THE PREVALENCE OF *LEPTOSPIRA* SPECIES IN FRESH WATER FISH IN SELECTED AREAS OF MOROGORO MUNICIPALITY, TANZANIA

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ABSTRACT

Leptospirosis is a neglected zoonotic disease of world wide public health importance. Rodents are major reservoirs of leptospira although wetland and aquatic migratory birds also carry and transmit leptospira. Studies on leptospirosis in fish are few in African countries, including Tanzania, despite favourable environment and abundant reservoirs, which can spread leptospires into aquatic habitats and infect fish. The objective of this study was to determine the presence of *Leptospira* spp in fish; the prevalent Leptospira serovars and whether they are related to serovars reported in animals; and their potential public health risk. A Multistage sampling technique was performed in Morogoro municipality, Tanzania, between November- 2016 and February -2017. Before the sampling, knowledge, attitude and practices (KAP) questionnaire was administered to assess the awareness of people (fishermen, fish mongers) on risk factors that might lead to the diversity of *Leptospira* species and water quality for pH , Dissolved Oxygen and Temperature was measured using Meter Sensor at Sample sites in Morogoro Municipality.Live catfish (n=193) were caught from five different locations, namely Sokoine University of Agriculture Magadu farm, Mzumbe University waste water pond, Morogoro Urban Water Supply and Sanitation Authority (MORUWASA)- Mafisa waste water ponds , Kingolwira Centre for Fish Farming and Fingering Production and Mkindo Fish Farmers in Morogoro Urban and mvomero districts Tanzania where blood and biopsy samples were collected. Microscopic agglutination test (MAT) was used to detect antibody against four leptospiral antigens, including local serovars Sokoine (serogroup Icterohaemorrhagiae); Hebdomadis (serogroup Hebdomadis); Kenya (serogroup Ballum) and Pomona (serogroup Pomona). Samples with MAT titers ≥ 1:160

were scored as higher values while samples with MAT titers ranging from 1:20 to1:80 were scored as lower values for seropositivity. Absence of agglutination titers was scored as negative. All MAT samples, including the low titre samples were subjected to PCR using Lepat and Sapro primers for the pathogenic and non-pathogenic species (saprophytic) respectively. Tissue samples were prepared by grinding freshly obtained kidneys of sharptooth catfish (*Clarias gariepinus*) for culturing in Fletchers medium. Dark-field microscopy readings of each of the tubes were performed at first,second, fourth, and sixth week after culturing at 20 X magnification to assess bacterial growth, of the total 193 samples tested, 29 (15 %) were positive with MAT. The major circulating leptospiral serovars were Pomona (11.4 %,), Keny*a* (1.55%), Hebdomadis (1.55%) and Sokoine (0.52%). By using PCR methods and of the 193 samples screened, 4 (2%) were positive for pathogenic leptospira. No isolation of leptospira was achieved from the kidney cultures. This study has shown a relatively high seroprevalence 15% of leptospirosis in common fresh water fish type, compared to warm blooded animals hence indicate an extended public health risk, of leptospirosis to fish handlers and consumers .To minmize leptospirosis transmission in Morogoro region, and Tanzania at large, where the disease is little known and thus neglected, it is recommended, to increase public awareness of leptospirosis in the general public, and particularly those groups at risk such as farmers, livestock keepers, fishermen, sewerage and abbatoir workers. Routine screening for leptospirosis should be considered in people engaged with occupational activities that increase contact with environments likely to contain leptospires. This is especially important in situation where malaria, typhoid and other common febrile diseases are ruled out; Isolation of leptospires from different hosts and sources should be emphasized in order to understand the sources of infections, infecting

serovars and developing a protocol for the diagnosis of this infectious disease appropriate for different areas. Rapid serological tests not involving live microorganisms for routine diagnosis is highly recommended.

DECLARATION

I, Mbambagwa Noel Alexander, declare that this dissertation is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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DEDICATION

To my parents for their love and encouragement

To my wife Prisca Furaha for her love and trust in me

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ABREVIATIONS AND SYMBOLS

bp	Base pair
^{0}C	Centigrade
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DO	Dissolved oxygen
EtBr	Ethidium bromide
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMJH	Ellinghausen, McCullough, Johnson and Harris
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LFA	Lateral flow assay
LPS	Lipopolysaccharides
MSAT	Macroscopic slide agglutination test
MAT	Microscopic agglutination test
µl	Microliter
Mg/L	Miligram per liter
MORUWASA	Morogoro Urban Water Supply and Sanitation Authority
KN	Kingolwira
M	Magada
MK	Mkindo

MZ	Mzumbe
OMPS	Outer membrane proteins
%	Percentage
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
KAP	Knowledge, Attitude and Practices
RDT	Rapid diagnostic test
r.p.m.	Revolution per minute
RNA	Ribonucleic acid
SUA	Sokoine University of Agriculture
spp	Species
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION.

1.1 Background Information.

Leptospirosis is a zoonotic disease caused by a spirochete bacterium of the genus Leptospira that affect humans and animals worldwide (Allan *et al*., 2015). Rodents are the major reservoirs of Leptospira bacteria (Biggs *et al*., 2011). Transmission of leptospirosis is facilitated by the survival of pathogenic leptospires in moist environments outside their mammalian hosts. Other wild animals and birds found in wetland areas may also carry and disseminate leptospires into the environment (Mgode *et al.*, 2014). Leptospires can live longer in water and moist soils at pH6–8, and have the ability to form biofilms during interaction with environmental bacteria (Barragán *et al*., 2011). There are pathogenic, saprophytic and intermediary leptospires found in natural environments and in animal hosts (Mgode *et al*., 2014a). Humans get leptospirosis through contaminated environments and /or by direct contact with infectious materials, especially urine and blood from infected animals. The disease is associated with certain occupational activities, such as rice and sugarcane farming, fishing and fish farming, livestock keeping, handling of animal products and water sports (Wasiński and Dutkiewicz 2013) . Clinical symptoms of leptospirosis in humans are variable (deVries *et al*., 2014), and may be easily mistaken for other febrie diseases like malaria.

The incidence of leptospirosis is significantly higher in countries with warm climate than in temperate regions due mainly to longer survival of leptospires in the environment in

warm, humid conditions. However, in most tropical countries there are greater possibilities for exposure of the human population to infected animals, whether livestock, domestic pets, or wild or feral animals (Orry *et al*.,2016). The disease is seasonal, with peak incidences occurring in summer or fall in temperate regions where the temperature is the limiting factor in survival of leptospires . Rapid dessication would otherwise prevent survival. Little is known about the mechanisms by which pathogenic leptospires persist in aqueous environments, outside the mammalian host. Both pathogenic and saprophytic strains can be isolated from rivers and lakes (Lehmann *et al*., 2014). Threatening leptospirosis infections with serogroup Icterohaemorrhagie have occurred in fish farmers, in late 1980 and early
1981 (Stuart, 1939).

Three cases of leptospirosis, including one death, due to strains of serogroup Icterohaemorrhagiae occurred amongst fish farmers in the United Kingdom. A preliminary serological survey of the workers, on the farm where two of the cases occurred, and on two other farms in the same area, suggested an appreciable occupational risk of leptospiral infection (Waitkins *et al,* 1986). Two of the three farms were also heavily infested with rats. Only one previous case of leptospirosis in a fish farmer had been reported in the United Kingdom; in 1968, when an Essex man recovered from serologically confirmed leptospirosis, serogroup Icterohaemorrhagiae.

Waterborne Leptospiral infections have also been reported in persons practicing kayaking, rafting, fishing (Bourhy *et al*., 2014). An interesting case was the diagnosis of

leptospirosis in cavers, who acquired the infection when crossing underground streams probably contaminated by rodent urine. An outbreak of leptospirosis among boys attending a Scout camp was reported in Belgium in August 2012 (Vein *et al.*, 2012).

Recent cases of leptospirosis have been reported on a Boy Scounting camping event in Luxembourg (Monahan *et al*., 2009) .The first serological report of leptospirosis in fish in Tanzania showed a prevalence of 54.3% in common fish types caught from the freshwater Mindu Dam in Morogoro Municipality. The report suggested a potential public health threat to fishermen and people who carry out water related occupations in Tanzania (Mgode *et al.*, 2014b) . The present study aimed at demonstrating the presence of Leptospira spp in fresh water fish from water bodies in Morogoro Municipality and surroundings by isolating the microorganisms and their characterization by serological and molecular methods, Using reference Leptospira serovars reported in animals and humans in this region (Machang'u *et al.*, 2004).

