MOLECULAR STUDIES OF BRUCELLOSIS IN SELECTED WILD ANIMAL SPECIES IN SERENGETI ECOSYSTEM, NORTHERN TANZANIA

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ABSTRACT

Brucellosis is a contagious bacterial zoonotic disease worldwide of public health and economic importance. The disease affects all species of livestock, wild animals and humans leading to severe economic losses in animals and permanent injury, disabling sequel and financial loss in humans. A cross-sectional study was carried out in Serengeti ecosystem between November 2017 and June 2018 to explore occurrence and magnitude of *Brucella* infection and circulating *Brucella* strains in wild animal species in Serengeti ecosystem, Northern Tanzania, using molecular techniques. The study also compared the effectiveness of different molecular techniques in detecting the *Brucella* strains in wild animals. The study used 189 whole blood, serum and amniotic fluid samples collected from seven different wild animal species. Wild animal species used were 46 buffaloes, 80 wildebeest, 25 zebra, 19 lions, 5 baboons, 10 impala and 4 hyenas. Most of the animals used in this study were female (96.3%), adults (99.5%) and those sampled from the Serengeti part of the ecosystem were 115 (60.3%). The tests used in the analysis were Multiplex polymerase chain reaction (AMOS PCR), Quantitative Real-Time PCR (RT-qPCR) and real-time speciation (RT-speciation) assay. The results indicated that out of 189 samples screened, DNA extracts from 12 (6.4%) and 24 (12.7%) were *Brucella* positive by AMOS PCR and RT-qPCR, respectively. The most affected wild animal species were lions (52.6%) and buffaloes (19.6%). A total of 16 (66.7%) out of 24 samples were confirmed as *B. abortus*. The other *Brucella* species identified were: *B. suis* (n=2; 8.3%), *B. melitensis* (n=2; 8.3%) and *B. ovis* (n=2; 8.3%). Two samples; one from buffalo and one from impala had three *Brucella* species each namely *B. melitensis*, *B. suis* and *B. ovis*. Overall comparison of the molecular tests showed better agreement and diagnostic performance with the real-time PCR techniques compared to the conventional AMOS PCR. The sensitivity of the AMOS PCR and RT-qPCR as compared to the real-time

speciation assay was 16.7% and 72.7% respectively, while the specificity was found to be 92% and 100% respectively. The detection of different strains of *B. abortus, B. suis, B. melitensis* and *B. ovis* in wild animals of Serengeti ecosystem implies that domestic animals and humans in the interface areas are at risk of acquiring the infection. The RT-qPCR is more superior in screening of *Brucella* than AMOS PCR. One health approach collaboration is important to establish the methods of brucellosis management in wild animals.

DECLARATION

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

_____ _____

Rosamystica Mkula Sambu Date

(MSc. PH & FS candidate)

The declaration above is confirmed by;

_____ _____

Dr. Hezron E. Nonga Date

(Supervisor)

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Life and its safeguard are a gift from God and without this gift human beings are zero and inexistent. May the Almighty God be praised for his consistent love to me which completed this academic journey and work realism. At this special moment, I remember my congregation of Immaculate Heart Sisters of Africa and all memberships who at different times opened me the door to higher learning. I frankly direct my appreciation to Dr. Hezron E. Nonga of the Department of Veterinary Medicine and Public Health my supervisor and guider, for giving me the vision to realize this dissertation, for his productive assessments, laboratory work and finally write this dissertation all along the way and for introducing me to the world of research. I real appreciate his presence. I acknowledge and thank Dr. Coletha Mathew for her helpful support, criticism, suggestions and assistance during the various stages of this work. I feel appreciative to thank Sokoine University of Agriculture and to remembrance the unspeakable value of all courses which were offered to me at the College of Veterinary and Biomedical Sciences, and in precise at the Department of Veterinary Medicine and Public Health. My lecturers' investments and inspiration to me as well as the collaboration with my colleagues were a great blessing to my knowledgeable. Special thanks to African Science Partnership for intervention Research Excellence (Afrique one-ASPIRE) project for its sincere financial support for second year studies costs and research. Thanks to Prof. Rudovick R. Kazwala, Principal Investigator of the Afrique one-ASPIRE Organization, Dr. Julius Keyyu and Dr. Jo Halliday who was part of Afrique one-ASPIRE team but were closer in playing their roles respectively to this work. Afrique One-ASPIRE fellow, Tanzania Veterinary Laboratory agency (TVLA) Temeke, Tanzania Wildlife Research Institute (TAWIRI) laboratory technicians, PREDICT laboratory workers, SACIDS laboratory workers and Kilimanjaro Clinical Research Institute (KCRI).

DEDICATION

This work is dedicated to lovely my deceased parents may the almighty God grant them peace and let their souls to rest in peace AMEN. My blood brothers and sister their prayers and success wishes are highly appreciated. Precious Lord may you grant them with intelligence and prudent. It has not been probable to find space to mention all people, I encompass my sincere thanks. To you all I say "*Thank you so much"*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYM

R-LPS	Rough lipopolysaccharide
®	Registered trademark
°C	Degrees Celsius
AMOS PCR	Multiplex Polymerase Chain Reaction
Bp	base pair
c-ELISA	competitive Enzyme Linked Immuno-Sorbent Assay
CFT	Complement Fixation Test
CT	Cycle time
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organisation of the United Nations
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
PGFG	Pulse field Gel electrophoresis
qPCR	Quantitative Real time PCR
RBPT	Rose Bengal Plate Test
RT-speciation	Real Time Polymerase Chain Reaction
S19	*Brucella abortus* strain 19
S-LPS	Smooth lipopolysaccharide
SUA	Sokoine University of Agriculture
TVLA	Tanzania Veterinary Laboratory Agency
USA	United States of America
WHO	World Health Organization
K	Kappa statistic
µ	Microliter

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Brucellosis is a worldwide problem of public health and economic importance. The disease affects all species of livestock, wild animals as well as humans, causing severe economic loss (Stack *et al.*, 2002). It is a disease of public health significance not only because of direct and indirect transmission of the disease from infected animals to humans, but also because it causes serious reduction of much needed animal productivity essential to human health and nutrition (Junaidu *et al.*, 2011). Brucellosis is one of the important re-emerging neglected tropical zoonoses largely due to lack of public awareness and yet it is one of the most important zoonotic infections, especially in pastoral and agro-pastoral farming systems in Africa (Arimi *et al.*, 2005; WHO, 2009).

Brucellosis is still an uncontrolled public health problem in many developing countries including Tanzania (Mantur and Amarnath, 2008; Matope *et al.*, 2010). The interest for brucellosis has increased since *Brucella* species were identified as a potential biological weapon (Blasco and Molina-Flores, 2011). For several decades brucellosis has been recognized as a significant public health problem in the Middle East, North of the Mediterranean countries, South and Central Asia, Central and South America. Brucellosis was reported for the first in 1859 in Malta (Lee *et al.*, 2009) and recent reports suggested that its incidence is increasing in domesticated ruminants, wild animals and humans (Refai, 2002). Veterinary researchers and policy-makers in sub-Saharan Africa have recognized the need to intensify research on these diseases and the need to develop tools for their control, initially targeting the African buffalo and the lion (*Panthera leo*) (Michel *et al.*, 2006).

The currently applied control measures for brucellosis may not be capable of reducing the levels of infection in ruminants (Hegazy *et al.,* 2009). In Tanzania, brucellosis has been reported to occur in cattle at the seroprevalence of 12.2% in Kilimanjaro (Mtui-Malamsha, 2001; Swai *et al.*, 2005), 12-14% in Eastern zone (Weinhaupl *et al.*, 2000), 2-90% in Morogoro (Minga and Balemba, 1990), 10.6% in Morogoro (Swai *et al*., 2009), 36.1% (Wankyo *et al.,* 2013), 5.2% in Dodoma (Kitaly, 1984), 3.2% in Arusha (Minja, 2002), 10.8% in Mwanza (Jiwa *et al.,* 1996) and 15.2% in Southern zone (Otaru, 1985). Brucellosis has also been reported in wild animals in some African countries, which include Kenya (Waghela and Karstad, 1986), South Africa (Bishop *et al.,* 1994), Zimbabwe (Madsen and Anderson, 1995) and Tanzania (Hamblin *et al.,* 1990; Fyumagwa *et al.,* 2009; Mellau *et al.,* 2009; Temba *et al.,* 2011).

In Tanzania *Brucella* infections in wild animals have been reported in topi, buffalo, impala, Thompson gazelle and wildebeest (Sachs *et al.,* 1968; Schiemann and Staak, 1971). Other studies reported brucellosis in livestock-wildlife interfaces in the Ngorongoro conservation area and Mikumi-Selous Ecosystem (Fyumagwa *et al.*, 2009; Mellau *et al.* 2009; Temba *et al*., 2011). Also studies have reported brucellosis in different wild animal species at prevalence of (10.5- 24%) in buffalo, 17% in wildebeest and 13% in general other wild animals like topi, impala and Thompson gazelle (Sachs *et al.,* 1968; Schiemann and Staak, 1971; Shirima, 2005; Fyumagwa *et al.,* 2009; Assenga *et al.,* 2015). Studies on brucellosis have also reported the disease in humans as an endemic problem especially pastoral and agropastoral communities who are constantly in contact with livestock and the tendency of eating raw or undercooked food of animal origin. The prevalence of brucellosis in some of the pastoral and agro pastoral communities in Tanzania ranges between 0.7% and 58.1% (Kunda *et al.*, 2005; Kunda, 2007; Swai, 2009; Mellau *et al.*, 2009; Kunda *et al.*, 2010; Wankyo, 2013). A study by Mellau *et al.* (2009) reported the

increase of human brucellosis from 35.6% in 2004 to 58.1% in 2005 in livestock-wildlife interface in Serengeti ecosystem which mostly is inhabited by pastoralists whose animals interacts with wild animals. This shows that the magnitude of brucellosis in humans and livestock is directly related to inhabiting closer to or within the wildlife-human-livestock interface areas. This proves the role of wild animals in the transmission dynamics of brucellosis. Nevertheless, surveys done in Tanzania show that the prevalence of brucellosis in cattle ranges between 2% and 90% (Staak and Protz, 1973; Kitaly, 1984; Otaru, 1985; Minga and Balemba, 1990; Jiwa *et al.,* 1996; Swai *et al*., 2009; Weinhaupl *et al.,* 2000; Mtui-Malamsha, 2001; Minja, 2002; Swai *et al.,* 2005; Karimuribo *et al.,* 2007; Temba, 2011; Chitupila *et al.,* 2015; Assenga *et al.,* 2015).

In wild animals, brucellosis can be a result of spillover from infected livestock or as a natural sustainable infection within susceptible wild animal population (Davis *et al.,* 1990; Bishop *et al.,* 1994). Studies have shown that there is no difference in the pathogenicity and transmission rate of *B. abortus* from cattle to cattle and from cattle to bison, suggesting the possibility of spillover of the infection between livestock and wild animals (Davis *et al.,* 1990). This is of concern regarding the impact of the infection on wild animals reproductive rates and the possibility of wild animals acting as sources of infection for domestic animals and humans Mathias *et al.* (1999) and Muma (2007) identified 23 isolates of *Brucella* species from rodents of the *Capybara* species (*Hydrochaeris hydrochaeris*), eight of which were *B. abortus* and 15 isolates were *B. suis*, suggesting that rodents are reservoirs and important in the epidemiology of *Brucella* infection in wild animals. The two *Brucella* species which are *B. abortus* and *B. suis* have been isolated worldwide from a great variety of wild animal species (Davis, 1990). The transmission of brucellosis among wild animals is highly dependent on species and social behaviors (Hellman *et al.,* 2002). Transmission rates are greater in highly social animals,

especially ungulates like wildebeest and buffaloes. In social ungulates the bacteria are spread through direct contact with discharge from the vagina, aborted foeti, and sexual intercourse. The disease may also spread when wild animals from an infected herd mingle with brucellosis-free herds (Godfroid *et al.*, 2002). Insects (face flies) play a minor role in transmission and maintenance of the infection in herds (Hirsh and Zee, 1999). Wild ungulates could also attain infection by ingesting contaminated pasture (Bishop *et al*., 1994). Carnivores such as wolves and foxes are thought to be exposed through the ingestion of infected animals, placentae or aborted foeti. Introduction of an infected individual is not a sufficient indicator of transmission of *Brucella* to other animals of their inheritor species. The probability of brucellosis becoming established and being sustainable in a species will be equal to or less than the probability of infection and in some cases will be close to zero because a combination of factors must be taken into account, including host susceptibility (or resistance), infectious dose, (repeated) contacts with infected animals, seasonal infectivity (calving), management and environmental factors (Godfroid *et al*., 2002).

Brucella species are non-motile, non-spore forming, aerobic, non-toxigenic and non-fermenting Gram-negative coccobacilli. Members of the genus *Brucella* are divided into six classical species, namely *Brucella melitensis*, *B. abortus, B. suis, B. canis*, *B. ovis* and *B. neotomae*. This classification is still widely used due to historical reasons and the overall pathogenicity to humans. Recently there have been newly identified *Brucella* species isolated from marine mammals' namely *B. ceti* and *B. pinnipedialis*, and are now included in the classification. These species *B. ceti* and *B. pinnipedialis* have recently been reported to cause human brucellosis (Mariana *et al.*, 2010). The most pathogenic *Brucella* species reported to date include *B. suis, B. melitensis, B. abortus* and *B. canis* which classically infect swine, goats, cattle, and dogs, respectively. However, infection with any

of the four species of *Brucella* may occur in all domesticated as well as wild animals. The *B. canis* is also pathogenic to humans but is of lesser importance since canines are mostly considered dead-end hosts. On the other hand, *B. ovis* and *B. neotomae* have never been reported to cause disease in humans (WHO, 2006). Among the four *Brucella* species known to cause disease in humans, *B. melitensis* is thought to be the most virulent and causes the most severe and acute cases of brucellosis, while *B. abortus* is reported to be the most widespread (Yingst *et al.*, 2010). Humans are infected with *Brucella* through consumption of contaminated raw milk, milk products, blood and meat (WHO, 2006). Acquiring infection through direct contact is a potential threat to occupational groups such as farmers who assist livestock deliveries, veterinarians, butchers, laboratory workers, milkers and inseminators (Minja, 2002).

