaemorrhagiae (both serovars) and Javanica).

Much greater serogroup specificity was observed in the convalescent serology data set (Figure 7.8). Correlations between serovars belonging to the same serogroup were no longer significant suggesting relative serovar specificity (e.g. serovar Australis (AusAus) and serovar Bratislava (AusBra) in the Australis serogroup ($r = 0.11$; $p > 0.05$). However, some significant correlations between serogroups remained, even after exclusion of the two hyper-reactive patients (**Ec** described in Chapter 7.3.8). Significant correlated reactivity was observed between serogroup Canicola and Tarassovi ($r = 0.79$; $p < 0.001$); Canicola and Celledoni ($r = 0.56$; $p < 0.001$); and Celledoni and Tarassovi ($r = 0.56$; $p < 0.01$) suggesting a potential association between all three serogroups. Weaker associations ($p < 0.05$) were observed between Autumnalis and Djasiman ($r = 0.37$); between Canicola and Djasiman ($r = 0.32$); and between Djasiman and Tarassovi ($r = 0.32$). However, no significant associations with any other serogroup were identified with the major predominant reactive serogroups Mini or Australis.

Correlogram for leptospirosis cases: Acute MAT titres (n = 71)

	AusAus	AusBra	AutAut	BalBal	BatBat	CanCan	CelCel	CynCyn	DjaDja	GriGri	HebBor	IctIct	IctMan	JavJav	MinGeo	PomPom	PyrAle	PyrPyr	SejWol
AusBra	0.84 ***																		
AutAut	0.30 *	0.38 **																	
BalBal	NA NA	NA NA	NA NA																
BatBat	−0.07	−0.05	−0.03	NA NA															
CanCan	−0.05	−0.03	0.82 ***	NA NA	−0.03														
CelCel	0.09	−0.00	−0.05	NA NA	0.00	−0.02													
CynCyn	−0.02	−0.03	0.84 ***	NA NA	0.01	0.95 ***	−0.00												
DjaDja	0.25 *	0.27 *	0.43 ***	NA NA	0.13	0.27 *	−0.00	0.35 **											
GriGri	0.81 ***	0.98 ***	0.43 ***	NA NA	−0.01	0.01	−0.01	0.02	0.31 *										
HebBor	−0.01	0.01	−0.04	NA NA	−0.05	−0.03	−0.03	−0.03	0.15	−0.01									
IctIct	−0.02	−0.02	0.86 ***	NA NA	−0.02	0.96 ***	−0.03	0.98 ***	0.30 *	0.02	−0.04								
IctMan	−0.03	−0.02	0.85 ***	NA NA	−0.03	0.97 ***	−0.02	0.98 ***	0.28 *	0.01	−0.03	0.99 ***							
JavJav	−0.04	−0.03	0.85 ***	NA NA	−0.03	0.97 ***	−0.02	0.98 ***	0.29 *	0.02	−0.02	0.99 ***	1.00 ***						
MinGeo	−0.04	−0.03	0.85 ***	NA NA	−0.03	0.97 ***	−0.02	0.98 ***	0.28 *	0.02	−0.03	0.99 ***	1.00 ***	1.00 ***					
PomPom	0.82 ***	0.98 ***	0.40 ***	NA NA	−0.03	−0.02	−0.02	−0.02	0.29 *	1.00 ***	−0.02	−0.01	−0.02	−0.01	−0.01				
PyrAle	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA			
PyrPyr	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA		
SejWol	−0.05	−0.03	0.83 ***	NA NA	−0.03	0.95 ***	−0.02	0.97 ***	0.27 *	0.01	−0.03	0.98 ***	0.98 ***	0.98 ***	0.99 ***	−0.02	NA NA	NA NA	
TarTar	−0.04	−0.03	0.85 ***	NA NA	−0.03	0.97 ***	−0.02	0.98 ***	0.29 *	0.02	−0.02	0.99 ***	1.00 ***	1.00 ***	1.00 ***	−0.01	NA NA	NA NA	0.98 ***

Figure 7.7: Matrix of Spearman's rank correlation coefficients (correlogram) for acute MAT titres from leptospirosis cases by test serovar.

See Table 7.2 for full serovar details Statistical significance of pairwise correlation coefficients (label) is shown by stars ($p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Shading corresponds to the numeric magnitude of the correlation coefficient ranging from -0.07 (white) to +1.00 (dark blue). NA = negative serogroups

Correlogram for leptospirosis cases: Convalescent MAT titres (n = 39)

	AusAus	AusBra	AutAut	BalBal	BatBat	CanCan	CelCel	CynCyn	DjaDja	GriGri	HebBor	IctIct	IctMan	JavJav	MinGeo	PomPom	PyrAle	PyrPyr	SejWol
AusBra	0.11																		
AutAut	0.13	0.19																	
BalBal	NA NA	NA NA	NA NA																
BatBat	NA NA	NA NA	NA NA	NA NA															
CanCan	0.06	−0.05	−0.08	NA NA	NA NA														
CelCel	−0.12	−0.18	−0.07	NA NA	NA NA	0.56 ***													
CynCyn	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA												
DjaDja	−0.12	0.27	0.37 *	NA NA	NA NA	0.32 *	0.26	NA NA											
GriGri	0.26	0.27	0.23	NA NA	NA NA	−0.04	−0.05	NA NA	−0.05										
HebBor	−0.06	−0.10	0.23	NA NA	NA NA	−0.04	−0.05	NA NA	−0.05	−0.03									
IctIct	−0.03	0.03	0.17	NA NA	NA NA	−0.06	−0.06	NA NA	0.12	−0.04	−0.04								
IctMan	0.26	0.27	0.52 **	NA NA	NA NA	−0.04	−0.05	NA NA	−0.05	−0.03	−0.03	0.20							
JavJav	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA						
MinGeo	−0.15	−0.10	−0.04	NA NA	NA NA	−0.09	−0.13	NA NA	−0.12	−0.06	−0.06	−0.08	−0.06	NA NA					
PomPom	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA				
PyrAle	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA			
PyrPyr	−0.06	−0.10	−0.06	NA NA	NA NA	−0.04	−0.05	NA NA	−0.05	−0.03	−0.03	−0.04	−0.03	NA NA	−0.06	NA NA	NA NA		
SejWol	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	NA NA	
TarTar	−0.08	−0.13	−0.08	NA NA	NA NA	0.79 ***	0.56 ***	NA NA	0.32 *	−0.04	−0.04	−0.06	−0.04	NA NA	−0.09	NA NA	NA NA	−0.04	NA NA

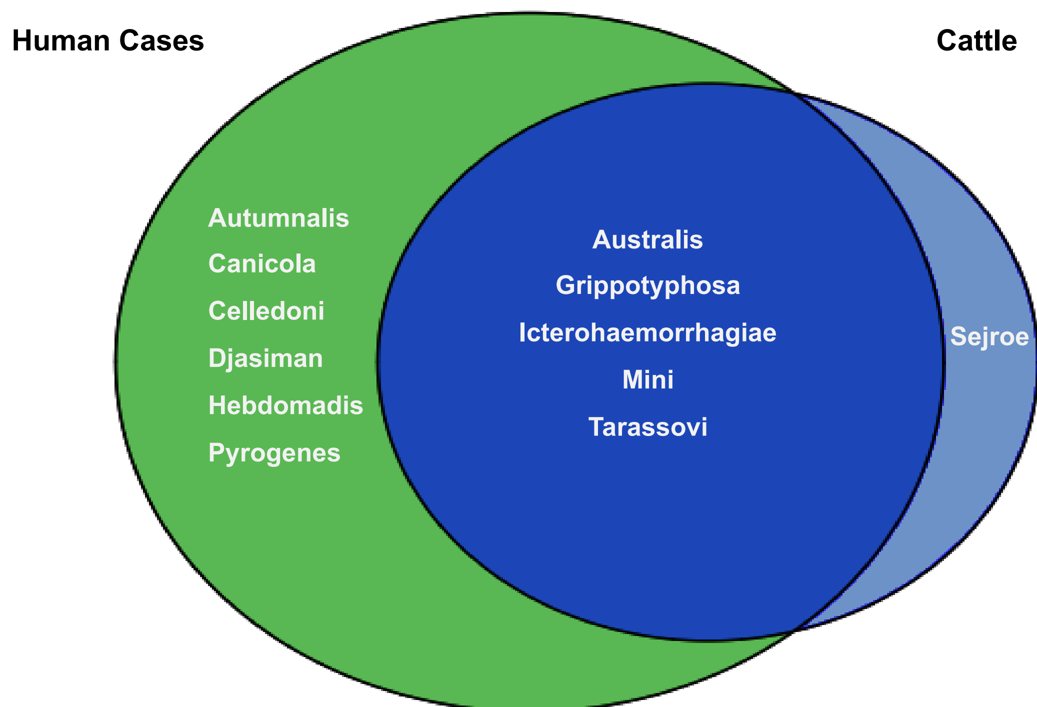
Figure 7.8: Matrix of Spearman's rank correlation coefficients (correlogram) for convalescent MAT titres from leptospirosis cases by test serovar

See Table 7.2 for full serovar details. Statistical significance of pairwise correlation coefficients (label) is shown by stars ($p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***)). Shading corresponds to the numeric magnitude of the correlation coefficient ranging from -0.18 (grey) to +0.79 (dark blue).

7.3.10 Comparison of serological results between people and cattle

Of 11 predominant serogroups observed in human cases, six serogroups - Australis, Grippotyphosa, Icterohaemorrhagiae, Mini and Tarassovi - were predominant reactive serogroups detected in cattle slaughtered at Moshi Municipal slaughter slabs (Chapter 6). The overlap between human and cattle predominant serogroups is shown in Figure 7.9. Mini was the most common predominant serogroup in both human cases (30.1%) and abattoir cattle (29.6%; Chapter 6.3.10).

Figure 7.9: Comparison of predominant reactive *Leptospira* serogroups between human cases and cattle sampled in Moshi abattoirs



7.4 Discussion

This chapter aimed to advance our knowledge of the epidemiology of human leptospirosis in northern Tanzania by bringing together molecular and serological data from a cohort of leptospirosis cases.

In-depth analysis of MAT serological profiles shows that seroreactivity to multiple serogroups is common in this patient cohort, particularly during the acute phase of infection. Convalescent serology showed much greater serogroup specificity and confirmed that two major *Leptospira* serogroups, Mini and Australis, are involved in human disease in northern Tanzania. None of the acute plasma or urine samples collected from serologically confirmed leptospirosis cases were positive by qPCR. A number of patient-related limiting factors including delays in seeking hospital health-care for severe febrile illness and widespread antibiotic use prior to presentation were identified that provide possible explanations for the lack of positive qPCR results in this study. Patient factors as well as logistical and diagnostic test limitations are therefore likely to limit the feasibility of qPCR as an acute-phase diagnostic test for *Leptospira* infection in Tanzania.

Considerable efforts were made to control for the influence of laboratory factors on the outcome of qPCR testing in this study. Firstly, two complementary qPCR approaches targeting different Leptospira genes (*lip*L32, 16S (*rrs*)) were used. Both qPCR assays have been robustly validated in the literature (Stoddard et al., 2009, Galloway and Hoffmaster, 2015, Bourhy et al., 2011, Smythe et al., 2002), and performed well in analytical validation studies described in this thesis (Chapter 4). Concurrent testing of appropriate clinical samples with two complementary assays has been shown to increase the probability of detecting DNA in an infected patient (Thaipadungpanit et al., 2011). The use of plasma rather than other blood derivatives was chosen as some studies have demonstrated relatively greater qPCR sensitivity in this sample type compared to serum or whole blood (Bourhy et al., 2011). The inclusion of urine samples in this study also increases the scope to detect infection, particularly in the later stages of infection (Haake and Levett, 2015). Finally, PCR inhibition has been described as a specific concern when testing urine samples (Burkardt, 2000, Schrader et al., 2012). The use of a human DNA control qPCR assay (*rnaseP*) run alongside the lipL32 TaqMan® qPCR allowed PCR inhibition to be monitored and accounted for (Stoddard et al., 2009). Only two urine samples failed to amplify human DNA and there was no evidence of amplification inhibition in any plasma sample (Table 7.4) detected in either plasma or urine samples. Therefore, the most parsimonious explanation for these results is that the amount of *Leptospira* DNA in these samples was less than the lower limit of detection (LLOD) of these qPCR assays

(Chapter 4). Factors related to sample timing or prior antibiotic treatment are considered to play an important role in the ability to detect *Leptospira* DNA in these leptospirosis cases.

Timing of patient sampling is critically important to obtain a diagnosis of *Leptospira* infection by qPCR (Picardeau et al., 2014) The leptospiraemic phase when *Leptospira* organisms may be detected in the blood of an infected patient is limited to the first five to seven days of clinical illness, hence blood samples for qPCR must be taken during this period (Figure 1.2) (Haake and Levett, 2015). Less than half of the leptospirosis cases included in this study presented at hospital within the first week of clinical illness, and only 22 of 58 cases with plasma samples available for qPCR were sampled during the likely acute leptospiraemic phase. For cases with urine samples available for testing, 24 of 37 patients presented at the hospital after the hypothetical time of onset of the leptospiruric phase. However, the majority of these patients had evidence of urinary antibacterial activity indicating prior antibiotic treatment, which may have reduced the number of *Leptospira* organisms shed in the urine.

Overall, prior antibiotic treatment is likely to be a major limiting factor in the ability to detect *Leptospira* DNA in samples from this patient cohort. In total, urinary antibacterial activity was detected in the urine of ~ 65% of leptospirosis cases, indicating that the majority of patients received antibiotic treatment prior to seeking health-care at either of the study hospitals. This was true even in patients that presented during the first week of clinical illness and in patients who did not report prior antibiotic use by questionnaire. Receiving antibiotic treatment early in the course of a clinical illness may eliminate *Leptospira* infection and reduce the number of patients experiencing secondary sequelae (Haake and Levett, 2015). However, early antibiotic treatment also decreases the probability of detecting *Leptospira* by qPCR, by reducing the bacterial load in the bloodstream in the acute phase and suppressing urinary shedding (Musso and La Scola, 2013). Antibiotic treatment prior to study enrolment is therefore considered as an important limiting factor in the outcome of plasma and urine qPCR testing in this patient cohort. Notably, substantial under reporting of antibiotic treatment was observed in this patient cohort. Only around one-third (32.3%) of patients with urinary antibacterial activity reported prior antibiotic use by questionnaire. A lack of awareness of previous treatment (or of willingness to share this information) may be at least partially explained by understanding health-care seeking behaviours in the study community.

The majority of leptospirosis cases came from the two Moshi Districts (Moshi Municipal and Moshi Rural) where a previous survey of health-seeking behaviour revealed that self-

medication[8] is preferred in this community as the initial response to a febrile disease episode (Panzner et al., 2016). Over 60% of study participants reported that they would try self-medication before seeking health-care at one of the study hospitals. Drugs for self-medication are often purchased from informal drug sellers without medical supervision in East Africa (Bigogo et al., 2010). Informal drug markets often deal with cocktails of unspecified compounds (locally known as 'dawa') that are marketed for particular symptoms or syndromes, such as fever and come with little information regarding their active ingredients. The use of generic drug cocktails may partially explain under-reporting of antibiotic use in the Moshi patients. However, antimicrobial products are also commonly available over the pharmacy counter in Moshi as are antimalarial drugs, many of which also contain antibacterial compounds (e.g. doxycycline, or Fansidar®, an antimalarial product that contains sulphonamide antibiotic (Murray et al., 2004, Basco, 2004)). As malaria is still considered the main cause of febrile disease in Tanzania (Chipwaza et al., 2014, Reyburn et al., 2004), over-the-counter antimalarial drugs are likely to be a common first choice for self-medication of a fever. Furthermore, little regulation or medical supervision for the prescription of antimicrobial products is currently in force in Tanzania (Goodman et al., 2007). Factors such as these may have contributed to the high prevalence of urinary antibacterial activity and under-reporting antibiotic use reporting in study patients. However, regardless of the cause, widespread antibiotic use will reduce the feasibility of qPCR testing for *Leptospira* infection in this community and should be accounted for when evaluating diagnostic test performance.

Given what we know about patient health-care seeking behaviour in northern Tanzania, even in the absence of positive qPCR results, the utility of implementing leptospirosis diagnosis by blood qPCR can now be estimated for this setting. Figures from this study suggest that 38% of leptospirosis cases that seek hospital health-care will do so in the first week of clinical illness. Of these, 64% will already have received antibacterial treatment and hence are not good candidates for testing. However, for approximately 14 cases out of every 100, qPCR still offers the possibility of a confirmatory diagnosis on an acute phase sample. If qPCR was used to test every patient with non-specific febrile disease that presented to the two study hospitals, this approach would detect one case of leptospirosis for every 100 patients tested (assuming that the true prevalence of disease is 8% (Biggs et al., 2011) and that the test has 100% diagnostic sensitivity). Investing in a diagnostic test that will benefit only 1% of patients seems hard to justify in a resource-limited setting.

[8] Self-medication is defined as deciding treatment autonomously for yourself or other members of your household without consulting a health-care professional

However, the lack of feasible diagnostic options for patient testing remains a major limiting factor for clinician awareness and disease surveillance on the continent. Continuing to explore acute phase test options for use in health-care settings in Africa should be an important research priority.

The second major goal of this chapter was to explore patterns and potential sources of human-infecting *Leptospira* types. Analysis of serological data from leptospirosis cases was performed to explore patterns in MAT serogroup titres and identify *Leptospira* serogroups important for human infection in northern Tanzania. Consistent with prevalent serogroups reported in the original analysis of leptospirosis patients (Biggs et al., 2011), three serogroups - Mini, Australis and Autumnalis – were predominant among the reactive serogroups from human cases. However, patients frequently demonstrated MAT titres to more than one *Leptospira* serovar and for some patients a single predominant reactive serogroup could not be determined due to high MAT titres to more than one serogroup. Currently, traditional methods of analysing serological data typically focus on the serogroup with the highest titre (i.e. the predominant reactive serogroups) or serogroup-specific seroprevalence (e.g. Table 7.1) (Goris and Hartskeerl, 2013). However, neither approach is able to account for cross-reactivity in serological profiles, which continues to be a major limiting factor in the use of serological data to determine infecting Leptospira serotypes in human cases.

Serological reactions against multiple *Leptospira* serovars or serogroups may occur due to a number of reasons including:

i) Co-infection with multiple different *Leptospira* serovars, which each generates their own specific antibody response;

ii) True cross-reactivity among closely related serovars; either between two serovars within a serogroup, or between serogroups within a complex (e.g. Sejroe-Hebdomadis-Mini complex and MAT cross-reactions between these three serogroups are common (Royal Tropical Institute (KIT), 2014, Kmety and Dikken, 1993);

iii) Specific immunological memory resulting from previous exposure that results in antibodies being produced to more than one *Leptospira* serovar in the event of infection or re-infection (Goris and Hartskeerl, 2013);

iv) Non-specific immune response seen in the early stages of an antibody response to *Leptospira* or as part of another infectious or non-infectious disease syndrome (e.g.

infection with other spirochaetes or autoimmune conditions), resulting in non-specific agglutination of multiple serovars (Goris et al., 2012).

Differentiating between serological reactivity to multiple serogroups as a result of co-infection and cross-reactivity as a consequence of a non-specific immune response is clearly necessary to determine important human-infecting serotypes of *Leptospira*. As yet, there are few methods that have been described to tackle this important question.

The heat maps and correlation matrices presented in this chapter represent an effort to explore patterns of serogroup cross-reactivity and serological reaction profiles in more depth. The two approaches used here are complementary. Heat maps allowed easier visualisation of complex seroreactivity patterns in the data set (Figure 7.5 and Figure 7.6) and correlation matrices offer quantitative estimations of correlated reactivity and can be used to improve the confidence of our infecting serogroup predictions. Heat maps have previously been used to explore serological data from epidemiological serosurveillance for leptospirosis (Lelu et al., 2015, Halliday, 2010), but to our knowledge have not been used to describe for MAT data from a cohort of patients with acute febrile disease.

Both approaches demonstrated that MAT serogroup specificity is much greater in convalescent serology than acute serology. The high degree of correlated reactivity observed in acute serum samples limits the utility of MAT data from acute samples to determine patient infecting serogroup (Figure 7.7). This pattern is widely reported in the literature and explains in part why data from acute-phase MAT is inconsistent in determining infecting serogroup (Haake and Levett, 2015, Levett, 2003). However, MAT titres performed on convalescent serum samples showed much greater serogroup specificity, demonstrated quantitatively by reduced correlation between serogroups displayed in the correlation matrices (Figure 7.7 and Figure 7.8). This result suggests that analysis of serological results from convalescent patients may provide valuable opportunities for investigating serogroup infection patterns in human leptospirosis.

In this patient cohort, a larger number of patients demonstrated titres to a single serogroup, most commonly to serogroups Mini, Australis or Celledoni in the convalescent serology data set than in the acute serology data. High titres to a variety of other serogroups were also seen although usually these co-occurred with reactivity to at least one other serogroup (e.g. Tarassovi or Icterohaemorrhagiae). Interestingly, very little seroreactivity to Autumnalis was seen in convalescent samples despite being one of the most common serogroups in the predominant reactive serogroup analysis presented in this chapter (Chapter 7.3.7) and in previous analysis of this dataset (Biggs et al., 2011). In the acute

serology dataset, MAT titres against serogroup Autumnalis were highly correlated with titres to many other serogroups (Figure 7.7), but were largely absent or very low in the convalescent serology dataset (Figure 7.6 and Figure 7.8). Therefore, in this patient cohort, reactivity to serogroup Autumnalis could be interpreted as a non-specific acute phase reaction rather than a true indicator of infecting serogroup.

Visualisation and clustering of patient MAT titres by heat map revealed a group of nine patients with substantial MAT reactivity (≥ 1:400) in their acute phase samples, but no MAT titres (< 1:100) in convalescent samples (Chapter 7.3.8; Group $\mathbf{A_C}$). This finding suggests that the specificity of the MAT to diagnose leptospirosis could be compromised when relying on single acute phase samples in Tanzania. Based on our current understanding, antibody titres typically remain elevated for months to years following *Leptospira* infection (Cumberland et al., 2001), although early antibiotic treatment has been reported to interfere with the immune response to infection and some specific serovars are also associated with a short duration of serological reactivity (Goris et al., 2013c, Musso and La Scola, 2013, Haake and Levett, 2015). Whilst a presumptive diagnosis of leptospirosis may be made on the basis of a single MAT titre ≥1:400 according to WHO guidelines, the broad applicability of this definition is still under debate (Goris et al., 2012, World Health Organization, 2011). Some studies advocate the use of a more conservative MAT cut-off titre for diagnosis in an endemic setting (Biggs et al., 2011, Cumberland et al., 1999, Cumberland et al., 2001). Local validation of appropriate case definitions is also recommended (Goris et al., 2012).

Regardless of the method of analysis, the data presented here indicates that least two serogroups of *Leptospira* – Mini and Australis – are important human infection in northern Tanzania. *Leptospira* serovars belonging to the serogroup Australis have been previously isolated from people and animals in East Africa (Chapter 2). Within the serogroup, *L. kirschneri* serovar Ramisi was originally isolated from the blood of patient from the Coastal Province of Kenya in the 1970s (de Geus, 1971, Dikken et al., 1979, de Geus et al., 1977a), and *L. interrogans* serovar Lora has also been isolated from multimammate mice (*Mastomys natalensis*) sampled in the Morogoro region of southern Tanzania (Mgode et al., 2015). Serological evidence of agglutinating antibodies to serovar Lora has also been previously reported in human seroprevalence studies in Tanzania (Assenga et al., 2015, Chipwaza et al., 2015) and in abattoir cattle in this study (Chapter 6 and Figure 7.9). Although both cattle and small mammals are implicated in the epidemiology of Australis infection in Tanzania, in other settings other animals such as pigs, dogs, hedgehogs and horses are considered major maintenance hosts of the Australis serovars, particularly of *L.*

interrogans serovar Bratislava (Ellis, 2015, Hamond et al., 2014). Given the high prevalence of Australis sero-reactivity in people, combined with the low seroprevalence in cattle in the abattoir study (Table 6.8) and absence of rodent *Leptospira* infection in this area (Chapter 5), it seems unlikely that cattle or rodents are the source of Australis infection for people in Moshi. Further investigation into other Australis hosts in the area is therefore warranted, particularly as pigs and dogs are both commonly kept in the two Moshi Districts (Table 5.3)

Very little is known about serogroup Mini in Tanzania. To date, *Leptospira* isolates of the serogroup Mini have not been reported anywhere else in mainland Africa, although isolates belonging to the Mini serogroup have been isolated from people, rodents and other small mammals on the nearby islands of Mayotte and Madagascar (Chapter 2) (Bourhy et al., 2010, Bourhy et al., 2014, Desvars et al., 2012, Dietrich et al., 2014). It is unclear how widespread human serological exposure to Mini is in Tanzania as this study is the first in the country to include serogroup Mini on MAT test panels (Table 7.1). However, exposure to serogroup Mini is common in cattle sampled in the abattoir surveillance component of this thesis (Chapter 6 and Figure 7.9), indicating that cattle are also to be involved in the epidemiology of Mini infection in the Kilimanjaro Region.

Overall, *Leptospira* exposure in people and cattle appears closely linked in Tanzania. Cattle sampled in abattoirs close to the study hospitals share a number of predominant serogroups with human leptospirosis cases (Figure 7.9). Serogroup Mini was the most prevalent serogroup in both cohorts. Published seroprevalence surveys also indicate that livestock infection may be a risk factor for human exposure. Milking cattle is a risk factor for human seroprevalence in Tanga (OR 3.44; 95% CI 1.76 – 6.75; $p < 0.001$) (Schoonman and Swai, 2009). Rural residence, which is often associated with livestock keeping, was also recognised as an important risk factor for human leptospirosis in this study cohort (Biggs et al., 2011).

However, attributing the sources of *Leptospira* infection for people requires more robust evidence than serological data can provide. Although serogroup patterns are a useful first step, most serogroups contain several distinct serovars (for example; serogroup Australis contains 14 known serovars (Royal Tropical Institute (KIT), 2014)). Direct demonstration and typing of the pathogen is necessary in both populations. Further work is needed to characterise infecting *Leptospira* in human cases and potential animal hosts to determine the sources and plausible transmission routes of human infections. Additional effort to empower clinicians to diagnose the aetiology of febrile illness in patients is particularly

important in areas such as Tanzania where malaria over-diagnosis is becoming endemic, and non-malarial causes of febrile illness often overlooked (Reyburn et al., 2004, Chandler et al., 2008, Crump et al., 2013). However, where human disease is recognised, livestock should be considered as a potentially important source of human infection.

8 Discussion

8.1 Investigating the epidemiology of leptospirosis in northern Tanzania: study conclusions

Leptospirosis is one of the most widespread zoonotic diseases around the world but many aspects of the disease remain poorly understood, particularly in Africa. The incidence of human disease remains poorly quantified for the continent as a whole, but robust evidence exists for the occurrence of acute human leptospirosis in many regions (Chapter 2)(Allan et al., 2015a) and East Africa in particular is predicted to have a high burden of human disease (Costa et al., 2015a). This study was established following the demonstration of leptospirosis as an important cause of non-malaria febrile illness (NMFI) in northern Tanzania (Crump et al., 2013). An integrated 'One Health' approach was taken to explore *Leptospira* infection in linked human and animal populations in Moshi, with an over-reaching goal of identifying possible animal sources of infection for people and informing future disease control strategies. The findings of this study have generated some novel and unexpected insights that both advance and challenge our understanding of the epidemiology of leptospirosis in this area. Although most of this thesis describes results from a specific site in Tanzania, the demonstration of widespread human and animal infection by the systematic review (Chapter 2) indicates that leptospirosis is an omnipresent health threat across Africa. Hence the findings of this thesis may have a broader relevance to the epidemiology of leptospirosis on the continent as a whole.

