POTENTIAL OF SNIFFER RATS IN DETECTION OF *BRUCELLA* SPECIES IN CATTLE

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ABSTRACT

Brucellosis is one of the most economically important bacterial zoonoses worldwide, affecting livestock, wild animals and humans. In Tanzania, brucellosis is in the list of six top priority zoonoses that the government is focusing to control although diagnostics remain to be a major constraint. An experimental study was carried out at an anti-persoonsmijnen ontmijnende product ontwikkeling abreviateted as APOPO which in English means anti-personnel landmines removal product development between may 2018 and august 2019 to train nine sniffer rats (*Cricetomys ansorgei*) to detect *Brucella abortus*. The study used the principle of operant conditioning in which sniffer rats were rewarded on sniffing *Brucella abortus* positive samples within two seconds. Cattle milk and aborted materials were the source of *Brucella* used in the study *Brucella* was grown on selective media and confirmed using morphological, biochemical and molecular methods. The isolated *Brucella abortus* strain was heat inactivated at 56^0C for 45 minutes before being presented to the sniffer rats. To ascertain the status of the sniffer rats regarding *Brucella* infection, the sniffer rats were screened for the presence of *Brucella* antibodies using Rose Bengal Test before and after the experiment. All sniffer rats were negative for *Brucella* antibodies before and after the experiment. Five days prior to the start of experimental testing, the baseline performance (sensitivity (se) and specificity (sp)) of the sniffer rats was assessed. On average, baseline sensitivity and specificity for the nine sniffer rats were 92% (range 82-98%) and 98% (range 95-99%) respectively. After finding the baseline performances, the sniffer rats were trained and subjected to three experimental tests to evaluate their ability to accurately detect positive samples (sensitivity) and discriminate against negative samples (specificity) using 10 positive and 90 negative samples. The average sensitivity for tests one, two and three were 93% (range 60-100%), 97% (range 90-100%) and 76% (range 60-90%) respectively, while average

specificity were 96% (range 88-98%), 93% (range 86-94%) and 98% (range 93-100%) respectively. Results from this study showed reasonably high sensitivity and specificity that suggests potential of sniffer rats to be used for diagnosis of *Brucella* infection. However, since this experiment used laboratory manipulated samples, further studies are needed: firstly, to explore its applicability in the clinical samples such as milk, blood and other tissues secondly, to evaluate components of *Brucella abortus* that the sniffer rats were sniffing and thirdly to evaluate their ability to detect other *Brucella* species.

DECLARATION

I, Raphael Raiton Mwampashi 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 in any other institution.

_____   _____

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(Supervisor)

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rat trainers whom I worked with in training the sniffer rats.

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LIST OF ABBREVIATIONS AND SYMBOLS

ALC	Automated Line Cage
	Anti-Persoonsmijnen Ontmijnende Product Ontwikkeling
APOPO	
°C	Degrees Celsius
c-ELISA	Competitive Enzyme Linked Immuno-Sorbent Assay
CO_2	Carbon Dioxide
CFT	Complement Fixation Test
CT	Cycle Time
DNA	Deoxyribonucleic Acid
FA	False Alarm
FALCON	Full Automated Line Cage
FAO	Food and Agriculture Organisation of the United Nations
	Hydrogen Sulphide
H_2S	
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
PGFG	Pulse field Gel electrophoresis
RBPT	Rose Bengal Plate Test
R-LPS	Rough Lipopolysaccharide
S19	*Brucella abortus* strain 19
SAT	Serum Agglutination Test
Se	Sensitivity
Sp	Specificity
Spp	Species
SUA	Sokoine University of Agriculture
TB	Tuberculosis
TTP	Thematic Training Program
USA	United States of America
WHO	World Health Organization
μ	Micro

CHAPTER ONE

1.0   INTRODUCTION

1.1   Background Information

Brucellosis is one of the most prevalent zoonotic and contagious diseases of huge public health prominence worldwide. It is the disease of major socio-economic importance to livestock keepers as it contributes to substantial reduction in herd productivity that compromises food security and their livelihood as they depend on their animals as a source of income through trade of surplus meat, dairy products and offspring (Poester *et al.*, 2010). This is vividly so in many African countries where the disease is widespread and control programmes are either non-existent or scarce (Schelling *et al.*, 2003). Few countries, however, such as Canada, Australia, New Zealand, Japan and some countries in Northern and Central Europe are officially free of the disease (WHO, 2006).

Brucellosis is caused by a bacterium of genus *Brucella*, which are small, non-motile, aerobic, facultative intracellular, Gram-negative coccobacilli. Currently, there are about twelve species in the genus *Brucella* that are known to cause disease worldwide (Scholz *et al.,* 2016). Each of these species has host preference, although some may infect more than one host species. *Brucella melitensis* preferentially infects sheep and goats, *B. abortus* (cattle), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs), *B. microti* (rodents- *Microtus arvalis*), *B. neotomae* (rodents - *Neotoma lepida*), *B. pinnipedialis* (pinnipeds), *B. ceti* (cetacean), *B. inopinata, B. papionis* (baboon) and *B. Vulpis* (Red fox) (Sánchez-Sarmiento *et al.*, 2019). Three of these species (*B. melitensis, B. abortus and B. suis*) are of great zoonotic and economic importance; preferentially they infect cattle, small ruminants and swine respectively as they infect humans and a variety of other mammals (Mathew *et al.,* 2015). In domestic animals, brucellosis is transmitted through

consumption of contaminated pasture and water. It can also be spread through artificial insemination, natural service from the infected bull and licking of infected placenta, young stock, foeti, or the genitalia of infected animals soon after abortion or normal delivery (Godfroid *et al.,* 2002). The disease is commonly spread into clean herds or flocks through the introduction of diseased animals which are either pregnant, that have recently delivered, or aborted (Shirima *et al*., 2005). Movement of animals between herds is said to be an important factor for transmission of *Brucella* infection in various regions of the world (Kabagambe *et al*., 2001). In human brucellosis is transmitted by direct or indirect contact with infected animals or their products (WHO, 2006).

Brucellosis is prevalent in many African countries although with varying prevalence (Karimuribo *et al*., 2007). In Tanzania, the first brucellosis report was reported in 1927 after an outbreak of abortions in cows in Arusha region (Swai *et al.,* 2010). Since then many studies have been carried out to show prevalence of the disease in livestock in various regions and production systems as the disease has continued to spread. Such studies have indicated that the prevalence of brucellosis in cattle in Tanzania ranges from 1% to 58.1% (Staak and Protz, 1973; Kitaly, 1984; Otaru, 1985; Minga and Balemba, 1990; Jiwa *et al.,* 1996;, 2002; Swai *et al*., 2005; Karimuribo *et al*., 2007; Swai *et al*., 2009; Temba, 2011; Chitupila *et al*., 2015; Assenga *et al.,* 2015).