Brucella abortus and *B. suis* have also been isolated world-wide from a great variety of wild animal species, such as bison *(Bison bison)*, elk/wapiti (*Cervus elaphus*), feral pigs (*Susscrofa*), wild boar (*Susscrofa*), European hares (*Lepuscapensis*), foxes (*Vulpesvulpes*), African buffalo *(Syncerus caffer)*, eland (*Taurotragus oryx*), waterbuck (*Kobus elipsiprymnus*), reindeer (*Rangifertarandus tarandus*), and caribou (*Rangifer tarandusgroenlandicus*) (OIE, 2000). Although *B. melitensis* is rarely reported in wild animals, cases were recently reported in Europe in chamois (*Rupicapra rupicapra*) and ibex (*Capra ibex*) in the Alps (OEI, 2010). A very important issue in the study of brucellosis in terrestrial wild animal is to distinguish between a spillover of infection from domestic animals and a sustainable infection in wild species (OEI, 2010).

Diagnosis of brucellosis can be gifted through direct methods (DNA detection/ tissue culture) and indirect methods (serology) (McGiven, 2003; Smits and Cutler, 2004; Godfroid *et al*., 2010). Direct smear microscopic examination is documented by Kaltungo

et al. (2014) to be one of the possible method used. Bacteria culture is another method of diagnosis but in most cases takes longer time (4 to 30 days) as compared to the other methods (Kaltungo *et al*., 2014). Different serological testing methods such as Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and Complement Fixation Test (CFT) are recommended methods in animals and humans (MacMillan, 1990; WHO, 2006; Lyimo, 2013). The immunological sensitive and specific techniques for *Brucella* infection include Enzyme Linked Immunosorbent Assay (ELISA), Polymerase Chain Reaction (PCR) (Morta *et al*., 2003; Lyimo, 2013).

Molecular techniques are numerous which are PCR-based assays and have been developed for the identification of *Brucella* to improve diagnostic capabilities (Bounaadja *et al*., 2009). The molecular techniques includes Plasmid profiling, mol% G+C content, Nucleotide sequencing, Restriction fragment length profiling (RFLP), Pulse field Gel electrophoresis (PFGE), Nucleic acid hybridization, Amplification techniques (signal amplification, probe amplification & target amplification (Whatmore *et al*., 2005). In the current study, molecular methods including AMOS PCR, RT-qPCR and RT-speciation assay (Whatmore *et al*., 2005; Muchowski *et al*., 2015).

Prevention and control of *Brucella* infection in domestic ruminants is based on vaccination, test and slaughter. All these the reactors remains a mystery in countries like Tanzania because of the grazing system, constant interactions with wild animals, lack of test and slaughter policy and lack of practical disease control programmes. The aim of this study was to establish the occurrence of brucellosis in selected wild animal species in order to identify the different strains of *Brucella* species circulating in wild animals in the Serengeti ecosystem.

1.2 Problem Statement and study Justification

Brucellosis is a zoonotic disease of public health importance worldwide and it is known to affect humans, domestic and wild animals. The disease causes a lot of sufferings in humans which may associate with high treatment and control costs. In domestic animals, frequent abortions are common, other forms of infertility and variable manifestations which associated with brucellosis. Several studies using serological tests have reported occurrences of brucellosis in humans and domestic ruminants in Tanzania. Limited studies on brucellosis have been done in wild animals and therefore, the magnitude of the infection may be underreported. This may partly be due to lack of knowledge that wild animals may equally get infected as domestic animals, high costs associated with sampling of wild animals, inadequate diagnostic protocols in wild animals and lack of appropriate reagents for diagnosis (Assenga *et al*., 2015). Wild animals are considered to be potential sources of infection to livestock and humans. Insufficient information on the status of *Brucella* infection in wild animals and the major circulating species can potentially affect any control strategies for brucellosis. Vaccination campaigns as among the control strategy for brucellosis is host specific and can be planned to attain maximum coverage in livestock and significantly reduce the incidence of livestock to livestock transmission as well as to human transmission if the circulating *Brucella* strains are known. However, if a sufficient reservoir of infection is maintained in wild animals, especially in wildlife-livestock-human interfaces of the Serengeti ecosystem, even the highest coverage of vaccination campaigns in livestock would only have a temporary effect on reducing incidences of brucellosis.

Furthermore, the recent reports of newly identified wild animal species are potentially source of infection to humans, and bridging the transmission through livestock hosts further complicates attempts at control strategies aimed at vaccination in the latter hosts.

The successful livestock vaccination campaigns could reduce the emergence of new (and potentially more virulent) strains of *Brucella* spilling over from wild animals to newly naïve intermediate and final hosts. The study was conducted to find out if there are new circulating *Brucella* strains in Serengeti ecosystem using molecular methods.

1.3 Objectives

1.3.1 Overall objective

The overall objective of this study was to investigate the role of wild animal species in maintenance and spread of brucellosis in the Serengeti ecosystem, northern Tanzania.

1.3.2 Specific objectives

i. To determine the occurrence of *Brucella* species in selected wild animals species using molecular techniques;
ii. To establish the prevalence of *Brucella* infection in selected wild animals species in the Serengeti ecosystem;
iii. To compare the effectiveness of conventional PCR against Real-time PCR in the detection of *Brucella* infection in wild animals.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition and Aetiology of Brucellosis

Brucellosis is a contagious bacterial zoonotic disease of humans, domestic and wild animals, which induces inflammation of the genital organs, abortion, sterility and localized granulomatous lesions in different organs. The disease is caused by a group of bacteria belonging to the genus *Brucella*, which are Gram-negative cocccobacili that possess surface antigens located on the lipopolysaccharide (Hirsh and Zee, 1999). Brucellosis in cattle is usually caused by biovars of *B. abortus* with biovar 1 being the most frequently isolated type in Zimbabwe and worldwide (Matope, 2009). Brucellosis has been referred to by many terms over time such as; Mediterranean fever, undulant fever or Bangs disease. It can be transmitted to humans by direct or indirect contact with infected animals or their products (WHO, 2006). Brucellosis was first diagnosed in humans by the bacteriologist Sir David Bruce (for whom the genus *Brucella* is named) by isolation of the causative organism from fatal cases in 1887 (David and Arthur, 1998).

In livestock, the disease leads to vital economic losses because of fruitful impairment caused by abortion, spontaneous abortion or weak calves and high fatality rates. Retained placenta, altered physiological condition, orchitis, redness and arthritis are common signs of infection, with high concentration shedding of the organisms in female internal reproductive organ discharges and in milk (Xavier *et al.,* 2009). In humans, brucellosis causes a febrile disease that may be associated with a broad spectrum of symptoms that may be fatal in some cases (Cutler *et al.,* 2005). The disease affects cattle, swine, sheep, goats, camels, dogs, wild ruminants and marine mammals. It is a very important zoonotic disease that causes vital fruitful losses in sexually mature animals (Wadood *et al.,* 2009).

Currently, there are ten species represented within the genus *Brucella* that have been reported worldwide; each could infect completely different host species. However every *Brucella* species contains a preference for its host species: *B. melitensis* (sheep and goats), *B. abortus* (cattle), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs), *B. microti* (rodents), *B. neotomae* (rodents), *B. pinnipedialis* (pinnipeds), *B. ceti* (cetacea), and *B. inopinata* (Xavier *et al.,* 2009; Scholz *et al.,* 2010). Three biotypes (1-3) are known in *B. melitensis*; eight biotypes (1-7, 9) in *B. abortus*; and five biotypes (1-5) in *B. suis* (Whatmore, 2009). All *Brucella* species are potentially pathogenic to humans, with the exceptions of *B. neotomae, B. microti,* and *B. ovis* (Bret *et al.,* 2008; Ahmed *et al.,* 2010; Mariana *et al.,* 2010; Silva *et al.,* 2011).

Optimum temperature for culture is 37ºC; however the organism can grow at a range of temperatures between 20ºC and 40ºC. Whereas the optimum pH scale ranges from 6.6 to 7.4, a few *Brucella* species need greenhouse gas for growth. Typical colonies may appear from two to thirty days of incubation, however a culture can be considered negative if there are no colonies visible after three weeks of incubation (Poester *et al.,* 2010). *Brucella* is fastidious bacterium that takes 1-2 weeks to be isolated on enriched media (Minja, 2002). *B. melitensis, B. suis, B. arbotus* and *B. neotomae* could occur as either smooth or rough strains expressing smooth-lipopolysaccharide (S-LPS) or rough-lipopolysaccharide (R-LPS) as major surface antigens, while *B. ovis* and *B. canis* are naturally rough strains (Shirima, 2005). *Brucella* is distinguished from most alternative pathogens as a result of it doesn't have "obvious virulence factors" like "capsules, fimbriae, flagella, exotoxins, exo-proteases, or alternative exoenzymes, cytolysins, resistance forms, matter variation, plasmids, or lysogenic phage. But recently, a sort IV secretion system has been shown as a very important contributor to virulence (Bret *et al.,* 2008).

Brucellosis is a common disease of the rural poor especially the pastoral and agro pastoral communities in developing countries typically found in Sub-Saharan Africa (Minja, 2002; Shirima, 2005). *Brucella abortus* (with 7 biovars) affects cattle and African buffaloes; *B. suis* (5 biovars) affects swine and reindeer but also cattle, *B. melitensis* (3 biovars) affects goats but can also infect sheep and cattle, *B. canis* affects dogs and *B. ovis* affects sheep (Gee *et al.,* 2004; Huber *et al.*, 2009).

2.2 *Brucella* Infection in Wild Animals

Brucella infections have been documented worldwide over the years in a great variety of terrestrial wild animal species and wide variety of marine mammals (Godfroid, 2002). Brucellosis has also been reported with seroprevalence of 50% in bison in Yellowstone National Park in USA (AVMA, 2007). In Tanzania *Brucella* infection has been reported in several wild animals in different ecosystems (Sachs *et al.,* 1968; Schiemann and Staak, 1971; Shirima, 2005; Fyumagwa *et al.,* 2009; Mellau *et al.*. 2009; Temba *et al*., 2011; Assenga *et al.,* 2015).

2.3 Epidemiology of Brucellosis

2.3.1 Distribution

Bovine brucellosis caused by *B. abortus* biovars is a disease of both economic and public health importance in many geographical regions of the world (Matope *et al*., 2010). Brucellosis has been reported worldwide some developed countries have managed to eradicate the disease (Muma *et al.,* 2006). Animal brucellosis is still endemic in Mediterranean countries, Africa, the Middle East, South Asia and Central and South America (Theegarten *et al.,* 2008). Bovine brucellosis is known to occur in 40 of the 55 African countries for which investigative reports are available, and the prevalence ranged from less than 1% in East Africa to 30% in West Africa (Bedard *et al.*, 1993). The disease

is common in sub-Sahara and is mainly found in dairy animals (Godfroid *et al.,* 2005; Pappas *et al*., 2006). Pathogenic species of *Brucella* are *B. abortus* (bovine brucellosis), *B. melitensis* (Ovine and Caprine brucellosis), and *B. suis* (Swine brucellosis). Brucellosis is well-documented by the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and also the World Organization for Animal Health (OIE), as the most widespread bacterial zoonoses in the world posing serious public health problems and extensive economic losses (Lopes *et al.,* 2010; Neta *et al.,* 2010; Yasmin *et al.,* 2011). Brucellosis is one of the most important and well-known bacterial zoonoses in the world (Lopes *et al.,* 2010; Swai and Schoonman, 2010). The disease is additionally described as a true zoonosis because all human infections are of animal origin (Kaoud *et al.,* 2010). Brucellosis is considered a re-emerging disease of special importance in countries East and North of the Mediterranean, South and Central Asia, Central and South America. Brucellosis is more important in female animals where it causes abortions (Hirsh and Zee, 1999). Although many countries have eradicated *B. abortus* from cattle, in some areas *B. melitensis* has emerged as a cause of abortions.

Infected wild animals like buffaloes and wildebeest keep on shedding the pathogen into the environment, which further spread the infection to other animals and humans. The *Brucella* from infected animals is secreted in placenta, fetal fluids, aborted fetuses, other uterine discharges, milk, feces, vaginal mucus, urine, semen and other body fluids which may also serve as sources of infections to humans (Blood *et al.*, 2007). The probability of brucellosis becoming established and being maintained in a species depends on a combination of factors including host susceptibility, infection dose, contact with infected animals, livestock management and environmental factors (Godfroid, 2002). The observation suggests that the infection causes infertility in susceptible population in wild animals and can have a significant impact on the population growth. While this may

represent a potential reservoir for infection to livestock, the actual level of risk to livestock remains unknown (Bishop *et al.*, 1994). Currently it is not known what level of infection exists in wild herbivores in Tanzania and its adverse effect on wild animals. Surveys carried out in the pastoralist tribes and in dairy sector in Tanzania revealed different prevalence ranges of brucellosis in different regions such as Kilimanjaro (Swai *et al.*, 2005), Morogoro and Dar es Salaam regions (Swai *et al*., 2009; Morogoro Temba, 2011) and in Iringa and Tanga regions (Karimulibo *et al.*, 2007). The interaction between wild animals, livestock and humans contributes to its persistence in Tanzania (Bouley *et al*., 2012; Assenga *et al*., 2015).