A number of key challenges exist that continue to limit our ability to monitor and control leptospirosis in Africa and in other parts of the world. For people, diagnosing leptospirosis in the event of an acute infection or disease outbreak still remains an enormous challenge. In Africa, improved performance and uptake of malaria diagnostic tests have highlighted the growing problem of malaria-negative febrile patients and the deficit of diagnostic tools for alternative aetiologies of febrile disease (Chappuis et al., 2013, Petti et al., 2006, Chandler et al., 2008). The non-specific nature of acute leptospirosis means that it is clinically indistinguishable from malaria and several other infectious causes of febrile illness (Haake and Levett, 2015). The lack of a highly sensitive and specific acute-phase test for human leptospirosis is a recognised problem within the leptospirosis community (Picardeau et al., 2014, Hartskeerl et al., 2011) and is likely to hinder attempts to monitor the disease in Africa. Studies have also shown that clinician awareness of leptospirosis and a variety of other causes of non-malarial febrile illness (NMFI) is lacking (Zhang et al.,

2016, Chipwaza et al., 2014). The scale of this problem was plainly demonstrated in the study of febrile illness from northern Tanzania that is linked with this thesis (Chapter 7). More than 60% of this cohort of febrile patients received a clinical diagnosis of (and treatment for) malaria but only 1.6% of patients were positive for acute malaria infection on laboratory tests (Crump et al., 2013). Leptospirosis was definitively diagnosed as the cause of illness in approximately 9% of these patients, but was not listed as a clinical diagnosis in any case (Crump et al., 2013).

Despite a high burden of disease in the local area (Biggs et al., 2013b), laboratory testing for *Leptospira* infection is not routinely performed in Moshi. For research studies, so far cases have been diagnosed retrospectively by MAT serology at international reference laboratories, with little scope to inform acute patient care. A reliable and practical diagnostic approach that could empower clinicians to diagnose leptospirosis in the acute phase of illness is clearly needed in this setting. Acute-phase serological tests have been trialled in children with febrile illness in central Tanzania, but showed relatively low diagnostic specificity when compared to MAT for confirmatory testing (Chipwaza et al., 2015). So far, direct detection methods of diagnosing human leptospirosis have not been successful at detecting infection in Tanzania (e.g. qPCR in this study (Chapter 7) and culture in on-going hospital febrile disease surveillance (John Crump and Renee Galloway, personal communication)). However, this failure to detect infection is not simply the result of poor test performance, but also reflects broader social issues surrounding health-care seeking behaviour and self-medication (Chapter 7). The widespread use of antibiotics in Tanzania may limit the utility of a direct detection test for *Leptospira.* Further work is needed not only to improve diagnosis and clinical management of leptospirosis but also to reduce the unregulated use of antimicrobials in these communities, which is of widespread concern with respect to antimicrobial resistance (Okeke et al., 2005, Leopold et al., 2014).

Although qPCR did not demonstrate good utility as a diagnostic test for human disease, the use of this testing method proved practical and productive in animal populations sampled in this study. qPCR was more sensitive than culture at detecting renal *Leptospira* infection and widespread application of the assays revealed some surprising and unusual patterns of infection. Infection was demonstrated in cattle, sheep and goats from across northern Tanzania but no evidence of *Leptospira* infection could be detected in peri-domestic rodents sampled from an area with a high burden of human disease (Chapters 5 and 6).

Worldwide, rodents are thought to be the most important source of human leptospirosis (Haake and Levett, 2015) and *Leptospira* infection in common invasive rodent species,

such as *Rattus rattus* or *Mus musculus* sampled in this study, is generally considered to be a ubiquitous phenomenon (Levett, 2001, Kosoy et al., 2015, Thiermann, 1983). However, based on the absence of infection in invasive rodents in this study, we can conclude that these rodent species are not always hosts of *Leptospira* infection in Tanzania and therefore are not always responsible for human disease. These findings were unexpected, particularly as many of the species tested in this study have been shown to be hosts of *Leptospira* infection in other settings (Rahelinirina et al., 2010, Felt et al., 2011, Mgode et al., 2005). However, similar results were also reported in a recent study of *R. rattus* in Niger (Dobigny et al., 2015) indicating that this is not a unique scenario. Further research is needed to explore whether this pattern of rodent infection occurs more widely in Tanzania and sub-Saharan Africa.

For the febrile patients of Moshi who became unwell in 2007-08 (Crump et al., 2013, Biggs et al., 2011), it still remains a possibility that rodents were involved in infection transmission. The rodent survey (2013-14) described in this thesis was performed five years after the original hospital surveillance. Data emerging from more recent studies in Moshi indicates that the incidence of acute human leptospirosis was substantially lower over the same time period. Between 2012-14, human leptospirosis incidence was estimated as 11-18 cases per 100,000 per year compared to 75-102 cases per 100,000 per year between 2007-08 (Maze et al., *in preparation*). These rather dramatic changes in incidence indicate that the dynamics of infection are unstable in this population and therefore it is possible that similar trends also occur in the local rodent populations. Longitudinal monitoring of both human and animal infection is advocated to better understand the epidemiology of infection in this area.

The absence of rodent infection in Moshi gave a strong indication that other animal sources of *Leptospira* are more important than rodents for the transmission of infection to people. Demonstration of *Leptospira* infection in ruminant livestock coupled with evidence of serogroup similarities between cattle and people with acute disease led to the hypothesis that ruminant livestock are an important source of *Leptospira* infection for people in northern Tanzania.

8.2 The livestock hypothesis: implications for disease control

Several strands of evidence from this study support a hypothesis of livestock to human *Leptospira* transmission in this setting. Firstly, the high prevalence of *Leptospira* infection in the kidneys and urine of cattle sampled in the Moshi slaughterhouses (Chapter 6.3.5 and 6.3.6) indicated that bovine *Leptospira* infection and urinary shedding is commonplace. Infection was demonstrated in cattle that originated from regions across the north of Tanzania. *Leptospira* infection was also demonstrated in sheep and goats, which are the most common ruminant livestock species kept by smallholders in the Moshi area (Table 5.4). Finally, analysis of serological data revealed similarities between predominant reactive serogroups seen in cattle and people with acute leptospirosis, providing the most robust evidence for a linkage between *Leptospira* infections in these two populations (Chapter 7.3.10). Collectively, this evidence led to the conclusion that ruminant livestock are an important reservoir of *Leptospira* infection for people in northern Tanzania, and that efforts to prevent and reduce human disease should also include control in ruminant livestock populations.

Identifying livestock as a major source of human infection rather than rodents has some crucial implications for the control strategies that may be appropriate (Hartskeerl et al., 2011). Disease interventions for leptospirosis, and zoonotic infectious diseases more broadly, can be considered in three broad groups: 1) control efforts that directly protect individual people from infection such as vaccination; 2) transmission blocking tactics that reduce the amount or types of contact that people have with a source of infection; or 3) interventions that control the disease 'at source' by reducing infection within a reservoir of infection (Haydon et al., 2002). In general, human vaccination for leptospirosis is not a very practical means of reducing human infection (Haake and Levett, 2015, Bharti et al., 2003), particularly in an area such as Tanzania where multiple serogroups appear to be circulating but remain poorly characterised. Globally, leptospirosis control efforts largely focus on either controlling infection in source populations (e.g. vaccination in cattle to reduce shedding (Bolin and Alt, 2001)) or blocking transmission between people and the source of infection (e.g. controlling flooding risks (Lau et al., 2010)). However, both of these strategies require a greater understanding of the dynamics of infection in a reservoir; for example, which animal hosts are critical for the maintenance of infection (Viana et al., 2014); and also the major routes of transmission routes from the source of infection to people (Haydon et al., 2002).

An extra complicating factor for the control of livestock to human *Leptospira* infection is that multiple types of *Leptospira* have been identified in ruminant livestock species in Tanzania. Serological data (Chapter 6) indicates that multiple serotypes of *Leptospira* are circulating in cattle populations and as yet, these serotypes have been poorly characterised. Two major genetic species have been detected in ruminant livestock (*L. borgpetersenii* and *L. kirschneri*) that are likely to have different dynamics within a reservoir due to their different environmental survival capabilities for example (Bulach et al., 2006). Therefore livestock-associated maintenance and transmission of *Leptospira* is likely to be complex and involve multiple host species. In northern Tanzania, the specific role of each livestock host of *Leptospira* is still poorly understood. However, based on the available evidence, plausible scenarios for infection maintenance and transmission can be proposed with different implications for disease control

8.2.1 Livestock maintenance of *Leptospira* infection in northern Tanzania

In the disease ecology literature, an infection reservoir has been defined as 'one or more epidemiologically connected populations (or environments) in which the pathogen can be permanently maintained, and from which infection is transmitted to the defined target population' (Haydon et al., 2002, Viana et al., 2014). In a particular setting, an infection reservoir may include a mixture of maintenance hosts (defined as a host population which is able to independently maintain infection over a prolonged period of time, without reintroductions (Viana et al., 2014)), non-maintenance hosts and contaminated environmental sources that all contribute to the transmission of infection to the target population. The role of different animal hosts within this reservoir has implications for designing effective disease control interventions (Haydon et al., 2002). A general framework for characterising reservoirs of disease has been laid out by (Viana et al., 2014), and can broadly be used to consider the potential roles of cattle in the reservoir of leptospirosis for people in northern Tanzania.

Firstly cattle in northern Tanzania could be **maintenance hosts** of a particular type of *Leptospira* bacteria that is permanently maintained within the cattle population by direct cow-to-cow transmission. Most simply, human infection would be acquired through direct contact with an infected cow. Therefore, reducing infection within cattle populations, for example by vaccination or treatment, would be an effective way to reduce transmission of infection to people.

Alternatively, cattle may be **non-maintenance hosts** of a particular type of *Leptospira* in northern Tanzania. This definition approximately translates to the 'incidental' host of *Leptospira* infection described in the leptospirosis literature (Levett, 2001). The outcome of *Leptospira* infection in a non-maintenance host may be less predictable than in a maintenance host and may vary by both the *Leptospira* type and the particular host species in question. In a non-maintenance host of *Leptospira,* infection and shedding may be short-term or transient and hence that host may make little contribution to the overall reservoir of infection. This kind of infection is often termed a 'dead-end' infection and is epitomised by the pattern of *Leptospira* infection in people (Haake and Levett, 2015). Alternatively, more persistent infection and shedding may be established in a non-maintenance host. In this case, the host could still be a source of infection for people (and other animals) but is unable to permanently maintain infection in the absence of re-introductions from other sources such as other livestock species or contaminated environmental sources. If cattle are in fact a non-maintenance host of a particular type *Leptospira* in northern Tanzania, reducing infection in cattle may still have direct benefits for human health by removing one potential source of infection. Furthermore, preventing or controlling infection in cattle may also have direct benefits to the animal, particularly if the serovar in question results in clinical disease. However, the design of an effective control program in this scenario should also consider the possibility of infection transmission from other animal hosts (i.e. not cattle) or environmental sources to people. Therefore, transmission-blocking strategies may be particularly relevant, especially where multiple animal host species are involved.

By the species of *Leptospira* detected in livestock in this study, we can hypothesise that cattle are the most likely maintenance hosts for *L. borgpetersenii* in northern Tanzania. This hypothesis is supported by the high proportion of bovine infections attributed to this species; the current lack of evidence for other livestock hosts of this *Leptospira* type; and the existing literature describing the epidemiology of *L. borgpetersenii* in cattle populations in other settings around the world (Ellis, 2015). However, the presence of *L. kirschneri* in multiple host species, coupled with the likelihood of better environmental survival of this pathogen (Bulach et al., 2006) implies that cattle are probably part of a more complex multi-host reservoir community for this *Leptospira* species. Based on this evidence we would therefore recommend that interventions for *L. borgpetersenii* are targeted at cattle populations alone, but control of *L. kirschneri* may require a broader multi-species or ecosystem approach. Fully characterising and understanding the complex dynamics of the relationship between a host and a particular *Leptospira* type is not possible by small-scale cross-sectional sampling as performed in this study. However, these

hypotheses represent a critical first step towards designing intervention trials and identifying priorities for future research.

8.2.2 Transmission of *Leptospira* from livestock to people in northern Tanzania

Assuming that cattle-to-human transmission of *Leptospira* does occur, minimising the contact of people with infected animals is another approach that may be useful in preventing human disease. This is likely to be a much greater challenge in a developing country setting like Tanzania where more than 75% of the working population is employed in agriculture (Food and Agriculture Organization of the United Nations, 2005), compared to in high-income countries like the UK where the industry employs just 1.5% of the population (Department for Environment Food and Rural Affairs et al., 2012).

Occupational exposure to infected livestock is an important risk factor for human leptospirosis in a number of settings (Mwachui et al., 2015). Some of the most robust data on occupational leptospirosis transmission comes from New Zealand where leptospirosis is an important work-related health threat for farmers and abattoir workers (Dreyfus et al., 2014a, Dreyfus et al., 2014b). In Moshi, figures from the abattoir study indicate that around 90 of the 550 or so cattle that pass through the Moshi slaughterhouses in an average week will be infected with *Leptospira* bacteria week (Figure 6.2). These animals may pose a considerable infection risk for slaughterers, butchers and Livestock Field Officers (LFOs) working in these settings.

However, in Tanzania, livestock keeping is more of a way of life in subsistence communities than a specific occupation. In the core Moshi study areas, ~ 38% of households reported keeping ruminant livestock, mostly sheep and goats (Table 5.3) and most respondents kept their livestock in zero-grazing units in close proximity to their home (data not shown). Contact with ruminant livestock and their waste is therefore a regular part of day-to-day life in this setting rather than an occupationally associated risk *per se*. Exposure through home slaughter may also be important in some settings (Qekwana and Oguttu, 2014, Ernest et al., 2009).

Finally, the possibility of exposure to *Leptospira* infection through the meat supply chain of bovine meat products remains a possibility. Oral transmission (i.e. eating contaminated meat) is not considered to be a major source of infection but butchering and preparing raw offal foodstuffs may pose a risk for transmission (Dreyfus et al., 2014a). Bovine kidneys

are a valuable food item for people in the Moshi area and as around 7% of cattle kidneys sampled in this study were positive for infection with pathogenic *Leptospira* bacteria, handling kidneys during food preparation is another potential route of infection for people.

Finally, indirect transmission of *Leptospira* infection from cattle to people through the environment may also play a role. This route is less likely to be important for *L. borgpetersenii* due to reduced environmental survival as previously mentioned (Bulach et al., 2006). Environmental contamination with infectious leptospires could occur anywhere that cattle urinate, from grazing areas and watering holes, to markets and slaughterhouse holding areas. However, quantifying and controlling environmental transmission of *Leptospira* is notoriously challenging (Munoz-Zanzi et al., 2014b, Faine, 1994) and controlling the infection in the host population may be a more feasible option.

8.2.3 The impact of leptospirosis on livestock health and productivity in Tanzania

Control of *Leptospira* in cattle populations may also be important for animal health and productivity as well as to prevent zoonotic transmission of infection to people. *L. borgpetersenii* serovar Hardjo, which was isolated from cattle in this study, is recognised as a commercially important infection in developed country settings and may result in abortion, infertility and reduced milk production (Dhaliwal et al., 1996a, Dhaliwal et al., 1996b, Thiermann, 1982). However most of the information on the clinical impact of bovine *Leptospira* infection comes from countries with intensive livestock production systems where *Bos taurus* breeds predominate. To date, there is very little field or experimental data describing the effects of *Leptospira* infection on *Bos indicus* breeds, which predominate in much of sub-Saharan Africa (Mwai et al., 2015). All the *Leptospira*-infected cattle reported by this study were humped Zebu cattle, a major sub-group of *B. indicus* cattle in that are commonly kept by subsistence farmers and pastoralist communities in East Africa (Mwai et al., 2015). Evidence generated by this study demonstrates that *B. indicus* cattle are susceptible to *Leptospira* infection but there is virtually no information in the literature regarding clinical leptospirosis or the dynamics of infection in a *B. indicus* host. Even the small number of studies reporting clinical leptospirosis in cattle in Africa identified by systematic review (Chapter 2.3.10) reported disease in *B. taurus* breeds (Burdin et al., 1958, Te Brugge and Dreyer, 1985). This lack of data may well be a consequence of under-reporting but it also may be the case that differences in disease susceptibility exist between the two bovine species. Between *B.*

taurus and *B. indicus* breeds, differences in resistance to tick infestations are reported (Piper et al., 2009) as well as varying susceptibility to parasitic diseases such as trypanosomiasis (Mwai et al., 2015). It is also possible that relative differences also occur in susceptibility to clinical leptospirosis, and more work is needed to explore the outcome of *Leptospira* infection in Tanzanian cattle.

Quantifying the effects of *Leptospira* infection in Tanzanian livestock-keeping communities is also an important priority to help assess the true impact of *Leptospira* infection. Overall, livestock play a major role in the socio-economic development and food security of many communities in sub-Saharan Africa (Otte and Chilonda, 2002) and in Tanzania (Food and Agriculture Organization of the United Nations, 2005). In subsistence farming communities, which depend on ruminant livestock for food, even small-scale production losses may have a considerable impact on food security (Sansoucy *et al.*, 1995). In particular, livestock infections that result in reduced milk production have been shown to have negative consequences for childhood nutrition (Shinsugi et al., 2015, Mosites et al., 2015). Furthermore, livestock reproductive failure through abortion or infertility may have a considerable detrimental impact on household income, which is particularly critical for small-scale or subsistence farmers (Grace et al., 2012a, Zinsstag et al., 2007). Zoonotic diseases like leptospirosis therefore have the potential to have many detrimental effects on the well-being of livestock-keeping communities (Maudlin et al., 2009) and controlling these diseases is advocated as one method of poverty alleviation for 'the world's rural poor' (Hotez, 2009, World Health Organization, 2006, Halliday et al., 2015).

Increasingly, the argument is also being made to include animal losses into a more comprehensive estimate of disease impact for zoonotic diseases (Molyneux et al., 2011, Zinsstag et al., 2007). Typically, estimates of the global burden of disease focus only human illness and death in the Disability Adjusted Life Years (DALY) metric (Murray et al., 2012, Torgerson et al., 2015) and do not account for the insidious effects of animal disease on human health. Accurately estimating these effects may be difficult where multiple production-limiting diseases are circulating. However, specifically for *Leptospira* infection, examples exist where either case-control approaches have been used to estimate the impact of infection in livestock (Ayanegui-Alcerreca et al., 2007), or vaccine trials have allowed the magnitude of *Leptospira*-attributable production losses to be quantified (Dhaliwal et al., 1996b). Similar approaches could be used in East Africa to improve our understanding of the full burden of disease in this setting.

8.3 Future directions for leptospirosis research in Tanzania

Over the course of this thesis, several key questions have been identified for future research on leptospirosis in Tanzania and elsewhere in Africa.

Question 1: How representative is Moshi of the incidence of human infection and the epidemiology of leptospirosis elsewhere in Tanzania?

An immediate question arising out of this research is whether this site in the north of Tanzania is unique in its high incidence of human disease and absence of *Leptospira* infection in rodents. In the initial hospital febrile surveillance study, the incidence of leptospirosis was estimated as 75 to 102 cases per 100,000 per annum (Biggs et al., 2013b), which is exceptionally high when compared to regional incidence estimates from around the world (Costa et al., 2015a). However, more recent data has indicated that the incidence of infection is not consistently as high as the initial figures implied (Maze et al., *in preparation*). Hence longitudinal monitoring at this site would allow more representative figures for human disease incidence to be calculated, as well as to monitor the long-term dynamics of infection in the rodent populations in this area. Sampling at other sites in Tanzania to cover a broader selection of populations, ecological and geographic settings and climate could also offer new insights into the extent of the problem in Tanzania, and the factors that drive high rates of infection.

Question 2: How can we improve our diagnosis of leptospirosis for people in Tanzania?

Timely diagnosis of infection remains a major stumbling block for leptospirosis. Without robust data on the prevalence and incidence of infection it is hard to raise the profile of the disease amongst many health threats (Abela-Ridder et al., 2010), or to measure the effect of any interventions that are trialled (Hartskeerl et al., 2011). In the absence of feasible and accessible diagnostic tests, the management of human cases will still rely on empirical treatment and clinicians will have few tools to improve their management of patients with NMFI (Chappuis et al., 2013).

The outcome of patients for severe illness may be improved by adherence to international syndromic treatment guidelines (Crump et al., 2011a) or by developing disease-specific diagnostic algorithms based on a combination of clinical signs, 'rule-out' testing for other infectious aetiologies where robust rapid tests do exist, and local risk factors for infection. In Moshi, risk factor analysis for acute human leptospirosis from the hospital study of

2012-14 is on-going and is hoped to reveal important insights into high risk scenarios for human *Leptospira* infection in this area.

Improved acute phase diagnostics for human leptospirosis remain an elusive goal for the global leptospirosis community (Hartskeerl et al., 2011) and the field is certainly open for innovative new solutions. Molecular advances have provided some important steps forward (Picardeau et al., 2014) but as this study shows, the performance of molecular diagnostic tests continue to be hampered by a number of external factors, in particular the accessibility of over-the-counter antimicrobial products in developing countries. Given the relatively low research investment in leptospirosis to date (Abela-Ridder et al., 2010), and the likely time scale for the development of a novel diagnostic test, perhaps the most immediately useful question to ask **how could we use our current diagnostic tools and therapeutic options more effectively**?

Timing of sampling and sample selection is critical to diagnosing infection by either serology or direct detection methods. Based on our understanding of the health-care seeking behaviours in Moshi, sampling patients at hospital may not be the most appropriate means of detecting early infection in this population. In future studies, implementing testing in community locations, such as pharmacies where patients obtain their first line of treatment for a fever, may be more appropriate to diagnose infection in the early stages of illness. Testing prior to antibiotic treatment would also give the best chance of obtaining more information about the infecting *Leptospira* type through either culture or PCR-based sequence analysis.

Understanding more about the background exposure and circulating serogroups in the healthy (i.e. non-febrile) human population could help us to improve the performance of available diagnostic tests in Tanzania and provide epidemiological data to underpin clinician assessment of prior probability of disease (Chappuis et al., 2013). Background prevalence and magnitude of MAT titres can inform locally appropriate serological case definitions and improve the specificity of case detection (Goris et al., 2012). Knowledge of locally circulating serovars can improve sensitivity of the MAT by selecting appropriate serogroups for inclusion on the test panel (Goris and Hartskeerl, 2013). So far, *L. borgpetersenii* serovar Hardjo has not been included on any diagnostic panels used to test patients in Moshi despite being isolated from cattle in slaughterhouses in the area. Including locally representative serovars on MAT panels may also help to improve the performance of the test in this setting.

Question 3: What factors drive transmission of *Leptospira* between livestock and people?

Risk factor analysis may help to improve our understanding of the factors that influence zoonotic transmission of *Leptospira* infection from animals to people in northern Tanzania. This work is underway both as part of on-going febrile disease surveillance in Moshi (mentioned above) and in a parallel analysis of data from a cross-sectional community surveillance study of *Leptospira* exposure in human and animal populations in the Kilimanjaro and Arusha Regions. Analysis of serological data generated by the cross-sectional project from linked human and cattle populations is already planned to follow-on from the work presented in this thesis.

Question 4: What factors influence the patterns of *Leptospira* infection in animal hosts in this setting?

One of the most intriguing questions to come out of this work was: why were the various rodent species trapped in and around households in the Moshi area negative for *Leptospira* infection? Several factors may influence the prevalence of infection in a rodent population including a range of host, population and environmental factors as discussed in Chapter 5. Longitudinal monitoring of rodent populations alongside long-term human disease surveillance would be fascinating study to explore whether human infection dynamics are also mirrored by the dynamics in the local rodent population. Expanding the study area to include a more diverse range of geographic or environmental settings could also shed new light on the factors that determine rodent infection in Tanzania.

Another enigma to arise from this study relates to the dominance of serogroup Mini in both human and cattle serological data. This *Leptospira* type was not isolated or well characterised by either genetic or serological methods by this study despite its prevalence in the cattle serological study. Virtually nothing is known about serogroup Mini infections from elsewhere in sub-Saharan Africa. To add further intrigue, reactivity to this serogroup was conspicuously absent from human cases identified by the 2012-14 febrile disease study (Maze et al., *in preparation*). More work is needed to understand the identity, maintenance hosts and transmission dynamics of serogroup Mini in this setting.

Finally, the source of serogroup Australis, which was the second most common serogroup in human serological data, has not been identified Moshi. Australis continues to be a dominant reactive serogroup in subsequent febrile disease surveillance in Moshi (Maze et

al., *in preparation*) and hence poses an important public health threat to people living in the area. Investigation of other maintenance hosts of serovars of this serogroup such as pigs and dogs is also an important next step in understanding the epidemiology of infection and identifying appropriate disease control targets.

Question 5: How can we tackle human leptospirosis 'at-source'?

Evaluating potential intervention strategies for human leptospirosis is an important future research question at this site. Various potential intervention strategies were discussed earlier in this chapter (Chapter 8.2.1) when discussing the potential role of livestock as hosts of infection in northern Tanzania. As well as evaluating the effectiveness of specific intervention to control human disease, intervention trials can also offer insights into the specific role of a particular animal host in the maintenance of infection (Haydon et al., 2002) and be used to quantify the impact of infection on the animal itself (Dhaliwal et al., 1996b).

Based on the conclusions of this thesis, evaluating the feasibility and effectiveness of livestock vaccinations in Tanzania would be an obvious starting point. Vaccinations do exist for several *Leptospira* serovars in ruminant livestock hosts including *L. borgpetersenii* serovar Hardjo, which was isolated in this study. At the moment, it is not clear how much human infection can be attributed to this *Leptospira* type. As mentioned above, the serovar was not included on MAT panels used in either the 2007-08 or 2012-14 febrile disease study. Virtually no reactivity to an alternative serovar of the serogroup Sejroe (the serogroup of *L. borgpetersenii* serovar Hardjo) was detected in the 2007-08 study (Chapter 7) but cases have been detected in the later cohort (Maze et al., *in preparation*). MAT testing of serum samples collected by cross-sectional survey will include *L. borgpetersenii* serovar Hardjo on the testing panel, which will allow the proportion of human exposure to this serovar to be quantified.

Tackling human leptospirosis 'at-source' requires an integrated, multidisciplinary approach to disease surveillance, control and prevention. A 'One Health' approach to controlling zoonotic disease is considered a more equitable approach to improving health in marginalised communities (Halliday et al., 2015, Zinsstag et al., 2007). It is important to note that like many other diseases that are characterised as 'neglected', leptospirosis is more accurately a disease of 'neglected communities' rather than a disease that warrants little international attention. Neglected diseases often highlight gross inequalities in

income, healthcare infrastructure or sanitation between communities and countries (Hotez, 2009, Molyneux et al., 2011) and like many other zoonotic infections, leptospirosis is strongly associated with poverty in both urban and rural populations (Lau et al., 2010, Abela-Ridder et al., 2010). Therefore, approaches that control the disease at source have the potential to have broader-reaching effects than improving treatment of individual cases for the minority of leptospirosis cases that reach a hospital.

In conclusion, the work presented here is only an incremental step towards understanding the epidemiology of leptospirosis in Tanzania, and indeed elsewhere in Africa, and many questions remain unanswered. The continent has a great deal to teach us about leptospirosis. Tackling the burden of leptospirosis both in Africa and on a global scale will require a universal effort to tackle poverty and health inequalities. In the meantime, general principles of leptospirosis epidemiology and control can be learned from better-characterised settings and applied to attempt to minimise the impact of this important but neglected disease.