There are several methods used in the diagnosis of *Brucella* infection in both humans and animals. Culturing and isolation of the agent is the gold standard for *Brucella* diagnosis but is costly, dangerous to personnel and takes longer time (4 to 30 days) as compared to the other diagnostic methods (Kaltungo *et al.,* 2014). On the hand, several serological assays are commonly used such as Rose Bengal Plate Test (RBPT), Enzyme Linked Immunosorbent Assay (ELISA), Complement Fixation Test (CFT) and Serum

Agglutination Test (SAT). These methods have varying specificities and sensitivities. Some tests such as RBT have low specificity and increased tendency to false positive results due to reactivity with other closely related bacteria. Majority of these tests fail the standard criteria for a resounding diagnosis, hence not recommended to be used alone in endemic areas (Ferreira *et al.,* 2003; Smirnova *et al.,* 2013). Currently, molecular techniques are being used in the diagnosis of brucellosis; however, these techniques are too expensive to be used widely as they require specialized machines along with skilled personnel (Poester *et al.,* 2010).

African giant pouched rats (*Cricetomys ansorgei*) had already been successfully trained to detect Tuberculosis (TB) since when an idea was developed in Tanzania, roughly 120 years after the TB-causing *Mycobacterium tuberculosis* was discovered by microscopy and bacterial culture. Today TB detection rats are one of the most advanced medical applications of animal scent detection (Edwards *et al*., 2017). Since their introduction into humanitarian activities, sniffer rats have shown more potential to be employed in research activities that are of more value to human (Poling *et al.,* 2010)

1.2 Problem Statement and Study Justification

Despite being pursued for over a decade, the diagnostics for *Brucella* infection in both humans and animals have shown to have several constrains including inaccuracy, time and cost to run them. This has negatively impacted on treatment, planning and delivering effective control programs of the disease. The same diagnostic challenges faced Tuberculosis (TB) control in Tanzania. Previously, sputum smear microscopy was the standard technique for diagnosing TB. This method was cheap but it was inaccurate and time consuming (Poling *et al.,* 2015). In an attempt to find alternative robust diagnostic method for TB in Tanzania, sniffer rats (*Cricetomys ansorgei*) were evaluated and

introduced in 2007. Since their introduction, the rats have been used in the diagnosis and detection of tuberculosis by sniffing sputum samples with great success to the extent that when compared to laboratory microscopy of technicians given the same mission, the rats have produced rapid output and increased new case detections by approximate 40% (Ellis *et al.*, 2017). If sniffer rats can detect TB with such a great success, there is a chance that they can be used for detection of other diseases. Therefore, this study explored the use of sniffer rats to detect *Brucella abortus* in laboratory prepared sample from cattle as an alternative to other diagnostic techniques for brucellosis diagnosis in order to enhance treatment, planning and delivering effective control program of the disease

1.3 Objectives

1.3.1 Overall objective

The main objective of this study was to investigate the potential of sniffer rats to be used as a diagnostic method for brucellosis.

1.3.2 Specific objective

i. To isolate *Brucella* spp. from clinical samples in order to prepare a strain to be used for training of sniffer rats.

ii. To train sniffer rats to detect *Brucella abortus* from inactivated culture materials and spiked faeces of cattle.

iii. To evaluate the sensitivity and specificity of the sniffer rats in the detection of *Brucella abortus* from laboratory prepared culture samples.

CHAPTER TWO

2.0   LITERATURE REVIEW

2.1   Definition and Aetiology of Brucellosis

Brucellosis is an infectious disease caused by bacteria that are gram negative, facultative intracellular micro-organisms that can infect many species of animals and humans. The bacteria are small, non-motile and non- sporulating aerobic coccobacilli 0.5-0.7 μm wide and 0.6-1.5 μm long (Mathew *et al.*, 2015). The disease 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), while the relationship between contagious bovine brucellosis and human brucellosis was confirmed by Meyer and Shaw in 1920 (Kumar *et al.,* 2015).

Brucella mico- organisms can be grown at the optimum temperature of 37ºC; however, the organism can grow at temperatures range of between 20ºC and 40ºC. Whereas the optimum pH scale ranges from 6.6 to 7.4, some *Brucella* spp require CO_2 for growth (Geresu and Kassa, 2016). 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 fourteen days of incubation. Most *Brucella* species are slow growing organisms on primary isolation, some of them requiring serum-enriched culture media, even laboratory report on isolation rates has shown to be between 20-50% (Poester *et al.,* 2010).

The survival of the organism in the contaminated environment following parturition or other vaginal discharges present after an infected animal contaminate the environment is influenced by prevailing environmental conditions (Nielson and Duncan, 1990). Bacteria survival outside a host is dependent on environmental factors including exposure to light,

humidity and temperature. *Brucella* can survive for approximately 5 hours on surface. The ability of members of the genus to persist outside the mammalian hosts is relatively high compared to most other non-sporing pathogenic bacteria; they can survive for a long time in both hot and cold environments, particularly with high moisture content (Kaltungo *et al.*, 2014). Therefore, the organisms survival and persistently causing infection especially so in tropical Africa where animal husbandry and management of the environmental practices is poor.

Despite their survival in harsh conditions, most common available disinfectants such as hypochlorite solutions, 70% ethanol, isopropanol, iodophors, phenolic disinfectants, formaldehyde and xylene are said to readily kill *Brucella* spp, however, the efficacy of these disinfectants is said to be decreased in the presence of organic matter and low temperatures (WHO, 2009). They can also be destroyed by moist heat of 121°C for at least 15 minutes, dry heat of 160-170°C for at least 1 hour, gamma irradiation and pasteurization (Kaltungo *et al.*, 2014).

Brucella is distinguished from most pathogens due to lack of 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). The organism is able to escape phagocytic killing through inhibiting the phagosome-lysosome fusion and reproducing inside macrophages (Young, 2005). *Brucella* species, except for *B. ovis* and *B. canis*, contain smooth lipopolysaccharide (SLPS) in their outer cell wall (Soler-Lloréns *et al.,* 2014). The presence of rough or smooth lipo-polysaccharide in their cell wall correlates with the virulence of the bacterium and smooth cell wall are generally more virulent as compared to rough cell

wall. *Brucella* species and their different biotypes are currently distinguished by differential tests based on serotyping, phage typing, dye sensitivity, CO_2 requirement, H_2S production, and metabolic properties (Kaltungo *et al.,* 2014). As the members of the genus *Brucella* reside within the cell they localize in lympho-reticular cells of the body and are said to have special tropism for the existence for sugar-rich organs such as the uterus during pregnancy, udder, testicles and the accessory sex glands of animals (De Figueiredo *et al.,* 2015).

2.2 Epidemiology of Brucellosis

2.2.1 Distribution

Brucellosis has been reported by FAO (2003) as the second most important zoonotic disease in the world after rabies. The disease is described as a true zoonosis because all infections in human are of animal origin (Kaoud *et al.,* 2010). The burden that the disease poses especially on low-income countries has led the World Health Organization (WHO) to classify it as one of the world's leading 'neglected zoonotic diseases (Franc *et al.,* 2018). Although it has been eradicated from other countries like New Zealand, Australia, Canada, Japan, Northern and Central Europe that are disease free (Diaz *et al.,* 2013; OIE Diagnostic Manual, 2012), brucellosis continues to be a major public and animal health problem in many regions of the world particularly where livestock are a major source of food and income (FAO, 2003).