2.3.2 Prevalence

Brucellosis has been reported in an exceedingly large range of African countries with a variation of prevalence in cattle (indigenous and dairy cow) like 3% in Malawi, 2.27% in Sudan, 4.2% in Ethiopia, 5.45-17.5% in Kenya, 9-61.8% in Egypt, 6.6-9.3% in Ghana,7-63% in Nigeria, 18.1% in Uganda, 22% in Mali, and 0.1% in Botswana (Minja, 2002). Brucellosis is endemic in Tanzania where the animal seroprevalence has been reported to range between 1 to 58.1%. Studies involving wild animals have indicated a seroprevalence ranging between 4.2% to 17% in buffaloes and 24% in wildebeest (Mellau *et al*., 2009). Brucellosis has been additionally documented worldwide in particular type of terrestrial life species similarly as in wide range of marine mammals (Mariana *et al.*, 2010). *Brucella* organism has been isolated in American buffalo, reindeer, and caribous in Canada, wild boars and brown hares in Europe (OIE, 2000).

The history of brucellosis in African country dates back to 1928 when samples of aborted oxen at Engarenanyuki in Arusha region of Tanzania were confirmed positive for brucellosis. Since then, a number of studies have been conducted to ascertain the illness

status in placental mammals. Surveys have shown the illness to occur in oxen in various regions and zones, with seroprevalence values varying significantly (Shirima, 2005). Brucellosis has been reported in Tanzania at the prevalence of 1-30% within the Northern zone (Mtui-Malamsha, 2001; Minja, 2002; Swai *et al.*, 2005; Shirima, 2005; Kunda *et al.*, 2010) and 12-14% in Eastern zone (Weinhaup *et al.*, 2000).

Brucellosis has been reported in a variety of domestic and wild animals, it is an important zoonosis causing undulant fever in humans (Radositits *et al.*, 2006). In addition, brucellosis has been observed in the domestic buffalo (*Bubalus bubalus*), American and European bison *(Bison bison*, *Bison bonasus*), yak (*Bos grunniens*), elk/wapiti (*Cervus elaphus*) and also occurs in the African buffalo (*Syncerus caffer*) and various African antelope species. The clinical manifestations of brucellosis in these animals are similar to those in cattle (Radositits *et al.*, 2006).

2.3.3 Transmission

Cattle are infected through licking of infected materials or the genital area of other infected cows or through ingestion of the disease-causing organism from contaminated water and pasture (Hirsh and Zee, 1999). The general rule is that brucellosis is carried from one herd to another by an infected animal and this mode of transmission occurs when an owner buys replacement cattle that are infected (Crawford *et al*., 1990). Humans can be infected by direct contact with infected animals, contaminated animal secretions, foeti and via retained placentas. They can also acquire infection by indirect transmission through consuming animal products mainly unpasteurized dairy products such as cheese and butter, as well as consuming blood and undercooked meat (Karimuribo *et al.*, 2007. It is an occupation risk for farmers, veterinary surgeons, and workers within the meat industry (Bertu *et al*., 2010). It is implicated as a major source of poor reproductive performance in

production animals in most parts of the world. Production losses to livestock farmers are inevitable as well as negative economic gain to the country at large (Chitupila *et al*., 2015). Aborted fetuses, placental membranes or fluids and other vaginal discharges present after an infected animal has aborted or calved are reported to be highly contaminated with infectious *Brucella* organisms (Godfroid *et al.,* 2002). Both wild and domestic animals are susceptible to infection with *Brucella* and may serve as carriers for other animals (Ahmad and Majali, 2005).

The disease may also spread when wild animals from an infected herd mingle with brucellosis-free herds (Godfroid *et al.*, 2002. In non-endemic countries with a successful eradication of animal brucellosis the disease is imported by travelling (Theegarten *et al.*, 2008). Brucellosis is commonly transmitted to susceptible animals by direct contact with infected animals or with an environment that has been contaminated with discharges from infected animals (Blood *et al*., 2007). This disease is transmitted by direct or indirect contact with infected execretors (Verger, 1985; Blood *et al*., 2007; Seleem *et al.,* 2010). Examples of human-to human transmission by tissue transplantation or sexual contact are occasionally reported but are insignificant (Corbel, 1997). This organism has also been implicated as a possible agent of bioterrorism (Valdezate *et al*., 2007). Transmission of brucellosis in terrestrial wild animals occurs through a spillover of infection from domestic animals and a sustainable infection in wild species (Truong *et al.,* 2011). Within wild animal species transmission has been observed to vary with species social behavior. Carnivore like wolves and foxes are thought to be exposed through consumption of infected animals, placenta or aborted fetuses. Ungulate animals reported to possess high rates, in these animals, brucellosis is transmitted through direct contact with discharge from the duct, aborted foeti and sexual activity. Wild ungulates conjointly acquire infection by uptake of contaminated pasture (Bishop *et al.,* 1994).

2.5 Diagnosis

2.5.1 Immunological tests

Detection of *Brucella* antibodies is another method of identifying the disease however; they are best joined with other tests. Immunological methods are frequently used for screening animals at herd level. Amid the tests used are Enzyme linked immunosorbent assay (ELISA), Complement fixation test (CFT), Rose Bengal plate test (RBPT) and Serum agglutination test (SAT) (Lyimo, 2013).

RBPT is an appropriate test for recognition of *Brucella* infested animals at a herd level. Its simplicity has made it a preferred test in the showing of animals to determine herd occurrence .It uses a standard that immunoglobulin M (IgM) antibody's competence to bind to antigens is significantly reduced when the pH is low (acidic) (WHO, 2006). It falls in a group of tests that are also known as the buffered *Brucella* antigen tests. It is a spot on test in which a stained antigen and antibodies are mixed on a glass plate. Positive reaction results into agglutinations (WHO, 2006; Ducrotoy *et al*., 2017).

ELISA tests are measured to be more sensitive and deliver more specificity than RBPT. However, there are cases that the tests fail to sense animals that were earlier picked by RBPT to be positive. Its' specificity is only marginally to that of CFT and RBPT. SAT is a simple and low-cost test in the diagnosis of Brucellosis. Nonetheless due to its insufficient sensitivity and specificity it has been dejected in the existence of other diagnostic tests (WHO, 2006).

2.5.2 Culture and isolation

Brucella organism isolation and identification from various tissues remains the most reliable means of diagnosis. The selection of tissue to culture is usually reliant on the type

of clinical signs that the animal is presenting (Poester *et al.,* 2010). The tissues however require careful and proper handling to prevent contamination but preserve the organisms as well. Among the preferred tissue from which samples for brucellosis diagnosis are acquired in animals are such as fetal membranes, hygroma fluids, vaginal discharges, aborted fetus, milk and semen. For cases that a postmortem is conducted then the preferred tissues are the udder, uterus (late term/ early post calving) and the reticulo-endothelial system tissues (OIE, 2009; Poester *et al*., 2010; Al-Garadi *et al*., 2011). Media selection depends on the type of tissue sample collected.

2.5.3 Molecular diagnostic techniques

These are modern diagnostic technique based on molecular biology. The *Brucella* organism can be identified directly from specimen hence shortening time required to isolate the pathogen (Whatmore *et al*., 2014). Molecular discovery of *Brucella* species can be through directly on clinical samples without previous isolation of the organism (Xavier *et al.,* 2010). The molecular techniques include Polymerase Chain Reaction (PCR), Restriction Endonuclease Analysis (REA), Restriction Endonuclease and Hybridisation which have been used for diagnosis and epidemiological studies of disease (Tenover, 1988; Ghassan *et al.,* 1996). Sequencing can help in characterization of different pathogen (Whatmore *et al.,* 2007; Whatmore *et al.,* 2008). It can provide both complementary and molecular epidemiological typing method based on specific genomic sequences (Whatmore, 2009). PCR diagnosis remains promising for the rapid diagnosis of acute but not chronic brucellosis since bacteriaemia is present only in the acute stages of infection (Sharma *et al*., 2008). In humans, due to its variable clinical features and lack of truly diagnostic tests, brucellosis remains a difficult disease to diagnose particularly in non-endemic countries with a low prevalence (Seleem *et al.,* 2010). PCR assays have been designed that are specific for the *Brucella* genus (Yingst *et al.,* 2010). Speciation by PCR

is possible, but it is not essential for initial diagnosis especially for outbreak detection (Yingst *et al.,* 2010). However these techniques are too expensive to be used widely, they are more and appropriate for differential diagnosis rather than for establishing prevalence of the disease.

The PCR involves three steps, namely, repeated rounds of denaturation, annealing of primers, and synthesis of DNA. A thermocycler machine is used to perform this reaction so that it can be programmed to change the temperatures quickly and accurately. Applications of the PCR are criminal investigations, DNA fingerprint, detection of pathogens, and analysis of DNA of early human species. In this study three types of polymerase chain reaction were used which are conventional multiplex AMOS PCR**,** RT-qPCR and RT-speciation assay. There are three major stages of conventional PCR, namely; DNA amplification stage, separation of PCR, and detection of products. Separation of DNA segments are typically done by agarose gel electrophoresis. The products are then stained with ethidium bromide. Finally, detection is achieved by visualization of bands onto gels under UV light. Therefore, the final results of conventional PCR are not expressed as numbers. Normally the conventional PCR is only able to detect a single parameter (Doosti and Ghasemi, 2011). Real-time PCR can detect the amplification products, as the products are synthesized. With the development of technology, PCR has become a very popular technique, especially for the detection and identification of bacteria in foods. The RT-speciation uses a florescent dye system and thermocycler equipped with fluorescent- detection capability (Glenn, 1997). The principal drawback of intercalator-based detection of PCR product accumulation is that both specific and nonspecific products generate signal (Whatmore *et al*., 2005).

These two types of PCR have similarities and different as follows: AMOS PCR is more time consuming as it uses gel electrophoresis to analyze the amplified PCR products. In contrast, RT-speciation is less time consuming as it can detect amplifications during the early phases of the reaction (Doosti and Ghasemi, 2011). Real-time PCR collects data at the exponential growth phase of PCR while traditional PCR collects data at end-point of the reaction (Doosti and Ghasemi, 2011). The end point results of the conventional PCR may not be very precise, but the results of the RT-speciation are very precise. Real-time PCR is more sensitive than conventional PCR. Conventional AMOS PCR has poor resolution while RT-speciation can detect very little changes due to the high resolution (Doosti and Ghasemi, 2011). End point detection of AMOS PCR has short dynamic range while RT-speciation detection has wide dynamic range. Unlike AMOS PCR, automated detection techniques are found in RT-speciation. Conventional AMOS PCR is highly sophisticated and labor intensive more than RT-speciation.

Real-time speciation assay systems are probe-based, rather than intercalator-based PCR product detection. The 5' nuclease assay provides a real-time method for detecting only specific amplification products. Cleavage of a target probe during PCR by the 5' nuclease activity of Taq DNA polymerase can be used to detect amplification of the target-specific product. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. RT-speciation has great advantages for estimating transcript levels in a variety of situations. The advantages include relative rapid assay times, reliability and ease of performing analyses.

2.6 Treatment and Control of Brucellosis

2.6.1 Treatment

Testing of livestock is cumbersome when dealing with farms located in remote areas or with animals from nomadic populations and migratory farmers (Abdoel *et al*., 2008). Treatment of brucellosis is not recommended in animals because the success rate is very low and expensive. Treatment in wild animals is almost impossible because it is expensive, time consuming and stressing to the animals (Godfroid, 2002). Tetracycline, rifampicin and the aminoglycosides such as streptomycin and gentamicin are effective against human brucellosis (Carter *et al.,* 1995).

2.6.2 Control of Brucellosis

There is general agreement that the most successful method for prevention and control of brucellosis in animals is through vaccination (Ibironke e*t al.,* 2008; Donev, 2010). While the ideal vaccine does not exist, the attenuated strains of *B. melitensis* strain Rev.1 for sheep and goats and *B. abortus* strain 19 have proven to be superior to all others. The non-agglutinogenic *B. abortus* strain RB51 has been used in the USA and some Latin American and sub Saharan countries with encouraging results (Ibironke *et al*., 2008). Vaccination against cattle brucellosis using S19 in Tanzania was adopted early 1980's (WHO, 2006). However vaccination was confined to government and parastatal dairy farm and no vaccination has been carried out in agro-pastoralist and pastoral animals (Shirima, 2005). The best way to deal with brucellosis in a herd is to vaccinate all heifers between 3 months and 10 months of age with strain 19 vaccines and to remove those which react positive to convectional serological tests (OIE, 2008).

The live vaccines have provoked unacceptable reactions in individuals sensitized by previous exposure to *Brucella* or if inadvertently administered by subcutaneous rather than percutaneous injection. Various preparations have been used, including the live attenuated

B. abortus strains 19-BA and 104M used in the Russia and China, in the cases of live vaccines, there were potentially serious reactogenic (Shang *et al.*, 2002). There are no licensed vaccines for humans. Consequently, since vaccination is among the potential means of controlling brucellosis in human then further research is required to discover vaccine preparation that will be safe for human, conveniently available and affordable especially to poor communities (Shang *et al.*, 2002; WHO, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out at Serengeti ecosystem in Northern Tanzania. The ecosystem is bordered in North by Kenyan border, which is between 1 and 3 degrees south (latitudes), and between 34 and 36 degrees east (longitudes) and it spreads to the Maasai Mara National Reserve. In the Southeast it is bordered by Ngorongoro Conservation area, while to the Southwest lies Maswa Game Reserve. To the West lay the Ikorongo and Grumeti Game Reserves and in Northeast and East is the Loliondo Game Control Area. Together, these border regions demarcate the larger Serengeti ecosystem. Figure 1 shows a map of the Serengeti ecosystem.