1.2 Problem Statement and Justification.

There is an increase in the spread of *Leptospira* spp in the environment, animals and humans (Wasiński and Dutkiewicz, 2013). Also, there is little knowledge on the public health significance among the population who are at the risk of exposure to the pathogen through their activities (Chipwaza *et al*., 2015). The relatively high seroprevalence of leptospirosis, in various animal hosts and humans, reported in Tanzania, suggests that this disease could be prevalent in aquatic hosts, such as freshwater fish (Mgode *et al.*, 2014a), which maintain the pathogen and disseminate it to humans.

Studies in Morogoro Tanzania have reported leptospirosis in fish and fish farmers (Mgode *et al.,* 2014b). Human leptospirosis cases associated with freshwater environment especially stagnant water and fish pond areas have been reported in UK (Gill *et al*., 1985). Such studies are lacking in countries such as Tanzania with a favorable environment for maintenance and dissemination of leptospirosis. The objectives of this study was to demonstrate the presence of Leptospira spp in fresh water fish in the Morogoro Municipality area by serological, cultural, and molecular techniques. The information obtained will add to previous serological reports and will be useful for promoting public awareness of this neglected zoonotic disease among people living around Morogoro Municipality

1.3 Objectives.

1.3.1 Main Objective.

To determine the prevalence of *Leptospira* spp in fresh water fish in selected areas of Morogoro Municipality, Tanzania.

1.3.2 Specific Objectives.

i. To demonstrate further, the presence of Leptospira spp in fresh water fish in Morogoro Municipality by serological and molecular techniques.
ii. To isolate and characterize leptospiral strains from fresh fish by cultural and molecular techniques.

1.4 Research Questions.

i. What are the species and serotypes/serovars of the genus Leptospira are present in fresh water fish using the micro agglutination assay and Polymarase Chain Reaction.

ii What are the limitation of identifying *Leptospira* spp from fresh water fish compared to mammalian species.

CHAPTER TWO

2.0 LITERATURE REVIEW.

2.1 Leptospiral species.

The leptospires are pathogenic spirochetes distributed worldwide (Binti and Shamhari, 2015). They are morphologically corkscrew-shaped. The microorganism belong to the family Leptospiraceae and the genus Leptospira, and differ from other spirochetes by the presence of terminal hooks. For the agent to survive, it requires long-fatty acids, vitamin B_1 and B_{12}. It grows well in a media of pH 7.4 (neutral) at 30^0C with maximum aeration (World Health Organization, 2003).The Leptospira spp do not survive in freezing conditions and can be easily killed by dehydration or temperatures in excess of 50^0C for 4 hours (Barragán *et al*., 2011) It is denatured or inactivated under 70% ethanol, detergents and acids. The genus Leptospira, was until recently classified into two species, *L.* interrogans and *L.* biflexa, comprising of pathogenic and non- pathogenic strains, respectively. Within each species, large numbers of serovars were differentiated using agglutinating antibodies. Serovar specificity is conferred by lipopolysaccharide (LPS) O antigens (Gill *et al*., 1985). More than 250 serovars of pathogenic leptospires have been described. Given the large number of serovars, antigenically related serovars are grouped into serogroups in serological testing.

Leptospires are currently classified into a number of species defined by their degree of genetic relatedness, determined by DNA reassociation (Pui *et al*., 2017). There are currently 14 named species, including pathogens (L. interrogans), non-pathogenic saprophytes (L. biflexa) and species of indeterminate pathogenicity (World Health Organization, 2010).

The leptospira survive longest in warm, humid, tropical and sub-tropical conditions, but also persist in temperate regions (Ningal *et al*., 2015). In 1886, Adolf Weil reported the leptospira as the causative agent of the disease in Japan in 1886, among affected coal miners (de Vries . *et al.,* 2014). The Leptospira spp cause infections in organs and tissue following contact with contaminated environment such as excretion of infected urine from hosts which then contaminate soil, water and food. Also, when intact skin is immersed in water for a long time the pathogens can readily penetrate the skin and enter the body (Mgode *et al*., 2015). Mucous membranes or braded skin and wounds offer the microorganisms easy entry into the host body. Inhalation of aerosols from contaminated urine can also cause infections. The infections pass through the blood stream to the body tissue and organs (Wasiński and Dutkiewicz, 2013).

A report on leptospirosis in fish in Tanzania showed a sero-prevalence of 54.3% in common fish types consumed by people in the Morogoro Municipality.This suggested infected fresh water fish to be a public health threat (Mgode *et al.,* 2014a). People engaging in fishing, rice farming and cultivation of vegetable around Morogoro Town are at risk of contracting leptospirosis (Mgode *et al.,* 2014b). Other vulnerable groups include: sugarcane farmers, fresh-fish traders, swimmers and other individuals with direct and/or indirect contact with wet environment contaminated with Leptospires from infected animals (Waitkins *et al.,* 1986). Workers in these environment requires protective gear to prevent infection.

Local Leptospira serovars Kenya and Sokoine that are predominant in rodents and cattle in Tanzania (Machang'u *et al*., 2004) appear to be the predominant serovars also

in tilapia and catfish species. This indicates relatedness or sharing of Leptospira pathogens between terrestrial mammals and aquatic organisms. Identification of the source of the widely circulating Leptospira serovars, such as Sokoine is necessary. This is possible through isolation from various hosts, including fish, and assessing the phylogenetic status of the isolates. The point source or hosts that are major reservoirs with transmitters of leptospirosis could be targeted in control strategies. The Seroprevalence of leptospirosis in fish exceeds that in terrestrial animals, such as cattle and rodents in Morogoro, which range from 10 to 20% (Mgode *et al.*, 2014a,b). Leptospirosis in catfish and tilapia is similar for the two reacting serovars Kenya and Sokoine which suggests that there is no host-specificity: There is a high probability of finding different infecting serogroups in freshwater fish species (host) from dams, rivers and lakes in Tanzania, and this could be useful in determining the burden of leptospirosis in this country.

2.2 Non- pathogenic Leptospiral species.

These feed on microscopic decaying organic matter in the water, and will survive and reproduce anywhere with suitable water conditions and a supply of organic material.The presence of saphrophytic leptospires in a body of water is not a sign that it is unclean or contaminated, in fact the bacteria prefer clean water without any parameter changes. It also has no bearing on the likely presence of pathogenic strains, however there is a theory that their widespread presence could explain why many fish and amphibians are immune to infection from the pathogenic strains – these species may have developed antibodies

because of constant exposure to the saphrophytes even though they were not made ill by them (Evangelista and Coburn, 2010)

Fish against the cause of stress (streses) temperature,pH, and Dissolved Oxygen will have a negative impact on the physiological changes of the fish body. These changes include growth disorders, productivity and all activities as a result of disrupted homeostatic mechanisms in the body

Studies have shown that when the water quality is affected by any stress, physiological changes will be reflected in values of one or more haematological parameters of aquatic organisms (Baumann *et al*., 2017). However, there is a possibility that fish blood might reveal conditions within the body of the fish long before there is any outward manifestation of disease.

2.3 Pathogenic leptospiral species.

These require a host in order to complete their life cycle, and whilst they can be cultivated in prepared serum they do not support stable colonies outside of the host. Survival and reproduction within the host animal is of course determined by the host's immune response and species. Pathogenic leptospira cause illness in their host, although of course they do not intend to do so – in fact many researchers argue that illness is a bad idea for leptospira (Haake and Levett, 2015) since a host that stays alive will be able to shed more of the bacteria in their urine. Outside a host animal, or the lab, it is believed that pathogenic leptospires have a close to zero reproductive output – in some cases limited binary fission has been seen, possibly caused by long-term absence of a host

species, but in general terms the bacteria do not multiply. Individuals can however survive for extended periods if the conditions are suitable. Where carrier hosts regularly add fresh urine to an environment the pathogenic colonies can maintain an almost continuous presence.

Pathogenic leptospires reproduce best at body temperature, but can survive over a wide range. They appear to be unable to tolerate temperatures over about 42°C, which can explain why bacterial growth is reduced or reversed in patients with very high fevers, with no reports of survivial when heated to over 55°C (Adler B. 2015). Cold is easier to tolerate, and they can be frozen (in ice or liquid nitrogen) and subsequently revived. Leptospires have been isolated from kidneys that have been deep-frozen as part of the food distribution chain. Their levels of activity and ability to reproduce drops when cooled below 10°C . L. Pomona was first isolated in the United States by (Baker and Little,1948), in Australia by (Sutherland *et al.* 1949). Subsequently L. Pomona infections in cattle were recorded in Italy (Babudieri, 1950). In Denmark 1956 , also recorded in Argentina 1948, New Zeland 1953, Canada 1957, Zaire 1955 and South Africa 1967.

2.4 Dissolved Oxygen (DO).

Dissolved Oxygen (DO) is the measures of gaseous oxygen (O_2) dissolved in an aqueous solution and it greatly depends on temperature as the higher the temperature, the lower the dissolved oxygen and vise versa.