The disease has a limited geographic distribution, but remains a major problem in the Mediterranean region, the Middle East, Central and Southeast Asia, and parts of Africa, Latin America and some Mediterranean countries in Europe (Donev *et al.,* 2010). Africa in particular, brucellosis has been reported from almost all countries in the continent (Refai, 2002). Though its distribution is worldwide; yet brucellosis is more common in

countries with poorly standardized animal and public health control programmes (Capasso, 2002). In Tanzania the disease is endemic and has been reported in almost all zones (Minga and Balemba, 1990; Weinhaupl *et al.,* 2000; Mtui-Malamsha, 2001; Minja, 2002; Swai *et al.,* 2005)

2.2.2 Prevalence

The disease is widespread in Africa and has been reported in most African countries although with varying prevalence (McDermott and Arimi, 2002). Following the first reported outbreak of bovine brucellosis in Tanzania in 1927 (Swai *et al.,* 2010) many studies have been carried out which indicate presence of the disease in different regions and production systems across the country with varying prevalence rates. There are some factors that can affect the prevalence of brucellosis, which can vary according to climatic conditions, geography, species, sex, age and diagnostic tests applied (Khan, 2007).

In the northern zone of Tanzania where intensive and extensive systems of production are common, the prevalence of brucellosis ranges from 1-30% (Mtui-Malamsha, 2001; Minja, 2002; Swai *et al.,* 2005), in Eastern zone with zero grazing and extensive system the range is 12-24% (Weinhaupl *et al.,* 2000). In Lake zone with traditional cattle production system, the range of 4-22.5% has been noted (Kagumba and Nandoka, 1978; Msanga *et al.,* 1986) in Central zone with extensive and intensive system the prevalence is 2-10.6% (Kitaly, 1984) while in Coastal region with extensive and intensive system the prevalence is 2-90.5% (Minga and Balemba, 1990; Swai, 1997; Weinhaupl *et al.,* 2000) and in Southern zone with extensive and intensive system of production the prevalence is 15.2% (Otaru, 1985).

2.2.3 Transmission

In livestock, brucellosis is normally spread to susceptible animals by direct contact with infected animals or with an environment that has been contaminated with discharges from infected animals (Abubakar *et al.,* 2012). Therefore, the disease is established into clean herds or flocks through the introduction of infected animals which are either pregnant, that have recently delivered, or aborted (Shirima *et al.,* 2005). Aborted foetuses, placental membranes or fluids and other vaginal discharges present after an infected animal has aborted or calved are all highly contaminated with infectious *Brucella* organisms. Animals may lick those materials or the genital area of other cows or ingest feed or water contaminated with the disease-causing organisms (Mangen *et al.,* 2002). The disease may also be spread when wild animals or animals from an affected herd mingle with brucellosis-free herds.

Transmission can also occur through direct contact with tissues or discharges from infected animals as full virulent *Brucellae* are highly invasive and capable of penetrating the mucosa or skin of the nose, throat, conjunctiva, urogenital tracts, teat canal and abraded skin (Davis *et al.,* 1990). Artificial insemination and natural mating with infected bulls can also transmit infection to cows at the time of service (Lim *et al.,* 2005; Temba, 2012). 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).

Brucellosis is usually considered as an occupational disease as it has shown to associate with group of individuals that are involved with animals in one way or another like in abattoir workers, veterinarians, laboratory technicians, hunters and livestock producers (Cadmus *et al.,* 2010). Consumption of contaminated products like unpasteurized milk

and other dairy products, undercooked meat and other animal products are the main source of infection in humans (WHO, 2006). Humans also get infected with *Brucellae* via contaminated mucous membranes and abraded skin. There is no indication that members of the genus *Brucella* are transmitted between people by casual contact under ordinary conditions. In recent times it has been shown that human-to-human transmission can occur transplacentally, via breastfeeding and in very rare cases through sexual intercourse, organ transplantation, exposure to contaminated material while assisting at a delivery, sexual intercourse and nursing infants and blood transfusions (Golshani and Buozari, 2017; Franc *et al.*, 2018).

2.3   Clinical Signs

Brucellosis is a severe disease for farmers since it does not only cause serious chronic disease in humans and suffering in their animals, but also a decreased production since the disease is associated with abortions and reproductive failures in the livestock (Enström *et al.,* 2017). The most obvious signs in pregnant animals are abortion or birth of weak calves (Franc *et al.*, 2015). Milk production may be reduced from changes in the normal lactation period caused by abortions and delayed conceptions. Not all infected cows abort, but those that do usually abort between the fifth and seventh month of pregnancy. Infected cows usually abort once, but a percentage will abort during additional pregnancies and calves born from later pregnancies may be weak and unhealthy. Calves from infected cows may have latent infections, i.e. infections that are not detected until they become pregnant, abort or give birth.

Even though their calves may appear healthy, infected cows continue to harbour and discharge infectious organisms and should be regarded as dangerous sources of the disease. Other signs of brucellosis include an apparent lowering of fertility with poor

conception rates, retained afterbirths with resulting uterine infections and (occasionally) enlarged, arthritic joints (WHO, 2006).

In humans, the most common clinical sign is undulant fever in which the temperature can vary. Typically the disease manifests with a range of non-specific clinical signs including malaise, fatigue, arthritis, night sweats with a peculiar odour, chills, arthralgia, myalgia and weakness (Kaltungo *et al.,* 2014). While in serious cases the disease may affect the musculoskeletal, cardiovascular and central nervous so, complications like orchitis, epididymitis, spondylitis, endocarditis and prostatitis may also be noticed (Abubakar *et al.,* 2012).

2.4   Diagnosis

Brucellosis diagnostics are based on bacteriological and molecular methods (direct tests) and serological in vitro and allergic in vivo methods (indirect tests). Despite the vigorous attempt for more than one century to come up with a definitive diagnostic technique for brucellosis, diagnosis still relies on the combination of several tests to avoid false negative results (Poester *et al.,* 2010).

2.4.1   Direct smear microscopic examination

Reports have shown that a presumptive bacteriological diagnosis of *Brucella* can be made by means of microscopic examination of stained smears from vaginal swabs, placenta or aborted foetuses, stained with the Stamp modification of the Ziehl-Neelsen staining method (Garin-Bastuji *et al.,* 2006). However, morphologically related microorganisms, such as *Chlamydophila abortus*, *Chlamydia psittaci* and *Coxiella burnetti* can mislead the diagnosis because of their superficial similarity (Poiester *et al.,* 2010).

2.4.2   Serological tests

Serological tests are crucial for laboratory diagnosis of brucellosis since most of control and eradication programs rely on these methods (Nielsen, 2002). Although several serological methods are currently available, these tests can be classified as screening tests, monitoring or epidemiological surveillance tests.