The Serengeti hosts the largest native mammal migrations in the world, which helps secure it as one of the Seven Natural Wonders of Africa as well as one of the ten natural travel wonders of the world. In addition, it is well known for its large lion population and is one of the best places to observe prides in their natural environment. The region contains the Serengeti National Park in Tanzania and several game reserves. Serengeti ecosystem has high multiplicity of different animals and plant species where by approximately 70 species of huge mammals and 500 bird species are found. The study area was selected because there is high interaction between wild animals, livestock and human beings.

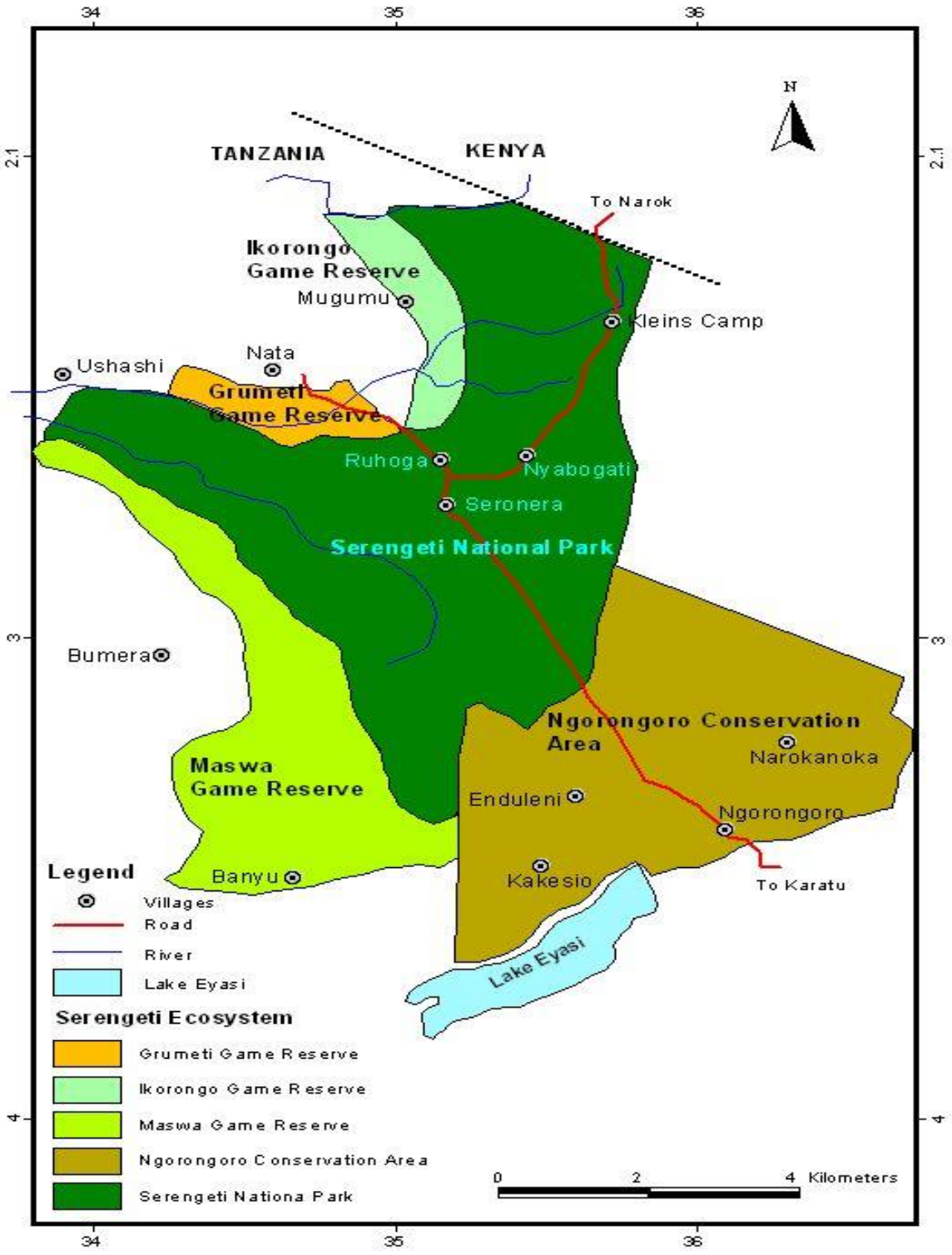

Figure 1: Map showing Serengeti ecosystem in Northern Tanzania

3.2 Study Design

The present study was of a cross sectional. The serum, whole blood and amniotic fluid samples used were collected and archived by other researchers between 2000 and 2017. Samples were collected from buffaloes, wildebeest, zebra, lions, baboon, impala and hyena. Amniotic fluid was collected from 26 wildebeest, whole blood from 155 animals selected for study and serum collected from 8 buffaloes. Samples were retrieved from the bank at Tanzania Wildlife Research Institute (TAWIRI) in Arusha and Serengeti which were stored at -20°C. Samples were stored in the cool box packed with ice packs and transferred to the College of Veterinary Medicine and Biomedical Sciences (CVMBS) laboratories and stored at -20°C until analysis.

3.3 Detection of *Brucella* Species in whole Blood, Serum and Amniotic Fluid by Polymerase Chain Reaction (PCR)

The detection of DNA of *Brucella* species in blood, serum and amniotic fluid by Polymerase Chain Reaction (PCR) was done as detailed in the subsequent sections.

3.3.1 DNA extraction from blood, serum and amniotic fluid

DNA extraction of *Brucella* was done as explained by the manufacturer of Zymo Research (ZR), USA Genomic DNA™ Tissue Mini Prepkit as described by Navarro *et al*. (2002). Briefly, 400 µl of Genomic Lysis Buffer was added to 200 µl of the source sample (whole blood, serum or amniotic fluid).The constituents of the PCR mix are shown in Table 1. The mixture was subjected to digestion, deactivation, washing and elution steps as per manufacturer's instructions. Working aliquots of all extracted DNA samples were adjusted at same concentration level i.e. 50 ng/l. Stock DNA samples were stored at -20 ºC freezer until the performance of PCR.

Table 1: Preparation of master mix PCR for the detection of *Brucella* in EDTA blood, serum and amniotic fluid samples

Components	1X (µl)	190x (µl)
Taq reaction buffer	2.5	475
dNTP	0.5	95
Taq polymerase	0.125	23.75
Primer cocktail	1	190
Nuclease free water	16.8	3192
Total	20	3945.75/190=20

3.3.2 Amplification of *Brucella* species DNA by Multiplex Conventional AMOS PCR

Recognition of the presence of *Brucella* spp. nucleic material in the eluted extract was detected using diagnostic AMOS PCR. Primers were used to amplify different base pair fragment that contained the target gene (Table 2). These primers were obtained from Bioline, Inc., (Taunton, MA, USA), as described by (Baily *et al*., 1992). All amplifications were performed in a total volume of 25 µl. The reaction mixtures containing primers were prepared and DNA sample was added. PCR was performed using a DNA polymerase procured from INQABA South Africa. The amplification conditions consisted of an initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation (30 seconds at 95°C), annealing (30 seconds at 55°C) and extension (90 seconds at 72°C), and a final extension at 72°C for 10 minutes on a thermal cycler (TaKaRa, Japan). After PCR, 5 µl of the PCR products was mixed with a 6x loading dye.

Table 2: Showing pairs of primers used to amplify the target region of *Brucella* spp present in the DNA extracts

Primers	Nucleotide sequences 5' to 3'	Concentration100x(µg/µl)
IS711-specific	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT-TGC	1.90
B. abortus specific	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	1.55
B. mellitenses specific	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	1.48
B. ovis specific	CGG-GTT-CTG-GCA-CCA-TCG-TCG	1.35
B. suis-biovar 1 specific	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG	1.48
B. suis-L specific	CGA-ACA-CGT-CGG-CAC-GCC-AGT-TCA	1.60
Suis-R specific	GCA-TCG-GCG-GGA-AAG-ACA-GCG-TTA-T	1.60

3.3.3 Preparation of agarose gel

Agarose gel was prepared by mixing 1 g of agarose powder (Invitrogen Carls bad, CA) in 100 ml of 0.5 × TBE buffer in a Pyrex conical flask to obtain a 1% concentration of the gel. The mixture was completely dissolved by boiling on a hot plate while hand shaking the conical flask. Agarose solution was left to cool at a temperature of 36°C set for about 15 minutes. This was followed by addition of 8 ml EZ-vision stain and hand shaken accordingly. The mixture was immediately poured into the horizontal electrophoresis casting equipment in the presence of a comb and was left out for about 40-60 minutes for solidification.

3.3.4 Loading of PCR products in agarose gel electrophoresis

A volume of 5 µl of the PCR products was mixed systematically with 1 µl of dark blue 6x loading dye (Promega, Madison-USA) on a laboratory parafilm. The PCR products were loaded in the wells of the agarose gel and 10 µl of 1 kb molecular weight marker (Promega, Madison, USA) was loaded in a parallel track on either side of the plate. The

horizontal gel electrophoresis was accomplished at a voltage of 120V for 120 minutes. The DNA bands were visualized by UV transluminator and photographed thereafter, finally the results were read and image captured using a gel documentation system (Gel doc EZ Imager, BioRed, USA). The RB 51 was used as positive control and nuclease free water was used as negative control for *Brucella* detection using AMOS PCR by observing different bands which appeared on agarose gel used to score *Brucella* species (Table 3).

Table 3: *Brucella* strains and predicted amplicons used for specie categorization

	Predicted amplicon sizes (bp)							
Brucella strain	1000	800	730	495	379	300	285	180
B. abortus biovars 1, 2 or 4	-	+	-	+	-	-	-	+
B. abortus strain 19	-	+	-	+	-	-	-	-
B. abortus strain RB51	-	+	-	+	-	+	-	+
B. melitensis biovars 1, 2, 3	-	+	+	-	-	-	-	+
B. ovis	+	+	-	-	-	-	-	+
B. suis biovar 1	-	+	-	-	+	-	+	+
B. suis biovars 2, 3, 4, 5	-	+	-	-	+	-	-	+
B. canis	-	+	-	-	+	-	-	+
B. neotomae	-	+	-	-	+	-	-	+
Marine mammal *Brucella* spp.	-	+	-	-	+	-	-	+
Non-*Brucella* spp.	-	+	-	-	-	-	-	-

Note: + *Brucella* strain present; - No *Brucella* strain detected

3.3.5 Methodology of Quantitative Real-Time PCR (RT-qPCR)

3.3.5.1 Amplification of *Brucella* species DNA by RT-qPCR

The RT-qPCR analysis was carried out as instructed by the manufacturer in the *Brucella* genus Genesig® standard kit based on two target genes which are IS711 and BCSP31 from the already extracted DNA template. A volume of 100 µM stock (µl) PCR reactions was used as described on table 4a and 4b respectively. The reactions followed the following steps: The samples were at 37ºC for 15 minutes followed by enzyme activation

at 95^{o}C for 2 minutes. This was then followed by 50 cycles of denaturation at 95^{o}C for 10 seconds. Data acquisition was done at 60^{o}C for 60 seconds.

The primers and probe to these targets were redesigned for the multiplex TaqMan format. The *B. abortus* primers and probe set targets the specific insertion of an IS*711* element downstream of the *alkB* gene (GenBank accession number AF148682), whereas the *B. melitensis* primers and probe set targets the insertion of an IS*711* element downstream of the same IS*711*reverse primer, while the forward primers target either *AlkB* (*B. abortus*) or BMEI1162 (*B. melitensis*). The *B. abortus* and *B. melitensis* TaqMan probes target the *alkB* and BMEI1162 gene, respectively. The *B. abortus* primers, 50x EXO IPC DNA (green), TE buffer and probe set targets the specific insertion of an IS*711* element downstream of the *alkB* gene (GenBank accession number AF148682) (Table 4a). The positivity criteria of the assay requires that a sample amplifies in both targets and below a set amplification cycle (<38) in order to be considered as positive for *Brucella*.

Table 4a: *IS711*primers/probe working dilution for *Brucella* species detection

Name	Sequence (5' to 3')	Volume of 100 µM stock (µl)
*IS711*forward primer	TGG-CTC-GGT-TGC-CAA-TAT- CAA	8
IS711 MGB probe primer	AAGCCAACACCCGGC	4
IS711 reverse primer	CGC-GCT-TGC-CTT-TCA-GGT	8
50x EXO IPC DNA (Green)		10
TE buffer		170
Total volume		200

All primers and TaqMan probes were designed using the multiplex TaqMan design feature of Beacon Designer software (Premier BioSoft International, Palo Alto, Calif.). For

Brucella spp. identification, the primers, TE buffer and probe target the *bcsp31* gene (GenBank accession number M20404) were used (Table 4b).