The relevance of monitoring the level of dissolved oxygen in water bodies is very important. For the African catfish, a farmer should try as much as possible to maintain

dissolved oxygen levels at between 4mg/liter to saturation levels in the water bodies.When DO level is consisitently between 1.5mg/liter to 5mg/liter, fish will be alive. This is simply because of the fact that fish breathe in oxygen for general body metabolism. DO is needed to help breakdown any potentially harmful metabolic waste into less harmful forms, e.g ammonia (NH_3) broken down into nitrites (NO_2) and then into nitrates (NO_3).

2.5 Temperature.

Unlike man that is warm blooded, fish are cold blooded. The metabolism which occurs in their bodies is greatly influenced by the water temperature.

For the African catfish, an acceptable temperature range is between 26ºC to 32ºC.

When water temperature in the ponds consistently stays between 16ºC and 26ºC, feed intake reduces and fish growth rate also drags tremendously.

Prolonged stress can open up the fish to opportunistic infections. When fish are consistently exposed to temperatures below 15ºC, fish growth will ultimately stop.The fish would die

Low temperature negatively affects rates at which wastes are converted in the water. However, when water temperature is above 32ºC, the resultant effect on the African Catfish in not good at all. This is because of the fact that Oxygen is not readily soluble in very warm water. High temperature in ponds/or water bodies will stress the fish and eventually lead to death.

2.6 pH.

pH can be defined as the negative logarithm of H+ (hydrogen ion) concentration in the water . Fishes can die when exposed to extreme or rapidly changing pH. pH can be altered by adding alum or acids like hydrochloric acid to reduce pH, or bases like sodium hydroxide to increase the pH . For the fish in the water bodies, acceptable pH value is between 6.5 to 7.5. When it is below 4, fish will die due to water acidity (Svobodová *et al*.,1993) .When pH is constantly between 4 to 6, fish will be alive, but, due to stress, will experience slow growth. Infact, for the observant fish farmer, low pH in pond water is an indication of high CO_2, in the water.

High pH values of between 9 to 11 in pond water will also retard fish growth. Fish will ultimately die when pH levels rise above 11. Low pH aids higher proportions of ionized ammonia which is less toxic to fish. The reverse is the case with high pH in water.

2.7 Transmission.

Leptospirosis occurs as a result of a complex interaction between humans, animal reservoirs, and the environment. Transmission in rural areas is related to increased rainfall, livestock holding and farming (Yesilmen and Icen, 2012). In urban areas, the transmission is usually rodent-borne and is associated with poor hygiene, inadequate waste disposal, and overcrowding, which are typical in urban slums in the developing world. Heavy rainfall and flooding are often, but not always associated

with leptospirosis.

The global epidemiology of leptospirosis has been reviewed (Cachy and Vinetz , 2005). In Latin and Central America; several outbreaks have been reported in humans. Brazil is more affected, with about 70% of cases of leptospirosis occurring in the urban areas (Romero *et al.,* 2010). managed to characterize 40 clinical samples suspected of leptospiral infection using molecular techniques. In Brazil, 39 cases were identified belong to serogroup Icterohaemorrhagiae (97.5%) and one to serogroup Sejroe (2.5%) . Nicaragua reported 1,980 cases of leptospirosis between the year 2004 and 2010 (Global Leptospirosis Environmental Action Network, 2013).

In Sri Lanka, leptospirosis was found to be a frequent cause of acute febrile illness in patients admitted to southern Sri Lanka hospitals. Of 889 patients with paired specimens, 120 and 241 had acute and chronic leptospirosis respectively. in this report, the serological method was used for diagnosis (Liu *et al.*, 2008).

France reported the highest number of cases in Europe in 2005. The disease incidence was of 1.7 to 7.9 cases per 100,000 (World Health Organization, 2003). The principal serogroups were Icterohaemorrhagiae (30% of cases), Australis, Grippotyphosa (23% of cases), Sejroe, Ballum, Hebdomadis, Canicola, Bataviae and Pomona (Global Leptospirosis Environmental Action Network, 2013).

In Africa there are few reports on leptospirosis. In Mauritius the burden of leptospirosis has been reported to be 2.5 cases per 100,000 inhabitants. The Seychelles were considered in the nineties as the hottest spot of leptospirosis in the world with an incidence of 101 cases per 100,000 (Bourhy *et al.,* 2014). The burden of leptospirosis has

been however lowered reaching an incidence rate of 17 per 100,000 inhabitants in 2013. However, case fatality rate remains high, estimated to be 16%. Mayotte is currently the high spot for leptospirosis in the Southwest Indian Ocean islands with an incidence rate ranging from 59 to 87 cases for 100,000 inhabitants in the last years (Bourhy *et al.*, 2010, de Vries *et al*., 2014). Risk factors for infections by leptospires in Mayotte are multiple, with exposure of the general population during daily activities (Bourhy *et al.*, 2014). In Nigeria and Ghana; the countrywide prevalence ranges between 20 and 35 % respectively (de Vries *et al.*, 2014). A seroprevalence of 15.7% in healthy people was demonstrated in five villages in the northeast of Gabon (de Vries *et al*., 2014).

In East Africa, human infection was first reported in Kenya by (Bourhy *et al.,* 2010, de Vries *et al*., 2014). In 1987, 7.4% of 353 healthy people in Nyanza Province and 16.9% of 130 in Coast province were found to have leptospiral antibodies. Currently, outbreak survey of malaria-negative fever in 21 patients in Malindi in Coast province of Kenya could not confirm leptospirosis (Biggs *et al.,* 2011).

In Ethiopia, a serological survey conducted in 2004 showed that 47.5% of 59 febrile patients in Wonji tested positive for leptospiral antibodies with the rapid diagnostic test LeptoTek Dri-Dottest (Bourhy *et al.*, 2014). Data from Sub-Saharan Africa, including Tanzania, remains scarce (de Vries *et al.,* 2014).

Leptospirosis has many different names including: "seven-day fever", "harvest fever", "field fever", "canefield fever", "mild fever", "rat catcher's yellows", "Fort Bragg fever", and "pretibial fever", depending to environment (Ningal *et al.,* 2015). The ability of the

leptopires to evade the immune defense is poorly understood but certain cellular proteins integral to the glycolytic pathway (anolase) are known to play an important role (Nogueira *et al.,* 2013). Other virulence factors have been identified that might contribute to the infections and disease including lipopolysaccharides (LPS), hemolysins, outer membrane proteins (OMPS) and other surface proteins as well as adhesion molecules. (Nogueira *et al.,* 2013).

2.8 Diagnosis of leptospirosis.

A patient is suspected to have acquired leptospirosis after experiencing symptoms for about 1 to 2 weeks of exposure to the carrier host. In fish, *Leptospira* spp may be found in the blood or tissue samples during the first phase of the illness. The diagnostic tests used are similar to those employed in humans, including immunofluorescence and immunohistochemical staining, culture, and ELISA (Gill *et al*, 1985). The disease can be confirmed through blood or tissue organ tests to detect the presence of leptospira, or by demonstrating the bacterial genome by polymerase chain reaction (PCR) (Mullis.,1983). In many cases, however, leptospirosis is diagnosed by the serological gold standard-microscopic agglutination test (MAT). However, this method may fail to reveal infection with certain leptospiral serovars, especially in a newly studied area where the prevalent (endemic) serovars are unknown, or in cases where antibody titres are low or absent (Cole *et al.*, 1973; Goris *et al.*, 2013) .

2.9 Microscopic agglutination test (MAT).

The MAT is a serial dilution of sera mixed with an equal volume of a well grown suspension of Leptospira serovars at a room temperature (24 to 30^{o} C) for a certain period of time. A 50% agglutination is the estimation of the end point titration of the reaction mixture read under the dark field microscope (Korver *et al.*, 1988). Principally, the test works as the sera react with homologous antigens on the surface of the bacteria and agglutinate them. The test was first described by Schuffner and Mochtar in 1926 and improved by others (Cole *et al*., 1973).

MAT detects antibody against Leptospira spp, usually after seven to ten days of the onset of the disease. Generally MAT detects IgG and IgM present in the sera. Higher agglutination titers are suggestive of current infection. Despite its limitations MAT is still considered as the diagnostic gold standard for leptospirosis (World Organisation of Animal Health, 2014).

2.10 Polymerase Chain Reaction (PCR).

The PCR is a technique allows the amplification of specific sequence of genomic materials. It was developed by (Mullis,1983), as a supplementary diagnostic tool to serology and cultural methods. A single use conventional PCR was introduced as a promising molecular detection method of Leptospira spp in biological materials in 1989 (Thaipadungpanit *et al*., 2011). At first, this method used a set of primers taken from genomic libraries to specifically detect serovars Hardjo Bovis (Van Eys *et al*.,1989). Adoption of the other primer pairs designed from varieties of genes including *rrs*, *rrl*,

flaB, gyrB, and *ompl* was used for detection of pathogenic leptospirosis (Ahmed *et al*., 2012). To increase sensitivity and specificity, other primer pair targeting repetitive element and nested PCR were introduced (Ahmed *et al*, 2012). Comparative: to cultural methods, PCR was superior (62% detection) to cultural methods culture (48% detection) (Romero *et al*., 2013). Other studies compared conventional PCR with MAT, whose high sensitivity was not clearly determined due to cross-contamination of MAT (Romero *et al*., 2010).