Appendix 1: Summary of eligible cohort and surveillance studies reporting human acute leptospirosis in Africa, 1930-2014

Citation	Study year(s)	Country	Setting and study design	Study inclusion criteria	Diagnostic tests	Patients enrolled (n)	Eligible cases* (n & %)
(Van Riel et al., 1956)	1952-54	Democratic Republic of Congo (DRC)	Hospital; retrospective cohort	Clinical suspicion of leptospirosis	Culture (blood) in Vervoort-Korthoff media; Agglutination-lysis (MAT)	45	27 (60·0%)
(Kolochine -Erber and Brygoo, 1956)	1954-55	Madagascar	Undefined; prospective cohort	Clinical suspicion of leptospirosis	Agglutination-lysis (MAT)	40	1 (2·5%)
(Forrester et al., 1969)	1961-62	Kenya	Hospital; prospective cohort	Febrile illness unexplained by malaria, dysentery or pneumonia.	MAT	67	6 (9·0%)
(Payet et al., 1966)	1964-65	Senegal	Hospital; prospective cohort	Clinical suspicion of leptospirosis; mostly defined by jaundice	Agglutination-lysis (MAT)	53	3 (5·7%)
(Silverie et al., 1968)	1966-67	Madagascar	Undefined; prospective cohort	Clinical suspicion of leptospirosis	Agglutination-lysis (MAT)	65	7 (10·8%)
(de Geus et	1967	Kenya	Hospital and	Febrile illness (temperature ≥	Culture (blood)	39	7 (17·9%)

al., 1969)			health centre; prospective cohort	38°C) without obvious cause; negative malaria smear or no response to anti-malarial treatment	in Fletcher's and Cox's media; MAT		
(Sankalé et al., 1973)	1967-72	Senegal	Hospital; retrospective cohort	Inpatients with serum samples tested for leptospirosis	Serum agglutination (MAT)	134	3 (2·2%)
(de Geus et al., 1977a)	1968-69	Kenya	Hospital outpatients and health centre; prospective cohort	Febrile illness (temperature ≥ 38.3°C) without obvious cause; negative malaria smear or no response to anti-malarial treatment [a]	Culture (blood) in Fletcher's media; MAT	91	10 (11·0%)
(de Geus et al., 1977b)	1969	Kenya	Hospital & outpatient department; prospective cohort & case-finding survey [b]	Febrile illness (temperature ≥ 38.3°C) without obvious cause; negative malaria smear or no response to anti-malarial treatment	Culture (blood) in Fletcher's media; MAT [c]	281	9 (3·2%)
(Kinebuchi and Afoakwa, 1973)	NA	Ghana	Hospital; prospective cohort	Clinical suspicion of leptospirosis, mostly defined by hepatitis or jaundice	Culture (blood) in Korthof's media; MAT	99	13 (13·1%)
(Hogerzeil et al., 1986)	1981-82	Ghana	Hospital outpatient department; prospective cohort	Group 1: Fever without obvious cause and/or any of the following; jaundice, muscle pains, meningism, conjunctival injection, albuminuria; negative malaria smear	Culture (blood and urine) in Fletcher's or EMJH media; MAT; IgM and IgG ELISA	Group 1: 88	Group 1: 4 (4·5%)

				Group 2: Jaundice		Group 2: 102	Group 2: 2 (2·0%)
(Delacollette et al., 1995)	1985-86	DRC	Hospital; prospective cohort	Inpatients with black or red urine with confirmed haemoglobinuria	ELISA (unspecified)	38	1 (2·6%)
(Pinn, 1992)	1988-90	Seychelles	Hospital; prospective cohort	Inpatients with clinical diagnosis of leptospirosis [d]	IgM ELISA	80	58 (72·5%)
(Collares-Pereira et al., 1997)	1993	Mozambique	Hospital outpatient department; prospective cohort	Outpatients aged 18-50 years with acute febrile illness without obvious cause; negative malaria smear.	MAT	43	1 (2·3%)
(Yersin et al., 1998)	1995-96	Seychelles	Nationwide health care providers; Prospective population-based surveillance	Fever or any of the following without obvious cause: myalgia, liver tenderness, jaundice, acute renal failure, bleeding tendency, radiographic lung infiltrates, or meningism	MAT; PCR (*rrs*)	125	75 (60·0%)
(Desvars et al., 2011)	1998-2008	Réunion	Hospital; retrospective population-based surveillance	Cases voluntarily reported to Centre National de References de Leptospiroses (Paris, France)	Culture (blood), media not specified; MAT; PCR (target not specified)	NA	613 cases
(Ismail et al., 2006)	1999-2003	Egypt	Hospital; retrospective cohort	Group 1: fever (temperature ≥38°C) for ≥3 days in the absence of diarrhoea, pneumonia, typhoid fever, brucellosis or established	IgM ELISA; MAT	Group 1:886 [e]	Group 1: 141 (15·9%)

				fever of unknown origin.			
				Group 2: acute hepatitis defined as signs of acute jaundice.		Group 2: 392 [f]	Group 2: 63 (16·1%)
(Renault et al., 2011)	2004-08	Réunion	Hospital; retrospective population-based surveillance	Hospitalised cases of leptospirosis cases in Réunion reported to the Regional Directorate for Health and Social Affairs/Regional Health Agency of the Indian Ocean.	Culture (sample and media not specified), MAT; PCR (target not specified); IgM ELISA	240	160 (66·7%)
(Pages et al., 2014)	2004-12	Réunion	Population-based surveillance	Confirmed or probable cases of leptospirosis in Réunion residents reported to the health watch platform of the French Regional Health Agency for the Indian Ocean.	Culture (sample and media not specified), MAT or PCR (target not specified); IgM ELISA	NA	405 cases
(Ari et al., 2011)	2005	Kenya	Community; prospective case-finding[g]	Community members with new onset febrile illness (temperature not defined) or joint pains	IgM ELISA	12	3 (25·0%)
(Bertherat et al., 2014)	2005	DRC	Community; retrospective case finding	Acute & convalescent patients with respiratory disease in a mining camp	MAT	82	8 (9·8%)
(Parker et al., 2006)	2005-2006	Egypt	Hospital; prospective cohort	Fever $\geq$ 2 days or admission temperature $\geq$38.5°C, aged $\geq$ 4 years without obvious cause of fever, such as diarrhoea, pneumonia, or	Culture (blood) in EMJH; MAT; PCR; IgM ELISA	981	194 (19·8%)

				clinical diagnosis of typhoid fever or brucellosis.			
(Parker et al., 2007)	2005-2006	Egypt	Hospital; prospective cohort	Fever ≥ 2 days or admission temperature ≥38.5°C, aged ≥ 4 years without obvious cause of fever; with laboratory evidence of co-infection with *Leptospira*, *Rickettsia typhi*, *Brucella*, or *Salmonella enterica* serogroup Typhi	Culture (blood) in EMJH; MAT; PCR (*lig*A)	187 [h]	152 (81·3%)
(Murray et al., 2008, Murray et al., 2011)	2005-2007	Egypt	Hospital; prospective cohort	Fever; aged ≥ 4 years without obvious cause of fever, such as diarrhoea, pneumonia, or clinical diagnosis of typhoid fever or brucellosis.	Culture (blood) in EMJH media; MAT; PCR (*lig*A)	2,441	98 (4·0%)
(Tagoe et al., 2010)	NA	Ghana	Hospital; prospective cohort	Fever ≥ 2 days and temperature ≥38.0°C; aged ≥ 4 years without obvious cause of fever	IgM ELISA; MAT	166	13 (7·8%)
(Biggs et al., 2011, Biggs et al., 2013a)	2007-08	Tanzania	Hospital; prospective cohort	Inpatients aged ≥13 years with fever (≥38.0°C oral) or inpatients aged 2 months to 12 years with history of fever within 48 hours or admission temperature ≥37.5ºC axillary ≥38.0ºC rectal.	MAT	831	70 (8·4%);
(Bourhy et al., 2012)	2007-08	Mayotte	Undefined; prospective	Fever (temperature ≥38°C) for ≤7days and headache	Culture (blood) in EMJH	388	53 (13·7%),

			cohort	and/or myalgia	media; PCR (*rrs*)		
(Bourhy et al., 2010)	2007-2010	Mayotte [k]	Undefined; population-based surveillance	Patients for which a blood sample was submitted for leptospirosis diagnosis to the Hospital Centre of Mayotte	Culture (blood) in EMJH media; PCR (*lbf1, lipL32, rrs*)	2,523	198 (7·8%)

Footnotes:

*Figures reported here are based on the number of acute leptospirosis cases that met the study case definitions and therefore may vary from the values reported in the original citations.

[a] Patients who refused hospital admission were not investigated.

[b] Methods describe a change to a case-finding survey partway through the study, but full details not available

[c] MAT performed in a subset of participants only

[d] Clinical diagnosis defined as ≥3 of the following: headache or fever (temperature not defined), evidence of liver inflammation (defined as jaundice, tender liver, and/or abnormal liver function tests), evidence of renal inflammation (haematuria and/or abnormal renal function), or evidence of muscle inflammation (tenderness and/or elevated creatine phosphokinase)

[e] All tested negative for *Salmonella enterica* serovar Typhi, *Brucella* spp., and *Rickettsia* spp.

[f] All tested negative for Hepatitis A, B, and C.

[g] In setting of outbreak of acute febrile illness in a well-defined population

[h] 187 patients were diagnosed with selected co-infections out of a total cohort of 1510 patients with non-specific febrile illness.

ϖ Taken ≥ 9 days of onset of illness

[k] Also report two imported cases from Comoros and Madagascar respectively

Appendix 2: Summary of eligible studies reporting *Leptospira* infection in animals in Africa, 1930 - 2014

Citation	Country; Study year(s)	Study design	Diagnostic tests	Animal Species tested	Animals tested (n)	Positive cases (n & prevalence)
(Brownlow and Dedeaux, 1964)	Egypt 1959	Wild animal surveillance	Culture (Kidney); Stuart's media	House mouse (*Mus musculus*)	44	2 (4·5%)
(Lazuga and Bonnefous, 1962)	Tunisia NA	Wild animal surveillance	Culture (Kidney); Korthoff's media	Norway rat (*Rattus norvegicus*)	919	57 (6·2%)
(Ball, 1966)	Kenya 1963	Wild animal surveillance	Culture (Kidney); Cox's media	Fringe-tailed gerbil (*Gerbilliscus robustus*)	113	4 (3·5%)
(Rademan et al., 1964)	South Africa 1963-1964	Wild animal surveillance	Culture (Kidney); Korthoff's media	Norway rat (*Rattus norvegicus*)	256	7 (2·7%)
(Chadli and Bakoss, 1965)	Tunisia 1964	Wild animal surveillance	Culture (Kidney); Korthoff's media	Norway rat (*Rattus norvegicus*)	130	11 (8·5%)
(Bakoss, 1969, Bakoss and Chadli, 1965)	Tunisia 1965	Abattoir surveillance and serovar typing	Culture (Kidney); Korthoff's media	Pigs (*Sus scrofa domesticus*)	185	2 (1·1%)
(Dikken et al., 1981)	Kenya 1967-1968	Serovar typing	Culture (Kidney); media NA.	Fringe-tailed gerbil (*Gerbilliscus robustus*)	NA	16 isolates obtained; breakdown not given
				African grass rat (*Arvicanthus niloticus*)	NA	
				South African pouched mouse (*Saccostomys campestris*)	NA	

(Maronpot et al., 1971)	Egypt NA	Domestic animal surveillance	Culture (Urine); Fletcher’s & Ellinghausen’s media	Dogs (*Canis lupis familiaris*)	68	2 (2·9%)
(Barsoum et al., 1973)	Egypt NA	Wild animal surveillance	Culture (Kidney & urine); Ellinghausen liquid media; Fletcher’s media	House mouse (*Mus musculus*)	95	7 (7·6%)
				Egyptian Mongoose (*Herpestes ichneumon*)	16	2 (12·5%)
				Red fox (*Vulpes vulpes*)	16	1 (6·3%)
(Van Rensburg, 1973)	South Africa NA	Animal disease outbreak	Culture (Kidney); Korthoff’s media	Pigs (*Sus scrofa domesticus*)	10	6 (60·0%)
(Diallo and Dennis, 1982)	Nigeria 1974-1976	Wild animal surveillance & abattoir sampling	Culture (Kidney); Ellinghausen-McCullough media	African grass rat (*Arvicanthus niloticus*)	221	8 (3·6%)
				Cattle (*Bos* sp.)	74	5 (6·8%)
(Tabel and Losos, 1979)	Kenya 1975-1976	Animal disease outbreak	Culture (Kidney); Korthoff’s media	Cattle (*Bos* sp.)	9	2 (22·2%)
(Le Bras et al., 1977)	Cameroon 1975-1976	Wild animal surveillance	Culture (Kidney); Korthoff’s media	Rusty-bellied brush-furred rat *Lophuromys sikapusi*)	NA	1 (NA)
(Herr et al., 1982)	South Africa 1980	Animal disease outbreak	Culture (Urine); Semi-solid Stuart’s & EMJH media	Cattle (*Bos* sp.)	20	10 (50·0%)
(Mugarula, 1984)	Tanzania 1980	Domestic animal surveillance	Culture (Urine); Korthoff’s media	Dogs (*Canis lupis familiaris*)	3693	48 (1·3%)

(Herr and Winnen, 1983)	Botswana NA	Animal disease outbreak	Culture (Urine); EMJH media	Cattle (*Bos* sp.)	40	1 (2·5%)
(Ezeh et al., 1989a, Ezeh et al., 1989b, Ezeh et al., 1990)	Nigeria 1984-1985	Abattoir sampling & serovar typing	Culture (Kidney); EMJH media	Cattle (*Bos* sp.)	525	6 (1·1%)
(Te Brugge and Dreyer, 1985)	South Africa NA	Animal disease outbreak	Culture (Urine); EMJH media	Cattle (*Bos* sp.)	19	3 (15·8%)
(de Lange et al., 1987)	South Africa NA	Animal disease outbreak	Culture (Kidney, renal lymph node, aborted foetuses); EMJH media	Pigs (*Sus scrofa domesticus*)	14	13 (92·9%)
(Hunter et al., 1987)	South Africa NA	Abattoir surveillance	Culture (Kidney); EMJH media	Pigs (*Sus scrofa domesticus*)	30	20 (66·6%)
(Feresu, 1992, Feresu et al., 1993, Feresu et al., 1998, Feresu et al., 1995, Feresu et al., 1994, Feresu et al., 1996, Feresu et al., 1999a)	Zimbabwe 1987-1988	Abattoir surveillance and serovar typing	Culture (Kidney); EMJH media	Cattle (*Bos* sp.)	480	50 (10·4%)
(Dalu and Feresu, 1997)	Zimbabwe 1995-1996	Wild animal surveillance	Culture (Kidney, Urine); EMJH media	Black rat (*Rattus rattus*)	293	46 [§]
				Multimammate mouse (*Mastomys natalensis*)	85	2 [§]
				House mouse (*Mus musculus*)	3	4 [§]

(Machang'u et al., 1997)	Tanzania NA	Abattoir surveillance	Culture (Urine); Fletcher's media	Cattle (*Bos* sp.)	1021	7 (0·1%)
(Gummow et al., 1999)	South Africa NA	Animal disease outbreak	Culture (Kidney, aborted foetuses, bovine urine); medium not stated	Pigs (*Sus scrofa domesticus*)	13	12 (92·3%)
				Cattle (*Bos* sp.)	12	3 (25·0%)
(Machang'u et al., 2002, Machang'u et al., 2004)	Tanzania NA	Serovar typing	Culture (Urine); Fletcher's media	Giant African pouched rat (*Cricetomys gambianus*)	83	8 (9.6%)
(Taylor et al., 2008)	South Africa 2004-2005	Wild animal surveillance	PCR (Kidney); 16S (*rrs*) (Murgia et al., 1997)	Norway rat (*Rattus norvegicus)*	63	8 (12·7%)
				House mouse (*Mus musculus*)	2	1 (50·0%)
				Black rat (*Rattus rattus*)	2	1 (50·0%)
(Zimmermann et al., 2007)	Guinea 2004	Human disease outbreak	PCR (Kidney): target not described	Rodents; various species[#]	330	5 (1.5%)
(Mgode et al., 2005)	Tanzania NA	Wild animal surveillance	PCR (Kidney); 16S (*rrs*) (Murgia et al., 1997)	Multimammate mice (*Mastomys* spp.)	18	PCR: 1 (6·3%)
			Culture (Kidney); Fletcher's media	Shrews (*Crocidura* spp.)	7	PCR: 2 (28·6%) Culture: 2 (28.6%)
(Mgode et al., 2006)	Tanzania NA	Serovar typing	Culture (Urine); Fletcher & EMJH media	Cattle (*Bos* sp.)	Not given	Not given

(Felt et al., 2011)	Egypt 2006-2007	Wild animal surveillance	PCR (Kidney); *lig A* & *lig B*(Palaniappan et al., 2005) Culture (Kidney, urine, blood); EMJH media.	Black rats (*Rattus rattus)*	100	PCR: 11 (11·0%)$
						Culture: 4 (4·0%)
(Desvars et al., 2012)	Mayotte 2007	Human disease outbreak	qPCR (Kidney); *lipL32*(Stoddard et al., 2009)	Black rats (*Rattus rattus*)	141	42 (29·8%)
(Kessy et al., 2010)	Tanzania 2007-2008	Abattoir surveillance	Culture (Kidney & urine); Fletcher's media	Pigs (*Sus scrofa domesticus*)	236	2 (0·8%)
(Halliday et al., 2013)	Kenya 2008	Wild animal surveillance	qPCR (Kidney); *secY* (Ahmed et al., 2009)	House mouse (*Mus musculus*)	194	37 (19·1%)
				Norway rat (*Rattus norvegicus*)	10	1 (10·0%)
				Black rat (*Rattus rattus*)	33	3 (9·1%)
(Rahelinirina et al., 2010)	Madagascar 2008-2009	Wild animal surveillance	Culture (Kidney* & urine); EMJH media qPCR (Kidney* & urine); *Hap1/lipL32*(Branger et al., 2005)	House mouse *(Mus musculus)*	55	PCR: 5 (10.0%)* Culture: 0 (0%)*
				Norway rat (*Rattus norvegicus*)	96	PCR: 39 (40·6%)* Culture: 6 (6·3%)*
				Black rat (*Rattus rattus*)	94	PCR: 27 (28.7%)* Culture: 3 (3·2%)*
				Asian house shrew (*Suncus murinus*)	23	PCR: 10 (43.5%)* Culture: 0 (0%)*

(Desvars et al., 2013c)	Réunion 2009	Wild and domestic animal surveillance	qPCR (Kidney; urine from bats only); *lipL32*(Stoddard et al., 2009)	House mouse (*Mus musculus*)	13^	11 (84.6%)
				Black rat (*Rattus rattus*)	76^	50 (65.8%)
				Norway rat (*Rattus norvegicus*)	6^	4 (66.6%)
				Asian House Shrew (*Suncus murinus*)	48^	15 (31.2%)
				Dog (*Canis lupis familiaris*)	24^	7 (29.2%)
				Cat (*Felis cattus*)	21^	6 (28.6%)
				Cattle (*Bos* sp.)	77^	14 (18.2%)
				Goat (*Capra aegagrus hircus*)	49^	13 (26.5%)
				Rusa Deer (*Rusa timorensis*)	32^	6 (18.8%)
				Pigs (*Sus scrofa domesticus*)	83^	13 (15.6%)
				Bats (*Mormopterus francoismoutoui*)	2^	2 (100%)
(Houemenou et al., 2013)	Benin 2009	Wild animal surveillance	qPCR (Kidney); *secY* (Ahmed et al., 2009)	Multimammate mice (*Mastomys* spp.)	12	4 (33.3%)

				Norway rats (*Rattus norvegicus*)	11	3 (27.3%)
				Black rats (*Rattus rattus*)	60	8 (13.3%)
				African giant shrew (*Crocidura olivieri*i)	6	1 (16.7%)
				Shrew (*Crocidura* spp.)	1	1 (100%)
(Jobbins et al., 2013)	Botswana 2009-2012	Wild animal surveillance	PCR (Kidney); 23S rDNA gene target (*rrl*) (Woo et al., 1997)	Banded mongoose (*Mungos mungo)*	41	17 (41.4%)
				Selous mongoose (*Paracynictis selousi*)	1	1 (100·0%)
(Lagadec et al., 2012)	Madagascar, Comoros NA	Wild animal surveillance	qPCR (pooled kidney, spleen & lung); 16S rRNA (*rrs*)(Smythe et al., 2002)	Bats; various species[&]	129	27 (20.9%)
Dietrich et al.(Dietrich et al., 2014)	Madagascar 2010-2012	Wild animal surveillance	qPCR (pooled kidney, spleen & lung); 16S rRNA (*rrs*)(Smythe et al., 2002)	Lesser tufted-tailed rat (*Eliurus minor*)	112	32 (28·6%)
				Cowan's shrew tenrec (*Microgale cowani*)	72	2 (2·8%)
				Dobson's shrew tenrec (*Microgale dobsoni*)	54	3 (5·6%)
				Lesser long-tailed shrew tenrec (*Microgale longicaudata*)	12	1 (8·3%)

				Major's long-tailed tenrec (*Microgale majori*)	10	2 (20·0%)
				Greater long-tailed shrew tenrec (*Microgale principula*)	6	2 (33·3%)
				Lowland streaked tenrec (*Hemicentetes semispinosus*)	4	1 (25·0%)
				Highland streaked tenrec (*Hemicentetes nigriceps*)	12	1 (8·3%)
				Bats; *Miniopterus* species [&&]	NA	6 (NA)
(Nimo Paintsil et al., 2013)	Ghana NA	Wild animal surveillance	PCR (kidney); not specificd	*Crocidura sp.*	NA	1 (NA)
(Hatem et al., 2014)	Egypt NA	Wild animal surveillance	Culture (Rats: kidney; cattle: blood, milk and/or urine); EMJH media	Rats (Species not stated)	200	9 (4·5%)
		Domestic animal surveillance		Cattle (*Bos* sp.)	625	7 (1·1%)

Footnotes: $ Numbers adjusted to report results for pathogenic *Leptospira* spp. only; § Prevalence of carriers cannot be calculated due to incomplete reporting)

Species include: *Rattus rattus, Mus musculus, Crocidura* spp., *Mastomys* spp.; individual species counts not given

* Only kidney results reported here. See original reference for full breakdown of positives by sample type; ^ Samples with PCR inhibition are excluded from denominator data

& Bat species include: *Chaerephon pusillus, Miniopterus gleni, M.griffithsi, M. griveaudi, M.mahafaliensis, Mormopterus francoismoutoui, Mormopterus jugularis, Mytotis goudoti, Otomops madagascariensis, Rousettus obliviosus, Triaenops furculus, Triaenops menamena*

&& *Miniopterus* species includes: *M. gleni, M. goudoti, M. griffithsi, M. mahafaliensis, M. majori, M. soroculus,*

Appendix 3: Rodent study participant information sheet and consent forms

Leptospirosis in northern Tanzania: investigating the role of rodents and cattle in a neglected public health problem

<u>Participant Information Sheet</u>

INTRODUCTION

You are being invited to take part in a research study because your household has been selected for inclusion in our study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please read this information sheet and the consent form carefully and take your time making your decision. As the study representative discusses this study with you, please ask him/her to explain any words or information that you do not clearly understand. We encourage you to talk with your family and friends before you decide to take part in this research study. The nature of the study, risks, inconveniences, discomforts, and other important information about the study are listed below.

WHO IS DOING THIS RESEARCH?

This research will be conducted by experts in human and animal health from Tanzania and from the UK. Prof. John A. Crump and Dr. Moshi K. Ntabaye from the Kilimanjaro Christian Medical Centre in Tanzania and Prof. Sarah Cleaveland and Dr. Kathryn Allan from the University of Glasgow in UK will conduct the study, with others working on their behalf. The sponsors of this study, the Wellcome Trust, UK will pay for this research.

WHO HAS REVIEWED THE STUDY?

The KCMC ethics committee, National Institute of Medical Research and Tanzanian Wildlife Research Institute have reviewed this study in Tanzania. The study has also been reviewed by the University of Glasgow in the UK.

WHY IS THIS STUDY BEING DONE?

The purpose of this study is to find out if the rodents in this area are carrying infections such as leptospirosis that may cause fever and illness in people. Illness with fever is very common in people seeking health care in this area. Leptospirosis has been identified in patients with fever at Kilimanjaro Christian Medical Centre (KCMC) and Mawenzi regional hospital in Moshi. It is important to learn more about the possible sources of this infection so that we can treat and prevent human disease more effectively.

WHY HAVE I BEEN CHOSEN?

This village has been selected at random from all the villages in the catchment area of KCMC. Your household is one of 20 households in this village that have also been randomly selected for study participation. Overall, around 600 households will be enrolled in this study over a 2-year period.

DO I HAVE TO TAKE PART?

It is up to you to decide whether or not to take part. If you do decide to take part, you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

HOW LONG WILL I BE IN THIS STUDY?

Your participation will be required for one to two weeks. You can choose to stop participating at any time without penalty.

WHAT IS INVOLVED IN THE STUDY?

If you agree to participate in this study, you will first be asked to sign this consent form. A study worker will ask then ask you some basic questions about you and the people that live in your household. We will also ask questions about the structure of your house and its

surroundings, the types of crops that you grown and the kind of animals that you come into contact with.

Study personnel will then place traps for rodents in and around your household. Traps will be checked daily and any trapped rodents will be removed and humanely killed before being tested for infections such as leptospirosis.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART IN THIS STUDY?

There are minimal physical risks associated with this study. For your safety, the traps placed within your household must not handled or moved by anyone other than project personnel. Members of the household are asked not to move or handle the traps in any way, or to handle any rodents that they see in or around these traps within their households. Study personnel will require access to your household twice daily for one week to place and check rodent traps.

ARE THERE BENEFITS TO TAKING PART IN THE STUDY?

Participating households will experience immediate benefit through the removal of a small number of pest rodents from households. Study personnel can also help to advice on methods of rodent control if this is not already in place. On a wider scale, the results of this study will help to improve medical care and laboratory diagnostics of infection in Tanzania. In the future, we hope that information gathered in this study will help to prevent human and animal disease in this area.

WHAT WILL HAPPEN TO THE RESULTS OF THE RESEARCH STUDY?

After rodent samples are taken, a number of tests will be run to help us understand more about leptospirosis and other diseases carried by rodents in this area. Similar tests are also being run on samples collected from cows in local abattoirs. Animal samples will firstly be tested at the Kilimanjaro Clinical Research Institute in Moshi. After this, additional tests may be run at one of collaborating institutions including Glasgow University, the Royal Tropical Institute (KIT) in Holland and Sokoine University of Agriculture in Morogoro, Tanzania. Samples will be stored for a minimum of 10 years and may be used by other

relevant research projects. Your name will not be on any of the samples taken from rodents so no one outside of the study team will be able to identify you from the samples. The results of these tests will then be analyzed alongside the data that we collect in the questionnaire survey to help us understand more these important infections.

The results of this study will be communicated to Tanzanian healthcare workers, doctors, veterinarians and government officials to help improve human and animal health in this area. In addition, the results of this study may be published in national or international scientific journals or other publications. However, no personal details will be released outside the study collaboration and you and your household will not be identified in any report or publication.

WILL MY INFORMATION BE KEPT CONFIDENTIAL?

All information that is collected about you and your household during the course of the research will be kept strictly confidential. Except when required by law, you will not be identified in the study records disclosed outside of KCMC or University of Glasgow. If study results are made available to other researchers, you will be assigned a unique code number so that you cannot be recognized from these results. .

HOW DO I WITHDRAW FROM THIS STUDY?

You may choose not to be in the study or, if you agree to be in the study, you may withdraw from the study at any time. If you withdraw from the study, no new data about you will be collected for study purposes other than data needed to keep track of your withdrawal. Your decision not to participate or to withdraw from the study will not involve any penalty or loss of benefits to which you are entitled. If you do decide to withdraw, we ask that you contact Dr Kathryn Allan at KCMC (write to: Dr. Kathryn Allan c/o Francis Karia, KCMC/Duke Collaboration, CCFCC Building, KCMC, PO Box 3010, Moshi) in writing and let her know that you are withdrawing from the study. At that time we will ask your permission to continue using all information about you that has already been collected as part of the study prior to your withdrawal.