Table 4b: *BCSP31* primer/probe working dilution for *Brucella* species detection

Name	Sequence (5' to 3')	Volume of 100 µM stock (µl)
BCSP31 forward primer	ATG-TAT-TGC-GCC-GTC-TGG	8
BCSP31 probe primer	AAATCTTCCACCTTGCCCTTGCCATCA	4
BCSP31 reverse primer	TGC-ATC-AGG-CGG-CGA-ATG	8
TE buffer		180
Total volume		200

3.3.5.2 Methodology of speciation by using RT-speciation

For *Brucella* spp. identification, the primers and probe targeting the *Bcsp31* gene (GenBank accession number M20404) as described by Probert *et al.* (2004) was used. The nucleic acid targets for *B. abortus* and *B. melitensis* identification the primer and probe target the *bcsp31* gene (GenBank accession number 20404). The *B. abortus* primers and probe set targets the specific insertion of an *IS711* element downstream of the *AlkB* gene (GenBank accession number AF148682) and *BMEI1162* (access no. NC_003317). The 50 µl multiplex PCR mixture consisted of: 1x AmpliTaq Gold buffer (Applied Biosystems, Foster City, Calif.), 6 mM $MgCl_2$, 2 mM of deoxynucleoside triphosphate blend (Applied Biosystems), a 200 nM concentration of each primer, a 100 nM concentration of each probe 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 5 µl of a DNA extract. Amplification and real-time fluorescence detection was performed on the iCycler real-time PCR detection system (Bio-Rad Laboratories, Hercules, Calif.). The RT-speciation PCR used the following parameters: 10-min denaturation and polymerase activation step at 95°C followed by 45 cycles of 95°C for 15 seconds and 57°C for 1

minute. Note that the positivity criteria of the assay requires that a sample amplifies in both targets and below a set amplification cycle (<38) in order to be considered as positive for *Brucella*.

Real-time speciation assay was used for confirmation of presumptive *Brucella* isolates. The assay was designed in a multiplex format that will allow the rapid identification of *Brucella* spp., *B. abortus*, and *B. melitensis* in a single test. Oligonucleotide primers and probes used in the real-time multiplex PCR assay for the speciation of *Brucella; B. abortus* and *B. melitensis* as shown in Table 5.

Table 5: Oligonucleotide primers and probes used in the RT-speciation of *Brucella; B. abortus, B. melitensis, B. ovis* and *B. suis*

PCR ID	Forward primer	Reverse primer	Probe
B. melitensis	AACAAGCGGCACCCCTAAAA	CATGCGCTATGATCTGGTTACG	CAGGAGTGTTTCGGCTCAGAATAATCCACA
B. abortus	GCGGCTTTTCTATCACGGTATTC	CATGCGCTATGATCTGGTTACG	CGCTCATGCTCGCCAGACTTCAATG
B. ovis	GCCTACGCTGAAACTTGCTTTTG	ATCCCCCCATCACCATAACCGAAG	
B. suis	TGCGCTATGATCTGGTTACGTT	AGCGCGGTTTTCTGAAGGT	

3.4 Ethical Consideration

This study was conducted in conformity with the ethical guidelines and the permission of conducting the study was obtained from the Vice Chancellor of Sokoine University of Agriculture (SUA) (Appendix 1). A research permit for study conduct in wild animal was provided by the Tanzania Wildlife Research Institute (TAWIRI) director (Appendix 2). All the information collected from the participants and the laboratory results obtained after

blood, serum and amniotic fluid samples analysis were kept under the custody of the researcher as confidential.

3.5 Data Analysis

Data was entered and coded in Microsoft Excel (2010) and then transformed into advanced mathematics and statistics software implemented in R® software (R foundation for statistical computing, Canada) analysis. Proportions of categorical variables like sex, age group, location and, sample type and animal species were computed. The chi-square (χ^2) test was used to compare age, sex, location, wild animal species as determinants of brucellosis positivity; population differences of p value < 0.05 were considered a significant. Cross tabulation was used to determine the diagnostic sensitivity and specificity of the AMOS PCR and RT-qPCR using the RT-speciation assay as the reference test for comparison. Cohen's two-rated Kappa scores for the independent tests were also calculated to evaluate agreement between individual tests without a reference test.

3.6 Comparison of Molecular Techniques Used for Detection of *Brucella* Strain

Detection of *Brucella* strains in positive samples used three molecular techniques. Conventional AMOS PCR used different pair of primers to detect different *Brucella* strain and RT-qPCR used *IS711* and *BCSP31* genes for detection of *Brucella* strain and down-stream speciation of *Brucella* positive samples targeted *AlkB* and *BMEI1162* to distinguish *B. abortus* and *B. melitensis*. The comparison of these molecular techniques was based on the specificity and sensitivity of each method considering positive and negative results using speciation data as reference diagnostic test.

CHAPTER FOUR

4.0 RESULTS

4.1 Description of Samples Used in the Analysis

A total of 189 whole blood, serum and amniotic fluid samples were collected from seven wild animal species and analysed. Table 6 summarizes the type of samples and the animal species involved.

Table 6: Wild animal species, types and number of samples analysed to determine *Brucella* species in Serengeti ecosystem Tanzania, 2017-2018

Wild animal species	Types and number of samples		
	Whole blood	Amniotic fluid	Serum
Buffaloes	38	0	8
Wildebeest	69	11	0
Zebra	25	0	0
Lions	19	0	0
Baboons	5	0	0
Impala	10	0	0
Hyenas	4	0	0
Total	170	11	8

4.2 Characteristics of Animal Species Used in the Study

A total of seven species of wild animals were used in the study. The dominant wild animal species sampled were wildebeest. Most of the animals were female (96.8%), adults (98.4%) and those sampled from the Serengeti area (60.9%). Table 7 summarizes the characteristics of animals and samples collected in Serengeti and Ngorongoro.

Table 7: Characteristics of animal species sampled for the study

Variable	Categories	Number	Percentage
Species	Buffaloes	46	24.3
	Wildebeest	80	42.3
	Zebra	25	13.2
	Lions	19	10.1
	Baboons	5	2.7
	Impala	10	5.3
	Hyenas	4	2.1
Sex	Female	183	96.8
	Male	6	3.2
Age (group)	Adult	186	98.4
	Sub-adult	3	1.6
Location of sample collection	Serengeti	115	60.9
	Ngorongoro	74	39.2

4.2 Detection of *Brucella* in Wild Animal Samples

4.2.1 Detection of *Brucella* using AMOS PCR

Of 189 samples screened, DNA extracts from 12 (6.3%) samples (9 whole blood, 1 serum and 2 amniotic fluid) from selected wild animal species were *Brucella* positive by AMOS PCR (Figure 2). The animal species that were found to be *Brucella* positive by AMOS PCR include: 1 (2.2%) buffalo, 3 (15.8%) lions, 4 (5%) wildebeest, 2 (20%) impala, 1 (4%) zebra and 1 (25%) hyena. The 12 positive samples met the criteria of amplifying PCR products of base pair characteristics typical of *B. abortus, B. melitensis* and *B. suis* on gel electrophoresis.

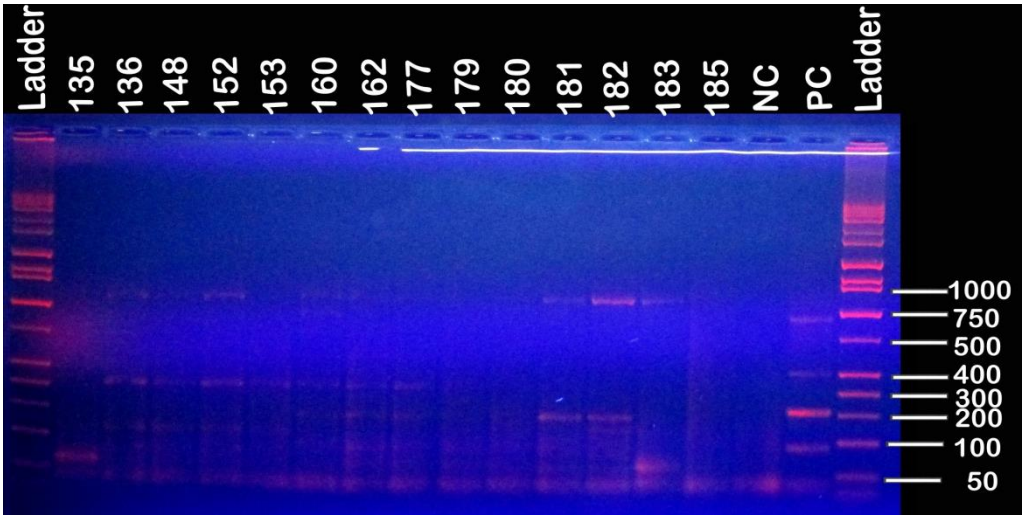

Figure 2: **Amplification of *Brucella* species-specific *IS711* gene using PCR. A 1% agarose gel electrophoresis of *Brucella* species specific *IS711* gene amplicon of different Bp from total DNA from whole blood, amniotic fluid and serum samples. LD is 1kb DNA ladder; Lane135, 136, 148, 152, 153, 160, 162, 177,179, 181, 180 and 182 are positive samples. Lane 183 and 185 are negative samples. Lane NC negative control containing nuclease free water. Lane PC positive control comprising DNA of *B. abortus* strain RB51.**

4.2.2 Detection of *Brucella* using real-time quantitative PCR

Two gene targets *Bcsp31* and *IS711* were used for detection of *Brucella* DNA by RT-qPCR in 189 samples (Table 8). The results indicate that 24 samples (12.7%) were positive for *Brucella* DNA. Figure 3 (a and b) illustrate the fluorescence against cycle time (CT) plot of amplifications on the Rotor Gene platform used to classify samples as positive for *Brucella* specie.

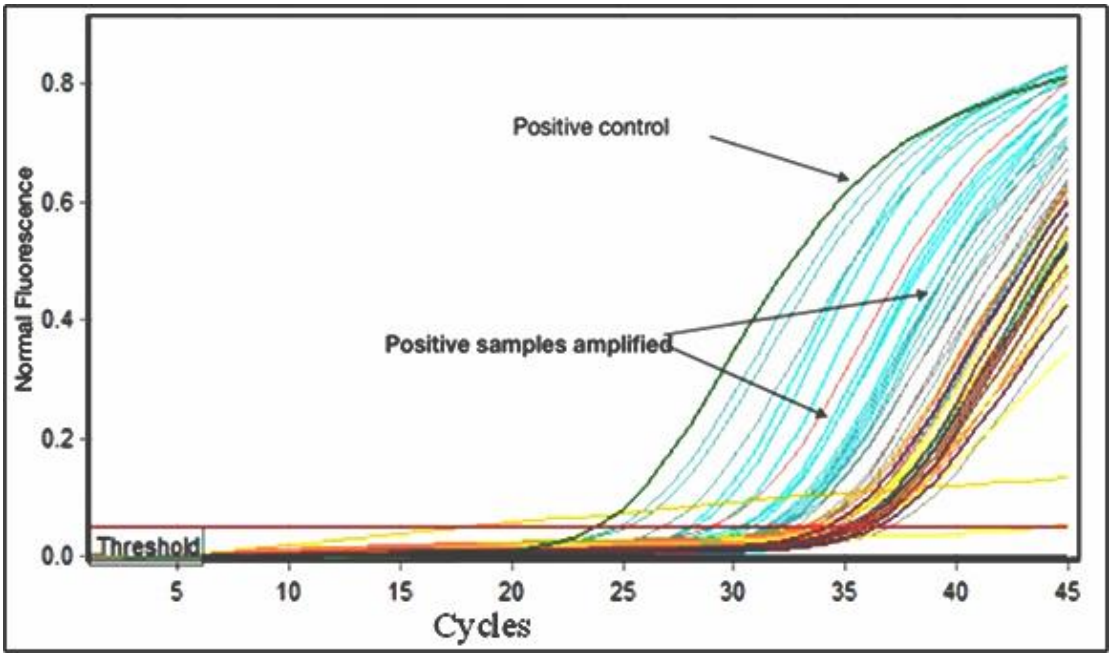

Figure 3a: Results of samples tested by qPCR using *IS711* on the Rotor Gene ® Q-series platform. Template DNA was run against a positive control of *B. abortus* template DNA and a negative template control of nuclease free water (not shown in image). Samples that amplified above the "Threshold" line (Red) and below a cycle time of 38 were considered as positive. All samples were subjected to double amplification on both targets with analysis of amplification curve times and duplication against both targets to determine *Brucella* positivity.

Figure 3b illustrates the amplification curves for same samples run against the *Bcsp31* gene marker.

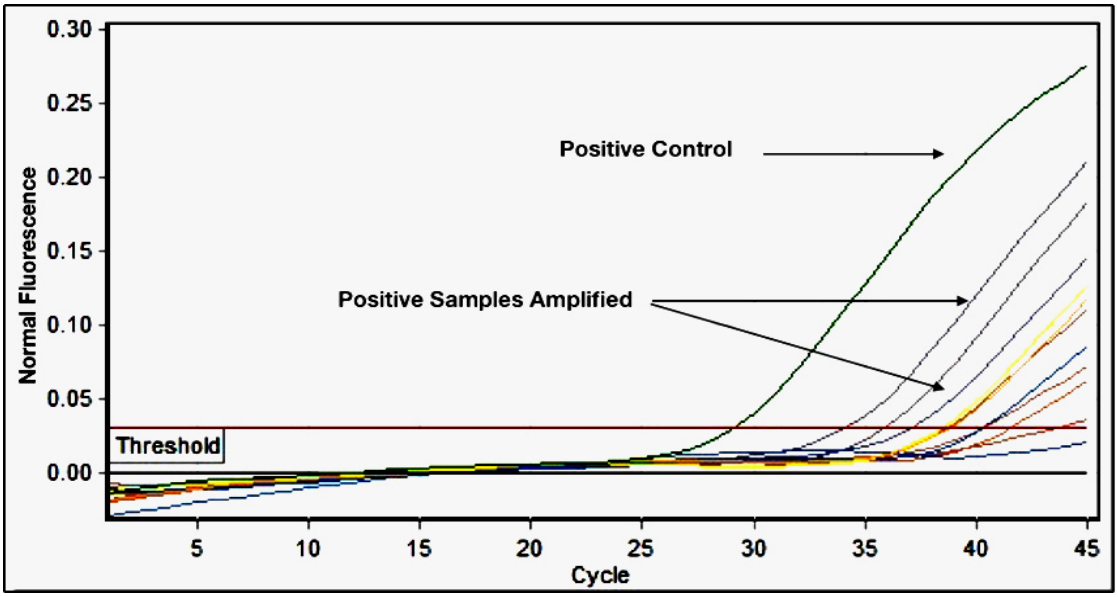

Figure 3b: Results of samples tested by RT-qPCR targeting *Bcsp31* on the Rotor Gene ® Q-series platform. Template DNA was run against a positive control of *B. abortus* template DNA and a negative template control of nuclease free water (not shown in image). Samples that amplified above the "Threshold" line (Red) and below a cycle time of 40 were considered as positive.