2.4.2.1   Slow agglutination test (SAT)

Particularly IgM under neutral pH bases the principle behind this test, which was one of the earlier developed serological tests for diagnosis of brucellosis, on bacterial antigen agglutination. Its reaction is slow contrary to the rapid agglutination tests as it requires an overnight incubation at 37°C (Nielsen, 2002). This test has low specificity and therefore it is not recommended by the OIE for bovine brucellosis diagnosis (Poester *et al*., 2010).

2.4.2.2   Complement fixation test

This test detects IgGl and IgM antibody classes, and is considered to be the most sensitive and the most accurate, enabling a distinction to be made between antibodies of vaccination and those of infection. However, this method has some disadvantages such as high cost, complexity for execution, and requirement for special equipment and trained laboratory personnel (Fensterbank, 1986).

2.4.2.3   Rose Bengal plate test

Rose Bengal plate test (RBPT) is a rapid plate agglutination test where drops of stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction (Smirnova *et al.,* 2013). It has a large measure of agreement on its use which is justified to the extent that the RBT is economical, simple and rapid and gives few false negative or false positive results, requiring verification by CF.

The test is an excellent screening test but may be oversensitive for diagnosis in individual animals, particularly vaccinated ones (WHO, 2006). The present World Health Organization (WHO) guidelines recommend the confirmation of the RBT by other assays. The drawbacks of RBT include low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozones make strongly positive sera appear negative in RBT.

2.4.2.4 Milk ring test

The MRT, is an agglutination test conducted on fresh milk collected from dairy cattle which detects IgM and IgA antibodies bound to fat globules, may have wide acceptability as it is cost effective, easy to perform and can allow screening of large number of cattle by using milk samples from tanks or pools from several cows in a short time (Cadmus *et al.,* 2008). This test is useful for monitoring cattle herds or areas free of brucellosis so it is classified as surveillance or monitoring test, a positive result indicates the presence of infected cattle in the herd so the test should be followed by serology (OIE manual, 2009).

2.4.2.5 Enzyme linked immunosorbent assay

The introduction of the ELISA technique has improved the sero-diagnosis of brucellosis as was found to be more sensitive than other serological tests (Batra *et al.,* 1998). ELISAs are divided into two categories, the indirect ELISA (iELISAs) and the competitive ELISA (cELISAs). Among the ELISA methods, the Competitive ELISA (c-ELISA) was found to be more robust and easy to perform compared to i-ELISA and has several diagnostic merits and these include high sensitivity and specificity, ability to differentiate vaccinated animals from naturally infected ones, or those infected with a cross-reacting organism and its use in areas where disease prevalence is low (Nielsen *et al.,* 1995; Gall *et al.,* 1998;

Biancifiori *et al.,* 2000). Additionally, the c-ELISA can be used on either serum or milk samples from different species (Saravi *et al.,* 1995; Vanzini *et al.,* 2001).

2.4.2.6 Fluorescence polarization assay

The fluorescence polarization assay (FPA) was initially developed for testing serum; however, the technology has been extended to testing whole blood and milk samples from individual animals. It is based on the rotational differences between a small soluble antigen molecule in solution and the antigen molecule complex with its antibody (Poester *et al.,* 2010). It measures the size of a fluorescent tagged molecule such as an antigen. The utilization of the O-side chain of LPS from *Brucella* species has shown encouraging results. This technique, which requires special reagents and reading equipment, is claimed to have advantages in sensitivity and specificity over other methods (Kaltungo *et al*., 2014). Evaluation has been limited and the procedure is not widely available. Also further information is required before its overall value can be assessed and it requires special equipment and it is not suitable for rapid and easy testing (Smirnova *et al.,* 2013).

2.4.2.7 Agar gel immunodiffusion test

The agar gel immunodiffusion test is based on precipitation of the antigen-antibody complex. This method is often used for the diagnosis of *B. ovis* infection. This test has a low cost, it is easily performed and it has sensitivity levels that are comparable to complement fixation. However, it has some disadvantages such as a marked decrease in sensitivity in chronic infections and high variability of the quality of commercially available antigens (Geresu and Kassa, 2016).

2.4.3 Culture and isolation

For the definitive diagnosis of brucellosis, culture and isolation of the bacteria is the

definitive test. The selection of tissue to culture is usually reliant on the type of clinical signs that the animal is presenting (Poester *et al.*, 2010). In this case, valid samples include aborted foetuses, fetal membranes, vaginal secretions, colostrum, milk, sperm, and fluid collected from arthritis or hygroma. The tissues however require careful and proper handling to prevent contamination but preserve the organisms as well. For cases that a post-mortem 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).

At slaughter, in order to confirm suspected cases of acute or chronic brucellosis, the preferred tissues are the genital and oropharyngeal lymph nodes, the spleen, and the mammary gland and associated lymph nodes. For the isolation of *Brucella* spp., the most commonly used medium is the Farrell medium, which contains antibiotics that are able to inhibit the growth of other bacteria present in clinical samples (Niels, 2003).

Brucella organisms are fastidious slow growers, hence are easily overgrown by other bacteria that often lead to misdiagnosis. In chronic cases, cultures may fail to grow due to low levels of bacteria. Stamp's modified Ziehl Nelsen staining is used to identify *Brucella* organisms as they stain red against a blue background when examined under a light microscope. The colonies appear small, singly or paired Coccobacilli. However, other organisms that cause abortions like *Chlamydia*, *Coxiella* and Norcardia spp are also acid-fast and stain the same colour (OIE Diagnostic Manual, 2012; Bishop *et al.*, 1994).

Brucella species are highly pathogenic to humans, hence all infected tissues, cultures and potentially contaminated materials must be handled under appropriate containment conditions (OIE Diagnostic Manual, 2012; Nielsen and Yu, 2010). Consequently,

laboratory personnel are highly at risk of contracting this zoonotic infection during these hazardous procedures that require high security laboratory facilities (biological containment level 3) and highly skilled personnel.

2.4.4 Molecular diagnostic techniques

The molecular techniques include Polymerase Chain Reaction (PCR), Restriction Endonuclease Analysis (REA), Restriction Endonuclease and Hybridisation that have been used for diagnosis and epidemiological studies of the disease (Tenover, 1988; Ghassan *et al.,* 1996). The technique is chosen based on the type of biological sample and the goal, i.e., diagnosis or molecular characterization or epidemiological survey (Geresu and Kassa, 2016). Most of them have their sensitivity ranging from 50% to 100% and specificity between 60% and 98%. The DNA extraction protocol, type of clinical sample, and detection limits of each protocol, are factors that can influence the efficiency of the technique (Mitka *et al.,* 2007). However, these techniques are too expensive to be used widely, they are more appropriate for differential diagnosis rather than for establishing prevalence of the disease.