WHAT ARE THE COSTS AND COMPENSATION?

There will be no additional costs to you as a result of being in this study and no compensation will be provided for your participation.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or if you have complaints, concerns or suggestions about the research, contact [the project representative – to be named and phone number given once known]. For questions about your rights as a research participant, or to discuss problems, concerns or suggestions related to the research, or to obtain information or offer input about the research, contact the KCMC Ethics Committee at +255-27-275-3909.

THANK YOU!

On behalf of all the project investigators, we would like to thank you for agreeing to take part in this study.

University of Glasgow | College of Medical, Veterinary & Life Sciences

Project Code Number: 57398/1

Subject Identification Number for this trial:

CONSENT FORM

Title of Project: Leptospirosis in northern Tanzania: the role of rodents and cattle in a neglected public health problem

Name of Researcher(s): Dr Moshi Ntabaye and Professor John Crump from the Kilimanjaro Christian Medical Centre, Tanzania**;** Professor Sarah Cleaveland and Dr Kathryn Allan from the University of Glasgow, UK.

STATEMENT OF CONSENT

"The purpose of this study, and the study procedures, risks and benefits have been explained to me. I have been allowed to ask questions, and my questions have been answered to my satisfaction. I have been told that I may contact the KCMC Ethics Committee at +255-27-275-3909 if I have questions about my rights as a research subject, to discuss problems, concerns, or suggestions related to the research, or to obtain information or offer input about the research. I confirm that I have read the information sheet dated 25th February 2013 (version 1.2). I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason and without my legal rights being affected. I agree to take part in this study."

______________________________	____________	______________________
Name of subject	Date	Signature
______________________________	____________	______________________
Name of Person taking consent	Date	Signature

Witness (if applicable)	Date	Signature

(1 copy for subject; 1 copy for researcher)

Appendix 4: Rodent study household questionnaire

RODENT PROJECT – HOUSEHOLD QUESTIONNAIRE

SECTION 1 - Interview details

Household ID: LCR-[_][_][_]

Interviewer ID (*initials*): [_][_][_]

1.3 Date of interview (dd/mm/yyyy) : [_][_]/[_][_]/[_][_][_][_]

1.4 Interview language: ☐ Kiswahili ☐ English ☐ Maa

Review details

1.5 Reviewer ID: [_][_][_]

1.6 Date of review (dd/mm/yyyy) : [_][_]/[_][_]/[_][_][_][_]

SECTION 2 – Respondent details

2.1 Sex: ☐ Male ☐ Female

2.2 Age in years [_][_][_]

2.3 What is your tribe?
Kabila lako?

☐ Chagga (*Mchagga*)
☐ Pare (*Mpare*)
☐ Maasai (*Mmaasai*)
☐ Sambaa (*Msambaa*)
☐ Other *Nyinginezo* __________________

2.4 How long have you lived in this village?
Ni muda gani umeishi katika kijiji hiki?

Units: ☐ years (*miaka*) ☐ months (*miesi*) ☐ days (*siku*)

Number: [_][_][_]

Household location

3.1 District : ☐ Moshi Urban

☐ Moshi Rural

3.2 Village : [_][_][_][_][_][_][_][_][_][_][_][_][_][_][_][_][_]

3.3 Subvillage : [_][_][_][_][_][_][_][_][_][_][_][_][_][_][_][_][_]

3.4 GPS location N/S Coord [_][_] . [_][_][_][_][_]

3.5 GPS location E/W Coord [_][_][_] . [_][_][_][_][_]

3.6 GPS location Altitude (m) [_][_][_][_]

3.7 Waypoint ID [_][_][_]

3.8 How long does it take to walk to the nearest stream/river?

Inachukua muda gani kutembea mpaka kwenye mto/mfereji wa karibu zaidi?

Time __________________________ Hours *Saa* / Minutes *Dakika*

3.9 How long does it take to walk to the nearest tarmac road?

Inachukua muda gani kutembea mpaka barabara ya lami iliyo karibu zaidi?

Time __________________________ Hours *Saa* / Minutes *Dakika*

Compound and household description

Please answer the following questions about the compound that you (the respondent) are part of.

4.1 How many adults (≥ 18 years) live in this household? [_][_][_]

Watu wangapi (≥ 18 miaka) wanaishi kaya hii?

4.2 How many children (< 18 years) live in this household? [_][_][_]

Watoto wangapi (< 18 miaka) wanaishi kaya hii?

4.3 How many sleeping rooms are used by the members of your house? [_][_]

Kuna vyumba vingapi katika nyumba yako?

4.4 Do you have any electricity at your house? ☐ Yes ☐ No

Una umeme wowote katika nyumba yako?

4.5 If yes, what kind? *Kama ndiyo, aina gani?*

☐ Grid *(Gridi)* ☐ Solar (*Solar*) ☐ Generator (*Jenerata*)

☐ Other *Nyinginezo* ____

4.6 What is the primary energy source used for cooking at this household? *(choose one only)*

Aina gani kuu (ya msingi) ya nishati inatumika kwa kupikia katika kaya hii? (chagua moja tu)

☐ Electricity *(Umeme)*

☐ Gas *(Gesi)*

☐ Kerosene *(Mafuta taa)*

☐ Cow dung (*Kinyesi cha ng'ombe*)

☐ Firewood (*Kuni)*

☐ Charcoal *(Mkaa)*

☐ Other *Nyinginezo* _______________________________________

4.7 What is the source of drinking water for members of this household?

Nini chanzo kikuu cha majo ya kunywa katika kaya hii?

Please indicate the primary water source used in the dry season and wet seasons (choose only one primary source in each season) and also indicate any other sources that are normally used during the dry and wet seasons.

Water source *Chanzo cha maji*	1° Dry season *Kiangazi*	1° Wet season *Mvua*	2° any season *Wakati wowote*
Piped water into the home *Yanayosukumwa kwa bomba mpaka ndani nyumbani*	1° ☐	1° ☐	☐
Public/communal well or standpipe *Kisima au pampu ya jumuia*	1° ☐	1° ☐	☐
River or stream (moving water) directly *Moja kwa moja kutoka mto au mfereji (majo yanatotembea)*	1° ☐	1° ☐	☐
Lake, pond, dam (standing water) directly *Moja kwa moja kutoka Ziwa, dimbwi, bwawa (maji yaliyosimama)*	1° ☐	1° ☐	☐
Private well or pump *Kisima au pampu ya binafsi*	1°☐	1° ☐	☐
From a spring *Kutoka katika chemchem*	1°☐	1° ☐	☐
Rainwater *Maji ya mvua*	1°☐	1° ☐	☐
Tanker truck *Tanki la gari*	1°☐	1° ☐	☐
Cart of wheelbarrow with small tank or drum *Mkokoteni na tanki dogo au madumu/pipa*	1°☐	1° ☐	☐
Bottled water *Maji ya chupa*	1°☐	1° ☐	☐
Other *Nyinginezo*____________________	1°☐	1° ☐	☐

4.8 Is the drinking water at this household treated (by filtering, boiling, chlorination, straining etc)?

Je maji ya kunywa katika kaya hii yanafanywa salama (kwa kuchuja, kuchemshwa, kuweka dawa ya shabu/klorine, kuchuja kwa nguo n.k.?)

☐ Always ☐ Often ☐ Infrequently ☐ Never

4.9 If Yes: how is it treated? (choose all that apply)

Kama ndiyo: unayafanya salama/takasa namna gani? (Chagua vote yanayohusika)

☐ Boiling (*Kuchemsha*)

☐ Strain it through a cloth (*Kuchujwa kwa nguo*)

☐ Adding disinfectant, such as chlorine or bleach (*Kuweka dawa kama shabu/klorine*)

☐ Sedimentation and decant (*Kuacha kwa muda yatwae/uchafu uende chini*)

☐ Filtering (*Kuchuiwa*)

☐ Solar disinfection (*Kufanya salama kwa jua*)

☐ Other *Nyinginezo*____

4.10 What is the primary source of bathing water in this household? *Nini chanzo kikuu cha maji ya kuoga katika kaya hii?*

Please indicate the primary water source used in the dry season and wet seasons (choose only one primary source in each case) and also indicate any other sources that are normally used during the dry and wet seasons.

Water source *Chanzo cha maji*	1° Dry season *Kiangazi*	1° Wet season *Mvua*	2° any season *Wakati wowote*
Piped water into the home *Yanayosukumwa kwa bomba mpaka ndani nyumbani*	1° ☐	1° ☐	☐
Public/communal well or standpipe *Kisima au pampu ya jumuia*	1° ☐	1° ☐	☐
River or stream (moving water) directly *Moja kwa moja kutoka mto au mfereji (majo yanatotembea)*	1° ☐	1° ☐	☐
Lake, pond, dam (standing water) directly *Moja kwa moja kutoka Ziwa, dimbwi, bwawa (maji yaliyosimama)*	1° ☐	1° ☐	☐
Private well or pump *Kisima au pampu ya binafsi*	1°☐	1° ☐	☐
From a spring *Kutoka katika chemchem*	1°☐	1° ☐	☐
Rainwater *Maji ya mvua*	1°☐	1° ☐	☐
Tanker truck *Tanki la gari*	1°☐	1° ☐	☐
Cart or wheelbarrow with small tank or drum *Mkokoteni na tanki dogo au madumu/pipa*	1°☐	1° ☐	☐
Bottled water *Maji ya chupa*	1°☐	1° ☐	☐
Other *Nyinginezo*____________________	1°☐	1° ☐	☐

4.11 What is the primary source of cleaning water (e.g. clothes/dishes) in this household?
Nini chanzo kikuu cha maji kufulia na kuoshea katika kaya hii?

Please indicate the primary water source used in the dry season and wet seasons (choose only one primary source in each case) and also indicate any other sources that are normally used during the dry and wet seasons.

Water source *Chanzo cha maji*	1° Dry season *Kiangazi*	1° Wet season *Mvua*	2° any season *Wakati wowote*
Piped water into the home *Yanayosukumwa kwa bomba mpaka ndani nyumbani*	1° ☐	1° ☐	☐
Public/communal well or standpipe *Kisima au pampu ya jumuia*	1° ☐	1° ☐	☐
River or stream (moving water) directly *Moja kwa moja kutoka mto au mfereji (majo yanatotembea)*	1° ☐	1° ☐	☐
Lake, pond, dam (standing water) directly *Moja kwa moja kutoka Ziwa, dimbwi, bwawa (maji yaliyosimama)*	1° ☐	1° ☐	☐
Private well or pump *Kisima au pampu ya binafsi*	1°☐	1° ☐	☐
From a spring *Kutoka katika chemchem*	1°☐	1° ☐	☐
Rainwater *Maji ya mvua*	1°☐	1° ☐	☐
Tanker truck *Tanki la gari*	1°☐	1° ☐	☐
Cart of wheelbarrow with small tank or drum *Mkokoteni na tanki dogo au madumu/pipa*	1°☐	1° ☐	☐
Bottled water *Maji ya chupa*	1°☐	1° ☐	☐
Other *Nyinginezo*___________________	1°☐	1° ☐	☐

4.12 What is the primary type of toilet system used by members of this household? (*choose only one*). *Aina gani ya mfumo wa choo ambao unatumika na wakazi wa kaya yako? (chagua moja tu)*

☐ Flush or pour toilet with septic tank, including squat toilet

Choo kwa kuvuta au cha kumwaga maji cha kuchuchumaa na mfumo wa shimo la maji taka

☐ Flush or pour toilet connected to sewer pipe, including squat toilet

Choo cha maji kilichounganishwa na bomba la maji taka, pamoja na choo cha kuchuchumaa

☐ Pit latrine with covering slab

Choo cha shimo kilichosakafiwa

☐ Pit latrine without covering slab

Choo cha shimo bila kusakafiwa

☐ Ventilated improved pit latrine (VIP)

Choo cha shimo bora chenya bomba la kutoa hewa chafu (VIP)

☐ Bucket or plastic bags

Ndoo au mifuko ya plastiki/Rambo

☐ No facilities or field or bush

Hakuna choo, kwenda porini

4.13 In the past 1 year (12 months), has there been standing water/flooding within this compound?

Katika mwaka uliopita, kumekuwa na maji yaliyosimama/mafuriko katika eneo la kaya hii?

☐ Yes ☐ No

4.14 What is the roof of your house made of? *(choose only one option)*

Nyumba yako imeezekwa na paa la aina gani? (Chagua jibu moja tu)

☐ Metal *Bati*

☐ Thatch *Nyasi*

☐ Wood *Mbao*

☐ Tiles *Vigae*

☐ Cement *Saruji*

☐ Other *Nyinginezo* ________________________________

4.15 What is the floor of your house made of? *(choose one option only)*

Sakafu ya nyuma yako imetengenezwa na nini? (Chagua jibu moja tu)

☐ Dirt or mud *Vumbi/tope*

☐ Dung *Kinyesi cha mifugo*

☐ Brick *Matofali*

☐ Cement *Saruji*

☐ Tile or linoleum *Vigae/sakafu ya mpira*

☐ Wood or planks *Mbao*

☐ Other *Nyinginezo* __________________________

4.16 What are the walls of your house made of? *(indicate all that apply)*

Ukuta wa nyumba yako umetengenezwa na nini? (chagua yote yanayohusika)

☐	Mud or manure	*Tope au kinyesi cha mifugo*
☐	Burnt brick	*Tofali zilizochomwa*
☐	Mud bricks	*Tofali za matope*
☐	Cement	*Saruji*
☐	Wood or planks	*Mbao*
☐	Stone	*Mawe*
☐	Thatch	*Nyasi*
☐	Other	*Nyinginezo* ___________________________

4.17 Do you have any of the following items in your household? *(choose all that apply)*

Kuna chochote kati ya hivi katika nyumba yako? (chagua vote yanayohusika)

If yes for any items, please enter the number of working items that are owned.

Please enter 00 in the Number of units field for items that are not owned at this household.

Asset *Chombo/kifaa*	Number of working units *Namba*
Ox plough *Jembe la n'gombe/Plau*	[_][_]
Ox cart *Mkokoteni wa n'gombe*	[_][_]
Bicycle *Baisikeli*	[_][_]
Motorbike *Piki piki*	[_][_]
Car *Gari*	[_][_]
Tractor *Trekta*	[_][_]
Mobile phone *Simu ya mkononi*	[_][_]
Radio *Redio*	[_][_]
Television *Luninga*	[_][_]
Sofa *Makochi*	[_][_]
Bed net *Chandarau*	[_][_]
Refrigerator *Jokofu (friji)*	[_][_]

Land and crops

5.1 What crops are grown around this compound (within 10 metres)? Choose all that apply.

Karibu kaya yako (katika metre 10), mimea gani inalima? Chagua yote yanayohusika

☐ Rice Mpunga

☐ Millet Mtama

☐ Sorghum Mtama

☐ Maize Mahindi

☐ Sesame Ufuta

☐ Cassava Muhugo

☐ Sweet potato Viazi vitamu

☐ Beans Maharage

☐ Cabbage Kabeji

☐ Lettuce Saladi

☐ Tomato Nyanya

☐ Banana Ndizi

☐ Cotton Pamba

☐ Coffee Kahawa

☐ Potato Viazi

☐ Avocado Parachichi

☐ Spinach Mchicha

☐ Sugarcane Miwa

☐ Other Nyinginezo (taja) ________________________________

____

Household livestock ownership and herd management

6.1 Are any animals of the following species kept within this compound?

Je kuna yeyote wa wanyama wafuatao wanatunzwa/fugwa katika eneo hili?

If Yes (for a given species), please record the total number of adult and juvenile animals that are currently kept at this compound (irrespective of who owns these animals).

For mammals: (Adult = > 1yr, Juvenile = 0-1 yr)

For birds: (Adult = adult plumage, Juvenile = non-adult plumage)

Species	Present?	Number of adults	Number of juveniles
Cattle *Ng'ombe*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Sheep *Kondoo*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Goats *Mbuzi*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Pigs *Nguruwe*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Horses *Farasi*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Donkeys *Punda*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Camels *Ngamia*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Cats *Paka*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Dogs *Mbwa*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Chickens *Kuku*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Ducks *Bata*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Rabbits *Sungura*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Pigeons *Ngiwa*	☐ Y ☐ N	[_][_][_]	[_][_][_]
Other *Nyinginezo* ________________________	☐ Y ☐ N	[_][_][_]	[_][_][_]

6.2 How do you graze your livestock during the day in the dry and wet seasons? (*choose one option for each species and season)*

Unachunga/lisha namna gani mifugo yako mchana wakati wa kiangazi na wakati wa mvua? (chagua jibu moja kwa kila aina ya msimu)

	Cattle *Ng'ombe*		Sheep *Kondoo*		Goats *Mbuzi*		Pigs *Nguruwe*	
	Dry *Kiangazi*	Wet *Mvua*	Dry *Kiangazi*	Wet *Mvua*	Dry *Kiangazi*	Wet *Mvua*	Dry *Kiangazi*	Wet *Mvua*
Free-ranging (no herdsman) *(Wanajichunga wenyewe hakuna mchunji)*	☐	☐	☐	☐	☐	☐	☐	☐
Herded (*Wanachungwa*)	☐	☐	☐	☐	☐	☐	☐	☐
Tethered (*Wanaofungwa*)	☐	☐	☐	☐	☐	☐	☐	☐
Not grazed *Wanakatiwa majani*	☐	☐	☐	☐	☐	☐	☐	☐
Other *Nyinginezo* _______________	☐	☐	☐	☐	☐	☐	☐	☐

6.3 Where are your adult livestock normally kept during the night in the dry and wet seasons? (*choose one option for each species and season)*

Wanyama wako wakubwa kwa kawaida wanawekwa wapi wakati wa usiku wakati wa msimu wa mvua na ukame? (chagua jibu moja tu kwa kila aina na msimu)

	Cattle *Ng'ombe*		Sheep *Kondoo*		Goats *Mbuzi*		Pigs *Nguruwe*	
	Dry *Kiangazi*	Wet *Mvua*	Dry *Kiangazi*	Wet *Mvua*	Dry *Kiangazi*	Wet *Mvua*	Dry *Kiangazi*	Wet *Mvua*
Not confined *Hawako sehemu moja*	☐	☐	☐	☐	☐	☐	☐	☐
Confined at the compound (<10 m from house) *Wanawekwa kwenya eneo kaya (< 10 m kwa nyumba)*	☐	☐	☐	☐	☐	☐	☐	☐
Confined at the compound (> 10m from house) *Wanawekwa kwenya eneo kaya (> 10 m kwa nyumba)*	☐	☐	☐	☐	☐	☐	☐	☐
Confined elsewhere (oustide the compound) *Wanawekwa sehemu nyingine*	☐	☐	☐	☐	☐	☐	☐	☐
Other *Nyinginezo* ____________________	☐	☐	☐	☐	☐	☐	☐	☐

Rodents around the compound

7.1 Have you seen rodents in your house in the past month? Please indicate the frequency of sightings

Je umewahi kuona panya katika nyumba yako katika mwezi uliopita? Tafadhali onyesha umewaona mara ngapi.

☐ Every day	*Kila siku*
☐ More than once a week	*Zaidi ya mara moja katika wiki*
☐ Less than once a week	*Pungufu ya mara moja kwa wiki*
☐ Never	*Haijatokea*

7.2 Have you seen evidence of rodents (e.g. faeces, urine, noises, rodent tracks, rodent damage) in your house in the past month? (Please indicate the frequency of sightings).

Je umewahi kuona panya au ushahidi wa panya (kama choo, mkojo, sauti, nija yake) katika nyumba yako katika mwezi uliopita? (Tafadhali onyesha umewaona mara ngapi).

☐ Every day	*Kila siku*
☐ More than once a week	*Zaidi ya mara moja katika wiki*
☐ Less than once a week	*Pungufu ya mara moja kwa wiki*
☐ Never	*Haijatokea*

7.3 Have you seen evidence of rodents (e.g. faeces, urine, noises, rodent tracks, rodent damage) in your kitchen or stored food in the past month? (Please indicate the frequency of sightings)

Je umewahi kuona panya au ushahidi wa panya (kama choo, mkojo, sauti, nija yake, vitu vilivyoharibiwa na panya) katika jiko lako au chakula kilichohifadhiwa katika mwezi uliopita? (Tafadhali onyesha umewaona mara ngapi)

☐ Every day | *Kila siku*
☐ More than once a week | *Zaidi ya mara moja katika wiki*
☐ Less than once a week | *Pungufu ya mara moja kwa wiki*
☐ Never | *Haijatokea*

7.4 Have you seen evidence of rodents (e.g. faeces, urine, noises, rodent tracks, rodent damage) in your compound in the past month? (Please indicate the frequency of sightings)

Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, njia yake, vitu vilivyoharibiwa na panya?) katika eneo lako katika mwezi uliopita? (Tafadhali onyesha umewaona mara ngapi)

☐ Every day | *Kila siku*
☐ More than once a week | *Zaidi ya mara moja katika wiki*
☐ Less than once a week | *Pungufu ya mara moja kwa wiki*
☐ Never | *Haijatokea*

7.5 Have you seen evidence of rodents (e.g. faeces, urine, noises, rodent tracks, rodent damage) in the fields around your compound in the past month? *(Please indicate the frequency of sightings)*

Je umewahi kuona ushahidi wa panya (kama choo, mkojo, sauti, nija yake vitu vilivyoharibiwa na panya?) katika shamba lako katika mwezi uliopita? (Tafadhali onyesha umewaona mara ngapi)

☐ Every day | *Kila siku*
☐ More than once a week | *Zaidi ya mara moja katika wiki*
☐ Less than once a week | *Pungufu ya mara moja kwa wiki*
☐ Never | *Haijatokea*
☐ Don’t have fields | *Hakuna shamba*

7.6 Do any members of this household do anything to control rodents?

Je, yeyote wa wakazi wa kaya hii anafanya chochote kuzuia hawa panya?

☐ Yes *Ndiyo* ☐ No *Hapana*

7.7 *If yes*, what type of rodent control do you use? *(choose all that apply)*

Kama ndiyo, njia gani huwa unatumia? (onyesha zote ziinazohusika)

☐ Mechanical (e.g. traps) *Kuwatega, kuwapiga (mfano: mitego)*

☐ Chemical (e.g. poisons) *Kutumia dawa/kemikali (mfano: sumu)*

☐ Biological (e.g. keeping predators) *Kutumia njia za kibiologia (mfano: kufuga paka)*

☐ Other *Nyinginezo*____

7.8 If yes for Chemical control; What is the name or brand of the poison that you use? *Kama ndiyo kwa kutumia sumu/madawa/kemikali kuwamaliza, nini jina au aina ya sumu/madawa/kemikali*?

____

7.9 How do you dispose of the carcasses of the rodents that you kill? *(choose all that apply) Unapata/unafanya nini mizoga ya panya ambao unawaua (onyesha yote yanayohusika)*

☐ Leave them where they die *Nawaacha walipokufa*

☐ Throw them into the bush *Kuwatupa porini*

☐ Burn *Choma moto*

☐ Bury *Kuwafukia*

☐ Feed to other animals *Kulishia wanyama wengine*

☐ Consume *Kuwala*

☐ Other *Nyinginezo*___________________________________

7.10 How many rodents do you see in the different seasons of the year?

Unawaona panya wangapi katika misimu tofauti ya mwaka?

Please tick one box (Many, Few, None or Don't know) for each season to indicate the numbers of rodents that you see in each season.

	Long rain *Mvua za muda mrefu*	Short rain *Mvua za muda mfupi*	Dry *Kiangazi*
Many *Wengi*	☐	☐	☐
Few *Wachache*	☐	☐	☐
None *Hakuna*	☐	☐	☐
Don't know *Hajui*	☐	☐	☐

END OF QUESTIONNAIRE

9 Glossary

Term	Definition
Analytical sensitivity	The dilution of a known quantity of pathogen DNA below which the assay fails to detected 95% of replicates.
Analytical specificity	The ability of an assay to detect a particular organism (rather than other organisms) in a sample.
Commensal rodent species	Rodents including *Rattus norvegicus, Rattus rattus* and *Mus musculus* that live in close proximity and dependence on people.
Compound	A discrete area often but not always marked by a boundary hedge that includes the buildings and open space used by one (or more) family who share cooking facilities.
Diagnostic sensitivity	A measure of the proportion of true positives within a population that are correctly identified by a diagnostic test.
Diagnostic specificity	A measure of the proportion of true negatives within a population that are correctly identified by a diagnostic test.
Household	A permanent structure where one or more inhabitants who share the same cooking facilities sleep over night.
Incidental host	A mammalian host that becomes incidentally infected with a *Leptospira* serovar that is not normally maintained by that particular species or population (Ko et al., 2009, Levett, 2001)
Maintenance host	A host that is able to maintain infection without re-introduction from other animal or environment sources of infection (Viana et al., 2014)
Predominant reactive serogroup	The serogroup with the single highest titre demonstrated by the microscopic agglutination test run on human or animal serum samples.
Reservoir of infection	One or more epidemiologically connected populations or environments in which a pathogen can be permanently maintained and from which infection is transmitted to the target population (Haydon et al., 2002)
Serogroup	Broadest classification of *Leptospira* bacteria, grouped on the basis of antigenic characteristics. Serogroups frequently encompass *Leptospira* serovars belonging to different species with similar

	serological reaction profiles (Levett, 2015).
Serovar	High-resolution taxonomic classification of *Leptospira* that now encompasses both genetic and serological characteristics of the bacterial sub-type (Levett, 2015).

10 List of references

A-ELGAYOUM, S. M. E., EL-FEKI, A. E.-K. A., MAHGOUB, B. A., EL-RAYAH, E.-A. & GIHA, H. A. 2009. Malaria overdiagnosis and burden of malaria misdiagnosis in the suburbs of central Sudan: special emphasis on artemisinin-based combination therapy era. *Diagnostic Microbiology and Infectious Disease,* 64**,** 20-26.

ABELA-RIDDER, B., SIKKEMA, R. & HARTSKEERL, R. A. 2010. Estimating the burden of human leptospirosis. *International Journal of Antimicrobial Agents,* 36**,** S5–S7.

ACESTOR, N., COOKSEY, R., NEWTON, P. N., MENARD, D., GUERIN, P. J., NAKAGAWA, J., CHRISTOPHEL, E., GONZALEZ, I. J. & BELL, D. 2012. Mapping the aetiology of non-malarial febrile illness in Southeast Asia through a systematic review--terra incognita impairing treatment policies. *PLoS One,* 7**,** e44269.

ADLER, B. 2015. History of Leptospirosis and *Leptospira*. *In:* ADLER, B. (ed.) *Leptospira and Leptospirosis.* Berlin: Springer

ADLER, B. & DE LA PENA MOCTEZUMA, A. 2010. *Leptospira* and leptospirosis. *Veterinary Microbiology,* 140**,** 287–96.

ADLER, B. & FAINE, S. 1977. Host immunological mechanisms in the resistance of mice to leptospiral infections. *Infection and immunity,* 17**,** 67-72.

ADLER, B., LO, M., SEEMANN, T. & MURRAY, G. L. 2011. Pathogenesis of leptospirosis: the influence of genomics. *Veterinary Microbiology,* 153**,** 73-81.

AGAMPODI, S. B., MATTHIAS, M. A., MORENO, A. C. & VINETZ, J. M. 2012. Utility of Quantitative Polymerase Chain Reaction in Leptospirosis Diagnosis: Association of Level of Leptospiremia and Clinical Manifestations in Sri Lanka. *Clinical Infectious Diseases,* 54**,** 1249-1255.

AGAMPODI, S. B., MORENO, A. C., VINETZ, J. M. & MATTHIAS, M. A. 2013. Utility and limitations of direct multi-locus sequence typing on qPCR-positive blood to determine infecting *Leptospira* strain. *The American Journal of Tropical Medicine and Hygiene,* 88**,** 184.