2.11 Treatment, Control and Prevention of Leptospirosis.

There is a lage number of antibiotics which can kill Leptospira spp such as penicillin. Doxycycline has been proven to be an appropriate antimicrobial agent for treatment of an individual suspected of leptospirosis. Azithromycin could be considered as an alternative treatment whenever doxycycline allergy or adverse conditions arise. **(**Kim *et al*., 2004**).** A vaccine against leptospirosis in humans was developed in the 1960s based on serogroup Icterohaemorrhagiae, strain Verdun, and used in a populated area with a high risk of the disease. However the diversity of serogroups rendered the vaccines a limited use (Global Leptospirosis Environmental Action Network, 2013). Good hygienic conditions, elimination of rodents, and proper disposal of infected animal carcasses are important practical ways of preventing leptospirosis in a community. (Allan *et al*, 2015).

2.12 Economic Significance of Leptospirosis.

Leptospirosis, is among the neglected zoonotic diseases, in many countries of the world, yet it has a big economic burden to humans such as, death, loss of productivity due to chronic illness, particularly in poor rural communities, and treatment costs (World

Health Organization, 2010). The disease reduces livestock productivity by causing losses primarily because of its effects on reproductive performance such as abortion, stillbirth or weak offspring as well as mortality. Leptospirosis also decreases milk production in livestock. In addition to the diseases, these effects cause food insecurity and aggravate poverty to communities (Radostitis *et al*, 2006; World Health Organization , 2010).

CHAPTER THREE

3.0 MATERIALS AND METHODS.

3.1 Study site description.

This research was carried out in fresh fish water bodies in the Morogoro Municipality area.The Municipality is located about 200 km west of Dar es Salaam city, and has a human population of about 315,886 (The United Republic of Tanzania, 2012). Latitude 8^{o} S to Longitude 37^{0}E The highest temperature is attained between November and December, with an average of 33^{0} C while the minimum temperature occurs between June and August, with the average temperatures of 16^{0} C. The mean relative humidity ranges between 37% and 66% and the total average annual rainfall ranges between 821 mm and 1,505 mm. The long rains occur between March and May and the short rains occur between October and December each year. Crop farming and fishing are the major economic activities around these water bodies (Sonaiya, 2013). In these environments the bacteria can survive for weeks, depending on weather conditions. Sokoine University of Agriculture Magadu farm, Mzumbe University waste water pond, Morogoro Urban Water Supply and Sanitation Authority (MORUWASA)- Mafisa waste water ponds , Kingolwira Centre for Fish Farming and Fingering Production and Mkindo Fish Farmers in Morogoro Urban and mvomero districts.

Certain *Leptospira* spp such as *L*. interrogans, have been shown to survive for longer periods outside a host than others .This is likely due to acquisition of genes through horizontal transfer, or loss of genes essential to pathways for environmental survival (

Picardeau *et al.,* 2008). Saprophytic species are more fastidious in the environment as their genome contains the pathways required for utilising alternative methods for acquiring metabolites (Picardeau *et al.,* 2008). To date, saprophytic strains have been isolated from water sources but never from a host (Storck *et al,* 2008).

3.2 Study Design.

A cross-sectional design was employed.

3.3 Sampling Technique.

Multistage sampling technique was performed at various stages;

Stage 1: Identifying of water bodies where fishing is done with the help of fishermen and field officers .

Stage2: Selection of the various fishermen and get the fish alive in ventilated plastic buckets to the laboratory for collection of blood and tissues (kidneys).

Stage 3: Before the sampling, knowledge, attitude and practices (KAP) questionnaire was administered to assess the awareness of people (fishermen, fish mongers) on risk factors that might lead to the diversity of *Leptospira* species.

3.4 Study Population.

Responders consisted of fishermen, and fish mongers mostly around the chosen 5 sites. Questionnaires were developed during the study and administered with the help of fishermen and field officers . Responders were personally contacted in the field, market places and in their homes. Total

number of responders was 75. Of 40 from Fishermen and 35 from fish mongers, 25 were males and 15 females Fishermen, 15 were male and 20 were female Fish mongers . Fishermen employed 25 male and 15 female of participants respectively. Fishermen and Fish mongers were dominated by males (40). Majority of responders were less Educated (26), the rest were higher educated. Most of responders were between 18 and 27 years of age (26) while the minimum ages of 28-37 were the minority (9). Approximately half of responders had less than 2years in their occupations

3.5 KAP Questionnaire and Measures.

A questionnaire was developed and administered based on previous (KAP) studies on leptospirosis (Mohd *et al* .,2012). The list of (KAP) questions is given in Appendix 2.The first part of the questionnaire focused on the socio-demographic characteristics of the respondents.The second part measured the prevailing Knowledge of the mode of transmission, epidemiology and symptoms, the third part of the questionnaire evaluated practices on disease prevention and the last part of questionnaire assessed attitude towards threat, and towards disease control. Knowledge, Attitude , Practices percentage scores for all respondents categorized by occupation was computed.

The total mean knowledge score for all respondents was 68.5%. The questions on leptospirosis transmission had the highest mean score at 81.38%, followed by questions related to symptoms, with a mean score of 77.43%. The questions on the epidemiology of leptospirosis had the lowest mean score at 44.32%. Fishermen had a total mean knowledge score of 67.70%, while Fish mongers scored 69.26%. However, the variation in their mean knowledge score was not that higher in their frequencies.

In terms of practices about leptospirosis, the total mean practices score for all respondents was 80.89%. Fisher men obtained a mean score of 79.97%, while Fish mongers scored 82.12%. The difference in their mean practices score did not show again a big variations. In terms of prevention practices related to leptospirosis, the total mean practice score for all respondents was 61.26%. The total mean attitude toward disease control had a mean score of 67.31%, while the attitude toward threat of diseases had a lower mean score of 56.68%. The total mean Attitude score on Fisher mongers (66.35%) was significantly higher than that of Fishermen (57.50%). With regard to item-specific threat, Fish mongers obtained a significantly higher mean attitude score (61.22%) for the use of protective gear and safety measures, compared with fishermen (53.26%). Fish mongers also obtained a significantly higher mean score for disease control measures (73.16%) compared with fisher men (62.89%) .

Figure 1: Dissolved Oxygen Sensor.

A dissolved oxygen sensor figure1 was obtained from the College of Animal Science, Aquaculture and Range Management: and attached to a meter for spot sampling and laboratory applications. This is the most commonly used method for assessing dissolved oxygen in water bodies (Katznelson, 2004). Since dissolved oxygen concentrations are affected by Temperature, the measurements for temperature were taken by a thermistor built in the sensor. Through mannual logging, the instrument could record water pH

3.6 Ethical Clearance.

This study was cleared by Institutional Review Board of Sokoine University of Agriculture. Ethical clearance was provided by the Sokoine University of Agriculture (Reference number SUA/VET/016/24).

3.7 Fish samples and diagnostic tests.

The diagnostic techniques utilized were Microscopic agglutination test (MAT), Polymerase chain reaction (PCR) for *Leptospira* spp, and cultivation of fish kidneys in Fletcher's medium . Live fish (n=193) caught by the fishermen from November, 2016 and February, 2017 using fishing, gill nets were randomly collected for the diagnostic tests from five different locations and transported in ventilated plastic buckets to the Sokoine University of Agriculture, Pest Management Centre. The sampling location were: Magadu Farm, Mzumbe University waste- water ponds, Morogoro Urban Water Supply and Sanitation Authority (MORUWASA) Mafisa waste water ponds , Kingolwira Centre for Fish Farming and Fingering Production and Mkindo Fish Farms. Samples of fish blood, and kidneys were collected. To obtain blood samples the fish were caught gently

in a small scoop net and then quickly taken out from the water and held firmly on a dissecting board with a piece of cloth covering the head and then blood samples from each fish were withdrawn from the caudal vein using a sterile syringe with needle. Whole blood withdrawal process took less than one minute per fish to avoid stressing the animal. Collected blood was gently emptied into sterile Eppendorf tubes of (3ml) and left to clot 30 min. For serological detection of Leptospira antibodies sera were separated from blood by centrifugation at 6,000g for 6min and there after stored at -20^{0}C until used. Kidney tissue samples were preserved in Eppendorf (3ml) tubes in 96% alcohol for molecular processes..