2.5 Treatment and Control of Brucellosis

2.5.1 Treatment

In animals, treatment of brucellosis is not recommended due to the fact that its success rate is very low and is also expensive. Following exposure to antibiotics such as penicillin and oxytetracycline, *Brucella* undergoes L-transformation that hinders serological detection and results in animal being carrier (Bishop *et al.,* 1994 cited by Temba, 2013). Unsuccessful treatments have been reported because the drugs are said to be unable to penetrate the cell membrane barrier due to the intracellular sequestration of the organisms in the lymph nodes, mammary glands and reproductive organs (Bishop *et al.,* 1994;

Radostits *et al.,* 2000). In humans, the essential component in the treatment of all forms of human brucellosis is the administration of effective antibiotics for an adequate length of time (WHO, 2006). Antibiotic treatment should be implemented at as early stage as possible, even in patients who appear to be showing a spontaneous improvement. A variety of antimicrobial drugs have been found to be effective against *Brucella.* A combination of drugs for several weeks is needed in order to treat and clear the organism since *Brucella* is an intracellular organism (Donev, 2010). In all cases it is important that the patient finishes the full course of medication because the risk of incomplete recovery and relapse is otherwise increased considerably (Smits and Kadri, 2005).

2.5.2   Control

Brucellosis control programs have been successful in eradicating the disease in animals in several countries based on various strategies, including vaccination and test and-slaughter of infected animals (Abubakar *et al.,* 2012). 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). The best way to deal with brucellosis in a herd is to vaccinate all heifers between 3 - 10 months of age with strain 19 vaccines and to remove those which react positive to serological tests (OIE, 2008).

There are no safe and effective vaccines for the prevention of human brucellosis, although vaccination played a significant role in the prevention of the disease, in conjunction with other measures, in the former USSR and China (WHO, 2006). 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).

In order to prevent brucellosis in humans, controlling or eliminating the disease from the animal population, avoiding consumption of raw milk, raw milk products and adopting hygienic practices should be considered. Also proper heat treatment of milk or milk products is important for effective prevention of brucellosis in humans (Abubakar *et al.,* 2012). Moreover, brucellosis must be included in public health education and public awareness programs, particularly in the rural areas and efforts should be directed towards preventive measures but not curative services (WHO, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

The study was carried out at the at anti-persoonsmijnen ontmijnende product ontwikkeling abreviateted as APOPO which in English means anti-personnel landmines removal product development training and research facility, SUA in Morogoro, Tanzania. The sniffer rats were housed and trained in a designated building containing kennels, lined with sets of spacious and ventilated interconnected cages, with plenty of wood shaving litter to absorb ammonia from urine and rat droppings. Each cage had a clay pot with bedding to simulate their natural underground nest, an untreated wooden tripod to gnaw on as well as regular enrichment toys. Samples used in culturing *Brucella* were collected from Kitengule Ranch in Kagera region which was purposeful selected, located in the north-western part of Tanzania on the western shore of Lake Victoria. The region lies just below the equator between latitudes 1º00' and 2º45' South and 30º25' and 32º40' East of Greenwich. The region neighbours Uganda, Rwanda and Burundi and lies across the lake from Kenya.

3.2 Study Design

The present study was an experimental study design. The study involved nine adult sniffer rats (*Cricetomys ansorgei*) which were available at the training facility, five being males and four females with age ranging from 3.6 -3.9 years. The sniffer rats were obtained from APOPO which has a breeding program that supplies sniffer rats for training, research and detection programs that are currently underway. The sniffer rats used were involved in another experiment that ended two weeks before the beginning of the current experiment.

3.3 Description of Sniffer Rats

APOPO's HeroRATs as they are known are African giant pouched rats (*Cricetomys ansorgei*) which comprise large muroid rodent found throughout most of sub-Saharan Africa. They are omnivorous, mainly nocturnal, have long heads with large ears and cheek pouches that are used to store and carry food. They have a long bare tail, which is white from the middle half to tip. Their body is covered with buff-colored, relatively long fur with pale under parts (Fig. 1). Their head and body lengths range from 25–30cm and tails ranging from 30–35 cm. These little heroes can weigh between 1 and 1.3 kg. They have highly developed sense of smell, intelligent and are easy to train. They are also too light to set off the landmines, locally sourced and widely available, easily transferable between trainers, cheap to feed, breed and maintain. They may live 6-8 years, indigenous to sub-Saharan Africa and therefore resistant to most tropical diseases.

Figure 1:   African giant pouched rat.

3.4 Field Clinical Sample Collection, Processing, Isolation, Identification and Confirmation of *Brucella*

3.4.1 Sample collection and processing

Blood, milk, faecal and aborted materials were collected from cattle. Sera were separated from blood and screened for *Brucella* antibodies using RBT test procedure as recommended by OIE (OIE, 2009). Briefly, 30µl of RBPT antigen and 30µl of the test serum were placed alongside on the glass plate and mixed thoroughly. After 4 minutes of rocking, any visible agglutination was considered as positive result. Milk samples were collected from seropositive cattle.

3.4.2 *Brucella* culture

Collected milk and aborted materials were processed for *Brucella* culture using Farrell's media. This media were prepared in preparation laboratory at SUA where a *Brucella* selective supplement SR0083A was added. The supplement contains polymyxin B 2500 IU, Bacitracin 12 500IU, Cyclohexamide 50.0mg, Nalidixic 2.5mg, Nystatin 50 000IU and Vancomycin 10mg. The medium was prepared by adding aseptically 10mls of 1:1 methanol: sterile distilled water to 1 vial to form an even suspension. It was then incubated for 15 minutes at 37°C, mixed thoroughly and immediately added to the vial content to 500 mls of sterile blood Agar Base (CM0271) prepared as directed, then, cooled to 5°C and enriched with 50 mls of inactivated horse serum (SR0035) and 25 ml of 10%w/v sterile solution of Glucose (LP0071). Then, mixed well and finally poured into sterile petri dishes.

The petri dishes were then kept in a refrigerator for 24 hours and then culture was done in a biosafety cabinet in duplicate, one set of petri dishes were kept under presence of oxygen while the other were kept under presence of carbon dioxide, container containing

CO_2 sachet (anaerobic condition) then incubated at 37°C. The plates were observed for any growth from three up to 14 days and those that did not show any growth after 14 days were discarded. In total six milk and aborted material samples were cultured.

3.4.3 Identification of *Brucella*

3.4.3.1 Microscopic examination

Colonies of bacteria that grew on the media after four days were Gram stained and subjected for microscopic examination. Using sterile wire loop, a drop of sterile water was picked and placed on a slide, a colony was picked also using sterile loop and gently stirred into the drop of sterile water on a slide, heat fixed and the smear allowed to air dry. The slide was then flooded with crystal violate and allowed to stay for one minute. Then tilted gently and rinsed the slide with tape water and then Lugose Iodine was added and allowed to stay for one minute, gently rinsed with tap water. Then, decolorized using acetone after that alcohol was applied drop by drop for five to 10 seconds until the alcohol runs almost clear and immediately rinsed with water. Finally flooded with counter-stain (safranin) and stayed for 45 seconds before being rinsed with tap water again. The slide was then air dried and examined using light-microscope under oil immersion.