Using the above set criteria, only 24 samples (12.7%) that amplified in both targets with sufficiently low cycle times to indicate sufficient target DNA in the template were deemed positive. The final positive sample types and distribution data are summarized in Table 8. The differences in percentages of *Brucella* detection according to age group, wild animal species and type of sample were statistically significant ($P<0.05$).

Table 8: Detection of *Brucella* and associated factors from collected samples (n = 32)

Variable	Categories	Number	Percentages	Pearson's χ^2	p-value
Sex	Female	30	16.4	4.36	0.0188*
	Male	2	33.3		
Age (group)	Adult	30	16.1	4.36	0.04*
	Sub-adult	2	66.7		
Location	Serengeti	23	20.0	0.27	0.03*
	Ngorongoro	9	12.2		
Species	Buffaloes	9	19.6		
	Wildebeest	4	5.0		
	Zebra	1	4.0		
	Lions	10	52.6	23.20	0.001*
	Baboons	0	0.0		
	Impala	6	60.0		
	Hyenas	0	0.0		
Sample type	Whole blood	29	17.1		
	Serum	1	12.5	6.21	0.04*
	Amniotic fluid	2	18.2		

* means statistically significant at $p<0.05$

4.2.3 Speciation of *Brucella* in collected samples by molecular techniques

A total of 24 samples that tested positive by RT-qPCR were subjected for further speciation using RT-speciation assay. By using conventional AMOS PCR and RT-qPCR the results show a number of *Brucella* species detected in samples. The AMOS PCR primers allowed detection of *Brucella* at the genus, species and biovar level, while the RT-qPCR only supported distinguishing to the species level for *B. abortus* and *B. melitensis.* The results indicate that 16 (66.7%) out of the 24 samples were identified as *B. abortus*, other *Brucella* species identified were 2 (8%) *B. suis, 2* (*8*%) *B. melitensis* and 2 (8%) *B. ovis* (Table 9). Two samples; one from buffalo and one from impala had three *Brucella* species each namely *B. melitensis, B. suis* and *B. ovis* implying that they had multiple infections.

Table 9: Speciation of *Brucella* in wild animals in Serengeti Ecosystem (n=189)

Wild animal	AMOS PCR	Quantity	RT-speciation	Quantity
Lions	*B. abortus biovar 1, 2 or 4*	3	*B. abortus*	6
Buffaloes	*B. abortus biovar 1, 2 or 4*	1	*B. abortus*	7
			B. melitensis	1
			B. ovis	1
			B. suis	1
Wildebeest	*B. suis biovar 1, 2, 3, 4, 5*	4	-	0
Zebra	*B. suis biovar 1, 2, 3, 4, 5*	1	*B. abortus*	1
Impala	*B. melitensis*	1	*B. abortus*	2
			B. melitensis	1
			B. suis	1
			B. ovis	1
Hyenas	*B. suis biovar 1, 2, 3, 4, 5*	1	-	0
Baboons	-	0	-	0

Each sample was run in a separate reaction tube against negative and positive controls on the PIKO real-time platform. The primers used in the RT-speciation allowed a multiplex detection of *Brucella* at the genus level targeting the *IS711* gene, followed by a nested step detecting the respective species of *Brucella* identified using the *Alk-B* gene for *B. abortus* and the *BMEI1162* gene for *B. melitensis.* The amplification graphs of 18 (9.5%) samples detected by the RT-speciation assay are as seen in the Figure 4.

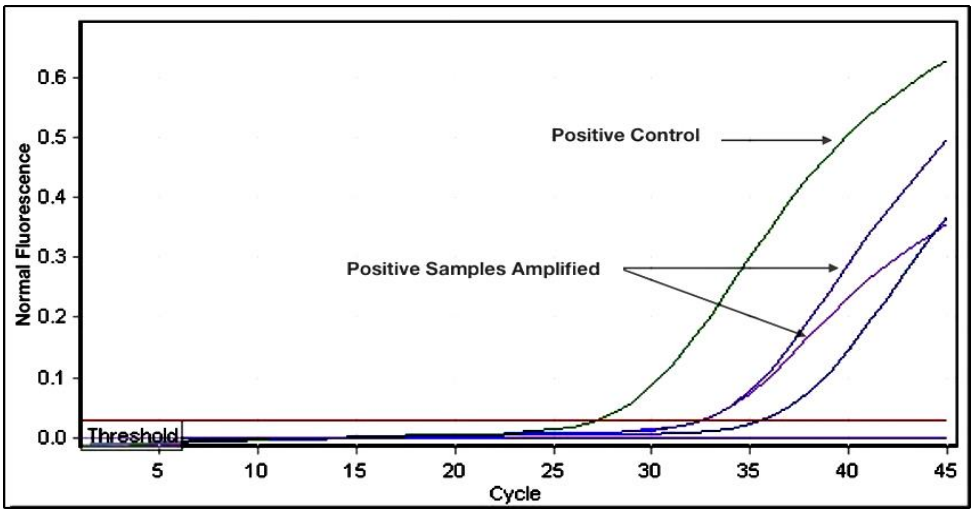

Figure 4: A graph output from the qPCR PIKO real time PCR machine corresponding to 18 (9.5%) samples that were run. Each sample reaction was run against a positive control (*B. abortus*) and a negative template control (nuclease free water, not shown).

4.3 Comparison of Molecular Techniques Used for Detection of *Brucella*

4.3.1 Comparison using real-time speciation assay as a reference test (sensitivity and specificity)

A comparative analysis for the detection of *Brucella* DNA in extracts from the wild animal samples using the conventional AMOS PCR and RT-qPCR techniques targeting the respective markers was done. The RT-speciation assay was used as a reference test since its design allows specific targeting of *Brucella* species identified in earlier screening tests. Using the RT-speciation assay as the reference test, AMOS PCR had a sensitivity of 12.5% and specificity of 94.2%, while the RT-qPCR assay had a sensitivity of 100% and specificity of 96.5%. Simple 2x2 cross tabulation analysis was done in R and the results of the diagnostic sensitivity and specificity are summarized in Table 10.

Table 10: Cross tabulation of the molecular tests used, with RT-speciation assay as the reference

	RT-speciation		
	Positive	Negative	Total
AMOS			
Positive	2 (0.125)	10 (0.058)	12
Negative	14 (0.875)	163 (0.942)	173
RT-qPCR			
Positive	16 (1.0)	6 (0.035)	22
Negative	0 (0)	167 (0.942)	167
Total	16	173	189

4.3.2 Kappa score comparison of molecular tests without a reference test

The comparison of tests against a reference standard is a robust tool for the determination of relative diagnostic performance. However, in the absence of a reference test, or as in this case where all the three tests were done in parallel, the level of agreement between any two tests is a more accurate determination of the positivity ratio detected in the population. Cohen's two-rater Kappa score detects the number of positive cells in each

test, irrespective of whether or not they were also detected in the comparative test. The Kappa score therefore compares the net positivity detection between any two tests. Overall, there was very little agreement between all the three techniques, with only 3 (1.6%) samples being detected by all test approaches. The Kappa score test showed that there was good agreement between the RT-qPCR and RT-speciation assay (0.82), while there was poor agreement between AMOS PCR and either of the RT speciation assays (0.075 and 0.04). The results of the scores for the molecular techniques are summarized in Table 11.

Table 11: Cohen's Kappa score comparison of molecular tests used

	Cohen's Kappa Score [95% CI range]		
Test	AMOS PCR	RT-qPCR	RT-speciation
AMOS	1	0.04 [-1.73; 1.80]	0.075 [-1.71; 1.87]
RT-qPCR	0.04 [-1.73; 1.80]	1	0.82 [-1.09; 2.75]
RT speciation	0.075 [-1.71; 1.87]	0.82 [-1.09; 2.75]	1

Key to Kappa scores:1 – perfect agreement; ≥0.75 – excellent agreement; 0.4<K<0.75 Fair - Good agreement; ≤0.4 – Poor agreement; 0 – No agreement

Overall, the comparison of the molecular tests showed better agreement and diagnostic performance with the RT-qPCR techniques compared to the AMOS PCR assay. The results of the comparison of molecular techniques are summarized in Table 12.

Table 12: Summary of the test characteristics for molecular assays used

Characteristic	AMOS PCR	RT-qPCR	RT-speciation
Sensitivity (%)*	12.5	100	100
Specificity (%)*	94.2	96.5	100
Kappa score [CI]	0.04 [-1.73; 1.80] [a]	0.82 [-1.09; 2.75] [b]	0.075 [-1.71; 1.87] [c]

[a] K AMOS PCR vs RT-qPCR; [b]RT-qPCR vs RT-speciation; [c] AMOS PCR vs RT-speciation
*Calculated using RT-speciation assay as the reference test

Overall, the comparison of the molecular tests showed better agreement and diagnostic performance with the RT-speciation techniques compared AMOS PCR assay. The results of the comparison of molecular techniques which used for speciation are summarized in Figure 5a (AMOS PCR) and 5b (RT-speciation) based on animal species tested positive.

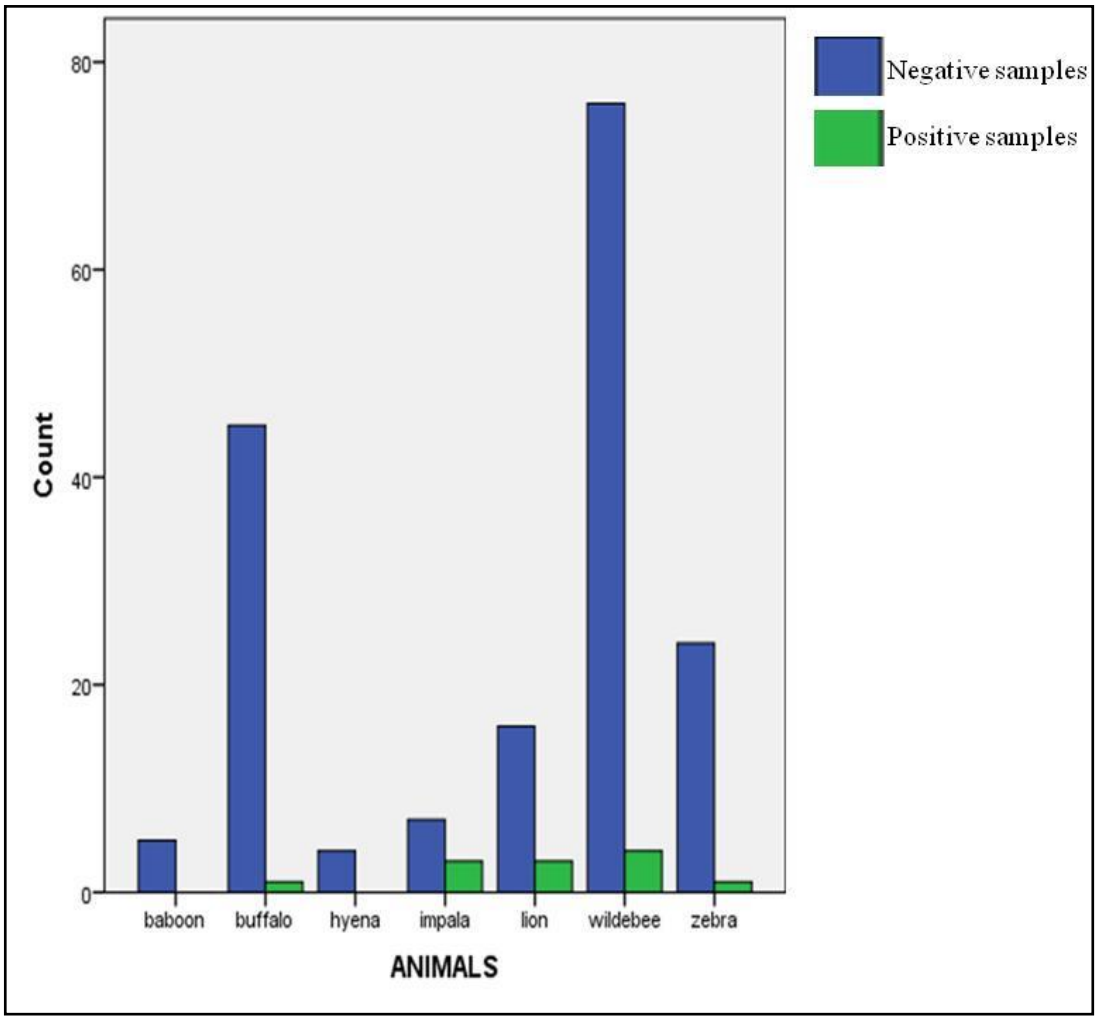

Figure 5a: Major animal species detected with *Brucella* are indicated with green colour by using AMOS PCR buffaloes, impala, lions, wildebeest, and Zebra are species tested positive.