AGAMPODI, S. B., PEACOCK, S. J., THEVANESAM, V., NUGEGODA, D. B., SMYTHE, L., THAIPADUNGPANIT, J., CRAIG, S. B., BURNS, M. A., DOHNT, M., BOONSILP, S., SENARATNE, T., KUMARA, A., PALIHAWADANA, P., PERERA, S. & VINETZ, J. M. 2011. Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the global burden of disease. *The American Journal of Tropical Medicine and Hygiene,* 85**,** 471-8.

AHMED, A., ENGELBERTS, M. F., BOER, K. R., AHMED, N. & HARTSKEERL, R. A. 2009. Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS One,* 4**,** e7093.

AHMED, A., VAN DER LINDEN, H., ENGELBERT, M. & HARTSKEERL, R. 2012. *Leptospira* Diagnostic SYBR Green real time PCR. Amsterdam: KIT Biomedical Research, Nationaal Referentie Laboratorium voor Leptospirosen (WHO, FAO, OIE).

ALLAN, K. J., BIGGS, H. M., HALLIDAY, J. E. B., KAZWALA, R. R., MARO, V. P., CLEAVELAND, S. & CRUMP, J. A. 2015a. 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**,** e0003899.

ALLAN, K. J., HALLIDAY, J. E. & CLEAVELAND, S. 2015b. Renewing the momentum for leptospirosis research in Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 109**,** 605-6.

ALLEYNE, G. A. 1987. Renal disease in Africa and the Caribbean: an overview. *Transplant Proceedings,* 19**,** 9-14.

AMEXO, M., TOLHURST, R., BARNISH, G. & BATES, I. 2004. Malaria misdiagnosis: effects on the poor and vulnerable. *The Lancet,* 364**,** 1896–98.

ANDRE-FONTAINE, G., AVIAT, F. & THORIN, C. 2015. Waterborne Leptospirosis: Survival and preservation of the virulence of pathogenic *Leptospira* spp. in fresh water. *Current Microbiology,* 71**,** 136-42.

APLIN, K. P., SUZUKI, H., CHINEN, A. A., CHESSER, R. T., TEN HAVE, J., DONNELLAN, S. C., AUSTIN, J., FROST, A., GONZALEZ, J. P., HERBRETEAU, V., CATZEFLIS, F., SOUBRIER, J., FANG, Y.-P., ROBINS, J., MATISOO-SMITH, E., BASTOS, A. D. S., MARYANTO, I., SINAGA, M. H., DENYS, C., VAN DEN BUSSCHE, R. A., CONROY, C., ROWE, K. & COOPER, A. 2011. Multiple Geographic Origins of Commensalism and Complex Dispersal History of Black Rats. *PLoS ONE,* 6**,** e26357.

APPLIED BIOSYSTEMS 2014. Real-time PCR Handbook. *In:* SCIENTIFIC, T. F. (ed.).

ARENT, Z., FRIZZELL, C., GILMORE, C., MACKIE, D. & ELLIS, W. A. 2013. Isolation of Leptospires from genital tract of sheep. *The Veterinary Record,* 173**,** 582.

ARI, M. D., GURACHA, A., FADEEL, M. A., NJUGUNA, C., NJENGA, M. K., KALANI, R., ABDI, H., WARFU, O., OMBALLA, V., TETTEH, C., BREIMAN, R. F., PIMENTEL, G. & FEIKIN, D. R. 2011. Challenges of Establishing the Correct Diagnosis of Outbreaks of Acute Febrile Illnesses in Africa: The Case of a Likely *Brucella* Outbreak among Nomadic Pastoralists, Northeast Kenya, March-July 2005. *The American Journal of Tropical Medicine and Hygiene,* 85**,** 909–912.

ASSENGA, J. A., MATEMBA, L. E., MULLER, S. K., MHAMPHI, G. G. & KAZWALA, R. R. 2015. Predominant Leptospiral Serogroups Circulating among Humans, Livestock and Wildlife in Katavi-Rukwa Ecosystem, Tanzania. *PLoS Neglected Tropical Diseases,* 9**,** e0003607.

ATHANAZIO, D. A., SILVA, E. F., SANTOS, C. S., ROCHA, G. M., VANNIER-SANTOS, M. A., MCBRIDE, A. J., KO, A. I. & REIS, M. G. 2008. *Rattus norvegicus* as a model for persistent renal colonization by pathogenic *Leptospira interrogans*. *Acta Tropica,* 105**,** 176-180.

AUBRY, P., BORDAHANDY, R., FERAH, T., MAILLOUX, M. & THOMAS, J. 1975. [An anademy of ictero-hemorrhagic leptospirosis in a military group in Algeria (author's translation)]. *Bulletin de la Societe de Pathologie Exotique et Filiales,* 68**,** 370–6.

AYANEGUI-ALCERRECA, M. A., WILSON, P. R., C.G., M., COLLINS-EMERSON, J., HEUER, C., MIDWINTER, A. & F., C.-A. 2007. Leptospirosis in farmed deer in New Zealand: A review. *New Zealand Veterinary Journal,* 55**,** 102-108.

BAHAMAN, A. R., IBRAHIM, A. L., STALLMAN, N. D. & TINNISWOOD, R. D. 1988. The bacteriological prevalence of leptospiral infection in cattle and buffaloes in West Malaysia. *Epidemiology and Infection,* 100**,** 239-46.

BAJANI, M. D., ASHFORD, D. A., BRAGG, S. L., WOODS, C. W., AYE, T., SPIEGEL, R. A., PLIKAYTIS, B. D., PERKINS, B. A., PHELAN, M., LEVETT, P. N. &

WEYANT, R. S. 2003. Evaluation of Four Commercially Available Rapid Serologic Tests for Diagnosis of Leptospirosis. *Journal of Clinical Microbiology,* 41**,** 803-809.

BAKOSS, P. 1969. *Leptospira tunis*, a new serotype of the Tarassovi group. *Archives de l'Institut Pasteur Tunis,* 46**,** 17–23.

BAKOSS, P. & CHADLI, A. 1965. The pig, reservoir of *Leptospira mitis* in Tunisia. *Archives de l'Institut Pasteur Tunis,* 42**,** 85–91.

BALL, M. G. 1966. Animal hosts of leptospires in Kenya and Uganda. *The American Journal of Tropical Medicine and Hygiene,* 15**,** 523–30.

BARSOUM, I. S., MOCH, R. W., BOTROS, B. A. & KAISER, M. N. 1973. Leptospires isolated from wild mammals in Egypt. *Tropical and Geographical Medicine,* 25**,** 362–64.

BASCO, L. K. 2004. Molecular epidemiology of malaria in Cameroon. XIX. Quality of antimalarial drugs used for self-medication. *The American Journal of Tropical Medicine and Hygiene,* 70**,** 245-250.

BELMAIN, S. Rats and Human Health in Africa: Proceedings of an international workshop on rodent-borne diseases and the RatZooMan research project. RatZooMan Workshop, 2006 Malenlane, Republic of South Africa. University of Greenwich, 46pp.

BENKIRANE, A., NOURY, S., HARTSKEERL, R. A., GORIS, M. G., AHMED, A. & NALLY, J. E. 2014. Preliminary Investigations on the Distribution of Leptospira Serovars in Domestic Animals in North-west Morocco. *Transboundary and Emerging Diseases*.

BENSON, D. A., KARSCH-MIZRACHI, I., CLARK, K., LIPMAN, D. J., OSTELL, J. & SAYERS, E. W. 2011. GenBank. *Nucleic Acids Research.*

BERLIOZ-ARTHAUD, A., KIEDRZYNSKI, T., SINGH, N., YVON, J. F., ROUALEN, G., COUDERT, C. & ULUIVITI, V. 2007. Multicentre survey of incidence and public health impact of leptospirosis in the Western Pacific. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 101**,** 714-21.

BERTHERAT, E., MUELLER, M. J., SHAKO, J. C. & PICARDEAU, M. 2014. Discovery of a leptospirosis cluster amidst a pneumonic plague outbreak in a miners' camp in the Democratic Republic of the Congo. *International Journal of Environmental Research and Public Health,* 11**,** 1824–33.

BHARTI, A. R., NALLY, J. E., RICALDI, J. N., MATTHIAS, M. A., DIAZ, M. M., LOVETT, M. A., LEVETT, P. N., GILMAN, R. H., WILLIG, M. R., GOTUZZO, E. & VINETZ, J. M. 2003. Leptospirosis: a zoonotic disease of global importance. *The Lancet Infectious Diseases,* 3**,** 757–771.

BHATTARAI, A., ALI, A. S., KACHUR, S. P., MARTENSSON, A., ABBAS, A. K., KHATIB, R., AL-MAFAZY, A. W., RAMSAN, M., ROTLLANT, G., GERSTENMAIER, J. F., MOLTENI, F., ABDULLA, S., MONTGOMERY, S. M., KANEKO, A. & BJORKMAN, A. 2007. Impact of artemisinin-based combination therapy and insecticide-treated nets on malaria burden in Zanzibar. *PLoS Medicine,* 4**,** e309.

BIASSONI, R. & RASO, A. 2014. *Quantitative Real-Time PCR*, Springer New York.

BIGGS, H. M., BUI, D. M., GALLOWAY, R. L., STODDARD, R. A., SHADOMY, S. V., MORRISSEY, A. B., BARTLETT, J. A., ONYANGO, J. J., MARO, V. P., KINABO, G. D., SAGANDA, W. & CRUMP, J. A. 2011. Leptospirosis among

hospitalized febrile patients in northern Tanzania. *The American Journal of Tropical Medicine and Hygiene,* 85**,** 275–81.

BIGGS, H. M., HERTZ, J. T., MUNISHI, O. M., GALLOWAY, R. L., MARKS, F., SAGANDA, W., MARO, V. P. & CRUMP, J. A. 2013a. Estimating leptospirosis incidence using hospital-based surveillance and a population-based health care utilization survey in Tanzania. *PLoS Negl Trop Dis,* 7**,** e2589.

BIGGS, H. M., HERTZ, J. T., MUNISHI, O. M., GALLOWAY, R. L., MARKS, F., SAGANDA, W., MARO, V. P. & CRUMP, J. A. 2013b. Estimating Leptospirosis Incidence Using Hospital-Based Surveillance and a Population-Based Health Care Utilization Survey in Tanzania. *PLoS Neglected Tropical Diseases,* 7**,** e2589.

BIGOGO, G., AUDI, A., AURA, B., AOL, G., BREIMAN, R. F. & FEIKIN, D. R. 2010. Health-seeking patterns among participants of population-based morbidity surveillance in rural western Kenya: implications for calculating disease rates. *International Journal of Infectious Diseases,* 14**,** e967-73.

BLACKMORE, D. & HATHAWAY, S. The nidality of zoonoses. Proceedings of the Second International Symposium on Veterinary Epidemiology and Economics, 1979. 207-213.

BLACKSELL, S. D., SHARMA, N. P., PHUMRATANAPRAPIN, W., JENJAROEN, K., PEACOCK, S. J., WHITE, N. J., PUKRITTAYAKAMEE, S. & DAY, N. P. 2007. Serological and blood culture investigations of Nepalese fever patients. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 101**,** 686-90.

BOLIN, C. A. & ALT, D. P. 2001. Use of a monovalent leptospiral vaccine to prevent renal colonization and urinary shedding in cattle exposed to *Leptospira borgpetersenii* serovar Hardjo. *American Journal of Veterinary Research,* 62**,** 995-1000.

BONILLA-SANTIAGO, R. & NALLY, J. E. 2011. Rat model of chronic leptospirosis. *Current Protocols in Microbiology.* 2011/03/15 ed.: Wiley Online.

BONNER, P. C., SCHMIDT, W.-P., BELMAIN, S. R., OSHIN, B., BAGLOLE, D. & BORCHERT, M. 2007. Poor housing quality increases risk of rodent infestation and Lassa fever in refugee camps of Sierra Leone. *The American Journal of Tropical Medicine and Hygiene,* 77**,** 169-175.

BOONSILP, S., THAIPADUNGPANIT, J., AMORNCHAI, P., WUTHIEKANUN, V., BAILEY, M. S., HOLDEN, M. T., ZHANG, C., JIANG, X., KOIZUMI, N., TAYLOR, K., GALLOWAY, R., HOFFMASTER, A. R., CRAIG, S., SMYTHE, L. D., HARTSKEERL, R. A., DAY, N. P., CHANTRATITA, N., FEIL, E. J., AANENSEN, D. M., SPRATT, B. G. & PEACOCK, S. J. 2013. A single multilocus sequence typing (MLST) scheme for seven pathogenic *Leptospira* species. *PLoS Neglected Tropical Diseases,* 7**,** e1954.

BOONSILP, S., THAIPADUNGPANIT, J., AMORNCHAI, P., WUTHIEKANUN, V., CHIERAKUL, W., LIMMATHUROTSAKUL, D., DAY, N. P. & PEACOCK, S. J. 2011. Molecular detection and speciation of pathogenic *Leptospira* spp. in blood from patients with culture-negative leptospirosis. *BMC Infectious Diseases,* 11**,** 338.

BOSTOEN, K. & CHALABI, Z. 2006. Optimization of household survey sampling without sample frames. *International Journal of Epidemiology,* 35**,** 751-755.

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**,** 2154-60.

BOURHY, P., COLLET, L., BRISSE, S. & PICARDEAU, M. 2014. *Leptospira mayottensis* sp. nov., a pathogenic *Leptospira* species isolated from humans. *International Journal of Systematic and Evolutionary Microbiology,* doi:10.1099/ijs.0.066597-0.

BOURHY, P., COLLET, L., CLEMENT, S., HUERRE, M., AVE, P., GIRY, C., PETTINELLI, F. & PICARDEAU, M. 2010. Isolation and characterization of new *Leptospira* genotypes from patients in mayotte (Indian Ocean). *PLoS Neglected Tropical Diseases,* 4**,** e724.

BOURHY, P., COLLET, L., LERNOUT, T., ZININI, F., HARTSKEERL, R. A., LINDEN, H. V. D., THIBERGE, J. M., DIANCOURT, L., BRISSE, S., GIRY, C., PETTINELLI, F. & PICARDEAU, M. 2012. Human *Leptospira* isolates circulating in Mayotte (Indian Ocean) have unique serological and molecular features. *Journal of Clinical Microbiology,* 50**,** 307–311.

BRANGER, C., BLANCHARD, B., FILLONNEAU, C., SUARD, I., AVIAT, F., CHEVALLIER, B. & ANDRÉ-FONTAINE, G. 2005. Polymerase chain reaction assay specific for pathogenic *Leptospira* based on the gene hap1 encoding the hemolysis-associated protein-1. *FEMS Microbiology Letters,* 243**,** 437–45.

BROUAT, C., TOLLENAERE, C., ESTOUP, A., LOISEAU, A., SOMMER, S., SOANANDRASANA, R., RAHALISON, L., RAJERISON, M., PIRY, S. & GOODMAN, S. 2014. Invasion genetics of a human commensal rodent: the black rat Rattus rattus in Madagascar. *Molecular Ecology,* 23**,** 4153-4167.

BROWNLOW, W. J. & DEDEAUX, J. D. 1964. Leptospirosis in animals of upper [southern] Egypt. *The American Journal of Tropical Medicine and Hygiene,* 13**,** 311–18.

BULACH, D. M., ZUERNER, R. L., WILSON, P., SEEMANN, T., MCGRATH, A., CULLEN, P. A., DAVIS, J., JOHNSON, M., KUCZEK, E., ALT, D. P., PETERSON-BURCH, B., COPPEL, R. L., ROOD, J. I., DAVIES, J. K. & ADLER, B. 2006. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 14560-5.

BURDIN, M. L., FROYD, G. & ASHFORD, W. A. 1958. Leptospirosis in Kenya due to *Leptospira grippotyphosa*. *The Veterinary Record,* 70**,** 830-835.

BURKARDT, H. J. 2000. Standardization and quality control of PCR analyses. *Clinical Chemistry and Laboratory Medicine,* 38**,** 87-91.

CAMERON, A. R. & BALDOCK, F. C. 1998. A new probability formula for surveys to substantiate freedom from disease. *Prev Vet Med,* 34**,** 1-17.

CERQUEIRA, G. M. & PICARDEAU, M. 2009. A century of *Leptospira* strain typing. *Infection, Genetics and Evolution,* 9**,** 760–768.

CHADLI, A. & BAKOSS, P. 1965. Enquete sur la leptospirose en Tunisie. *Archives de l'Institut Pasteur de Tunis,* 42**,** 45–58.

CHANDLER, C. I. R., JONES, C., BONIFACE, G., JUMA, K., REYBURN, H. & WHITTY, C. J. M. 2008. Guidelines and mindlines: why do clinical staff over-diagnose malaria in Tanzania? A qualitative study. *Malaria Journal,* 7**,** 53.

CHAPPUIS, F., ALIROL, E., D'ACREMONT, V., BOTTIEAU, E. & YANSOUNI, C. P. 2013. Rapid diagnostic tests for non-malarial febrile illness in the tropics. *Clinical Microbiology and Infection,* 19**,** 422-31.

CHEN, H. 2015. VennDiagram: Generate high-resolution Venn and Euler plots Version 1.6.16 ed.

CHIPWAZA, B., MHAMPHI, G. G., NGATUNGA, S. D., SELEMANI, M., AMURI, M., MUGASA, J. P. & GWAKISA, P. S. 2015. Prevalence of bacterial febrile illnesses in children in Kilosa district, Tanzania. *PLoS Neglected Tropical Diseases,* 9**,** e0003750.

CHIPWAZA, B., MUGASA, J. P., MAYUMANA, I., AMURI, M., MAKUNGU, C. & GWAKISA, P. S. 2014. Community Knowledge and Attitudes and Health Workers' Practices regarding Non-malaria Febrile Illnesses in Eastern Tanzania. *PloS Neglected Tropical Diseases,* 8**,** e2896.

CLEAVELAND, S., BORNER, M. & GISLASON, M. 2014. Ecology and conservation: contributions to One Health. *Revue Scientifique et Techique (International Office of Epizootics),* 33**,** 615–627.

CLEAVELAND, S., SHAW, D., MFINANGA, S., SHIRIMA, G. M., KAZWALA, R., EBLATE, E. & SHARP, M. 2007. *Mycobacterium bovis* in rural Tanzania: Risk factors for infection in human and cattle populations. *Tuberculosis,* 87.

COLLARES-PEREIRA, M., GOMES, A. C., PRASSAD, M., VAZ, R. G., FERRINHO, P., STANEK, G. & ROSARIO, V. E. 1997. Preliminary survey of Leptospirosis and Lyme disease amongst febrile patients attending community hospital ambulatory care in Maputo, Mozambique. *Central African Journal of Medicine,* 43**,** 234–8.

CORTIZO, P., LOUREIRO, A. P., MARTINS, G., DO RODRIGUES, P. R., FARIA, B. P., LILENBAUM, W. & DEMINICIS, B. B. 2015. Risk factors to incidental leptospirosis and its role on the reproduction of ewes and goats of Espirito Santo state, Brazil. *Tropical Animal Health and Production,* 47**,** 231-5.

COSSON, J.-F., PICARDEAU, M., MIELCAREK, M., TATARD, C., CHAVAL, Y., SUPUTTAMONGKOL, Y., BUCHY, P., JITTAPALAPONG, S., HERBRETEAU, V. & MORAND, S. 2014. Epidemiology of *Leptospira* Transmitted by Rodents in Southeast Asia. *PLoS Neglected Tropical Diseases,* 8**,** e2902.

COSTA, F., HAGAN, J. E., CALCAGNO, J., KANE, M., TORGERSON, P., MARTINEZ-SILVEIRA, M. S., STEIN, C., ABELA-RIDDER, B. & KO, A. I. 2015a. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. *PLoS Neglected Tropical Diseases,* 9**,** e0003898.

COSTA, F., PORTER, F. H., RODRIGUES, G., FARIAS, H., DE FARIA, M. T., WUNDER, E. A., OSIKOWICZ, L. M., KOSOY, M. Y., REIS, M. G., KO, A. I. & CHILDS, J. E. 2014. Infections by *Leptospira interrogans*, Seoul virus, and *Bartonella* spp. among Norway rats (*Rattus norvegicus*) from the urban slum environment in Brazil. *Vector Borne and Zoonotic Diseases,* 14**,** 33-40.

COSTA, F., WUNDER, E. A., JR., DE OLIVEIRA, D., BISHT, V., RODRIGUES, G., REIS, M. G., KO, A. I., BEGON, M. & CHILDS, J. E. 2015b. Patterns in *Leptospira* Shedding in Norway Rats (*Rattus norvegicus*) from Brazilian Slum Communities at High Risk of Disease Transmission. *PLoS Neglected Tropical Diseases,* 9**,** e0003819.

COUSINS, D. V., ELLIS, T. M., PARKINSON, J. & MCGLASHAN, C. H. 1989. Evidence for sheep as a maintenance host for *Leptospira interrogans* serovar Hardjo. *The Veterinary Record,* 124**,** 123-4.

COUTINHO, M. L., MATSUNAGA, J., WANG, L. C., DE LA PENA MOCTEZUMA, A., LEWIS, M. S., BABBITT, J. T., ALEIXO, J. A. & HAAKE, D. A. 2014. Kinetics of *Leptospira interrogans* infection in hamsters after intradermal and subcutaneous challenge. *PLoS Neglected Tropical Diseases,* 8**,** e3307.

COX, T., SMYTHE, L. & LEUNG, L.-P. 2005. Flying foxes as carriers of pathogenic *Leptospira* species. *Journal of Wildlife Diseases,* 41**,** 753-757.

CRUMP, J. A., GOVE, S. & PARRY, C. M. 2011a. Management of adolescents and adults with febrile illness in resource limited areas. *The British Medical Journal,* 343**,** d4847.

CRUMP, J. A. & KIRK, M. D. 2015. Estimating the Burden of Febrile Illnesses. *PLoS Neglected Tropical Diseases,* 9**,** e0004040.

CRUMP, J. A., MORRISSEY, A. B., NICHOLSON, W. L., MASSUNG, R. F., STODDARD, R. A., GALLOWAY, R. L., OOI, E. E., MARO, V. P., SAGANDA, W., KINABO, G. D., MUIRURI, C. & BARTLETT, J. A. 2013. Etiology of severe non-malaria febrile illness in northern Tanzania: a prospective cohort study. *PLoS Neglected Tropical Diseases,* 7**,** e2324. doi:10.1371/journal.pntd.0002324.

CRUMP, J. A., RAMADHANI, H. O., MORRISSEY, A. B., MSUYA, L. J., YANG, L. Y., CHOW, S. C., MORPETH, S. C., REYBURN, H., NJAU, B. N., SHAW, A. V., DIEFENTHAL, H. C., BARTLETT, J. A., SHAO, J. F., SCHIMANA, W., CUNNINGHAM, C. K. & KINABO, G. D. 2011b. Invasive bacterial and fungal infections among hospitalized HIV-infected and HIV-uninfected children and infants in northern Tanzania. *Tropical Medicine & International Health,* 16**,** 830-7.

CRUZ, L. S., VARGAS, R. & LOPES, A. A. 2009. Leptospirosis: a worldwide resurgent zoonosis and important cause of acute renal failure and death in developing nations. *Ethnicity and Disease,* 19**,** 37–41.

CUMBERLAND, P., EVERARD, C. O. & LEVETT, P. N. 1999. Assessment of the efficacy of an IgM-ELISA and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. *The American Journal of Tropical Medicine and Hygiene,* 61**,** 731–34.

CUMBERLAND, P., EVERARD, C. O. R., WHEELER, J. G. & LEVETT, P. N. 2001. Persistence of Anti-Leptospiral IgM, IgG and Agglutinating Antibodies in Patients Presenting with Acute Febrile Illness in Barbados 1979-1989. *European Journal of Epidemiology,* 17**,** 601-608.

CUNNINGHAM, D. & MOORS, P. 1996. *A guide to the identification and collection of New Zealand Rodents,* Wellington, New Zealand Wildlife Service Department.

D'ACREMONT, V., LENGELER, C. & GENTON, B. 2010. Reduction in the proportion of fevers associated with *Plasmodium falciparum* parasitaemia in Africa: a systematic review. *Malaria Journal,* 9**,** 240.

DA SILVA, E. F., FELIX, S. R., CERQUEIRA, G. M., FAGUNDES, M. Q., NETO, A. C., GRASSMANN, A. A., AMARAL, M. G., GALLINA, T. & DELLAGOSTIN, O. A. 2010. Preliminary characterization of Mus musculus-derived pathogenic strains of Leptospira borgpetersenii serogroup Ballum in a hamster model. *The American Journal of Tropical Medicine and Hygiene,* 83**,** 336-7.

DALU, J. M. & FERESU, S. B. 1997. Domestic rodents as reservoirs of pathogenic *Leptospira* on two city of Harare farms: Preliminary results of bacteriological and serological studies. *Belgian Journal of Zoology,* 127**,** 105–12.

DAVIS, S. & CALVET, E. 2005. Fluctuating rodent populations and risk to humans from rodent-borne zoonoses. *Vector-Borne & Zoonotic Diseases,* 5**,** 305-314.

DE GEUS, A. 1971. *Human leptospirosis in rural Kenya.* PhD, University of Amsterdam.

DE GEUS, A., KRANENDONK, O. & BOHLANDER, H. J. 1969. Clinical leptospirosis in Kwale District, Coast Province, Kenya. *East African Medical Journal,* 46**,** 491–6.

DE GEUS, A., WOLFF, J. W. & TIMMER, V. E. 1977a. Clinical leptospirosis in Kenya (1): a clinical study in Kwale District, Coast Province. *East African Medical Journal,* 54**,** 115–24.

DE GEUS, A., WOLFF, J. W. & TIMMER, V. E. 1977b. Clinical leptospirosis in Kenya (2): A field study in Nyanza Province. *East African Medical Journal,* 54**,** 125–32.

DE LANGE, J. F., GUMMOW, B., TURNER, G. V. & REDMAN, A. R. 1987. The isolation of *Leptospira interrogans* serovar Pomona and related serological findings associated with a mixed farming unit in the Transvaal. *Onderstepoort Journal of Veterinary Research,* 54**,** 119–21.

DE VRIES, S. G., VISSER, B. J., NAGEL, I. M., GORIS, M. G., HARTSKEERL, R. A. & GROBUSCH, M. P. 2014. Leptospirosis in Sub-Saharan Africa: a systematic review. *International Journal of Infectious Diseases,* 28c**,** 47–64.

DELACOLLETTE, C., TAELMAN, H. & WERY, M. 1995. An etiologic study of hemoglobinuria and blackwater fever in the Kivu Mountains, Zaire. *Annales de la Societe Belge de Medecine Tropicale,* 75**,** 51–63.

DEPARTMENT FOR ENVIRONMENT FOOD AND RURAL AFFAIRS, DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT (NORTHERN IRELAND), THE SCOTTISH GOVERNMENT RURAL AND ENVIRONMENT RESEARCH AND ANALYSIS DIRECTORATE & WELSH ASSEMBLY GOVERNMENT THE DEPARTMENT FOR RURAL AFFAIRS AND HERITAGE 2012. Agriculture in the United Kingdom.

DESVARS, A., JEGO, S., CHIROLEU, F., BOURHY, P., CARDINALE, E. & MICHAULT, A. 2011. Seasonality of human leptospirosis in Reunion Island (Indian Ocean) and its association with meteorological data. *PLoS One***,** e20377.

DESVARS, A., MICHAULT, A. & BOURHY, P. 2013a. Leptospirosis in the western Indian Ocean islands: what is known so far? *Veterinary Research,* 44**,** doi: 10.1186/1297-9716-44-80.

DESVARS, A., MICHAULT, A. & CHIROLEU, F. 2013b. Influence of risk factors on renal leptospiral load in naturally infected wild black rats. *Acta Tropica,* 125**,** 258-61.