3.4.3.2 Biochemical properties evaluation of the *Brucella* catalase test

A drop of catalase reagent was picked using a sterile wire loop and placed on a microscopic glass slide then followed by a colony from the growth and mixed them. The mixture was allowed to react and the reaction was observed. A positive reaction was indicated by gas bubble formation within minutes while for negative reaction there were no gas bubbles formations.

Oxidase test

Using a sterile loop, colonies were picked from the growth plates and smeared on the edge of the test sticks. The sticks were then left to stand on the table for about two to three minutes and colour changes were noted and recorded. For positive reaction the colour changed from white to dark blue while with negative reaction there were no colour changes. Sterile normal saline was used as a negative control while *Brucella abortus* S19 vaccine strain was used as a positive control.

3.4.4 Detection of *Brucella* spp. from colonies using polymerase chain reaction

The detection of DNA of *Brucella* species from colonies obtained after culture by Polymerase Chain Reaction (PCR) was done as detailed in the subsequent sections.

3.4.4.1 DNA extraction from colonies

DNA extraction was done by boiling method in which 200 µl of dionized water was mixed with a colony of *Brucella* which was picked from the plate using sterile wire loop into an ependorf and mixed thoroughly. The mixture was boiled into water bath heated at 98°C for 15 minutes and allowed to cool. After cooling 100 µl of supernatant were picked and used in DNA identification.

3.4.4.2 Preparation of PCR master mix

The reaction mixtures were prepared with constituents as shown in Table 1.

Table 1: **Preparation of PCR master mix for detection of *Brucella* from colonies obtained after culturing milk and placental fluid samples**

Components	1X (µl)	14x (µl)
Taq reaction buffer	2.5	35
MgCl$_2$	0.75	10.5
Dntp	0.5	7
Taq polymerase	0.1	1.4
Primer dtail	2	28
Nuclease free water	14.15	198.1
Template	5	
Total	25	

3.4.4.3 Amplification of *Brucella* species DNA by AMOS PCR

Detection of the presence of *Brucella* spp. nucleic acid material in the eluted extract was done using AMOS PCR. Primers were used to amplify different base pair fragment that contained the target gene (Table 2). All amplifications were performed in a total volume of 25 µl. The reaction mixtures containing primers were prepared, DNA sample was added and PCR was performed. 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. After PCR, 5 µl of the PCR products was mixed with a 6x loading dye.

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

Primers	Nucleotide sequences 5' to 3'	Concentration100 x (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

3.4.4.4   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℃ that could be touched by hand. 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.

3.4.4.5   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

multiplex (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**

Brucella strain	Predicted amplicon sizes (bp)							
	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

3.5 Pre - Screening of the Sniffer Rats

Prior to training the sniffer rats with *Brucella abortus* sniffing, the nine sniffer rats used in this experiment were screened for brucellosis. This was done in order to be sure that before the experiment took place they were free of the disease or not. Blood samples were collected from the tail using plain vacutainer tubes and centrifuged to obtain serum. All serum samples were then screened using RBPT for *Brucella* antibodies according to the test procedure recommended by OIE (OIE, 2009).

3.6 Preparation of Sample for Training Sniffer Rats

After obtaining *Brucella abortus* samples from the culture, 10mls of distilled water was mixed with 10 colonies from the growth plate to form a solution and then the solution was heat inactivated at 56^0C for 45 minutes in a water bath. From a resulting solution 500 µl

were pipetted and spread into a nutrient agar medium which was prepared in a 40 mls plastic container. Each of these plastic containers had 5 mls of nutrient agar poured into them and incubated overnight. The medium was used as background for the sniffer rats to be able to differentiate the odour produced by *Brucella abortus* and that produced by the medium.

Similar to positive samples, the negative samples were prepared in the same way as the positive ones; 10 mls of distilled water was mixed with ten loop full *Brucella* selective media to form the solution. From the resulting solution 500 µl were pipetted into the container with 5 mls of nutrient agar medium, this was used as negative control. The transfer of bacteria into the containers was done in a biosafety cabinet every morning of the training session and after that the containers were labelled and packed into a plastic bag transported to APOPO via cool box for sniffer rats training as shown in Fig. 2.

A   **B**

Figure 2:   **Containers (A) with the media only and containers (B) with the media and inactivated *Brucella* sample inside.**

3.7 Rat Training Facility

The rats were trained in fully automated line cage (FALCON) (Fig. 3), like the automated line cage (ALC). This facility is used for various stages of odour discrimination training by the research team at APOPO. The FALCON is made of a rectangular chamber measuring 210 x 41 x 52 cm, with hinged top panels and glass walls which is mounted on four 96 cm high legs. As with other line cages, 10 circular holes, 30 mm in diameter, are

evenly spaced along the cage floor. Each hole is fitted with a through-beam (infrared) photoelectric sensor and covered by an aluminum plate that can be slid open by underlying solenoids when the rat places its nose in the hole (breaks the photobeam). The aluminum plate can be programmed to automatically close after the sniffer rat removes its nose from the hole (typically with a time delay to prevent pinching the rat).

The first hole opens upon the start of the session and each subsequent hole opens after the infrared beam is broken (by the rat inserting its nose) into the hole immediately preceding it. Timing and duration of the beam break (such as when the sniffer rat is evaluating an odour sample placed below the hole) is monitored by the controlling software. The cage is fitted with a pellet dispenser and magazine (ENV-203-94, Med Associates, Georgia, VT) located on the left side of the cage, which delivers an adjustable number of pellets (5TCY OmniTreat^{TM}) via a 20 cm long plastic tube attached to a 25 cm^2 hole located above the chamber floor. Pellet delivery can be triggered by manual depression of a handheld button (usually during early training), or by a custom designed software (REST_LIMS, programmed using MS Visual Basic) which sends the relevant signal to the apparatus from a computer connected via USB.

As with all other line cages, aluminium cassettes measuring 192 x 8 x 45 cm, with 10, 40-mm diameter holes (note – hole diameter differs from other line cages) positioned in correspondence with the 10 holes in the floor of the cage, can be loaded with sample containers. The pre-loaded cassettes fit into a hinged bracket that swings up and locks into position underneath the cage.

Computer   pellet dispenser

Figure 3: Fully automated line cage system.

3.8 Evaluation Criteria during Training

For the sniffer rats to be considered successful in the particular stage of training they were supposed to obtain a score of 80% or above on each session and test for three consecutive days. At least seven rats had to pass this score line, this was considered by looking on the sensitivity and specificity of indirect tests for the diagnosis of cattle brucellosis as published in literature ranging from 76.9-100% and 77.4-100% respectively by Godfroid *et al.* (2010). Also each rat had a maximum of 30 minutes to complete a session in a cage, more than that was considered as failure and the rat was withdrawn from the cage.