Figure 2: Fishermen, catching fish from a constructed pond MORUWASA

3.8 Detection methods.

3.8.1 Seroprevalence of leptospirosis in fishes

The MAT was carried out as described by Cole *et al.,* (1973) using live antigens of four Leptospira serovars. The serovars were; Sokoine (serogroup Icterohaemorrhagiae); Hebdomadis (serogroup Hebdomadis); Kenya (serogroup Ballum) and Pomona (serogroup Pomona). Detection of *Leptospira* spp antibodies was performed according to (World Health Organization, 2003). Using the four serovars of *Leptospira* spp as antigens. The serovars were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) culture medium and incubated at 30^0 C for 4 to 7 days before being used as live antigen in MAT. Culture purity and density were checked using the dark field microscope by estimating the number of leptospires per field, that corresponded to about of 3×10^8 leptospires/ml.

The screening of fish serum samples was done by initially diluting volumes of 50 µl culture at 1:10 to 1:80 with phosphate buffered saline (pH 7) in U–bottomed microtitre plates and mixed with equal volumes of each antigen. The plates with serum–antigen mixture were then incubated at 30^0 C for 4 h before examining for agglutination of the leptospires under dark field microscope. Titer values of initially reactive were determined by futher diluting to 1:20480. MAT titers ≥ 1:160 were scored as higher titer values, while titers between 1:20 and 1:80 were scored as lower titer values. Absence of agglutination was scored as negative. The final titre was the titre that presented an agglutination of at least 50% of the spirochetes (Faine *et al.,* 2000).

3.8.2 Leptospira isolation from kidney tissues.

Tissue samples (n= 193) were prepared by grinding freshly obtained kidneys of sharptooth catfish (*Clarias gariepinus*)` For culturing in Fletcher's medium, the Kidney pulp were processed according to (Cole *et al*.,1973). Briefly about 0.5 ml of the kidney homogenates were inoculated into a tube containing semi-solid Fletcher's medium (Difco®) with 0.15% agar, supplemented with 100µg of 5-fluorouracil/mL and 1% sterile rabbit serum. The cultures were then incubated at 30◦C for 16 weeks and examined for leptospiral growth at seven-day intervals by the dark field microscopy as described by (Forster *et al*,. 2013). Dark-field microscopy readings of each of the tubes were performed at first, second, fourth, and sixth week after culturing at 20× magnification to assess bacterial growth.

Figure 3: The kidney tissue culture obtained from fish using Dark-field microscopy at Morogoro Municipality- Tanzania.

3.8.3 Identification of pathogenic and non pathogenic *Leptospira* spp by PCR.

DNA was extracted from preserved kidney tissues using the Quick- gDNA™ Blood MiniPrep kit (Catalog No. D3072 & D3073. Zymoresearch DNA and RNA purification), as per manufacturer instructions, by adding 200µl genomic lysis buffer to all samples to make a ratio of (4:1) then mixed by vortexing for 6s , then let to stand 10min at room temperature. The mixture were transferred to Zymo-Spin IIC™ Column[2] in a collection tube and then centrifuged at 10,000 xg for one minute. The Zymo-Spin™ was then transfered to a new collection tube. Then a total of 200µl of DNA Pre-Wash buffer was added to the column and centrifuged at 10,000 xg for one minute. A total of 500µl of g-DNA Wash buffer was then added to the spin column and again centrifuged at 10,000 x g for one minute. The spin column was then transferred to a clean microcentrifuge tube, a total of 100µl DNA elution buffer was added followed by incubation at room temperature for 5min and then centrifuged at 14000 rpm for 30s to elute the DNA. The eluted DNA was stored at $\leq 20^{O}$C for future use. The PCR was carried out using reported sets of primers Lepat1/Lepat2 and Sapro1/Sapro2 as described by (Mgode *et al.,* 2006). Briefly, the PCR consisted of Lepat 1 (5'-GAG-TCT-GGG-ATA-ACT-TT-3') and Lepat 2 (5'-TCA-CAT-CG(CT)-TGC-TTA-TTT-T-3') primer pair for pathogenic leptospira; which targets 16SrRNA gene of 330bp and Sapro 1 (5'-AGAAAT-TTG-TGC-TAA-TAC-CGA-ATG-T-3') and Sapro 2 (5'-GGC-GTC-GCT-GCT-TCA-GGC-TTT-CG-3') primer pair for non-pathogenic leptospira; which targets 16SrRNA gene of 240 bp. These primers can discriminate pathogenic and saprophytic leptospira in test samples.

DNA of known pathogenic *Leptospira* spp (serovar Sokoine, Pomona, Hebdomadis, Kenya), and non-pathogenic (saprophytic) spp (serogroup Semaranga) was used as the control.

The reactions mix consisted 10 mM Tris-HCl, pH 8.3,50 mM KCl, 200mM of each deoxynucleoside triphosphates (dNTP), 0.5µM of each primer, 5µl template DNA with modified MgCl concentration (2 mM) and DNA polymerase (1 U). The PCR condition for pathogenic Leptospira were : initial denaturation at 93^0 C for 3 min then 35 cycles of denaturation at 93^0 C for 1min, primer annealing at 48^0 C for 1 min, DNA extension at 72^0C for 1min, and further 10 min extension after the last cycle. Saprophytic PCR condition were : heat denaturation at 93^0 C for 3min, then 35 cycles of heat denaturation at 93^0 C for one minute, primer annealing at 63^0 C for 1.5min, DNA extension at 72^0 C for 2min. After the last cycle extension continued for further 10min. The PCR amplification conditions for pathogenic and saprophytic leptospira were used. The PCR products were visualized after performing agarose gel electrophoresis. Briefly, 1.5 % agarose gel was prepared and pre-stained using Ethidium bromide. Electrophoresis of DNA was performed at 100V for 60min, followed by visualization of PCR product using a gel documentation gel documentation system as described by (Muller *et al*., 2016)

3.8.4 Data anlyisis:

Data analysis was run using the modules available in Microsoft Exel 2010 for the prevalence of leptospira for each of the study location.

CHAPTER FOUR

4.0 RESULTS.

Table 1: Socio-demographic characteristics of questionnaire respondents.

Socio-Demographics	Fishermen n=40 Mean	Fish mongers n=35 Mean	All Respondent n=75 Mean
Gender	Count %	Count %	Count %
Male	25(62.5)	15(42.86)	40(53.33)
Female	15(37.5)	20(57.14)	35(46.67)
Age (in Years)			26(34.66)
	18-27 20(50)	6(17.14)	
	28-37 5(12.5)	4(11.42)	9(12.00)
	38-47 12(30)	5(14.3)	17(22.67)
	Above 48 3(7.5)	20(57.14)	23(30.67)
Education	16 (40)	10(28.57)	26(34.67)
Elementary			
High school	14 (35)	12(34.29)	26(34.67)
College	10(25)	13(37.14)	23(30.66)
	< 2 21(52.5)	13(37.14)	34(45.33)
Years in main	2-5 5(12.5)	5(14.29)	10(13.33)
Occupation	5-6 6(15)	3(8.57)	9(12)
	>10 8(20)	14(40)	22(29.33)
	<10 20(50)	13(37.14)	33(44)
Number of fish collected from each	10-50 12(30)	10(28.57)	22(29.33)
source of water	50> 8(20)	12(34.29)	20(26.67)

Total of 75 participants responded to the survey questionnaire; 40 from Fishermen and 35 from fish mongers, 25 were males and 15 females Fishermen, 15 were male and 20 were female Fish mongers. Fishermen employed 62.5% and 37.5% of participants respectively. Fishermen and Fish mongers were dominated by males (53.3%). Majority of responders were less Educated (34.67%), the rest were higher educated. Most of responders were between 18 and 27 years of age (34.66%) while the minimum ages of 28-37 were the minority (12%). Approximately half of responders had less than 2years in their occupations

Table 2: Knowledge, attitude, and practice percentage scores of all respondents categorized by occupation.

	Fisher men n=40	Fish mongers n=35	All respondent n=75
Total mean Knowledge Score	Mean=67.70	Mean=69.26	68.50
Mode of Transmission	79.61	83.77	81.39
Epidemiology	44.52	44.06	44.32
Symptoms	75.56	79.56	77.43
Total Practice score On disease Prevention	79.97	82.12	80.89
Total Attitude score	57.40	66.35	61.26
Towards threat	53.26	61.22	56.68
Towards disease control	62.89	73.16	67.31

Table 2: Presents the knowledge, attitude, and practices percentage scores for all respondents categorized by occupation. The total mean knowledge score for all respondents was 68.5%. The questions on leptospirosis transmission had the highest mean score at 81.38%, followed by questions related to symptoms, with a mean score of 77.43%. The questions on the epidemiology of leptospirosis had the lowest mean score at 44.32%. Fishermen had a total mean knowledge score of 67.70%, while Fish mongers scored 69.26%. However, the variation in their mean knowledge score was not that higher in their frequencies.