Figure 5b: illustrates *Brucella* positive wild animal species detected by RT-speciation assay.

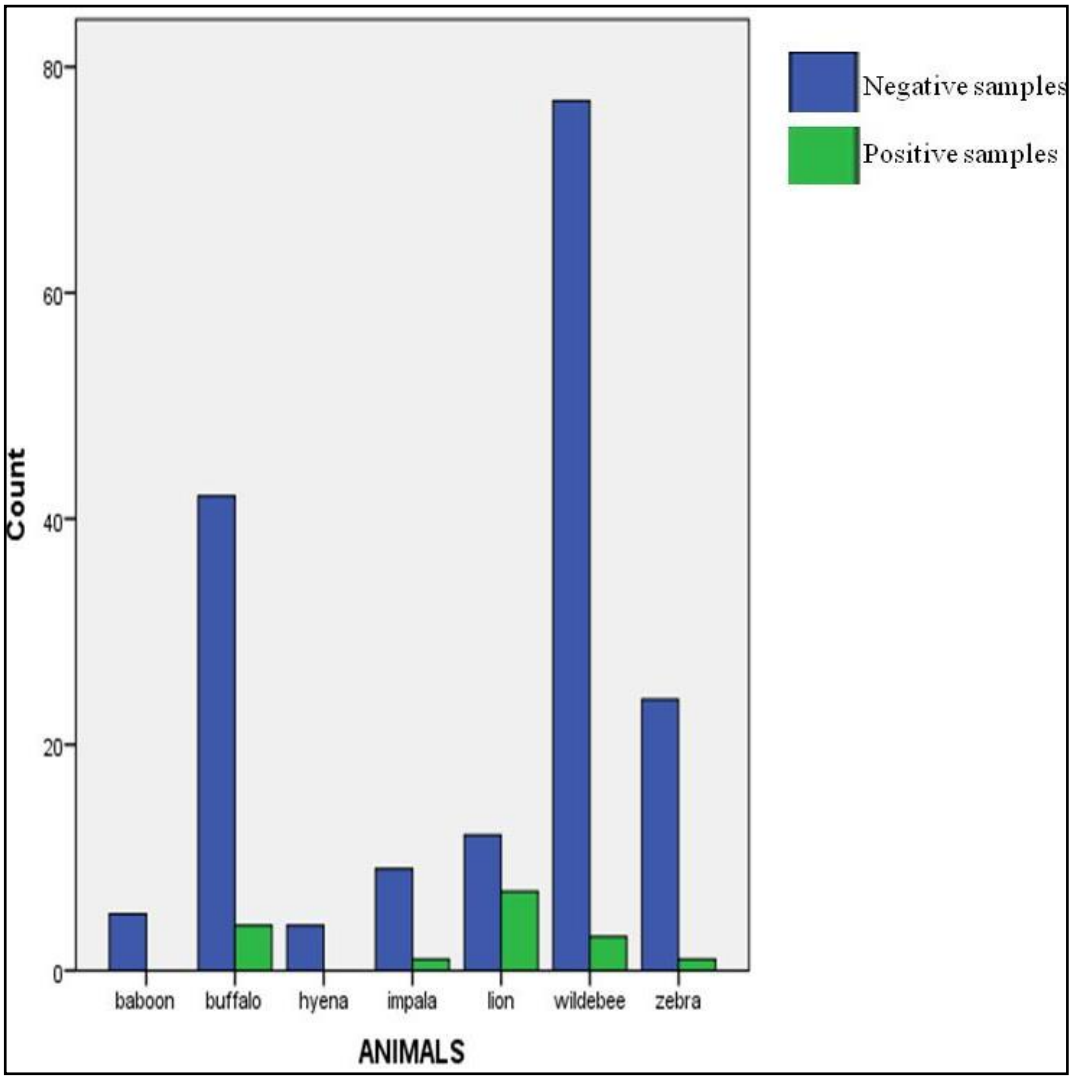

Figure 5b: Major animal species detected with *Brucella* are indicated with green color by using RT-speciation assay buffalo, impala, lion, wildebeest, and Zebra are species tested positive.

CHAPTER FIVE

5.0 DISCUSSION

The present cross-sectional study was carried out to determine the occurrence and magnitude of *Brucella* infection in 189 wild animals in the Serengeti ecosystem using molecular methods. The study also compared the effectiveness of conventional AMOS PCR against RT-qPCR in the detection of *Brucella* infection in wild animals. Generally, it was found that of 189 animals screened, 12 (6.4%) and 24 (12.7%) were *Brucella* positive by AMOS PCR and RT speciation respectively. The most affected wild animal species were lions (52.6%) and buffaloes (19.6%). *Brucella abortus* was the mostly identified species in wild animals, nevertheless *B. suis*, *B. melitensis* and *B. ovis* were also detected at rather low levels. This shows that all the four *Brucella* species are harbored in wild animals and are the source of infection in domestic animals and humans especially in wildlife-livestock human interface areas. There was better agreement and diagnostic performance with the RT-qPCR techniques compared to the conventional AMOS PCR. The RT-qPCR showed high sensitivity (100%) and specificity of 96.5% hence the more superior method in screening of *Brucella* than multiplex AMOS PCR assay. Generally, diagnosis of brucellosis by PCR was more sensitive if compared with previously reported methods like blood cultures and serological tests (Navarro *et al.*, 2002; Mariri *et al.*, 2009). Although the molecular methods may have some limitations in terms of costs, availability of equipments and reagents as well as personnel, there are time effective and more reliable methods for diagnosis of brucellosis in animals and humans.

The current study established that of the 189 screened wild animals 6.4% and 12.7% by AMOS PCR and RT-qPCR respectively were infected with different species of *Brucella*. This magnitude of infection is within the previously reported *Brucella*

infection rates in wild animals in Tanzania that ranges between 10.5% and 17% (Sachs *et al.,* 1968; Schiemann and Staak, 1971; Shirima, 2005; Fyumagwa *et al.,* 2009; Assenga *et al.,* 2015). However, these previous studies had screened *Brucella* infection in wild animals using serological tests; they showed the reality on what is existing in the wild animals. The observed magnitude of infection of wild animals during this study is high and gives evidences of their involvement in the transmission of *Brucella* in livestock and humans in the interface areas of Serengeti ecosystem. For example, result of brucellosis surveys in Tanzania show that the prevalence in cattle ranges between 2% and 90% with the highest infection rates realized in the interface areas (Minga and Balemba, 1990; Jiwa *et al.,* 1996; Swai, 1997; Weinhaupl *et al.,* 2000; Mtui-Malamsha, 2001; Minja, 2002; Swai *et al.,* 2005; Karimuribo *et al.,* 2007; Temba, 2011; Chitupila *et al.,* 2015; Assenga *et al.,* 2015). The prevalence of brucellosis in some of the pastoral and agro pastoral communities in Tanzania ranges between 0.7% and 58.1% (Kunda *et al.,* 2005; Kunda, 2007; Swai, 2009; Mellau *et al.,* 2009; Kunda *et al.,* 2010; Wankyo, 2013). The *Brucella* infections in humans is reported to be increasing in livestock-wildlife interface areas inhabited by pastoralists whose animals interacts with wild animals (Mellau *et al.,* 2009; Temba, 2011; Assenga *et al.,* 2015). This further supports that wild animals are the reservoirs of *Brucella* and keep on shedding the infection to the environment where humans and domestic animals get infected. However, other studies elsewhere demonstrated the presence of brucellosis in the wild animals in different areas (Mohan *et al*., 1996; OIE, 2000; Rajala, 2016; Matope *et al*., 2010; Wareh, 2015).

Most of the *Brucella* positive samples observed during the current study were detected in female animals 30 (16.4%) different from the previous report in Tanzania by Assenga *et al*. (2015) and elsewhere in Africa (Matope *et al*., 2010). Nevertheless, there is no controlled study that has been conducted on the relative susceptibility of female and male

cattle to brucellosis. However, a study by Degefu *et al.* (2011) in cattle proposes that bulls are more resistant than sexually mature heifers and cows. A study by Ferede *et al.* (2011) discovered that *Brucella* infections are limited to testes in male animals and this result into non reactors or reactors displaying low antibody titers. The same authors associated less susceptibility of male animals to *Brucella* infection with lack of erythritol (Ferede *et al.*, 2011).

The study further found that *Brucella* infection rates were high in lions (52.6%) and buffaloes (19.6%). The reasons are not clear but probably lions are carnivores and are likely to prey on *Brucella* infected animals like buffaloes. However, the high infection rates observed in buffaloes may be due to *B. abortus* being the common species in the ecosystem and is known to mostly affect bovine animals. Other studies have recorded seroprevalence in buffalo of Serengeti National Park and Tarangire National Park to range between 37% and 67%, respectively (Schiemann and Staak, 1971; Anderson, 1988).

In the present study, *B. abortus* biovars 1, 2, and 4 were detected in 11.1% of buffalo from Serengeti by using AMOS PCR and *B. abortus* was detected in 7 (77.8%) in buffaloes from Serengeti by using RT-speciation assay PCR. *Brucella abortus* biovars 1, 2, and 4 was detected again in 3 (30.0%) lion from Serengeti by using AMOS PCR. *Brucella abortus* was detected from 6 (60%) lions by using RT-speciation assay. Amongst the six lions, two were male sub-adult. Various studies report that serologically, young animals tested positive due to maternal antibodies, and at a later time they tested negative (Blood *et al*., 2007). Calves from seropositive dams had been reported to be usually seropositive for up to 4-6 months due to colostrum antibodies and later test negative (Blood *et al*., 2007). It was also observed that there was higher infection of *Brucella* 23 (20%) in Serengeti National Park compared to (12.2 %) Ngorongoro Conservation areas due to the

reason that Serengeti is niche area of lions and buffaloes which are more sedately at their habitant and hence contamination of environment is likely to be high. In addition, in Ngorongoro conservation area there are high rate of interactions between domestic animals and wild animals. It is likely that vaccination against brucellosis is done in domestic cattle a situation indirectly may minimize the spread of the disease from domestic to the wild animals.

It was established that *B. suis* biovar 1, 2, 3, 4 and 5 was detected in wildebeest 4 (5 %) from Serengeti and Ngorongoro, 1 (4%) in zebra, 1 (25%) in hyena, 1 (2%) in buffalo, 1(10%) in impala from Serengeti (Table 9). Interestingly, this is the first brucellosis study in wild animals to be conducted by using molecular technique and the first to detect *Brucella* species in zebra and hyena in Tanzania. Using RT-speciation assay *B. abortus, B. suis* were detected in the same zebra indicating occurrence of multiple infection. Normally wildebeest immigrate from Serengeti to Masai Mara looking for pastures during dry season and come back when pastures are available, a practice likely to introduce the *Brucella* contaminations in the Serengeti ecosystem. Zebra is always intermingling with wildebeest during grazing; living together in close association and this behavior could be the basis for the transmission of the pathogens amongst the wild animals. The study detected *B. suis* in hyena which also was detected in wildebeest, buffalo, impala and zebra. Scavenging behavior of hyena of eating leftovers or cadavers left by lions could be the source of infection to this species. Serologic survey conducted in Kenya in blue wildebeest (*Connochaetes taurinus* Burchell) in the Masai Mara area found that antibodies of *Brucella* spp. at 18% (Waghela and Karstad, 1983). The findings of *Brucella* in zebra and hyena suggest that more studies have to be done in different wild animals to better determine the *Brucella* strains affecting such uncommon wild animals at Serengeti ecosystem.

The current study detected *B. melitensis* in one (2/10) impala from Serengeti by AMOS PCR while *B. abortus* in (2/10), *B. suis* in (1/10) and *B. ovis* in (1/10) impala by RT-speciation. Previous studies show that there were high infection of *B. melintesis* and *B. abortus* in antelope in Kafue flat area in Zambia because cattle were sharing source of water with wild animals during dry season in June up September (Muma *et al.,* 2011; Arimi, 2002). Infection of *B. melitensis* is reported to be less common in sub-Saharan African countries in camel, sheep and goats (Gwida *et al*., 2011;Wareth *et al*., 2015) and in cattle (Rajala, 2016). *Brucella melitensis* is the host specific bacteria and normally affect sheep and goat which resemble with impala (Blood *et al*., 2007). *Brucella melitensis* have been reported in red deer and Iberian wild goat (Rajala, 2016). This study reports for the first time the occurrence of *B. melitensis* and *B. abortus* in impala using molecular techniques. *Brucella melitensis* is known to be a classical zoonotic bacteria, its detection in impala found in the interface areas of Serengeti ecosystem entails that it is also likely that the bacteria is available in domestic small ruminants and humans.

The current study detected *B. ovis* in one (1/10) impala and in one (1/46) buffalo from Serengeti by RT-speciation. Previous studies show that there were high infection of *B. ovis* and is one of the leading causes of infertility in ovine which is normally characterized by epididymitis, orchitis and testicular atrophy in rams. These were detected in rams and goats flock in the State of Minas Gerais, Brazil (Xavier *et al*., 2014). This study reports for the first time the occurrence of *B. ovis* in impala found in Serengeti ecosystem suggestive its possible availability in domestic small ruminants and humans.