3.9 Training Sniffer Rats

3.9.1 Socialization and clicker trainings

Before being trained in *Brucella* detection the sniffer rats had undergone socialization and clicker trainings under TB detection program. The former training stage is essential for the sniffer rats to have human interaction and habituation to novel environments. In this stage handlers gently carry them around, introducing them to sights, sounds, smells and noises, to adapt them to the training environment and their handlers. The later training

stage was implemented after passing the socialisation tests, the sniffer rats first have to hear clickers and receive rewards in order for them to associate the sound with food and later be motivated to carry out trained actions such as searching out the target scent. Therefore, this stage of training is critical to all later stages of training.

3.9.2 Indication training

This stage teaches the sniffer rat to hold its nose in the hole containing a target sample (*Brucella abortus*) for as long as it takes to produce the click. The goal of indication training was to train the sniffer rat to approach and sniff a hole in the fully automatic line cage (Falcon cage) containing target samples, this helped to establish the target odour that the sniffer rat was being trained to detect. So, the response was for the sniffer rat to hold its nose in the hole containing the target sample for progressively longer durations (2 seconds) to produce a click sound through which the sniffer rat was rewarded by getting (food) three food pellets. The training started with 12 target samples (*Bucella abortus* collected and inactivated at day 10) in a three holes' indication training using four bars and they did it for two weeks.

3.9.3 Discrimination training

The goal of discrimination training was to establish the smell of target sample as a discriminative stimulus. That is, the sniffer rat had to learn that indication behaviour (holding its nose in the hole for two seconds) was only rewarded when it occurs in the presence of a *Brucella abortus* sample. This was accomplished by a procedure called differential reinforcement where indications over holes containing *Brucella abortus* samples were reinforced while indications over holes containing *Brucella* negative samples were ignored. In this training, the sniffer rats started with 30 holes' discrimination training in which ten samples were positive and twenty were negative

samples. The training took place for three weeks and in all these sessions the *Brucella abortus* used was collected on day 10 after culturing. On day 14 before the completion of 30-hole discrimination training, new target samples were introduced.

The new target samples were collected on day seven and 12 and their distribution was four samples from day ten colonies, three samples from day seven colonies and the last three samples from 12-day colonies making a total of ten positive samples. These samples were used during training, while on the test session another target sample was introduced. The idea here was that, different odour may be produced by *Brucella* colonies depending on their age due to availability of food and waste produced in a media where growth was taking place. At the start of this session ten bars containing three samples each were used and then later changed to three bars containing ten samples each followed. The pattern on how the negative and positive samples were placed was determined by computer-based program which allocate the target samples at different positions daily. After completing the 30 holes' discrimination training, 50 holes discrimination training followed which included 10 positive samples and 40 negative samples and they did it for 14 days. On the process two blinds were introduced on this phase the blind samples were just the same as the normal targets (positive samples) only they were entered in the software differently so that they do not show up for the trainers on the computer.

On day ten of this session, new non target samples (*Escherichia coli*) were introduced to check if the rats were detecting other smell other that the one from *Brucella abortus*. *E.coli* samples were prepared on the same way as the other negative samples; i.e. ten colonies of *E. coli* were picked and mixed with 10mls of distilled water to form a solution. The resulting solution was heat inactivated at 56°C for 45minutes in a water bath and then allowed to cool. On the day of their use, 500µl of the solution was pipette into

sample containers filled with 5mls of nutrient agar. The transfer of bacterium into the containers was done in the lab early in the morning of the training session and after that the containers were labelled and packed into a plastic bag transported to APOPO via cool box for sniffer rats training. On successful completion of this session, the rats were moved to one hundred holes' discrimination training. During this session, the number of target samples remained the same as the previous session i.e. ten *Brucella abortus* samples (the two blind samples inclusive) collected from colonies that were seven, 10 and 12 days old. The remaining 90 holes contained non target samples including 22 *E.coli* samples. This training was done for 19 days and day 20 of this session the first test session was conducted.

3.9.4 Testing and evaluation of rat's performance

3.9.4.1 Test one

The test was conducted using samples that were prepared by other personnel as they were prepared daily. The idea here was that sniffer rats have a tendency of picking a smell or any other thing done by one person daily, so preparing samples by a different person was introduced to verify if the performance of the rats was due to this myth. So the preparation of the samples was done normally except that it was done by a different person who was not doing it in daily basis. After this test the sniffer rats returned to two days' baseline training session before subjected to the second test.

3.9.4.2 Test two

The second test session was done three days after the completion of test one. This test involved addition of new three target sample which was *Brucella abortus* collected on day five and 18 non-target samples (blood agar) on the 90 non target samples. Note that the negative sample (non target) i.e. blood agar introduced here, was once used in the first

two sessions which were not included in this study. After the test, the sniffer rats were subjected to a normal training session for one day followed by the third test.

3.9.4.3 Test three

This was the final test which included additional of new target, three spiked faecal samples and 18 non target (fecal) samples. This test was done two days after the completion of second tests. A spatula was used to pick faeces, a full picked spatula was mixed with 1mls of distilled water and the resulting solution was introduced into one plastic container mixed thoroughly and taken to the rats. There was not heat inactivation with the faecal samples and the containers did not contain any media. So the preparation was done in the morning of the training session and after that the containers were labelled and packed into a plastic bag transported to APOPO via cool box for sniffer rats training. After this test the sniffer rats were trained for five days with samples spiked on faecal matter.

3.10 Post - Screening of the Sniffer Rats

Immediately after completion of the training the sniffer rats were screened for *Brucella* infection. About 2mls of blood was collected in a plain vacutainer tube from each rat and serum was harvested. All collected serum samples were then screened using RBT for presence of *Brucella* antibodies.

3.11 Ethical Consideration

This study was conducted in conformity with the ethical guidelines and the permission of conducting the study obtained from the Vice Chancellor of Sokoine University of Agriculture (SUA) (Appendix 1). All field and laboratory results obtained after blood,

serum, aborted fluids and milk samples analysis were kept under the custody of the researcher as confidential.

3.12   Data Analysis

Data was entered and coded in Microsoft Excel (2010) and then transformed into mathematics and statistics software implemented in SPSS for analysis.

CHAPTER FOUR

4.0   RESULTS

4.1   Isolation and Identification of *Brucella* spp

Brucella spp. was cultured from collected samples. Six samples were cultured, four of them grew and microscopically they were tiny, Gram negative, coccobacilli arranged as single or short rods, though they were sometimes in pairs or small groups (Fig. 4).
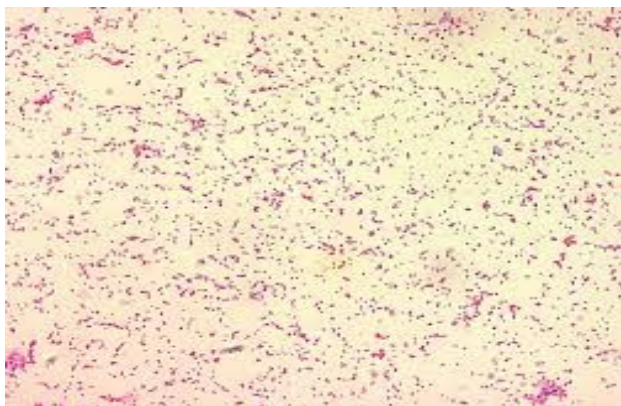
Figure 4: Gram staining and morphologic features of *Brucella* spp.