In terms of practices about leptospirosis, the total mean practices score for all respondents was 80.89%. Fisher men obtained a mean score of 79.97%, while Fish mongers scored 82.12%. The difference in their mean practices score did not show again a big variations. In terms of prevention practices related to leptospirosis, the total mean

practice score for all respondents was 61.26%. The total mean attitude toward disease control had a mean score of 67.31%, while the attitude toward threat of diseases had a lower mean score of 56.68%. The total mean Attitude score on Fisher mongers (66.35%) was significantly higher than that of Fishermen (57.50%) . With regard to item-specific threat , Fish mongers obtained a significantly higher mean attitude score (61.22%) for the use of protective gear and safety measures, compared with fishermen (53.26%). Fish mongers also obtained a significantly higher mean score for disease control measures (73.16%) compared with fisher men (62.89%) .

Table 3: PH, Dissolved Oxygen and Temperature at Sample sites in Morogoro Municipality.

Water Source Units	PH	Dissolved Oxygen (DO) Mg/L	Temperature(0C)
KINGOLWIRA- FARM	10.64	9.07	28.48
MKINDO FISH FARMERS	8.31	4.08	24.56
MZUMBE PONDS	8.42	4.76	25.14
MORUWASA-MAFISA PONDS	8.52	2.13	26.26
SUA -MAGADU FARM	7.76	6.08	24.63

Studies have shown that when the water quality is affected by any stress, physiological changes will be reflected in values of one or more haematological parameters of aquatic organisms . However, there is a possibility that fish blood might reveal conditions within the body of the fish leading to unable bacterial growth and survival under these stress.

4.2 pH , Dissolved Oxygen and Temperature (Water quality).

The maximum level of water Temperature was 28.48^{o}C and minimum level 24.56^{o}C while the maximum level of pH measured in all water ponds/or bodies was 10.64,

followed with minimum level pH 7.7 and the maximum Dissolved Oxygen measured was 9.07 and minimum 2.13

4.1 Microscopic agglutination test (MAT).

Out of a total of 193 catfish screened for leptospirosis by using MAT, 29 (15%) were seropositive for Leptospira serovars Kenya, Sokoine, Hebdomadis and Pomona. The major circulating (prevalent) Leptospira serovars were serovar pomona (11.4%), serovar Kenya (1.55%), Serovar Hebdomadis (1.55%) and serovar Sokoine (0.52%). Each Sampled site had a seropositive fish. Positive agglutination titers ≥ 1:160 were detected for serovar Pomona and lower titres for the other serovars. The titers and the numbers of subjects for each serovar were as shown in Table 4.

Table 4: Leptospira seropositive fish isolated Morogoro Municipality from five study sites.

Study site	Titers					Positive	Negative	Total
	1:20	1:40	1:80	1:160	1:320			
MAGADU	5	2	2	1	0	10(19.6%)	41	51
MZUMBE	0	1	1	0	0	2(15.4%)	11	13
MORUWASA	1	2	1	1	0	5(12.2%)	36	41
KINGOLWIRA	3	2	2	0	1	8(18.6%)	35	43
MKINDO	3	1	0	0	0	4(8.9%)	41	45
Total	12	8	6	2	1	29	164	193

The prevalent Leptospira serovars were serovars Pomona (11.4%), Kenya (1.55%), Hebdomadis (1.55%) and Sokoine (0.52%) Pomona titre was highest titres (1:320) in one catfish obtained from Kingolwira Centre for Fish Farming and Fingering Production ponds.

Table 5: Seroprevalence of leptospira in catfish from five locations, Morogoro Municipality Tanzania.

LOCATION	Samlpes (n)	Sokoine	Kenya	Pomona	Hebdomadis
MAGADU	51	1*	0	7	2*
MZUMBE	13	0	0	2	0
MORUWASA	41	0	0	4	1
KINGOLWILA	43	0	2	6	0
MKINDO	45	0	1	3	0
TOTAL	**193**	**1**	**3**	**22**	**3**
		0.52%	1.55%	11.40%	1.55%

* Three specimens that reacted with Serovar Pomona were also positive for serovar Hebdomadis (2) and Sokoine (1) suggesting a cross-reaction.

Leptospirosis prevalence was high in all locations for serovar Pomona where by 22 of the 193 tested catfish were positive (11.40%) followed by serovars Kenya and Hebdomadis whereas 3 of the 193 tested catfish were positive (1.55%) and Sokoine serovars one of 193 (0.52%) was leptospirosis positive.

Table 6: Serovars and their corresponding agglutination titers from study locations, Morogoro Municipality.

	Titres					
Serovars	1:20	1:40	1:80	1:160	1:320	TOTAL
Sokoine	1*	0	0	0	0	1
Kenya	2	1	0	0	0	3
Pomona	8	6	5	2	1	22
Hebdomadis	1*	1	1*	0	0	3
TOTAL	**12**	**8**	**6**	**2**	**1**	**29**

* Indicate that specimens reacted with serovar more than once .The prevalent serovars were serovar Pomona (11.4%), Kenya (1.55%), Hebdomadis (1.55%) and Sokoine (0.52%). Serovar Pomona reacted with highest titres (1:320) in one catfish sample, and is considered to be the most circulating serovar The agglutination titres across serovars showed a cross reactivity of Sokoine and Hebdomadis and Pomona.

Table 7: Diagnostic results per location in study site.

Study site	Test Positive	Test Negative	Total
MAGADU	10	41	51
MZUMBE	2	11	13
MORUWASA	5	36	41
KINGOLWIRA	8	35	43
MKINDO	4	41	45
Total	29	164	193

Test evaluation.

a) Measuring accuracy of a test (Sensitivity and Specificity).

These are two basic measures of inherent accuracy of a diagnostic test**.(** Irwig *et al***,** 2002)

Sensitivity: The proportion of subjects with disease that test positive.Is the probability that a test result will be positive when the disease is present(true positive rate)

Specificity: The proportion of subjects without disease that test negative.Is the probability that a test result will be negative when the disease is not present(true negative rate)

b) Interpretation of Diagnostic Tests (Calculating the Predictive Values)

-Predictive values evaluate a test´s ability to correctly identify the condtio of interest:

Positive predictive value:The proportion of test positive subjects which have the disease.Is the probability that the disease is present when the test is positive.

Negative predictive value: The proportion of test negative subjects which do not have the disease.Is the probability that the disease is not present when the test is negative.

Study site Sensitivity: Magadu =10/29, Mzumbe =2/29, Moruwasa=5/29, Kingolwira=8/29, Mkindo =4/29.

Study site Specificity: Magadu= 41/164, Mzumbe=11/164, Moruwasa= 36/164, Kingolwira=35/164, Mkindo = 41/164.

Study site Positive Predictive Value: Magadu = 10/51, Mzumbe 2/13 , Moruwasa = 5/41, Kingolwira =8/43, Mkindo =4/45.

Study site Negative Predictive value: Magadu =41/51, Mzumbe =11/13, Moruwasa= 36/41, Kingolwira= 35/43, Mkindo= 41/45.

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4.3 Isolation of leptospires from kidney tissue homogenates.

Kidney culture of the 193 sharptooth catfish (*Clarias gariepinus*) yielded no leptospira isolates:.

4.4 PCR of kidney tissues.

Out of 193 catfish tested, none were PCR positive generating a 240 base pair product with the Sapro 1 and Sapro2 primers (Figure4). None of the samples were PCR positive with Sapro primers. The finding was consistent with the absence of non-pathogenic leptospires.

Out of 193 fish tested, four (2%) were PCR positive generating a 330 base pair product with the Lepat1 and Lepat2 primers (Figure 4).The finding was consistent with the presence of pathogenic leptospires.