Whole blood, amniotic fluids and serum were used in detection of *Brucella* species in selected wild animals in Serengeti ecosystem. The study observed that the use of blood in detection of *Brucella* by using molecular technique was insignificant when compared to

the amniotic fluids and serum. The study shows that 29 (17.1%) of blood used were *Brucella* positive, while 2 (18.2%) amniotic fluid were *Brucella* positive and 1 (18.2%) of serum were *Brucella* positive. These results are in agreement with other studies in the detection of *Brucella* spp. in blood and serum. A study by Zerva *et al*. (2011) shows that sensitivity of molecular technique is higher with serum samples (94%) than whole blood samples (61%). During the current study, the specificity of molecular technique was excellent (100%) with serum samples. In a different study, reproductive organs such as uterus, testicles and accessory glands are reported to be good samples in detection of *Brucella* by using molecular techniques (Vladimira *et al*., 2009). For the first time in Tanzania, *Brucella* spp. have been detected in amionic and serum using molecular techniques. This has provided a unique perspective contribution to the epidemiology of brucellosis and in science in general. More studies are recommended by using serum and amniotic fluids in detection of *Brucella* in wild animals.

AMOS PCR amplifies simultaneously two or more unique target sequences in a sample. In this study AMOS PCR was used to amplify IS 711 gene using one set of primers to amplify positive control samples to verify the integrity of the PCR while the second set of primer targeted the DNA sequence of interest. Absence of control amplicon indicated that PCR conditions were not correct and the PCR conditions had to be reset. Studies show that AMOS PCR can be used to detect various organisms in a single specimen. AMOS PCR, had a specific base pair to each strain. Some studies show that, AMOS PCR is able to differentiate positive results of *Brucella* natural infection from positive results due to *Brucella* vaccine (Doosti and Ghasemi, 2011). AMOS PCR can also be used in brucellosis eradication program without any supplement to other diagnostic (Doosti and Ghasemi, 2011). AMOS PCR has some limitations which include unsuitability for testing RB 51 vaccine, which is rough strains of *Brucella*, and hence can give false negative (Doosti and

Ghasemi, 2011); takes longer time in preparation and production of results; may be source of contamination to laboratory technician and environment and needs man power on sample loading in gel casting pan and needs high quality sample for good results. Samples used in this study were archived, some collected in 2000. Hence results obtained showed multiple bands, with little quantity of DNA. In view of this a RT-qPCR was employed for the purpose of confirming the samples for *Brucella* spp.

The RT-qPCR was used to quantify the amount of nucleic acid (DNA) in the sample. The method involves a competition between the target nucleic acid and the competitive DNA for amplification process. The RT-qPCR required the same primer pair as AMOS PCR and was added in known primer concentrations. Two gene targets *Bcsp31* and *IS711* were used for detection of *Brucella* DNA in 189 samples. Twenty two (11.6%) samples were *Brucella* positive by using *Bcsp31* and *IS711* primers. Gene *IS711*produces many copies of *Brucella* in the host DNA and hence become very sensitive in detecting *Brucella* (Foster *et al*., 2008; Vladimira *et al*., 2009). The results presented in this study indicate that IS*711* RT-qPCR assay was specific and sensitive tool for detection of *Brucella* spp. infections in wild animals. For this reason, the study proposes to use IS711 gene as a complementary tool in brucellosis screening programs and for confirmation of diagnosis in doubtful cases *BCSP31*gene can be used.

The RT-speciation was used for confirmation of presumptive *Brucella* isolates in different samples. The assay was designed in a multiplex format that was allowing the rapid identification of *B. abortus, B. melitensis, B.suis and B.ovis* in a single test which could not be done by AMOS PCR and RT-qPCR. The RT-speciation was used to determine the prevalence of *Brucella* spp. and to differentiate *B. melitensis, B.suis, and B.ovis B. abortus* in selected wild animal species in Serengeti ecosystem. Results obtained from RT-

speciation shows that presence of *B. abortus* in samples was 66.7%, suggesting that it is a common *Brucella* species circulating in Serengeti ecosystem. RT-speciation PCR does not require electrophoresis analysis, and it has little chance of causing contaminations as conventional PCR (Doosti and Ghasemi, 2011). Although RT-speciation has a lot of strength in detecting *Brucella* infection to specie level, it was observed in this study that the technique has bias in speciation because was specie specific. Detection of the strain is decided by the primers of interest. This is the first study to report on identification and differentiation of *B. abortus, B. melitensis, B.suis, and B.ovis* by RT-speciation technique in Tanzania. Some studies suggest that, this technique could be potentially useful for the diagnosis of brucellosis since it could detect the bacteria in pauci bacillary samples and even in samples highly contaminated with other microorganisms (Bricker, 2002 and Pilar *et al.,* 2010). Therefore, molecular diagnostic techniques such as RT-speciation are simpler, faster, less hazardous and usually more sensitive, developed for *Brucella* detection (Bricker, 2002). The RT-speciation assay is also reported to be easy to use, producing results faster than conventional PCR systems while reducing DNA contamination risks (Newby *et al.,* 2003; Doosti and Ghasemi, 2011).

Comparison of three molecular techniques used in detection of *Brucella* in wild animal samples was done. The study found that the RT-qPCR assay was the most sensitive, detecting 24 (12**.7**%) of the samples tested as containing *Brucella* DNA. This sensitivity had been reported by other studies as a justification for the preferred use of the RT-qPCR assay to screen unknown or previously untested samples (Probert *et al.*, 2004; Doosti and Ghasemi, 2011; Whatmore *et al.*, 2014). Using the real-time multiplex, nested, speciation assay (Probert *et al.*, 2004) as the reference test, the sensitivity and specificity of the AMOS PCR and RT-qPCR were found to be 12.5% against 94.2% and 100% against 96.5% respectively. Previous studies that have

compared these two technique platforms (Probert *et al.*, 2004, Doosti and Ghasemi, 2011), have reported similar diagnostic performance of the two techniques in the detection of *Brucella* DNA.

However, both the real-time assays apply similar primer targets and downstream design essentially making them nested within one another. In order to bypass this bias in analysis, all the three molecular techniques were further subjected to Cohen's two-rated Kappa test for agreement in net positivity detection. The AMOS PCR was again found to poorly agree with both the RT-qPCR assay (0.04 [-1.73; 1.80]) and only slightly better with the RT-speciation assay (0.075 [-1.71; 1.87]). The RT-qPCR assay however had very good agreement (0.82 [-1.09; 2.75]). However, due to the small number of positive samples detected, none of the scores were statistically significant. The observations in this study are consistent with those reported in other studies (Probert *at el*., 2004) implying the superior performance of the RT-qPCR over the conventional AMOS PCR in the detection of *Brucella* DNA. Further studies could potentially be performed to look into the varied performance of the techniques on pre-screened samples as well as DNA from different sample sources for the robust detection of *Brucella* DNA.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Based on the above findings the following are the conclusions that can be made;

i. The prevalence of *Brucella* infection was 6.4% and 12.7% by AMOS PCR and RT-qPCR respectively.

ii. The most affected wild animal species were lions and buffaloes based on RT-speciation hence are more involved in spreading the infection to other animal species in the Serengeti ecosystem.

iii. *Brucella abortus* was the most commonly identified species in wild animals, nevertheless *B. suis, B. melitensis* and *B. ovis* were also detected.

iv. There was better agreement in diagnostic performance with the RT-qPCR techniques compared to the conventional AMOS PCR assay.

v. The RT-qPCR showed high sensitivity (72.7%) and specificity of 100%.

vi. The RT-qPCR is more superior in screening of *Brucella* in the field than AMOS PCR.

vii. The Serengeti ecosystem may be the major source of spreading of *Brucella* pathogen in wildlife-domestic animal-human interface areas of Serengeti ecosystem.

viii. For the first time, molecular technique was used to diagnose *Brucella* species in wild animals by using different technique and different samples in Tanzania.

ix. In this study comparison of the test characteristics of the three molecular techniques showed good agreement between the RT-speciation, and poor agreement with the AMOS PCR.

6.2 Recommendations

Based on the above conclusions the following are the recommendations;

i. Molecular technique should be used in detecting *Brucella* species in wild animals so that to detect more species of *Brucella* circulating in Serengeti ecosystem.

ii. Samples to be used in molecular technique should be of high quality especially when AMOS PCR because the technique is very sensitive to contaminated samples and may lead to wrong results.

iii. From this study, should be considered that, even if *Brucella* is host specific, cross infection can occur in different animals. Example from this study by using AMOS PCR it was found that hyena and lion they also harbor the pathogen according to their eating habit.

iv. One health approach: Collaboration is important to plot the methods of brucellosis management, through standardization of diagnostic techniques, instrumentation with diagnostic kits of Veterinary Investigation Centers, wildlife laboratories for active surveillances.

v. Collaborations of various stakeholders like veterinary department, health department, local authorities, national park authorities, natural resource and tourism department as well as livestock keepers.

vi. It is possible to control brucellosis in livestock by vaccination, culling of infected animals and improved hygiene but practically difficult in wild animals.

vii. Public health education: from this study management of brucellosis want public education to be aware that wild animals can be source of infection to human and livestock that, public campaign utilizing all means that of public information available to deliver information to the target groups.

6.3 Limitations of the Study

With all good achievement in this study there were some limitations which are:

i. Since some of the achieved samples were stored for a long time probably the results obtained after samples analysis by using AMOS PCR were not good since most bands produced were faint and multiple. This was overcome by use of more than one laboratory technique in order to be assured with the results obtained.

ii. Though the samples were well labeled, it was not clear as to whether each sample belonged to one animal or may be other animals had given more than one sample e.g. blood and amniotic fluid.

iii. Shortage of power supply during laboratory work caused destruction of some materials example agarose preparation and during running of PCR. This compromised the smooth performance laboratory work.

iv. By use of molecular techniques for detection of *Brucella* infection in wild animals, it is not easy to establish the nature of infection e.g. acute, subacute or chronic.

v. High cost in doing research in wild animals was limitation especially on immobilization of animals. This necessitated this study to use archived samples.

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APPENDICES

Appendix 1: Vice Chancellor's Letter

CLEARANCE PERMIT FOR CONDUCTING RESEARCH IN TANZANIA

SOKOINE UNIVERSITY OF AGRICULTURE
OFFICE OF THE VICE-CHANCELLOR
P.O. Box 3000 CHUO KIKUU, MOROGORO, TANZANIA
Phone: 255-023-2640006/7/8/9, Direct VC: 2640015; Fax: 2640021;
Email: vc@suanet.ac.tz; vc2004sua@yahoo.com

Our Ref. SUA/DRPSG/R/126/3/93 18 August, 2017

District Executive Director,
Serengeti District
P.O. Box 176
SERENGETI

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by University Act Number 7 of 2005 and SUA Charter of 2007 which became operational on 1st January 2007 repealing Act Number 6 of 1984. One of the mission objectives is to generate and apply knowledge through research. For this reason the staff and researchers undertake research activities from time to time.

To facilitate the research function, the Vice-Chancellor of the Sokoine University of Agriculture (SUA) is empowered to issue research clearance to both staff, students and researchers of SUA onbehalf of the Government of Tanzania and the Tanzania Commission for Science and Technology.

The purpose of this letter is to introduce to you **Rosamystica Mkula Sambu** a bonafide **MSc. (Public Health and Food Safety)** student with registration number **MPH/E/2016/0002** of SUA. By this letter **Sambu** has been granted clearance to conduct research in the country. The title of the research in question is **"The role of Wildlife animals in Transmission of Brucellosis in Human and Livestock in Serengeti Ecosystem Northern Tanzania.".**

The period for which this permission has been granted is from **September, 2017 to December 2017.** The research will be conducted in **Serengeti and Ngorongoro District.**

Should some of these areas/institutions/offices be restricted, you are requested to kindly advice the researcher(s) on alternative areas/institutions/offices which could be visited. In case you may require further information on the researcher please contact me.

We thank you in advance for your cooperation and facilitation of this research activity.

Yours sincerely,

Prof. Raphael T. Chibunda
VICE-CHANCELLOR

VICE CHANCELLOR
SOKOINE UNIVERSITY OF AGRICULTUR
P. O. Box 3000
MOROGORO, TANZANIA

Copy to:– **Rosamystica Mkula Sambu - Researchers**

Appendix 2: A research permit provided by the Tanzania Wildlife Research Institute (TAWIRI)

Tanzania Wildlife Research Institute

Head Office P.O. Box 661, Arusha, Tanzania
Tel.: +255 (0) 27 254 9571 / 254 8240; Fax + 255 (0) 27 254 8240
E-mail: info@tawiri.or.tz
Website: www.tawiri.or.tz

Our Ref: TWRI/RS-57/VOL.IV/85/72

Your Ref:

Date: 25th August, 2017

Dr. Nonga, H.E.
Supervisor for the PhD Student
Sokoine University of Agriculture
College of Veterinary and Medical Sciences
Department of Veterinary Medicine and Public Health
P.O. Box 3021
MOROGORO

RE: REQUEST FOR WILDLIFE SAMPLES COLLECTED FROM SERENGETI ECOSYSTEM FROM TAWIRI SAMPLES BANK FOR USE IN MSC. RESEARCH PROJECT

Kindly refer to the heading above and your letter with Ref. DVMPH/MPH/2016/0002 dated 03 July 2017.

Please note that TAWIRI has received your request for wildlife samples for your M.Sc. student Sambu, Rosamystica Mkula and we are sorting to come up with the list of available stocked samples in TAWIRI bank which were collected from different animals from Serengeti Ecosystem. You will be notified on availability of samples immediately after completion of this exercise. The student will be required to pay sample fees depending on number of available samples.

Yours sincerely,
TANZANIA WILDLIFE RESEARCH INSTITUTE

Dr. Angela Mwakatobe
FOR: DIRECTOR GENERAL