DESVARS, A., NAZE, F., BENNEVEAU, A., CARDINALE, E. & MICHAULT, A. 2013c. Endemicity of leptospirosis in domestic and wild animal species from Reunion Island (Indian Ocean). *Epidemiology & Infection,* 1**,** 1–12.

DESVARS, A., NAZE, F., VOURC'H, G., CARDINALE, E., PICARDEAU, M., MICHAULT, A. & BOURHY, P. 2012. Similarities in *Leptospira* serogroup and species distribution in animals and humans in the Indian Ocean island of Mayotte. *The American Journal of Tropical Medicine and Hygiene,* 87**,** 134–40.

DHALIWAL, G. S., MURRAY, R. D., DOBSON, H., MONTGOMERY, J. & ELLIS, W. A. 1996a. Effect of *Leptospira interrogans* serovar Hardjo infection on milk yield in endemically infected dairy herds. *The Veterinary Record,* 139**,** 319-20.

DHALIWAL, G. S., MURRAY, R. D., DOBSON, H., MONTGOMERY, J. & ELLIS, W. A. 1996b. Effect of vaccination against *Leptospira interrogans* serovar Hardjo on milk production and fertility in dairy cattle. *The Veterinary Record,* 138**,** 334-5.

DIALLO, A. A. & DENNIS, S. M. 1982. Bacteriological survey of leptospirosis in Zaria, Nigeria. *Tropical and Geographical Medicine,* 34**,** 29–34.

DIETRICH, M., WILKINSON, D. A., SOARIMALALA, V., GOODMAN, S. M., DELLAGI, K. & TORTOSA, P. 2014. Diversification of an emerging pathogen in a biodiversity hotspot: *Leptospira* in endemic small mammals of Madagascar. *Molecular Ecology,* 23**,** 2783–2796.

DIKKEN, H., KMETY, E., DE GEUS, A. & TIMMER, V. E. 1979. A new serovar in the Australis serogroup. *Tropical and Geographical Medicine,* 31**,** 263-67.

DIKKEN, H., TIMMER, V. E. & NJENGA, R. 1981. Three new leptospiral serovars from Kenya. *Tropical and Geographical Medicine,* 33**,** 343–6.

DOBIGNY, G., GARBA, M., TATARD, C., LOISEAU, A., GALAN, M., KADAOURE, I., ROSSI, J. P., PICARDEAU, M. & BERTHERAT, E. 2015. Urban Market Gardening and Rodent-Borne Pathogenic *Leptospira* in Arid Zones: A Case Study in Niamey, Niger. *PLoS Neglected Tropical Diseases,* 9**,** e0004097.

DREYFUS, A., BENSCHOP, J., COLLINS-EMERSON, J., WILSON, P., BAKER, M. G. & HEUER, C. 2014a. Sero-prevalence and risk factors for leptospirosis in abattoir workers in New Zealand. *International Journal of Environmental Research and Public Health,* 11**,** 1756-75.

DREYFUS, A., HEUER, C., WILSON, P., COLLINS-EMERSON, J., BAKER, M. G. & BENSCHOP, J. 2014b. Risk of infection and associated influenza-like disease among abattoir workers due to two *Leptospira* species. *Epidemiology & Infection*, 1-11.

DURACK, J., BURKE, T. P. & PORTNOY, D. A. 2015. A prl mutation in *Sec*Y suppresses secretion and virulence defects of *Listeria monocytogenes sec*A2 mutants. *Journal of Bacteriology,* 197**,** 932-42.

ELDER, J. K. & WARD, W. H. 1978. The prevalence and distribution of leptospiral titres in cattle and pigs in Queensland. *Australian Veterinary Journal,* 54**,** 297-300.

ELLIS, W. A. 1984. Bovine leptospirosis in the tropics: Prevalence, pathogenesis and control. *Preventive Veterinary Medicine,* 2**,** 411-421.

ELLIS, W. A. 1994. Leptospirosis as a cause of reproductive failure. *The Veterinary Clinics Of North America. Food Animal Practice,* 10**,** 463–78.

ELLIS, W. A. 2010. Control of canine leptospirosis in Europe: time for a change? *The Veterinary Record,* 167**,** 602-5.

ELLIS, W. A. 2015. Animal Leptospirosis. *In:* ADLER, B. (ed.) *Leptospira and Leptospirosis.* 2014/11/13 ed. Berlin: Springer.

ELLIS, W. A., O'BRIEN, J. J., NEILL, S. D. & BRYSON, D. G. 1986. Bovine leptospirosis: experimental serovar Hardjo infection. *Veterinary Microbiology,* 11**,** 293-9.

ERNEST, E., NONGA, H. E., KASSUKU, A. A. & KAZWALA, R. R. 2009. Hydatidosis of slaughtered animals in Ngorongoro district of Arusha region, Tanzania. *Tropical Animal Health and Production,* 41**,** 1179-85.

EVANGELISTA, K. V. & COBURN, J. 2010. *Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. *Future Microbiology,* 5**,** 1413–25.

EVERARD, C. O., BENNETT, S., EDWARDS, C. N., NICHOLSON, G. D., HASSELL, T. A., CARRINGTON, D. G. & EVERARD, J. D. 1992. An investigation of some risk factors for severe leptospirosis on Barbados. *The Journal of Tropical Medicine and Hygiene,* 95**,** 13-22.

EVERARD, J. & EVERARD, C. 1993. Leptospirosis in the Caribbean. *Reviews in Medical Microbiology,* 4**,** 114.

EVERITT, B. 2005. *An R and S-PLUS companion to multivariate analysis,* London, Springer-Verlag.

EZEH, A. O., ELLIS, W. A., KMETY, E., ADESIYUN, A. A. & ADDO, P. B. 1989a. Bacteriological examination of bovine kidneys for leptospires in Plateau State, Nigeria. *Revue Scientifique et Techique (International Office of Epizootics),* 8**,** 1005–8.

EZEH, A. O., KMETY, E., ELLIS, W. A., ADDO, P. B. & ADESIYUN, A. A. 1989b. Characterisation of leptospires isolated from cattle and man in Plateau State, Nigeria. *Revue Scientifique et Techique (International Office of Epizootics),* 8**,** 1009–20.

EZEH, A. O., KMETY, E., ELLIS, W. A., ADDO, P. B. & ADESIYUN, A. A. 1990. A new leptospiral serovar in the Pyrogenes serogroup isolated in Nigeria. *Revue Scientifique et Techique (International Office of Epizootics),* 9**,** 1195–96.

FABER, N. A., CRAWFORD, M., LEFEBVRE, R. B., BUYUKMIHCI, N. C., MADIGAN, J. E. & WILLITS, N. H. 2000. Detection of *Leptospira* spp. in the aqueous humor of horses with naturally acquired recurrent uveitis. *Journal of Clinical Microbiology,* 38**,** 2731-2733.

FAINE, S. 1994. *Leptospira and leptospirosis*, CRC Press Inc.

FANG, F., COLLINS-EMERSON, J. M., CULLUM, A., HEUER, C., WILSON, P. R. & BENSCHOP, J. 2014a. Shedding and Seroprevalence of Pathogenic *Leptospira* spp. in Sheep and Cattle at a New Zealand Abattoir. *Zoonoses and Public Health,* 62**,** 258-68.

FANG, F., COLLINS-EMERSON, J. M., HEUER, C., HILL, F. I., TISDALL, D. J., WILSON, P. R. & BENSCHOP, J. 2014b. Interlaboratory and between-specimen comparisons of diagnostic tests for leptospirosis in sheep and cattle. *Journal of Veterinary Diagnostic Investigation,* 26**,** 734-47.

FEIKIN, D. R., OLACK, B., BIGOGO, G. M., AUDI, A., COSMAS, L., AURA, B., BURKE, H., NJENGA, M. K., WILLIAMSON, J. & BREIMAN, R. F. 2011. The burden of common infectious disease syndromes at the clinic and household level from population-based surveillance in rural and urban Kenya. *PLoS One,* 6**,** e16085.

FELT, S. A., WASFY, M. O., EL-TRAS, W. F., SAMIR, A., RAHAMAN, B. A., BOSHRA, M., PARKER, T. M., HATEM, M. E., EL-BASSIOUNY, A. A., MURRAY, C. K. & PIMENTEL, G. 2011. Cross-species surveillance of *Leptospira* in domestic and peri-domestic animals in Mahalla City, Gharbeya

Governorate, Egypt. *The American Journal of Tropical Medicine and Hygiene,* 84**,** 420–25.

FERESU, S. B. 1992. Isolation of *Leptospira interrogans* from kidneys of Zimbabwe beef cattle. *The Veterinary Record,* 130**,** 446–48.

FERESU, S. B., BOLIN, C. A. & KORVER, H. 1993. A new leptospiral serovar in the Icterohaemorrhagiae serogroup isolated from an ox in Zimbabwe. *International Journal of Systematic Bacteriology,* 43**,** 179–82.

FERESU, S. B., BOLIN, C. A. & KORVER, H. 1998. A new leptospiral serovar, Ngavi, in the Tarassovi serogroup isolated from Zimbabwe oxen. *International Journal of Systematic Bacteriology,* 48**,** 207–13.

FERESU, S. B., BOLIN, C. A., KORVER, H. & KEMP, H. V. D. 1995. Identification of leptospires of the Pomona and Grippotyphosa serogroups isolated from cattle in Zimbabwe. *Research in Veterinary Science,* 59**,** 92–94.

FERESU, S. B., BOLIN, C. A., KORVER, H. & TERPSTRA, W. J. 1994. Classification of leptospires of the Pyrogenes serogroup isolated from cattle in Zimbabwe by cross-agglutinin absorption and restriction fragment length polymorphism analysis. *International Journal of Systematic Bacteriology,* 44**,** 541–46.

FERESU, S. B., BOLIN, C. A., VAN DE KEMP, H. & KORVER, H. 1999a. Identification of a serogroup Bataviae *Leptospira* strain isolated from an ox in Zimbabwe. *Zentralblatt fur Bakteriologie,* 289**,** 19–29.

FERESU, S. B., KORVER, H., RIQUELME, N., BARANTON, G. & BOLIN, C. A. 1996. Two new leptospiral serovars in the Hebdomadis serogroup isolated from Zimbabwe cattle. *International Journal of Systematic Bacteriology,* 46**,** 694–98.

FERESU, S. B., STEIGERWALT, A. G. & BRENNER, D. J. 1999b. DNA relatedness of *Leptospira* strains isolated from beef cattle in Zimbabwe. *International Journal of Systematic Bacteriology,* 49 Pt 3**,** 1111-7.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS 2005. *Livestock Sector Brief: United Republic of Tanzania,* Rome, Italy, Food and Agriculture Organization of the United Nations (FAO).

FORRESTER, A. T., KRANENDONK, O., TURNER, L. H., WOLFF, J. W. & BOHLANDER, H. J. 1969. Serological evidence of human leptospirosis in Kenya. *East African Medical Journal,* 46**,** 497–506.

FOUTS, D. E., MATTHIAS, M. A., ADHIKARLA, H., ADLER, B., AMORIM-SANTOS, L., BERG, D. E., BULACH, D., BUSCHIAZZO, A., CHANG, Y.-F., GALLOWAY, R. L., HAAKE, D. A., HAFT, D. H., HARTSKEERL, R., KO, A. I., LEVETT, P. N., MATSUNAGA, J., MECHALY, A. E., MONK, J. M., NASCIMENTO, A. L. T., NELSON, K. E., PALSSON, B., PEACOCK, S. J., PICARDEAU, M., RICALDI, J. N., THAIPANDUNGPANIT, J., WUNDER, E. A., JR., YANG, X. F., ZHANG, J.-J. & VINETZ, J. M. 2016. What Makes a Bacterial Species Pathogenic? Comparative genomic analysis of the genus *Leptospira*. *PLoS Neglected Tropical Diseases,* 10**,** e0004403.

GALLOWAY, R. L. & HOFFMASTER, A. R. 2015. Optimization of LipL32 PCR assay for increased sensitivity in diagnosing leptospirosis. *Diagnostic Microbiology and Infectious Disease,* 82**,** 199-200.

GANOZA, C. A., MATTHIAS, M. A., SAITO, M., CESPEDES, M., GOTUZZO, E. & VINETZ, J. M. 2010. Asymptomatic Renal Colonization of Humans in the Peruvian Amazon by *Leptospira*. *PLoS Neglected Tropical Diseases,* 4**,** e612.

GASEM, M. H., WAGENAAR, J. F., GORIS, M. G., ADI, M. S., ISBANDRIO, B. B., HARTSKEERL, R. A., ROLAIN, J. M., RAOULT, D. & VAN GORP, E. C. 2009. Murine typhus and leptospirosis as causes of acute undifferentiated fever, Indonesia. *Emerging Infectious Diseases,* 15**,** 975–77.

GEAR, J., WOLSTENHOLME, B., JACKSON, A., CHESLER, E. & BRUECKNER, R. M. 1958. Leptospirosis in South Africa: The occurrence of cases of leptospiral meningo-encephalitis on the Witwatersrand. *South African Medical Journal,* 32**,** 94–100.

GOLDSTEIN, R. E. 2010. Canine leptospirosis. *The Veterinary Clinics Of North America. Small Animal Practice,* 40**,** 1091–101.

GOMARD, Y., DIETRICH, M., WIESEKE, N., RAMASINDRAZANA, B., LAGADEC, E., GOODMAN, S. M., DELLAGI, K. & TORTOSA, P. 2016. Malagasy bats shelter a considerable genetic diversity of pathogenic *Leptospira* suggesting notable host-specificity patterns. *FEMS Microbiology Ecology,* 92**,** pii: fiw037.

GONZALEZ, S., GEYMONAT, J. P., HERNANDEZ, E., MARQUES, J. M., SCHELOTTO, F. & VARELA, G. 2013. Usefulness of real-time PCR assay targeting *lip*L32 gene for diagnosis of human leptospirosis in Uruguay. *The Journal of Infection in Developing Countries,* 7**,** 941-5.

GOODMAN, C., KACHUR, S. P., ABDULLA, S., BLOLAND, P. & MILLS, A. 2007. Drug shop regulation and malaria treatment in Tanzania—why do shops break the rules, and does it matter? *Health Policy and Planning,* 22**,** 393-403.

GORIS, M. G. & HARTSKEERL, R. A. 2013. Leptospirosis serodiagnosis by the microscopic agglutination test. *Current Protocols in Microbiology.* Wiley Online.

GORIS, M. G., KIKKEN, V., STRAETEMANS, M., ALBA, S., GOEIJENBIER, M., VAN GORP, E. C., BOER, K. R., WAGENAAR, J. F. & HARTSKEERL, R. A. 2013a. Towards the burden of human leptospirosis: duration of acute illness and occurrence of post-leptospirosis symptoms of patients in the Netherlands. *PLoS One,* 8**,** e76549.

GORIS, M. G., LEEFLANG, M. M., LODEN, M., WAGENAAR, J. F., KLATSER, P. R., HARTSKEERL, R. A. & BOER, K. R. 2013b. Prospective evaluation of three rapid diagnostic tests for diagnosis of human leptospirosis. *PLoS Neglected Tropical Diseases,* 7**,** e2290.

GORIS, M. G. A., BOER, K. R., BOUMAN-STRIJKER, M., HARTSKEERL, R., LUCAS, C. & LEEFLANG, M. M. G. 2011. Serological laboratory tests for diagnosis of human leptospirosis in patients presenting with clinical symptoms. *Cochrane Library***,** 1–11.

GORIS, M. G. A., BOER, K. R., DUARTE, T. A. T. E., KLIFFEN, S. J. & HARTSKEERL, R. A. 2013c. Human Leptospirosis Trends, the Netherlands, 1925–2008. *Emerging Infectious Diseases,* 19**,** 371-378.

GORIS, M. G. A., LEEFLANG, M. M. G., BOER, K. R., GOEIJENBIER, M., VAN GORP, E. C. M., WAGENAAR, J. & HARTSKEERL, R. A. 2012. Establishment of Valid Laboratory Case Definition for Human Leptospirosis. *Bacteriology and Parasitology,* 3**,** 1000132.

GRACE, D., GILBERT, J., RANDOLPH, T. & KANG'ETHE, E. 2012a. The multiple burdens of zoonotic disease and an Ecohealth approach to their assessment. *Tropical Animal Health and Production,* 44 Suppl 1**,** S67-73.

GRACE, D., MUTUA, F., OCHUNGO, P., KRUSKA, R., JONES, K., BRIERLEY, L., LAPAR, L., SAID, M., HERRERO, M. & PHUC, P. 2012b. Mapping of poverty and likely zoonoses hotspots. *Report to the Department for International Development, UK.* International Livestock Research Institute.

GRATZ, N. G. & ARATA, A. A. 1975. Problems associated with the control of rodents in tropical Africa. *Bulletin of the World Health Organization,* 52**,** 697-706.

GRAVEKAMP, C., VAN DE KEMP, H., FRANZEN, M., CARRINGTON, D., SCHOONE, G., VAN EYS, G., EVERARD, C., HARTSKEERL, R. & TERPSTRA, W. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Journal of General Microbiology,* 139**,** 1691–1700.

GRESHAM, A. 2003. Infectious reproductive disease in pigs. *In Practice,* 25**,** 466-473.

GUMMOW, B., MYBURGH, J. G., THOMPSON, P. N., VAN DER LUGT, J. J. & SPENCER, B. T. 1999. Three case studies involving *Leptospira interrogans* serovar Pomona infection in mixed farming units. *Journal of the South African Veterinary Association,* 70**,** 29–34.

HAAKE, D. A., CHAO, G., ZUERNER, R. L., BARNETT, J. K., BARNETT, D., MAZEL, M., MATSUNAGA, J., LEVETT, P. N. & BOLIN, C. A. 2000. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infection and Immunity,* 68**,** 2276-2285.

HAAKE, D. A., DUNDOO, M., CADER, R., KUBAK, B. M., HARTSKEERL, R. A., SEJVAR, J. J. & ASHFORD, D. A. 2002. Leptospirosis, water sports, and chemoprophylaxis. *Clinical Infectious Diseases,* 34**,** e40-3.

HAAKE, D. A. & LEVETT, P. N. 2015. Leptospirosis in humans. *In:* ADLER, B. (ed.) *Leptospira and Leptospirosis.* 2014/11/13 ed. Berlin: Springer.

HALLIDAY, J., DABORN, C., AUTY, H., MTEMA, Z., LEMBO, T., BAREND, M., HANDEL, I., KNOBEL, D., HAMPSON, K. & CLEAVELAND, S. 2012. Bringing together emerging and endemic zoonoses surveillance: shared challenges and a common solution. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 367**,** 2872–2880.

HALLIDAY, J. E., KNOBEL, D. L., ALLAN, K. J., BRONSVOORT, B. M. D. C., HANDEL, I., AGWANDA, B., CUTLER, S. J., OLACK, B., AHMED, A. & HARTSKEERL, R. A. 2013. Urban leptospirosis in Africa: a cross-sectional survey of *Leptospira* infection in rodents in the Kibera urban settlement, Nairobi, Kenya. *The American Journal of Tropical Medicine and Hygiene,* 89**,** 1095–1102.

HALLIDAY, J. E. B. 2010. *Animal Sentinel Surveillance: Evaluating domestic dogs as sentinels for zoonotic pathogen surveillance.* PhD, University of Edinburgh.

HALLIDAY, J. E. B., ALLAN, K. J., EKWEM, D., CLEAVELAND, S., KAZWALA, R. R. & CRUMP, J. A. 2015. Endemic zoonoses in the tropics: a public health problem hiding in plain sight. *The Veterinary Record,* 176**,** 220–25.

HAMOND, C., MARTINS, G., BREMONT, S., MEDEIROS, M. A., BOURHY, P. & LILENBAUM, W. 2014. Predominance of *Leptospira interrogans* serovar Bratislava DNA in vaginal fluid of mares suggests sexual transmission of leptospirosis. *Animal Reproduction Science,* 151**,** 275-9.

HAMOND, C., PESTANA, C. P., MEDEIROS, M. A. & LILENBAUM, W. 2016. Genotyping of *Leptospira* directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. *Epidemiology & Infection,* 144**,** 72-75.

HARRELL, F. E., DUPONT, C. & AL., E. 2016. Hmisc: Harrell Miscellaneous. Version 3.17-4 ed.

HARTSKEERL, R., COLLARES-PEREIRA, M. & ELLIS, W. 2011. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infection,* 17**,** 494–501.

HARTSKEERL, R., SMITS, H. L., KORVER, H., GORIS, M. & TERPSTRA, W. J. 2006. International course on laboratory methods for the diagnosis of leptospirosis. 5th ed. The Netherlands: Royal Tropical Institute.

HATEM, M. E., ATA, N. S., ABDOU, A. M., IBRAHIM, E. S., BAKRY, M. A. & SAMIR, A. 2014. Surveillance of bovine leptospirosis: isolation and serodiagnosis. *Global Veterinaria,* 13**,** 127–132.

HAYDON, D. T., CLEAVELAND, S., TAYLOR, L. H. & LAURENSON, M. K. 2002. Identifying reservoirs of infection: a conceptual and practical challenge. *Emerging Infectious Diseases,* 8**,** 1468-1473.

HERR, S., RILEY, A. E., NESER, J. A., ROUX, D. & DE LANGE, J. D. 1982. *Leptospira interrogans* ssp Pomona Associated with Abortion in Cattle: Isolation Methods and Laboratory Animal Histopathology. *Onderstepoort Journal of Veterinary Research,* 49**,** 57–62.

HERR, S. & WINNEN, G. M. 1983. First isolation of *Leptospira interrogans* serovar Pomona from cattle in Botswana. *Journal of the South African Veterinary Association,* 54**,** 83–84.

HIGGINS, J. P. & GREEN, S. 2008. *Cochrane handbook for systematic reviews of interventions*, Wiley Online Library.

HIMSWORTH, C. G., BIDULKA, J., PARSONS, K. L., FENG, A. Y. T., TANG, P., JARDINE, C. M., KERR, T., MAK, S., ROBINSON, J. & PATRICK, D. M. 2013. Ecology of *Leptospira interrogans* in Norway Rats (*Rattus norvegicus*) in an Inner-City Neighborhood of Vancouver, Canada. *PLoS Neglected Tropical Diseases,* 7**,** e2270.

HOGERZEIL, H. V., DE GEUS, A., TERPSTRA, W. J., KORVER, H. & LIGTHART, G. S. 1986. Leptospirosis in rural Ghana: Part 2. Current leptospirosis. *Tropical and Geographical Medicine,* 38**,** 408–14.

HOLT, J., DAVIS, S. & LEIRS, H. 2006. A model of Leptospirosis infection in an African rodent to determine risk to humans: Seasonal fluctuations and the impact of rodent control. *Acta Tropica,* 99**,** 218-225.

HOME OFFICE 2014. *Guidance on the Operation of the Animals (Scientific Procedures) Act 1986,* London, Home Office

HOPEWELL, S., CLARKE, M., LEFEBVRE, C. & SCHERER, R. 2007. Handsearching versus electronic searching to identify reports of randomized trials. *Cochrane Database Syst Rev***,** Mr000001.

HOTEZ, P. J., FENWICK, ALAN, SAVIOLI, LORENZO, MOLYNEUX, DAVID H. 2009. Rescuing the bottom billion through control of neglected tropical diseases. *The Lancet,* 373**,** 1570–1575.

HOTEZ, P. J., MOLYNEUX, D. H., FENWICK, A., KUMARESAN, J., SACHS, S. E., SACHS, J. D. & SAVIOLI, L. 2007. Control of neglected tropical diseases. *New England Journal of Medicine,* 357**,** 1018-1027.

HOUEMENOU, G., AHMED, A., LIBOIS, R. & HARTSKEERL, R. A. 2013. *Leptospira* spp. prevalence in small mammal populations in Cotonou, Benin. *ISRN Epidemiology,* http://dx.doi.org/10.5402/2013/502638.

HUNTER, P., VAN DER VYVER, F. H., SELMER-OLSEN, A., HENTON, M. M., HERR, S. & DE LANGE, J. F. 1987. Leptospirosis as a cause of "white spot" kidneys in South African pig abattoirs. *Onderstepoort Journal of Veterinary Research,* 54**,** 59–62.

IDO, Y., HOKI, R., ITO, H. & WANI, H. 1917. The rat as a carrier of Spirochaeta Icterohaemorrhagiae, the causative agent of Weil's Disease (Spirochaetosis Icterohaemorrhagica) *The Journal of Experimental Medicine,* 26**,** 341-53.

ISMAIL, T. F., WASFY, M. O., ABDUL-RAHMAN, B., MURRAY, C. K., HOSPENTHAL, D. R., ABDEL-FADEEL, M., ABDEL-MAKSOUD, M., SAMIR, A., HATEM, M. E., KLENA, J., PIMENTEL, G., EL-SAYED, N. & HAJJEH, R. 2006. Retrospective serosurvey of leptospirosis among patients with acute febrile illness and hepatitis in Egypt. *The American Journal of Tropical medicine and Hygiene,* 75**,** 1085–89.

JOBBINS, S., SANDERSON, C. & ALEXANDER, K. 2013. *Leptospira interrogans* at the Human-Wildlife Interface in Northern Botswana: A Newly Identified Public Health Threat. *Zoonoses and Public Health,* 61**,** 113-23.

JOLLEY, K. A. & MAIDEN, M. C. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics,* 11**,** 1-11.

JOLLEY, K. A. & MAIDEN, M. C. 2016. *Leptospira spp. MLST Databases* [Online]. University of Oxford. Available: http://pubmlst/org/leptospira [Accessed].

KARIMURIBO, E. D., SWAI, E. S. & KYAKAISHO, P. K. 2008. Investigation of a syndrome characterised by passage of red urine in smallholder dairy cattle in East Usambara Mountains, Tanzania. *Journal of the South African Veterinary Association,* 79**,** 89-94.

KENDALL, E. A., LAROCQUE, R. C., BUI, D. M., GALLOWAY, R., ARI, M. D., GOSWAMI, D., BREIMAN, R. F., LUBY, S. & BROOKS, W. A. 2010. Leptospirosis as a cause of fever in urban Bangladesh. *The American Journal of Tropical Medicine and Hygiene,* 82**,** 1127–30.

KESSY, M. J., MACHANG'U, R. S. & SWAI, E. S. 2010. A microbiological and serological study of leptospirosis among pigs in the Morogoro municipality, Tanzania. *Tropical Animal Health and Production,* 42**,** 523–30.

KINEBUCHI, H. & AFOAKWA, S. N. 1973. Leptospirosis in Ghana. *Ghana Med J,* 12**,** 190–93.

KINGDON, J. 1997. *The Kingdon Field Guide to African Mammals*, Bloomsbury Publishing.

KLOPPER, J. F. 1969. [Leptospirosis--a discussion of 4 cases]. *South African Medical Journal,* 43**,** 1138–40.

KMETY, E. & DIKKEN, H. 1993. *Classification of the species Leptospira interrogans and history of its serovars,* Groningen - Netherlands, University Press Groningen.

KO, A. I., GOARANT, C. & PICARDEAU, M. 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. *Nature Reviews Microbiology,* 7**,** 736–747.

KOKO, J., MOUSSAVOU, A., ORIMA, C., SEILHAN, C., LEMBA-ABAKA, A. & DAMAS, S. 2001. Leptospirosis in children in Libreville: first case report, a difficult diagnosis. *Bulletin de la Societe de Pathologie Exotique,* 94**,** 394–96.

KOLOCHINE-ERBER, B. & BRYGOO, E. R. 1956. Investigation of the leptospiroses on Madagascar. *Bulletin de la Societe de Pathologie Exotique,* 49**,** 686–698.

KOSOY, M., KHLYAP, L., COSSON, J. F. & MORAND, S. 2015. Aboriginal and invasive rats of genus rattus as hosts of infectious agents. *Vector Borne and Zoonotic Diseases,* 15**,** 3-12.