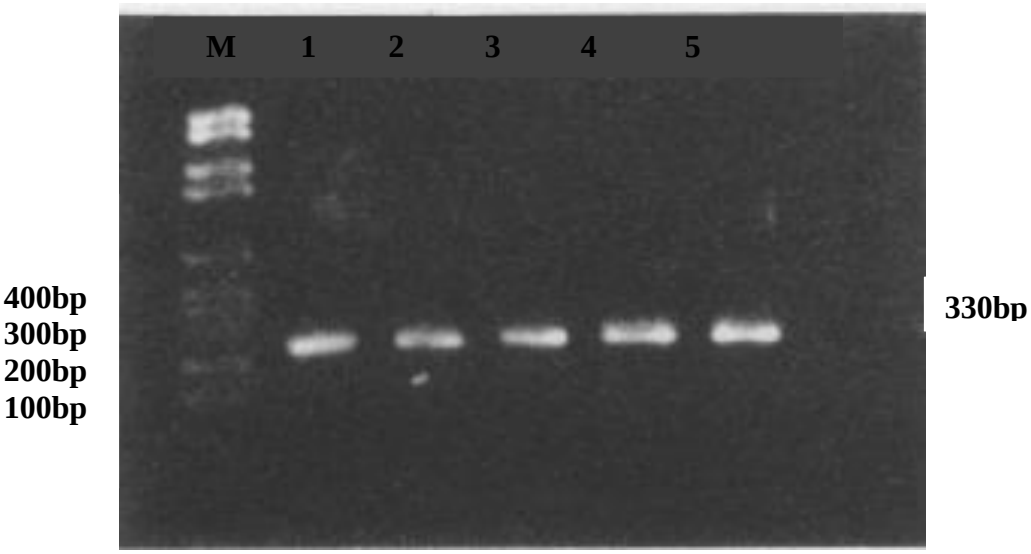

Figure 4: -PCR products of DNA from kidneys of catfish (*Clarias gariepinus*). Lanes (2, 3 , 4 and 5) show the with primers Lepat1/Lepat 2. Sokoine is the control for pathogenic lane (1), and lane M is the DNA ladder. The products were separated by electrophoresis in 1.5% agarose and stained with ethidium bromide

CHAPTER FIVE

5.0 DISCUSSION.

This study is the first attempt to describe the awareness on knowledge, attitude and practices of leptospirosis during Fresh water fishing as well as using/ or without using proper protective gear, among Fishermen and Fish mongers living in the communities of Morogoro Municipality -Tanzania. The total mean practice score (80.89%) of all respondents was higher than their total mean knowledge (68.50%) and attitude (61.28%) scores. The results may imply that a highly positive practice toward one's ability to control the disease is not sufficient alone to transform behavioral attitude. Positive practice should be complemented with knowledge to enhance the ability of individuals to integrate prevention and control measures into attitude.

Water quality-the maximum level of water Temperature was 28.48^{o}C and minimum level 24.56^{o}C.Thus the Pathogenic leptospires reproduce best at body temperature, but can survive over a wide range,they appear to be unable to tolerate temperatures over about 42°C (Adler, 2015) and their levels of activity and ability to reproduce drops when cooled below 10°C. African catfish, an acceptable temperature range is between 26ºC to 32ºC. The maximum level of pH measured in all water ponds/or bodies was 10.64, followed with minimum level pH 7.7. For the fish in the water bodies, acceptable pH value is between 6.5 to 7.5. When it is below 4, fish will die due to water acidity (Svobodová *et al*.,1993) . The Fish ultimately die when pH levels rise above 11.

Monitoring the level of dissolved oxygen in water bodies is very important, during the study maximum Dissolved Oxygen measured was 9.07 and minimum 2.13. For the African catfish, a farmer should try as much as possible to maintain dissolved oxygen levels at between 4mg/liter to saturation levels in the water bodies.When DO level is consisitently between 1.5mg/liter to 5mg/liter, fish will be alive.

Isolation of leptospires from kidney tissue homogenates. The negative results in isolation of leptospires could have been due to contamination of the cultures with less fastidious and faster growing microorganisms such as bacilli in spite of the fact that contamination was kept at minimum by deploying selective growth inhibitors 5-fluorouracil in the culture medium.

This study, basing on MAT and PCR results, reveals a predominance 15% of Pomona serovar in sharptooth African catfish (*Clarias gariepinus*)` from five different fresh water locations found in the Morogoro Municipality area. A prevalence of 11.40% has been observed in this common fish type largely consumed by people in this region suggesting a potential public health threat. People engaged in rice farming and cultivating vegetables around Magadu Farm and Mzumbe University waste water ponds, Morogoro Urban Water Supply and Sanitation Authority (MORUWASA) Mafisa waste water ponds , Kingolwira Centre for Fish Farming and Fingerling Production and Mkindo Fish Farmers area are at risk of contracting this disease. Other vulnerable groups include. Sugarcane farmers, fishermen, fresh-fish traders, swimmers and other individuals with direct and/or indirect contact with wet environments contaminated with leptospires from

infected animals. Workers in these environments require protective gears to prevent infection (Waitkins , 1986).

L. Pomona was first isolated in the United States by (Baker and Little,1948), in Australia by (Sutherland *et al.* 1949). Subsequently L. Pomona infections in cattle were recorded in Italy (Babudieri, 1950), and in Denmark 1956 , also recorded in Argentina 1948, New Zeland 1953, Canada 1957, Zaire 1955 and South Africa 1967

Leptospira serovar pomon*a* were the predominant serovars also in rodents, pigs and cattle in Tanzania (Machang'u *et al*., 2004; Mgode *et al*., 2006) . This indicates possible sharing of Leptospira pathogens between fish and terrestrial mammals, such as rodents, pigs and cattle; and found in the same location. Identification of the point source of the widely circulating Leptospira serovars, such as Pomona which is found in a broad range of species is necessary. This is possible through isolation of the pathogen from potential hosts, including fish, and assessing the phylogenetic status of the isolates. The point source or hosts that are major reservoirs and transmitters of leptospirosis could be then targeted in control strategies.

African catfish is found in lakes, streams, rivers, swamps and flood plains, many of which are subject to seasonal drying. The most common habitats are flood plain swamps and pools where they can survive during the dry season(s) due to their accessory air breathing organs (Bruton *at el.,*1988) . *Clarias gariepinus* undertake lateral migrations from the larger water bodies, in which they feed and mature at about the age of 12

months, to temporarily flooded marginal areas in order to breed. The reproductive migrations typically take place shortly after the onset of the rainy season(s). (Clay *et a*l., 1979).

The prevalence of leptospirosis in fish in this study is higher than that reported in terrestrial animals such as cattle, pigs and rodents in Morogoro, which starts is around 10 % (Machang'u *et al*., 2006). Although this study reports on fish specimens from five different locations, its findings may reflect the leptospirosis prevalence in other fresh water bodies found in Tanzania and in the great lakes region of East and Central Africa. Further studies with larger sample size and broader geographical coverage, to include the major sources of catfish consumed in Tanzania, is required to determine the extent of potential human health risks due to leptospirosis.

Efforts to isolate the Leptospira spp from fishes need to be continued to determine the infecting serovars, and their characterization to the molecular level. The diversity of potential reservoir hosts of Leptospira spp in Tanzania, (including rodents, bats and domestic animals) as reported by (Machang'u *et al*., 1997); (Machang'u *et al*., 2004); and Mgode *et al*., (2006, 2014), complemented with this report involving catfish, further call for the need to enhance public awareness of this emerging zoonotic disease.This is important to protect the health of vulnerable occupational groups and consumers of fish and fish products.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS.

6.1 Conclusion.

This study has shown a relatively high seroprevalence 15% of leptospirosis in common fresh water fish type, compared to warm blooded animals, hence it indicates an extended public health risk of leptospirosis to fish handlers and consumers .

6.2 Recommendations.

To minmize leptospirosis, transmission in Morogoro region, and Tanzania at large, where the disease is little known and thus neglected, it is recommended (i) To increase public awareness of leptospirosis in the general public, and particularly those groups at risk such as farmers, livestock keepers, fishermen, sewerage and abbatoir workers. (ii) routine screening for leptospirosis should be considered in people engaged with occupational activities that increase contact with environments likely to contain leptospires. This is especially important in situation where malaria, typhoid and other common febrile diseases are ruled out; and (iii) isolation of leptospires from different hosts and sources should be emphasized in order to understand the sources of infections, infecting serovars and developing a protocol for the diagnosis of this infections disease appropriate for different areas. (iv) Rapid serological diagnostic tests not involving live microorganisms for routine diagnosis should be developed.

6.3 Sudy limitations.

Important limitation to my study included frequent fluctuation of power (electricity) and a contamination of cultures.

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APPENDICES

Appendix 1: Ethical clearance certificate for conducting animal related research in Tanzania

SOKOINE UNIVERSITY OF AGRICULTURE
FACULTY OF VETERINARY MEDICINE
P.O BOX 3015 CHUO KIKUU MOROGORO TANZANIA
TEL. 255 23 2 603511/4; DIR. 255 23 2 604542
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TELEGRAMS "UNIAGRIC" MOROGORO
E-Mail: deanfvm@suanet.ac.tz

Our Ref: SUA/FVM/R.1/9 You Ref: SUA/VET/016/24 Date: July 25th 2016

Mr. Mbambagwa Noel Alexander,
Department of Veterinary Microbiology, Parasitology and Immunology
P. O. Box 3015
Morogoro

Dear, Mbambagwa Noel Alexander,

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING ANIMAL RELATED RESEARCH IN TANZANIA

Kindly refer to the above heading.

We are delighted to inform you that the proposal submitted for ethical Review by the Faculty Research Publication and Ethics Committee with guidelines from the CODE OF CONDUCT FOR RESEARCH ETHICS of Sokoine University Agriculture has been evaluated and recommended for approval.
We therefore certify that your research entitled:"The Prevalence of *leptospira species* in fresh water fish in selected areas of morogoro municipality".
to conducted in Tanzania and in which you are the Principal Investigator has been granted ethical clearance. Reference number for your ethical approval is SUA/VET/016/ 24

As principal Investigator you must ensure that the following conditions are fulfilled:

i. MUST observe that the approval is valid for two years; 1st AUGUST 2016 to 31st JULY 2018 subject to submission of satisfactory annual reports by 31st December of each year of your research.
ii. MUST observe and adhere to the approved protocols when dealing with study subjects. In cases where you will be compelled to make changes **during the implementation of** your project, please make sure that you submit an amendment for review and approval.

The committee congratulates your team for this innovative project and looks forward to its success.

Yours sincerely,
Prof. Robinson Mdegela, (PhD)
Chairman,
Faculty Research, Publication & Ethics Committee

Dr. Huruma Tuntufye, (PhD)
Secretary,
Faculty Research, Publication & Ethics Committee

Appendix 2: Fishermen and Fish Mongers Questionaire

PART A: GENERAL DEMOGRAPHIC AND SOCIAL ECONOMIC CHARACTERISTICS

Record Number…….. Date of interview: _____ /_____ /_____

District : …… Ward: ……Street/ Village : ……..
Interviewer's name: ……
Interviewee name……
Phone number……
Start time _____ Finish time _____

PART B: Social Economic Characteristics

NO	QUESTIONS	CODING ANSWERS
1	Sex	1. Male: 2. Female:
2	Age of respondent (in years)	Years…….
3	Highest level of education	1. Elementary : 2 High school: 3 College
4	Marital status	1: Married: 2 Never Married: 3. Widowed: 4. Divorced
5	Main occupation of the respondent	(a)Fish mongers (b) Fishermen
6	Number of fish collected (/day)	(a) <10 (b) 10-50 (c) 50>
7	Years in fish fishing?	(a)<2 (b) 2-5(c) 5-6 (d) >10
8	What is the source of water for your fish?	(a)Uluguru mountain(b) House hold(c) Mindu dam (d)Hospital, House hold, Uluguru mountain …… …… ……
9	Are you fishing other fish species	1. Yes 2. No
10	If Yes in qn (9), specify the fish species	(1).Tilapia 2. Catfish 3. Eelfish

PART C: The following statements identify your practices in regard to fishing practices, fish trading , drinking water and biosecurity.

I. Fishing practices

11a. Do you do Fresh water fishing? 1.Yes I do 2. No I do not ( )

……
……
……

11b. Is it often?
1.Yes 2. No ( )

12. If using fishing gear (observation), how is it constructed (1) Poor constructed (2) well contructed ( )

II: Trading fish

13. How do you handle your fish?

(a) Locally (b) Professionally

14. What type of fish are you selling? (1)Tilapia (b) Catfish (c) Tilapia, catfish and Eelfish
15. Are you processing your fish before selling them? Yes ( ) No ( )

III: Drinking water

16. Do you always drink clean water (boiled water, tap water, bottled water)? Yes ( ) No ( )

17. Where do you obtain water for drinking?(a) Tap water(b) River(c)Tap water, River and bottled water

iv: Biosecurity

18. Do you Camping beside lakes/rivers? Yes ( ) No ( )

19. If yes in qn (18), how many times? (a) Once (b) Twice (c) More than Twice

20. Do you bathe or soak in water sources (such as canals, Pond, Lake, swamps, and creeks)? Yes ( ) No ( )
……
……
21. For how long (time)? (a) > 1hr (b)> 2hrs (c) >3hrs
22. Are you Wald in mud barefooted while having wounds/scratches on your legs and/or feet?
Yes ( ) No ( )
.....
23. How do you walk in (material)? (1) Bare foot and hands (2) protected with gear

24. Do you clean environment surrounding fishing area? Yes ( ) No ( )

25. Do you contact urine of cattle, rats, and/or pigs every day? (1) Yes ( ) (2) No ( )

26. Which material are you use after contact with urine (clean water, soap)? (1) Clean water(2) Soap

27.Do you dissect your fish while wearing gloves? Yes ( ) No ( )
……
……
…….....
28. If yes qn 27 which type of gloves?

STREET	FM NO.	TYPE OF WATER BODIES	NO. OF FISH /FISHERMAN

THANK YOU FOR YOUR COOPERATION

Appendix 3: One Health Workforce Institutional Animal Care And Use Committee (IACUC)

Client Consent Form

Animal Subject

If privately owned animals are used by University investigators for research or education, the owner
or client must sign a consent form. The animal ethical IACUC or local animal ethical review body
must review and approve the "client consent form" prior to the investigator obtaining the client's
consent.

Investigators may create their own consent form, but it must include the following elements:

☐ Department and college that will be conducting the study.

☐ Title of the study.

☐ Description of the purpose of the study and the benefits.

☐ Description in detail of the procedures that will be performed on the animals.

☐ Describe the risks, if any, to the animal or the client. Include risks of not using alternative,
established treatments.

☐ Indication if there will be any compensation for participating in the study.

☐ Indication if there will be, or if there will not be compensation in case of problems resulting from
the study.

☐ Costs that will be incurred by the client. (general types of charges, rather than actual fee amounts)

☐ Information that participation is voluntary and that they may withdraw at any time.

☐ The contact person if there are problems, including a phone number. Details of likely problems or
symptoms that the investigator or other veterinarian needs to be notified of should be included.

☐ Lines for Investigator and client signatures and dates.

☐ Be translated into the local language and administered and explained in the local language.

Example consent form:

[note that this is a *sample* and should be altered to accurately reflect your *individual* study or

educational activity]

Client Consent Form

Department of < *Veterinary Clinical Sciences, etc.* **>**

Study Title: < *title here* >

You are invited to participate in a research/educational study < *brief purpose in lay terms* > because

< *explain how the animal was identified* >. This study is being conducted by < *PI name and credentials*

and list department if you haven't listed it above >. We ask that you read this form and ask any

questions prior to agreeing to participate in this study.

Background and Purpose:

ONE HEATH WORKFORCE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

< *summary of the study, in lay terms, that includes whether treatment is experimental, placebocontrolled,*

etc. >

Study Procedures:

< *Describe, in lay language, the tasks and procedures to be followed and indicate if any procedures*

are "experimental". Describe assignment to study groups, length of time for participation and

frequency of proceduresinclude

a table of study visits if applicable. Indicate if study participation

involves withholding of standard treatment. Quantities such as blood volume to be drawn should be

listed in lay language equivalents >

Risks of study participation:

< *should correspond to your IACUC section. Use lay terms and include risks of withholding standard*

treatment, if applicable >

Benefits of study participation:

The benefits to study participation are: < *Describe any direct benefit to the subject or benefit to others,*

which may reasonably be expected from the research. If there is no direct benefit to the subject,

declare that fact. Note that payment to owners is not considered a benefit of participation. >

Study costs/compensation:
< Indicate whether or not there are costs to the owner for participation in the study. E.g.: "There is no
cost to you for participating in this study." AND/OR "You will be responsible for <list>." AND/OR
"All costs associated with <list> will be covered by the study ." AND/OR *"Payments to owners should*
be for time and inconvenience" >

Confidentiality:
All results will be confidential. Information about your animal may be used in scientific presentations
and/or publication. However, **no** personal or identifying information about you or your animal will be
released. Your animal's record for the study may, however, be reviewed by *[Indicate if the*
drug/device manufacturer, study sponsor, etc. may have access.] and by departments at the University
with appropriate regulatory oversight. *[Indicate whether study information will be recorded in the*
subject's medical record. If any study data will be transmitted via the Internet, indicate what
provisions for protection of privacy are in place.] To these extents, confidentiality is not absolute.
< State if study data will be encrypted according to current University policy for protection of
confidentiality. >

Voluntary Participation:
Participation in this study/educational exercise is voluntary. Your decision whether or not to participate
in this study/educational exercise will not affect your current or future relations with the University,
your veterinarian or the community . If you decide to participate, you are free to withdraw **at any time**
without affecting those relationships.

Please do not hesitate to contact us if you have any questions or concerns about this study.

Investigator(s): Dr. – Phone number & email:
ONE HEATH WORKFORCE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)
Dr. – Phone number & email:
Clinical Research Technician: Name – Phone number & email:
I have read the above information. I have asked questions and have received answers. I consent to
participate in the study. *[Avoid statements that begin with "I understand...".]*

Client Signature Date
Attending Veterinarian or Technician Date
This consent form will be kept by the researcher for at least three years beyond the end of the study.
The title of the study should appear at the top of every page.