KUMAR, S., STECHER, G. & TAMURA, K. 2015. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution, Submitted*.

LAGADEC, E., GOMARD, Y., GUERNIER, V., DIETRICH, M., PASCALIS, H., TEMMAM, S., RAMASINDRAZANA, B., GOODMAN, S. M., TORTOSA, P. & DELLAGI, K. 2012. Pathogenic *Leptospira* spp. in Bats, Madagascar and Union of the Comoros. *Emerging Infectious Diseases,* 18**,** 1696–97.

LAHSEN, A. O., RACHIDI, T., SODQI, M., ABADA, R. & MARHOUM, K. E. 2010. [Facial palsy associated with leptospirosis]. *Medecine et Maladies Infectieuses,* 40**,** 716–17.

LAU, C. L., SMYTHE, L. D., CRAIG, S. B. & WEINSTEIN, P. 2010. Climate change, flooding, urbanisation and leptospirosis: fuelling the fire? *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 104**,** 631–638.

LAU, C. L., WATSON, C. H., LOWRY, J. H., DAVID, M. C., CRAIG, S. B., WYNWOOD, S. J., KAMA, M. & NILLES, E. J. 2016. Human Leptospirosis Infection in Fiji: An Eco-epidemiological Approach to Identifying Risk Factors and Environmental Drivers for Transmission. *PLoS Neglected Tropical Diseases,* 10**,** e0004405.

LAZUGA, K. & BONNEFOUS, S. 1962. Contribution to the study of leptospirosis in rats in the city of Tunis [English and German summ.]. *Archives de l'Institut Pasteur Tunis,* 39**,** 49–63.

LE BRAS, J., GUYER, B., SULZER, C. & MAILLOUX, M. 1977. [Anademic focus of leptospirosis at Fondem (U.R. of Cameroon)]. *Bulletin de la Societe de Pathologie Exotique Filiales,* 70**,** 569–83.

LEARY, S., UNDERWOOD, W., ANTHONY, R., CARTNER, S., COREY, D., GRANDIN, T., GREENACRE, C., GWALTNEY-BRANT, S., MCCRAKIN, M. A., MEYER, R., MILLER, D., SHEARER, J. & YANONG, R. 2014. The AVMA Guidelines for the Euthanasia of Animals. 2013 ed. Schaumburg, IL: American Veterinary Medical Association.

LEGRIS, T., JAFFAR-BANDJEE, M. C., FAVRE, O., LEFRANCOIS, N., GENIN, R., RAGOT, C., FERNANDEZ, C. & REBOUX, A. H. 2014. Ameboma: an unusual cause of gastrointestinal bleeding during severe leptospirosis. *BMC Infectious Diseases,* 14**,** 299–305.

LEIRS, H., VERHAGEN, R., VERHEYEN, W., MWANJABE, P. & MBISE, T. 1996. Forecasting rodent outbreaks in Africa: an ecological basis for Mastomys control in Tanzania. *Journal of Applied Ecology***,** 937-943.

LELU, M., MUNOZ-ZANZI, C., HIGGINS, B. & GALLOWAY, R. 2015. Seroepidemiology of leptospirosis in dogs from rural and slum communities of Los Rios Region, Chile. *BMC Veterinary Research,* 11**,** 31.

LEONARD, F. C., QUINN, P. J., ELLIS, W. A. & O'FARRELL, K. 1992. Duration of urinary excretion of leptospires by cattle naturally or experimentally infected with *Leptospira interrogans* serovar Hardjo. *The Veterinary Record,* 131**,** 435-9.

LEOPOLD, S. J., VAN LETH, F., TAREKEGN, H. & SCHULTSZ, C. 2014. Antimicrobial drug resistance among clinically relevant bacterial isolates in sub-Saharan Africa: a systematic review. *Journal of Antimicrobial Chemotherapy,* 69**,** 2337-53.

LEVETT, P. N. 2001. Leptospirosis. *Clinical Microbiology Reviews,* 14**,** 296–326.

LEVETT, P. N. 2003. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. *Clinical Infectious Diseases,* 36**,** 447–52.

LEVETT, P. N. 2015. Systematics of *Leptospiraceae*. *In:* ADLER, B. (ed.) *Leptospira and Leptospirosis.* Berlin: Springer-Verlag

LILENBAUM, W. & MARTINS, G. 2014. Leptospirosis in cattle: a challenging scenario for the understanding of the epidemiology. *Transboundary and Emerging Diseases,* 61**,** 63-68.

LILENBAUM, W. & SOUZA, G. N. 2003. Factors associated with bovine leptospirosis in Rio de Janeiro, Brazil. *Research in Veterinary Science,* 75**,** 249-51.

LILENBAUM, W., VARGES, R., BRANDAO, F. Z., CORTEZ, A., DE SOUZA, S. O., BRANDAO, P. E., RICHTZENHAIN, L. J. & VASCONCELLOS, S. A. 2008. Detection of *Leptospira* spp. in semen and vaginal fluids of goats and sheep by polymerase chain reaction. *Theriogenology,* 69**,** 837-42.

LIMMATHUROTSAKUL, D., TURNER, E. L., WUTHIEKANUN, V., THAIPADUNGPANIT, J., SUPUTTAMONGKOL, Y., CHIERAKUL, W., SMYTHE, L. D., DAY, N. P., COOPER, B. & PEACOCK, S. J. 2012. Fool's gold: Why imperfect reference tests are undermining the evaluation of novel diagnostics: a reevaluation of 5 diagnostic tests for leptospirosis. *Clinical Infectious Diseases,* 55**,** 322-31.

LIU, Y. C., HUANG, W. K., HUANG, T. S. & KUNIN, C. M. 1999. Detection of antimicrobial activity in urine for epidemiologic studies of antibiotic use. *Journal of Clinical Epidemiology,* 52**,** 539-45.

LLOYD-SMITH, J. O., CROSS, P. C., BRIGGS, C. J., DAUGHERTY, M., GETZ, W. M., LATTO, J., SANCHEZ, M. S., SMITH, A. B. & SWEI, A. 2005. Should we expect population thresholds for wildlife disease? *Trends in Ecology and Evolution,* 20**,** 511-9.

MACHANG'U, R., MGODE, G., ASENGA, J., MHAMPHI, G., HARTSKEERL, R., GORIS, M., COX, C., WEETJENS, B. & VERHAGEN, R. 2002. Characterisation of *Leptospira* isolates from captive giant African pouched rats, Cricetomys gambianus. *ACIAR Monograph Series,* 96**,** 40–42.

MACHANG'U, R. S., MGODE, G. & MPANDUJI, D. 1997. Leptospirosis in animals and humans in selected areas of Tanzania. *Belgian Journal of Zoology,* 127**,** 97–104.

MACHANG'U, R. S., MGODE, G. F., ASSENGA, J., MHAMPHI, G., WEETJENS, B., COX, C., VERHAGEN, R., SONDIJ, S., GORIS, M. G. & HARTSKEERL, R. A. 2004. Serological and molecular characterization of *Leptospira* serovar Kenya from captive African giant pouched rats (*Cricetomys gambianus*) from Morogoro Tanzania. *FEMS Immunology & Medical Microbiology,* 41**,** 117–21.

MAGNE, M. C., ONDOUNDA, M., MBETHE, L. G., MOUNGUENGUI, D. & NZENZE, J. R. 2013. [Leptospirosis in Libreville (Gabon): four cases]. *Medecine et Sante Tropicales,* 23**,** 347–50.

MAILLOUX, C. H., MAILLOUX, M., GIRAUDEAU, P., GILOT, Y. & SAINT-ANDRE, P. 1974. [First case of confirmed leptospirosis in Mali]. *Bulletin de la Societe de Pathologie Exotique Filiales,* 67**,** 498–503.

MAILLOUX, M. 1967. Leptospiroses in Morocco: New data [Engl. sum.]. *Bulletin de la Societe de Pathologie Exotique,* 58**,** 841–47.

MAILLOUX, M. 1971. [Simultaneous cases of leptospirosis in a family in Morocco]. *Bulletin de la Societe de Pathologie Exotique Filiales,* 64**,** 42–45.

MANOCK, S. R., JACOBSEN, K. H., DE BRAVO, N. B., RUSSELL, K. L., NEGRETE, M., OLSON, J. G., SANCHEZ, J. L., BLAIR, P. J., SMALLIGAN, R. D., QUIST, B. K., ESPIN, J. F., ESPINOZA, W. R., MACCORMICK, F., FLEMING, L. C. & KOCHEL, T. 2009. Etiology of acute undifferentiated febrile illness in the Amazon basin of Ecuador. *The American Journal of Tropical Medicine and Hygiene,* 81**,** 146–51.

MARIANELLI, C., TARANTINO, M., ASTARITA, S., MARTUCCIELLO, A., CAPUANO, F. & GALIERO, G. 2007. Molecular detection of *Leptospira* species in aborted fetuses of water buffalo. *The Veterinary Record,* 161**,** 310-2.

MARONPOT, R. R., BARSOUM, I. S. & EZZAT, E. 1971. Canine leptospirosis in Cairo. *Journal of Infectious Diseases,* 123**,** 548–50.

MARTINEZ SANCHEZ, R., PEREZ SIERRA, A., BARO SUAREZ, M., ALVAREZ, A. M., MENENDEZ HERNANDEZ, J., DIAZ GONZALEZ, M., CRUZ DE LA PAZ, R., DE LOS REYES, G., MONTOYA BATISTA, B., SIERRA GONZALEZ, G., ARMESTO DEL RIO, M., SALTAREN COBAS, A. & SABOURNIN RAMOS, O. 2000. [Evaluation of the effectiveness of a new vaccine against human leptospirosis in groups at risk]. *Revista Panamericana de Salud Publica,* 8**,** 385-92.

MARTINS, G. & LILENBAUM, W. 2014. Leptospirosis in sheep and goats under tropical conditions. *Tropical Animal Health and Production,* 46**,** 11-7.

MASI, E., PINO, F. A., MARIA DAS GRA√SSA, S. S., GENEHR, L., ALBUQUERQUE, J. O. M. M., BANCHER, A. A. M. & ALVES, J. C. M. 2010. Socioeconomic and environmental risk factors for urban rodent infestation in Sao Paulo, Brazil. *Journal of Pest Science,* 83**,** 231-241.

MATTHIAS, M. A. & LEVETT, P. N. 2002. Leptospiral carriage by mice and mongooses on the island of Barbados. *West Indian Medical Journal,* 51**,** 10-3.

MAUDLIN, I., EISLER, M. C. & WELBURN, S. C. 2009. Neglected and endemic zoonoses. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364**,** 2777–87.

MAZE, M. J., BIGGS, H. M., RUBACH, M. P., GALLOWAY, R., CASH-GOLDWASSER, S., ALLAN, K. J., HALLIDAY, J. E. B., HERTZ, J. T., SAGANDA, W., LWEZAULA, B. F., CLEAVELAND, S., MMBAGA, B. T., MARO, V. P. & CRUMP, J. A. *in preparation*. Comparison of the incidence of acute leptospirosis in the Kilimanjaro Region of Tanzania between 2007-08 and 2012-14.

MAZE, S. S. & KIRSCH, R. E. 1981. Leptospirosis Experience at Groote Schuur Hospital South Africa 1969-1979. *South African Medical Journal,* 59**,** 33–36.

MAZET, J. A. K., CLIFFORD, D. L., COPPOLILLO, P. B., DEOLALIKAR, A. B., ERICKSON, J. D. & KAZWALA, R. R. 2009. A "One Health" approach to address emerging zoonoses: The HALI project in Tanzania. *PLoS Medicine,* 6**,** e1000190.

MCBRIDE, A. J., ATHANAZIO, D. A., REIS, M. G. & KO, A. I. 2005. Leptospirosis. *Current Opinion in Infectious Diseases,* 18**,** 376–86.

MCKENNA, S., KEEFE, G. P., BARKEMA, H., MCCLURE, J., VANLEEUWEN, J. A., HANNA, P. & SOCKETT, D. 2004. Cow-level prevalence of paratuberculosis in culled dairy cows in Atlantic Canada and Maine. *Journal of Dairy Science,* 87**,** 3770-3777.

MEERBURG, B. G., SINGLETON, G. R. & KIJLSTRA, A. 2009. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology,* 35**,** 221-270.

MERIEN, F., AMOURIAUX, P., PEROLAT, P., BARANTON, G. & SAINT GIRONS, I. 1992. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. *Journal of Clinical Microbiology,* 30**,** 2219-24.

MERIEN, F., PORTNOI, D., BOURHY, P., CHARAVAY, F., BERLIOZ-ARTHAUD, A. & BARANTON, G. 2005. A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. *FEMS Microbiology Letters,* 249**,** 139-147.

MGODE, G. F., KATAKWEBA, A. S., MHAMPHI, G. G., FWALO, F., BAHARI, M., MDANGI, M., KILONZO, B. S. & MULUNGU, L. S. 2014. Prevalence of leptospirosis and toxoplasmosis: a study of rodents and shrews in cultivated and fallow land, Morogoro rural district, Tanzania. *Tanzania Journal of Health Research,* 16**,** 250-5.

MGODE, G. F., MACHANG'U, R. S., GORIS, M. G., ENGELBERT, M., SONDIJ, S. & HARTSKEERL, R. A. 2006. New *Leptospira* serovar Sokoine of serogroup Icterohaemorrhagiae from cattle in Tanzania. *International Journal of Systematic and Evolutionary Microbiology,* 56**,** 593–597.

MGODE, G. F., MACHANG'U, R. S., MHAMPHI, G. G., KATAKWEBA, A., MULUNGU, L. S., DURNEZ, L., LEIRS, H., HARTSKEERL, R. A. & BELMAIN, S. R. 2015. *Leptospira* Serovars for Diagnosis of Leptospirosis in Humans and Animals in Africa: Common Leptospira Isolates and Reservoir Hosts. *PLoS Neglected Tropical Diseases,* 9**,** e0004251.

MGODE, G. F., MHAMPHI, G., KATAKWEBA, A., PAEMELAERE, E., WILLEKENS, N., LEIRS, H., MACHANG'U, R. S. & HARTSKEERL, R. A. 2005. PCR detection of *Leptospira* DNA in rodents and insectivores from Tanzania. *Belgian Journal of Zoology,* 135**,** 17–19.

MILLIGAN, P. & BENNETT, S. 2004. Comparison of two cluster sampling methods for health surveys in developing countries. *International Journal of Epidemiology,* 33**,** 469-476.

MMBANDO, B. P., VESTERGAARD, L. S., KITUA, A. Y., LEMNGE, M. M., THEANDER, T. G. & LUSINGU, J. P. A. 2010. A progressive declining in the burden of malaria in north-eastern Tanzania. *Malaria Journal,* 9**,** 216-216.

MOHER, D., LIBERATI, A., TETZLAFF, J., ALTMAN, D. G. & GROUP, T. P. 2009. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *The British Medical Journal,* 339**,** 336–39.

MOLYNEUX, D., HALLAJ, Z., KEUSCH, G. T., MCMANUS, D. P., NGOWI, H., CLEAVELAND, S., RAMOS-JIMENEZ, P., GOTUZZO, E., KAR, K. &

SANCHEZ, A. 2011. Zoonoses and marginalised infectious diseases of poverty: Where do we stand. *Parasites and Vectors,* 4**,** 106–112.

MONAHAN, A. M., CALLANAN, J. J. & NALLY, J. E. 2009. Host-Pathogen Interactions in the Kidney during Chronic Leptospirosis. *Veterinary Pathology,* 46**,** 792-799.

MOREY, R. E., GALLOWAY, R. L., BRAGG, S. L., STEIGERWALT, A. G., MAYER, L. W. & LEVETT, P. N. 2006. Species-specific identification of Leptospiraceae by 16S rRNA gene sequencing. *Journal of Clinical Microbiology,* 44**,** 3510-3516.

MOSELEY, M., RAJERISON, M., GARIN, B., PIERTNEY, S., RAHELINIRINA, S. & TELFER, S. *in preparation*. Mixed infections of *Leptospira* in a diverse reservoir community in Madagascar: implications for transmission and emergence of a neglected zoonotic pathogen.

MOSITES, E. M., RABINOWITZ, P. M., THUMBI, S. M., MONTGOMERY, J. M., PALMER, G. H., MAY, S., ROWHANI-RAHBAR, A., NEUHOUSER, M. L. & WALSON, J. L. 2015. The Relationship between Livestock Ownership and Child Stunting in Three Countries in Eastern Africa Using National Survey Data. *PLoS One,* 10**,** e0136686.

MUGARULA, D. R. 1984. Canine leptospirosis in Tabora township [Tanzania]. *Bulletin of Animal Health and Production in Africa,* 32**,** 99–101.

MUNOZ-ZANZI, C., MASON, M., ENCINA, C., GONZALEZ, M. & BERG, S. 2014a. Household characteristics associated with rodent presence and *Leptospira* infection in rural and urban communities from Southern Chile. *The American Journal of Tropical Medicine and Hygiene,* 90**,** 497-506.

MUNOZ-ZANZI, C., MASON, M. R., ENCINA, C., ASTROZA, A. & ROMERO, A. 2014b. Leptospira contamination in household and environmental water in rural communities in southern Chile. *International Journal of Environmental Research and Public Health,* 11**,** 6666-80.

MURGIA, R., RIQUELME, N., BARANTON, G. & CINCO, M. 1997. Oligonucleotides specific for pathogenic and saprophytic *Leptospira* occurring in water. *FEMS Microbiology Letters,* 148**,** 27–34.

MURRAY, C. J., VOS, T., LOZANO, R., NAGHAVI, M., FLAXMAN, A. D., MICHAUD, C., EZZATI, M., SHIBUYA, K., SALOMON, J. A., ABDALLA, S., ABOYANS, V., ABRAHAM, J., ACKERMAN, I., AGGARWAL, R., AHN, S. Y., ALI, M. K., ALVARADO, M., ANDERSON, H. R., ANDERSON, L. M., ANDREWS, K. G., ATKINSON, C., BADDOUR, L. M., BAHALIM, A. N., BARKER-COLLO, S., BARRERO, L. H., BARTELS, D. H., BASANEZ, M. G., BAXTER, A., BELL, M. L., BENJAMIN, E. J., BENNETT, D., BERNABE, E., BHALLA, K., BHANDARI, B., BIKBOV, B., BIN ABDULHAK, A., BIRBECK, G., BLACK, J. A., BLENCOWE, H., BLORE, J. D., BLYTH, F., BOLLIGER, I., BONAVENTURE, A., BOUFOUS, S., BOURNE, R., BOUSSINESQ, M., BRAITHWAITE, T., BRAYNE, C., BRIDGETT, L., BROOKER, S., BROOKS, P., BRUGHA, T. S., BRYAN-HANCOCK, C., BUCELLO, C., BUCHBINDER, R., BUCKLE, G., BUDKE, C. M., BURCH, M., BURNEY, P., BURSTEIN, R., CALABRIA, B., CAMPBELL, B., CANTER, C. E., CARABIN, H., CARAPETIS, J., CARMONA, L., CELLA, C., CHARLSON, F., CHEN, H., CHENG, A. T., CHOU, D., CHUGH, S. S., COFFENG, L. E., COLAN, S. D., COLQUHOUN, S., COLSON, K. E., CONDON, J., CONNOR, M. D., COOPER, L. T., CORRIERE, M., CORTINOVIS, M., DE VACCARO, K. C., COUSER, W., COWIE, B. C., CRIQUI, M. H., CROSS, M., DABHADKAR, K. C., DAHIYA, M.,

DAHODWALA, N., DAMSERE-DERRY, J., DANAEI, G., DAVIS, A., DE LEO, D., DEGENHARDT, L., DELLAVALLE, R., DELOSSANTOS, A., DENENBERG, J., DERRETT, S., DES JARLAIS, D. C., DHARMARATNE, S. D., et al. 2012. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *The Lancet,* 380**,** 2197-223.

MURRAY, C. K., ELLIS, M. W. & HOSPENTHAL, D. R. 2004. Susceptibility of *Leptospira* serovars to antimalarial agents. *The American Journal of Tropical Medicine and Hygiene,* 71**,** 685-6.

MURRAY, C. K., GRAY, M. R., MENDE, K., PARKER, T. M., SAMIR, A., RAHMAN, B. A., HABASHY, E. E., HOSPENTHAL, D. R. & PIMENTEL, G. 2011. Use of patient-specific *Leptospira* isolates in the diagnosis of leptospirosis employing microscopic agglutination testing (MAT). *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 105**,** 209–13.

MURRAY, C. K., GRAY, M. R., MENDE, K., PIMENTEL, G., PARKER, T., SAMIR, A., RAHMAN, B. A. & HOSPENTHAL, D. R. 2009. Evaluation of the Use of Egyptian *Leptospira* Isolates in the Microscopic Agglutination Testing (Mat) Diagnosis of Leptospirosis. *The American Journal of Tropical Medicine and Hygiene,* 81**,** 76.

MURRAY, C. K., PIMENTEL, G., PARKER, T., BECKIUS, M. L., SAMIR, A., RHMAN, B. A., MENDE, K., GALLOWAY, R. L. & HOSPENTHAL, D. R. 2008. Antimicrobial Susceptibilities of Clinical Human Isolates of *Leptospira* from Egypt. *The American Journal of Tropical Medicine and Hygiene,* 79**,** 78.

MURRAY, G. L. 2013. The lipoprotein LipL32, an enigma of leptospiral biology. *Veterinary Microbiology,* 162**,** 305-314.

MUSSO, D. & LA SCOLA, B. 2013. Laboratory diagnosis of leptospirosis: A challenge. *Journal of Microbiology, Immunology and Infection,* 46**,** 245-252.

MWACHUI, M. A., CRUMP, L., HARTSKEERL, R., ZINSSTAG, J. & HATTENDORF, J. 2015. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLoS Neglected Tropical Diseases,* 9**,** e0003843.

MWAI, O., HANOTTE, O., KWON, Y.-J. & CHO, S. 2015. African Indigenous Cattle: Unique Genetic Resources in a Rapidly Changing World. *Asian-Australasian Journal of Animal Sciences,* 28**,** 911-921.

NASCIMENTO, A. L., VERJOVSKI-ALMEIDA, S., VAN SLUYS, M. A., MONTEIRO-VITORELLO, C. B., CAMARGO, L. E., DIGIAMPIETRI, L. A., HARSTKEERL, R. A., HO, P. L., MARQUES, M. V., OLIVEIRA, M. C., SETUBAL, J. C., HAAKE, D. A. & MARTINS, E. A. 2004. Genome features of *Leptospira interrogans* serovar Copenhageni. *Brazilian Journal of Medical and Biological Research,* 37**,** 459-77.

NELSON, L. & CLARK, F. 1973. Correction for Sprung Traps in Catch/Effort Calculations of Trapping Results. *Journal of Mammology,* 54**,** 295-298.

NEWMAN, R. C. & COHEN, H. L. 1962. Weil's disease in Cape Town. A case report. *South African Medical Journal,* 36**,** 851–853.

NILOOFA, R., FERNANDO, N., DE SILVA, N. L., KARUNANAYAKE, L., WICKRAMASINGHE, H., DIKMADUGODA, N., PREMAWANSA, G., WICKRAMASINGHE, R., DE SILVA, H. J., PREMAWANSA, S., RAJAPAKSE, S. & HANDUNNETTI, S. 2015. Diagnosis of Leptospirosis: Comparison between

Microscopic Agglutination Test, IgM-ELISA and IgM Rapid Immunochromatography Test. *PLoS One,* 10**,** e0129236.

NIMO PAINTSIL, S. C., FICHET-CALVET, E., MOHAREB, E., MORALES, M., BONNEY, J. H., OBIRI-DANSO, K., AMPOFO, W. K., SCHOEPP, R. J. & KRONMANN, K. C. 2013. Rodent species and their correlation with human seropositivity for zoonotic infections in Ghana. *The American Journal of Tropical Medicine and Hygiene,* 89 (Suppl 1)**,** 422.

O'DOHERTY, E., SAYERS, R., L, O. G. & SHALLOO, L. 2015. Effect of exposure to *Neospora caninum*, *Salmonella*, and *Leptospira interrogans* serovar Hardjo on the economic performance of Irish dairy herds. *Journal of Dairy Science,* 98**,** 2789-800.

O'MEARA, W. P., BEJON, P., MWANGI, T. W., OKIRO, E. A., PESHU, N., SNOW, R. W., NEWTON, C. R. & MARSH, K. 2008. Effect of a fall in malaria transmission on morbidity and mortality in Kilifi, Kenya. *The Lancet,* 372**,** 1555-62.

O'MEARA, W. P., MANGENI, J. N., STEKETEE, R. & GREENWOOD, B. 2010. Changes in the burden of malaria in sub-Saharan Africa. *The Lancet Infectious Diseases,* 10**,** 545–55.

ODONTSETSEG, N., SAKODA, Y. & KIDA, H. 2005. Serological evidence of the persistence of infection with *Leptospira interrogans* serovar Hardjo in cattle in Mongolia. *Microbiology and Immunology,* 49**,** 865-9.

OKEKE, I. N., LAXMINARAYAN, R., BHUTTA, Z. A., DUSE, A. G., JENKINS, P., O'BRIEN, T. F., PABLOS-MENDEZ, A. & KLUGMAN, K. P. 2005. Antimicrobial resistance in developing countries. Part I: recent trends and current status. *The Lancet Infectious Diseases,* 5**,** 481-493.

OKIRO, E. A., HAY, S. I., GIKANDI, P. W., SHARIF, S. K., NOOR, A. M., PESHU, N., MARSH, K. & SNOW, R. W. 2007. The decline in paediatric malaria admissions on the coast of Kenya. *Malaria Journal,* 6**,** 151-151.

OLIVEIRA, F., AZEVEDO, S. S., PINHEIRO, S. R., BATISTA, C. S., MORAES, Z. M., SOUZA, G. O., GONÇALES, A. P. & VASCONCELLOS, S. A. 2010. Risk factors associated with leptospirosis in cows in the state of Bahia, northeastern Brazil. *Pesquisa Veterinária Brasileira,* 30**,** 398-402.

OTTE, M. J. & CHILONDA, P. 2002. *Cattle and small ruminant production systems in sub-Saharan Africa: a systematic review,* Rome, Food and Agriculture Organisation of the United Nations.

PAGES, F., POLYCARPE, D., DEHECQ, J. S., PICARDEAU, M., CAILLERE, N., JAFFAR-BANDJEE, M. C., MICHAULT, A. & FILLEUL, L. 2014. Human leptospirosis on Reunion Island: past and current burden. *International Journal of Environmental Research and Public Health,* 11**,** 968–982.

PALANIAPPAN, R. U., CHANG, Y.-F., CHANG, C.-F., PAN, M., YANG, C., HARPENDING, P., MCDONOUGH, S. P., DUBOVI, E., DIVERS, T. & QU, J. 2005. Evaluation of *lig*-based conventional and real time PCR for the detection of pathogenic leptospires. *Molecular and Cellular Probes,* 19**,** 111–17.

PANZNER, U., PAK, G. D., AABY, P., ADU-SARKODIE, Y., ALI, M., ASEFFA, A., BAKER, S., BJERREGAARD-ANDERSEN, M., CRUMP, J. A., DEERIN, J., CRUZ ESPINOZA, L. M., GASMELSEED, N., HERINIAINA, J. N., HERTZ, J. T., IM, J., VON KALCKREUTH, V., KEDDY, K. H., LANKOANDE, B., LOFBERG, S., MEYER, C. G., ORESTO, M. M., PARK, J. K., PARK, S. E., RAKOTOZANDRINDRAINY, R., SARPONG, N., SOURA, A. B., GASSAMA

SOW, A., TALL, A., TEFERI, M., WORKU, A., YESHITELA, B., WIERZBA, T. F. & MARKS, F. 2016. Utilization of Healthcare in the Typhoid Fever Surveillance in Africa Program. *Clinical Infectious Diseases,* 62 Suppl 1**,** S56-68.

PAPPAS, G., PAPADIMITRIOU, P., SIOZOPOULOU, V., CHRISTOU, L. & AKRITIDIS, N. 2008. The globalization of leptospirosis: worldwide incidence trends. *International Journal of Infectious Diseases,* 12**,** 351-357.

PARKER, T. M., ISMAIL, T., FADEEL, M. A., MAKSOUD, M. A., MORCOS, M., NEWIRE, E., WASFY, M. O., MURRAY, C. K., PIMENTEL, G., EL-SAYED, N. & HAJJEH, R. 2006. Laboratory-based surveillance for acute febrile illness in Egypt: A focus on leptospirosis. *The American Journal of Tropical Medicine and Hygiene,* 75**,** 18.

PARKER, T. M., MURRAY, C. K., RICHARDS, A. L., SAMIR, A., ISMAIL, T., FADEEL, M. A., JIANG, J., WASFY, M. O. & PIMENTEL, G. 2007. Concurrent infections in acute febrile illness patients in Egypt. *The American Journal of Tropical Medicine and Hygiene,* 77**,** 390–92.

PAYET, M., PENE, P., DOROLLE, C. L., BONNARDOT, R., BERNOU, J. C. & RAHMI, R. 1965. Hyos leptospirosis in Dakar [Engl. summ.]. *Bulletin de la Societe de Pathologie Exotique,* 58**,** 54–59.

PAYET, M., PENE, P., SANKALE, M., BAYET, R., BONNARDOT, R., BERNOU, J. C. & FRAMENT, V. 1966. [Leptospirosis in Dakar]. *Bulletin de la Societe de Pathologie Exotique Filiales,* 59**,** 207–17.

PEREZ, J. & GOARANT, C. 2010. Rapid *Leptospira* identification by direct sequencing of the diagnostic PCR products in New Caledonia. *BMC Microbiology,* 10**,** 325.

PERRET, J. L., MOUSSAVOU-KOMBILA, J. B., DUONG, T. H., BERTHONNEAU, J. P. & NGUEMBY-MBINA, C. 1994. [First clinical and serological description of leptospirosis in Gabon]. *Bulletin de la Societe de Pathologie Exotique,* 87**,** 181–82.

PERRY, B. & GRACE, D. 2009. The impacts of livestock diseases and their control on growth and development processes that are pro-poor. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364**,** 2643–2655.

PETTI, C. A., POLAGE, C. R., QUINN, T. C., RONALD, A. R. & SANDE, M. A. 2006. Laboratory medicine in Africa: a barrier to effective health care. *Clinical Infectious Diseases,* 42**,** 377–82.

PICARDEAU, M. 2015. Genomics, Proteomics, and Genetics of *Leptospira*. *In:* ADLER, B. (ed.) *Leptospira and Leptospirosis.* Berlin: Springer-Verlag

PICARDEAU, M., BERTHERAT, E., JANCLOES, M., SKOULOUDIS, A. N., DURSKI, K. & HARTSKEERL, R. A. 2014. Rapid tests for diagnosis of leptospirosis: Current tools and emerging technologies. *Diagnostic Microbiology and Infectious Disease,* 78**,** 1–8.

PICARDEAU, M., BULACH, D. M., BOUCHIER, C., ZUERNER, R. L., ZIDANE, N., WILSON, P. J., CRENO, S., KUCZEK, E. S., BOMMEZZADRI, S., DAVIS, J. C., MCGRATH, A., JOHNSON, M. J., BOURSAUX-EUDE, C., SEEMANN, T., ROUY, Z., COPPEL, R. L., ROOD, J. I., LAJUS, A., DAVIES, J. K., MEDIGUE, C. & ADLER, B. 2008. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS One,* 3**,** e1607.

PINN, T. G. 1992. Leptospirosis in the Seychelles. *Medical Journal of Australia,* 156**,** 163–67.

PIPER, E. K., JONSSON, N. N., GONDRO, C., LEW-TABOR, A. E., MOOLHUIJZEN, P., VANCE, M. E. & JACKSON, L. A. 2009. Immunological profiles of *Bos taurus* and *Bos indicus* cattle infested with the cattle tick, *Rhipicephalus* (*Boophilus*) *microplus*. *Clinical and Vaccine Immunology,* 16**,** 1074-1086.

QEKWANA, N. D. & OGUTTU, J. W. 2014. Assessment of food safety risks associated with preslaughter activities during the traditional slaughter of goats in Gauteng, South Africa. *Journal of Food Protection,* 77**,** 1031-7.

QIAGEN 2012. *QIAamp® DNA Mini and Blood Mini Handbook*.

R CORE TEAM 2015. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

RADEMAN, J., STEYTLER, J. G. & WRIGHT, N. 1964. First isolations of Leptospirae in Cape Town. *South African Medical Journal,* 38**,** 694–96.

RADOSTITS, O., GAY, C., BLOOD, D. & HINCHCLIFF, K. 2000. Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses WB Saunders. *New York, USA*.

RAHELINIRINA, S., LEON, A., HARSTSKEERL, R. A., SERTOUR, N., AHMED, A., RAHARIMANANA, C., FERQUEL, E., GARNIER, M., CHARTIER, L., DUPLANTIER, J. M., RAHALISON, L. & CORNET, M. 2010. First isolation and direct evidence for the existence of large small-mammal reservoirs of *Leptospira* sp. in Madagascar. *PLoS One,* 5**,** e14111.

REIS, R. B., RIBEIRO, G. S., FELZEMBURGH, R. D. M., SANTANA, F. S., MOHR, S. & MELENDEZ, A. X. T. O., ET AL 2008. Impact of Environment and Social Gradient on *Leptospira* infection in Urban Slums. *PLoS Neglected Tropical Diseases,* 2**,** e228. doi:10.1371/journal.pntd.0000228.

RENAULT, P., BOIDIN, E., D'ORTENZIO, E., BALLEYDIER, E., DANIEL, B. & FILLEUL, L. 2011. Surveillance épidémiologique de la leptospirose à la Réunion, 2004-2008: possible impact de l'épidémie de chikungunya sur la létalité de la leptospirose. *Bulletin de la Societe de Pathologie Exotique,* 104**,** 148–52.

REYBURN, H., MBATIA, R., DRAKELY, C., CARNEIRO, I., MWAKASUNGULA, E., MWERINDE, O., SAGANDA, K., SHAO, J., KITUA, A., OLOMI, R., GREENWOOD, B. M. & WHITTY, C. J. 2004. Overdiagnosis of malaria in patients with severe febrile illness in Tanzania: a prospective study. *The British Medical Journal,* 329**,** doi: 10.1136/bmj.38251.658229.55.

ROJAS, P., MONAHAN, A. M., SCHULLER, S., MILLER, I. S., MARKEY, B. K. & NALLY, J. E. 2010. Detection and quantification of leptospires in urine of dogs: a maintenance host for the zoonotic disease leptospirosis. *European Journal of Clinical Microbiology and Infectious Diseases,* 29**,** 1305-9.

ROYAL TROPICAL INSTITUTE (KIT). 2014. *Leptospira library* [Online]. Amsterdam: KIT Biomedical Research. Available: http://www.kit.nl/NET/Leptospirosis/LeptospiraLibrary/ [Accessed 2015].

RUTHERFORD, M. E., MULHOLLAND, K. & HILL, P. C. 2010. How access to health care relates to under-five mortality in sub-Saharan Africa: systematic review. *Tropical Medicine & International Health,* 15**,** 508–519.

RUWANPURA, R., RATHNAWEERA, A., HETTIARACHCHI, M., DHAHANAYAKE, K. & AMARARATNE, S. 2012. Severe pulmonary leptospirois associated with high fatality rate: an autopsy series in Galle, southern Sri Lanka. *Medical Journal of Malaysia,* 67**,** 595-600.

RYAN, E. G., LEONARD, N., O'GRADY, L., DOHERTY, M. L. & MORE, S. J. 2012. Herd-level risk factors associated with *Leptospira* Hardjo seroprevalence in Beef/Suckler herds in the Republic of Ireland. *Irish Veterinary Journal,* 65**,** 6.

SAAH, A. J. & HOOVER, D. R. 1997. "Sensitivity" and "specificity" reconsidered: the meaning of these terms in analytical and diagnostic settings. *Annals of Internal Medicine,* 126**,** 91-4.

SAINT GIRONS, I., NORRIS, S. J., GÖBEL, U., MEYER, J., WALKER, E. M. & ZUERNER, R. 1992. Genome structure of spirochetes. *Research in Microbiology,* 143**,** 615-621.

SAKUNDARNO, M., BERTOLATTI, D., MAYCOCK, B., SPICKETT, J. & DHALIWAL, S. 2014. Risk factors for leptospirosis infection in humans and implications for public health intervention in Indonesia and the Asia-Pacific region. *Asia Pacific Journal of Public Health,* 26**,** 15-32.

SALGADO, M., OTTO, B., SANDOVAL, E., REINHARDT, G. & BOQVIST, S. 2014. A cross sectional observational study to estimate herd level risk factors for *Leptospira* spp. serovars in small holder dairy cattle farms in southern Chile. *BMC Veterinary Research,* 10**,** 126.

SAMSON, R. I. & PILLAY, T. S. 1966. Leptospirosis in Cape Town. *South African Medical Journal,* 40**,** 1010–11.

SANKALÉ, M., SOW, A. M., RUSCHER, H. & SARRAT, H. 1973. [Leptospirosis in the hospital environment in Dakar (results of a new survey)]. *Bulletin de la Societe Medicale d'Afrique Noir de Langue Francaise,* 18**,** 227–35.

SARKAR, U., NASCIMENTO, S. F., BARBOSA, R., MARTINS, R., NUEVO, H., KALOFONOS, I., KALAFANOS, I., GRUNSTEIN, I., FLANNERY, B. & DIAS, J. 2002. Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. *The American Journal of Tropical Medicine and Hygiene,* 66**,** 605-610.

SCHELLING, E., GRACE, D., WILLINGHAM III, A. L. & RANDOLPH, T. 2007. Research approaches for improved pro-poor control of zoonoses. *Food and Nutrition Bulletin,* 28**,** 345S–356S.

SCHOONMAN, L. & SWAI, E. S. 2009. Risk factors associated with the seroprevalence of leptospirosis, amongst at-risk groups in and around Tanga city, Tanzania. *Annals of Tropical Medicine and Parasitology,* 103**,** 711-8.

SCHOONMAN, L. & SWAI, E. S. 2010. Herd- and animal-level risk factors for bovine leptospirosis in Tanga region of Tanzania. *Tropical Animal Health and Production,* 42**,** 1565-72.

SCHRADER, C., SCHIELKE, A., ELLERBROEK, L. & JOHNE, R. 2012. PCR inhibitors–occurrence, properties and removal. *Journal of Applied Microbiology,* 113**,** 1014-1026.

SERGEANT, E. S. G. 2016. *Epitools epidemiological calculators* [Online]. AusVet Animal Health Services and Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease Available: http://epitool.ausvet.com.au [Accessed].

SHINSUGI, C., MATSUMURA, M., KARAMA, M., TANAKA, J., CHANGOMA, M. & KANEKO, S. 2015. Factors associated with stunting among children according to the level of food insecurity in the household: a cross-sectional study in a rural community of Southeastern Kenya. *BMC Public Health,* 15**,** 441.

SILVERIE, R., MONNIER, M. & LATASTE-DOROLLE, C. 1968. Leptospirosis in Madagascar. Human, bovine and porcine leptospiroses of the southern region. *Bulletin de la Societe de Pathologie Exotique,* 61**,** 346–59.

SIMPSON, F. G., GREEN, K. A., HAUG, G. J. & BROOKES, D. L. 1998. Leptospirosis associated with severe pulmonary haemorrhage in Far North Queensland. *Medical Journal of Australia,* 169**,** 151-3.

SMYTHE, L. D., SMITH, I. L., SMITH, G. A., DOHNT, M. F., SYMONDS, M. L., BARNETT, L. J. & MCKAY, D. B. 2002. A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infectious Diseases,* 2**,** 13.

SMYTHE, L. D., WUTHIEKANUN, V., CHIERAKUL, W., SUPUTTAMONGKOL, Y., TIENGRIM, S., DOHNT, M. F., SYMONDS, M. L., SLACK, A. T., APIWATTANAPORN, A., CHUEASUWANCHAI, S., DAY, N. P. & PEACOCK, S. J. 2009. The microscopic agglutination test (MAT) is an unreliable predictor of infecting *Leptospira* serovar in Thailand. *Am J Trop Med Hyg,* 81**,** 695-7.

SOORS, W., DKHIMI, F. & CRIEL, B. 2013. Lack of access to health care for African indigents: a social exclusion perspective. *International Journal for Equity in Health,* 12**,** 91.

STODDARD, R. A. 2013. Detection of pathogenic *Leptospira* spp. through real-time PCR (qPCR) targeting the LipL32 gene *In:* WILKS, M. (ed.) *PCR Detection of Microbial Pathogens.* Second Edition ed.

STODDARD, R. A., GEE, J. E., WILKINS, P. P., MCCAUSTLAND, K. & HOFFMASTER, A. R. 2009. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagnostic Microbiology and Infectious Disease,* 64**,** 247–55.

SUBHARAT, S., WILSON, P., HEUER, C. & COLLINS-EMERSON, J. 2012. Longitudinal serological survey and herd-level risk factors for *Leptospira* spp. serovars Hardjo-bovis and Pomona on deer farms with sheep and/or beef cattle. *New Zealand Veterinary Journal,* 60**,** 215-22.

SUPUTTAMONGKOL, Y., NIWATTAYAKUL, K., SUTTINONT, C., LOSUWANALUK, K., LIMPAIBOON, R., CHIERAKUL, W., WUTHIEKANUN, V., TRIENGRIM, S., CHENCHITTIKUL, M. & WHITE, N. J. 2004. An Open, Randomized, Controlled Trial of Penicillin, Doxycycline, and Cefotaxime for Patients with Severe Leptospirosis. *Clinical Infectious Diseases,* 39**,** 1417–24.

SUTTINONT, C., LOSUWANALUK, K., NIWATAYAKUL, K., HOONTRAKUL, S., INTARANONGPAI, W., SILPASAKORN, S., SUWANCHAROEN, D., PANLAR, P., SAISONGKORH, W., ROLAIN, J. M., RAOULT, D. & SUPUTTAMONGKOL, Y. 2006. Causes of acute, undifferentiated, febrile illness in rural Thailand: results of a prospective observational study. *Annals of Tropical Medicine and Parasitology,* 100**,** 363–70.

SWAI, E. S. & SCHOONMAN, L. 2012. A survey of zoonotic diseases in trade cattle slaughtered at Tanga city abattoir: a cause of public health concern. *Asian Pacific Journal of Tropical Biomedicine,* 2**,** 55-60.

SWAI, E. S., SCHOONMAN, L. & MACHANG'U, R. 2005. Prevalence and factors associated with of bovine leptospirosis in small scale dairy farms in Tanga Region, Tanzania. *Bulletin of Animal Health and Production in Africa,* 53**,** 51-59.

SYKES, J., HARTMANN, K., LUNN, K. F., MOORE, G., STODDARD, R. & GOLDSTEIN, R. 2011. 2010 ACVIM small animal consensus statement on

leptospirosis: diagnosis, epidemiology, treatment, and prevention. *Journal of Veterinary Internal Medicine,* 25**,** 1-13.

TABEL, H. & LOSOS, G. 1979. Report on an outbreak of bovine leptospirosis in Kenya due to *Leptospira grippotyphosa*. *Bulletin of Animal Health and Production in Africa,* 27**,** 61–64.

TAGOE, J. A., PUPLAMPU, N., ODOOM, S. C., ABDUL-RAHMAN, B., HABASHY, E. E., PIMENTEL, B., KRONMANN, K., KORAM, K., WILSON, M., ABDEL, M., NYARKO, E. & AGBENOHEVI, P. 2010. Serosurvey of Leptospirosis among Patients with Acute Febrile Illness in Accra. *The American Journal of Tropical Medicine and Hygiene,* 83**,** 306.

TAKAFUJI, E. T., KIRKPATRICK, J. W., MILLER, R. N., KARWACKI, J. J., KELLEY, P. W., GRAY, M. R., MCNEILL, K. M., TIMBOE, H. L., KANE, R. E. & SANCHEZ, J. L. 1984. An efficacy trial of doxycycline chemoprophylaxis against leptospirosis. *New England Journal of Medicine,* 310**,** 497-500.

TAMURA, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Molecular Biology and Evolution,* 9**,** 678-87.

TANZANIA NATIONAL BUREAU OF STATISTICS 2002. Population and Housing Census.

TANZANIA NATIONAL BUREAU OF STATISTICS 2012. Population and Housing Census.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & BELMAIN, S. 2008. Understanding and managing sanitary risks due to rodent zoonoses in an African city: beyond the Boston Model. *Integrative Zoology,* 3**,** 38–50.

TE BRUGGE, L. A. & DREYER, T. 1985. *Leptospira-Interrogans* serovar Hardjo Associated with Bovine Abortion in South Africa. *Onderstepoort Journal of Veterinary Research,* 52**,** 51–52.

THAIPADUNGPANIT, J., CHIERAKUL, W., WUTHIEKANUN, V., LIMMATHUROTSAKUL, D., AMORNCHAI, P., BOONSLIP, S., SMYTHE, L. D., LIMPAIBOON, R., HOFFMASTER, A. R. & DAY, N. 2011. Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and *s*L32 genes for human leptospirosis in Thailand: a case-control study. *PLoS One,* 6**,** e16236.

THAIPADUNGPANIT, J., WUTHIEKANUN, V., CHIERAKUL, W., SMYTHE, L. D., PETKANCHANAPONG, W., LIMPAIBOON, R., APIWATANAPORN, A., SLACK, A. T., SUPUTTAMONGKOL, Y., WHITE, N. J., FEIL, E. J., DAY, N. P. & PEACOCK, S. J. 2007. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. *PLoS Neglected Tropical Diseases,* 1**,** e56.

THE FIELD MUSEUM. 2011. *Mammals of Tanzania: Order Rodentia* [Online]. Chicago. Available: http://archive.fieldmuseum.org/tanzania/ [Accessed 2013 to 2015].

THEUERKAUF, J., PEREZ, J., TAUGAMOA, A., NIUTOUA, I., LABROUSSE, D., GULA, R., BOGDANOWICZ, W., JOURDAN, H. & GOARANT, C. 2013. Leptospirosis risk increases with changes in species composition of rat populations. *Naturwissenschaften,* 100**,** 385-8.

THIERMANN, A. B. 1982. Experimental leptospiral infections in pregnant cattle with organisms of the Hebdomadis serogroup. *American Journal of Veterinary Research,* 43**,** 780-4.

THIERMANN, A. B. 1983. Bovine leptospirosis: bacteriologic versus serologic diagnosis of cows at slaughter. *American Journal of Veterinary Research,* 44**,** 2244-5.

THRUSFIELD, M. 1995. *Veterinary Epidemiology,* Oxford, Blackwell Science.

TIBARY, A., FITE, C., ANOUASSI, A. & SGHIRI, A. 2006. Infectious causes of reproductive loss in camelids. *Theriogenology,* 66**,** 633-47.

TORGERSON, P. R., HAGAN, J. E., COSTA, F., CALCAGNO, J., KANE, M., MARTINEZ-SILVEIRA, M. S., GORIS, M. G., STEIN, C., KO, A. I. & ABELA-RIDDER, B. 2015. Global Burden of Leptospirosis: Estimated in Terms of Disability Adjusted Life Years. *PLoS Neglected Tropical Diseases,* 9**,** e0004122.

UNITED NATIONS STATISTICS DIVISION. 2012. *Composition of macro geographical (continental) regions, geographical sub-regions, and selected economic and other groupings* [Online]. Available: http://unstats.un.org/unsd/methods/m49/m49regin.htm [Accessed 3rd February 2014].

URI GENOMICS & SEQUENCING CENTER. 2004. *Calculator for determining the number of copies of a template* [Online]. Available: cels.uri.edu/gsc.cndna.html [Accessed].

VALLEE, E., HEUER, C., COLLINS-EMERSON, J. M., BENSCHOP, J. & WILSON, P. R. 2015. Serological patterns, antibody half-life and shedding in urine of Leptospira spp. in naturally exposed sheep. *New Zealand Veterinary Journal,* 63**,** 301-12.

VAN DE WEYER, L. M., HENDRICK, S., ROSENGREN, L. & WALDNER, C. L. 2011. Leptospirosis in beef herds from western Canada: serum antibody titers and vaccination practices. *Canadian Veterinary Journal,* 52**,** 619-26.

VAN RENSBURG, W. J. J. 1973. Isolation of *Leptospira-Canicola* in Pigs and Dogs in South Africa. *Journal of the South African Veterinary Association,* 44**,** 435–36.

VAN RIEL, J., SZPAJSHENDLER, L. & VAN RIEL, M. 1956. Clinical, bacteriological and epidemiological study of a new focus of leptospirosis in the Belgian Congo. *Bulletin de la Societe de Pathologie Exotique Filiales,* 49**,** 118–43.

VAN SCHAIK, G., SCHUKKEN, Y. H., NIELEN, M., DIJKHUIZEN, A. A., BARKEMA, H. W. & BENEDICTUS, G. 2002. Probability of and risk factors for introduction of infectious diseases into Dutch SPF dairy farms: a cohort study. *Preventive Veterinary Medicine,* 54**,** 279-89.

VIANA, M., MANCY, R., BIEK, R., CLEAVELAND, S., CROSS, P. C., LLOYD-SMITH, J. O. & HAYDON, D. T. 2014. Assembling evidence for identifying reservoirs of infection. *Trends in Ecology & Evolution,* 29**,** 270-279.

VICTORIA, B., AHMED, A., ZUERNER, R. L., AHMED, N., BULACH, D. M., QUINTEIRO, J. & HARTSKEERL, R. A. 2008. Conservation of the S10-spc-Œ± locus within otherwise highly plastic genomes provides phylogenetic insight into the genus *Leptospira*. *PLoS ONE,* 3**,** e2752.

VINETZ, J. M. 2003. A mountain out of a molehill: do we treat acute leptospirosis, and if so, with what? *Clinical Infectious Diseases,* 36**,** 1514-1515.

WARNES, G., BOLKER, B., BONENAKKER, L., GENTLEMAN, R., HUBER, W., LIAW, A., LUMLEY, T., MAECHLER, M., MAGNUSSON, A., MOELLER, S., SCHWARTZ, M. & VENABLES, B. 2015. gplots: Various R programming tools for plotting data. Version 2.17.0 ed.

WATSON, J. T., GAYER, M. & CONNOLLY, M. A. 2007. Epidemics after natural disasters. *Emerging Infectious Diseases,* 13**,** 1-5.

WILLIAMS, D. & WINDEN, S. V. 2014. Risk factors associated with high bulk milk antibody levels to common pathogens in UK dairies. *The Veterinary Record,* 174**,** 580.

WILSON, D. E. & REEDER, D. M. 2005. *Mammal Species of the World: A Taxonomic and Geographic Reference*, Johns Hopkins University Press.

WOO, T. H., PATEL, B. K., SMYTHE, L. D., SYMONDS, M. L., NORRIS, M. A. & DOHNT, M. F. 1997. Identification of pathogenic *Leptospira* genospecies by continuous monitoring of fluorogenic hybridization probes during rapid-cycle PCR. *Journal of Clinical Microbiology,* 35**,** 3140–46.

WORLD HEALTH ORGANIZATION 2006. The Control of Neglected Disease: a route to poverty alleviation. *World Health Organization: Report of a joint WHO/DFID-AHP meeting, 20-21 September 2005.* Geneva.

WORLD HEALTH ORGANIZATION 2011. Report of the Second Meeting of the Leptospirosis Burden Epidermiology Reference Group. Geneva: World Health Organization.

WORLD ORGANISATION FOR ANIMAL HEALTH (OIE) 2008. *OIE Manual of Diagnostic Tests and Vaccines for Terestrial Animals*

WORLD ORGANISATION FOR ANIMAL HEALTH (OIE) 2014. Leptospirosis *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.*

WRIGHT, K. 2015. corrgram: Plot a Correlogram. Version 1.8 ed.

WUTHIEKANUN, V., AMORNCHAI, P., LANGLA, S., WHITE, N. J., DAY, N. P., LIMMATHUROTSAKUL, D. & PEACOCK, S. J. 2015. Antimicrobial Disk Susceptibility Testing of *Leptospira* spp. Using Leptospira Vanaporn Wuthiekanun (LVW) Agar. *The American Journal of Tropical Medicine and Hygiene,* 93**,** 241-3.

XU, Y., ZHU, Y., WANG, Y., CHANG, Y.-F., ZHANG, Y., JIANG, X., ZHUANG, X., ZHU, Y., ZHANG, J., ZENG, L., YANG, M., LI, S., WANG, S., YE, Q., XIN, X., ZHAO, G., ZHENG, H., GUO, X. & WANG, J. 2016. Whole genome sequencing revealed host adaptation-focused genomic plasticity of pathogenic *Leptospira*. *Scientific Reports,* 6**,** 20020.

YAN, K. T., ELLIS, W. A., MACKIE, D. P., TAYLOR, M. J., MCDOWELL, S. W. J. & MONTGOMERY, J. M. 1999. Development of an ELISA to detect antibodies to a protective lipopolysaccharide fraction of *Leptospira borgpetersenii* serovar Hardjo in cattle. *Veterinary Microbiology,* 69**,** 173-187.

YE, Y., MADISE, N., NDUGWA, R., OCHOLA, S. & SNOW, R. W. 2009. Fever treatment in the absence of malaria transmission in an urban informal settlement in Nairobi, Kenya. *Malaria Journal,* 8**,** 160.

YERSIN, C., BOVET, P., MERIEN, F., WONG, T., PANOWSKY, J. & PEROLAT, P. 1998. Human leptospirosis in the Seychelles (Indian Ocean): a population-based study. *The American Journal of Tropical Medicine and Hygiene,* 59**,** 933–40.

ZALTZMAN, M., KALLENBACH, J. M., GOSS, G. D., LEWIS, M., ZWI, S. & GEAR, J. H. S. 1981. Adult Respiratory Distress Syndrome in *Leptospira-Canicola* Infection. *The British Medical Journal,* 283**,** 519–20.

ZHANG, H. L., MNZAVA, K. W., MITCHELL, S. T., MELUBO, M. L., KIBONA, T. J., CLEAVELAND, S., KAZWALA, R. R., CRUMP, J. A., SHARP, J. P. & HALLIDAY, J. E. B. 2016. Mixed Methods Survey of Zoonotic Disease Awareness and Practice among Animal and Human Healthcare Providers in Moshi, Tanzania. *PLoS Neglected Tropical Diseases,* 10**,** e0004476.

ZHANG, Y., LOU, X. L., YANG, H. L., GUO, X. K., ZHANG, X. Y., HE, P. & JIANG, X. C. 2012. Establishment of a leptospirosis model in guinea pigs using an epicutaneous inoculations route. *BMC Infectious Diseases,* 12**,** 20.

ZIMMERMANN, S., TER MEULEN, A., CALVET, E., KOIVOGUI, L., SYLLA, O., GORIS, M., HARTSKEERL, R. & TER MEULEN, J. 2007. Seroprevalence and reservoirs of leptospirosis in Conakry (Guinea). *International Journal of Antimicrobial Agents,* 29**,** S49.

ZINSSTAG, J., SCHELLING, E., ROTH, F., BONFOH, B., DE SAVIGNY, D. & TANNER, M. 2007. Human benefits of animal interventions for zoonosis control. *Emerging Infectious Diseases,* 13**,** 527.

ZINSSTAG, J., SCHELLING, E., WALTNER-TOEWS, D. & TANNER, M. 2011. From “one medicine” to “one health” and systemic approaches to health and well-being. *Preventive Veterinary Medicine,* 101**,** 148-156.