Biochemical analysis of the isolates using catalase and oxidase tests indicated that they were oxidase positive (Fig. 5) and catalase positive (Fig. 6).

Positive control (Black/dark blue)

Negative control (No colour change)
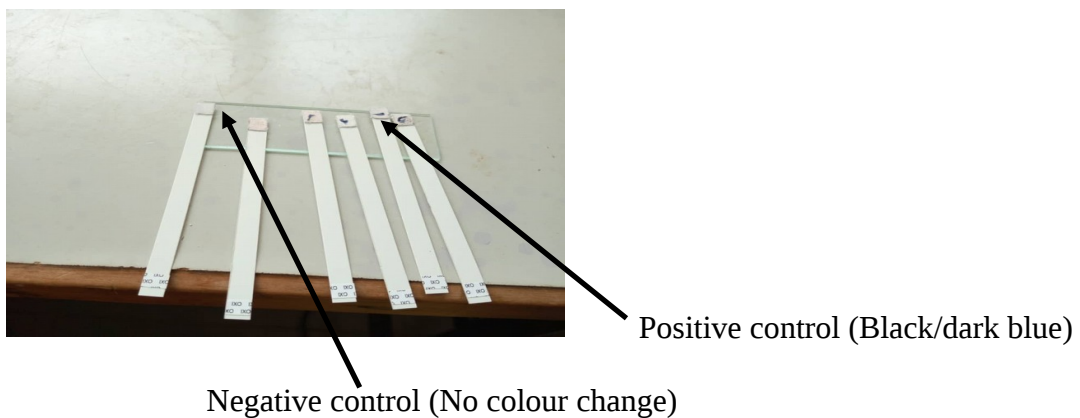
Figure 5:   Positive oxidase reaction indicated by a black/dark blue colour.

Foam formation (positive control)

No changes (Negative control)
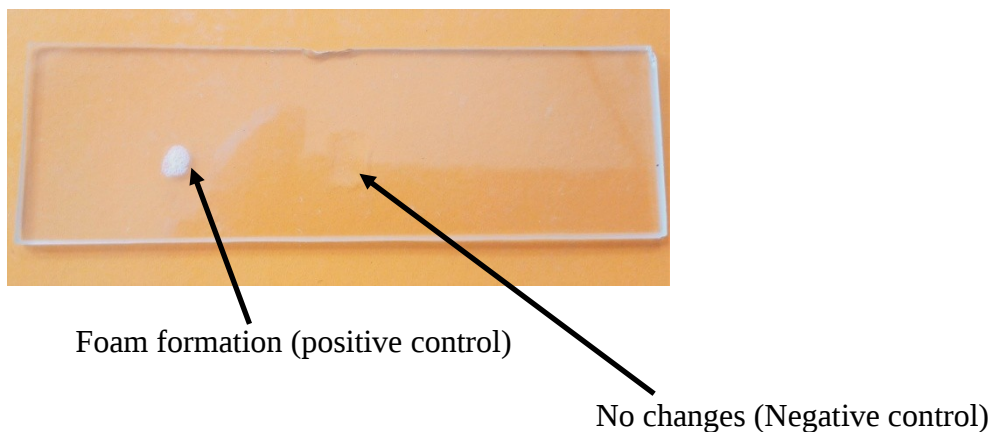
Figure 6:   Gas bubbles/foam formation as a positive result on catalase test.

AMOS PCR targeting IS*711* gene of *Brucella* spp. indicated presence of *Brucella* DNA in the samples collected as indicated in the gel photo below (Fig. 7).
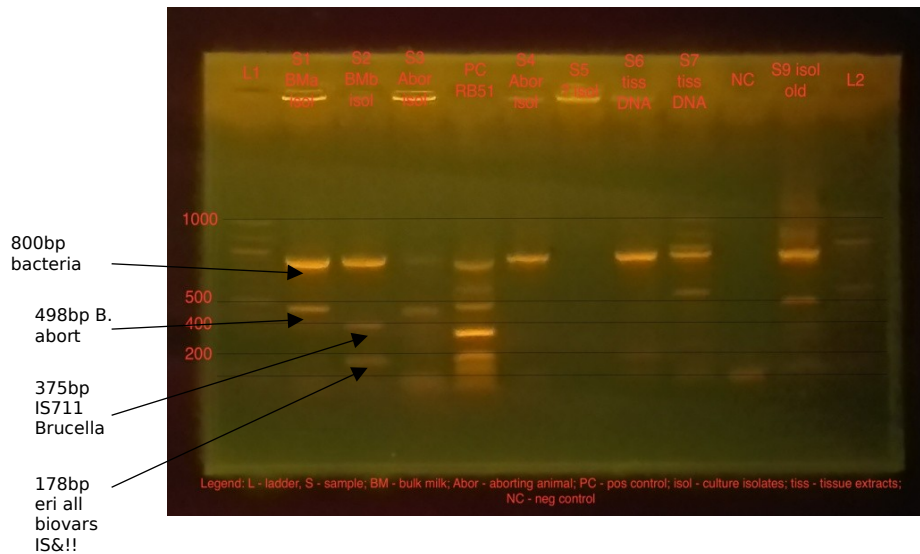
Figure 7: **Amplification of *Brucella abortus* IS711 gene using PCR. A 1% agarose gel electrophoresis of *Brucella abortus* IS711 gene amplicon of 495 bp from total DNA of cow milk and aborted material. Lane L1 and L2; 1kb DNA ladder; Lane; S1 and S2, bulky milk samples; Lane; S3 and S4, aborted material; Lane RB51, positive control; Lane; S6 and S7, tissue isolates; Lane, NC, negative control containing water.**

4.2 Pre and Post Screening of the Rats

Both pre and post screening of serum samples from rats used in the current experiment indicated absence of *Brucella* antibodies (Fig. 8).

Positive control

Figure 8: Negative results of seventeen rat's sera on RBPT.

4.2.1 Performance of the trained rats

Nine rats were trained to detect *Brucella* spp. Seven (78%) rats successfully completed the training and tests while the remaining two (22%) completed the training but failed to complete their tests. One of the two rats (Angela) failed in test one and two while the other rat (Stewart) failed in test two. They both failed because they were unable to complete the task within 30 minutes in a given test session while in the cage.

4.2.2 Baseline sensitivity and specificity

Baseline data on rat's accuracy (sensitivity and specificity) was collected over five consecutive days prior to actual tests. The rats showed an average sensitivity of 92% with range of 82-98 % (Table 4) and average specificity of 98% ranging from 95-99% (Table 5).

Table 4: **Baseline data on sensitivity (hits) of the rats in detection of *Brucella* spp. on five-day sessions prior to testing**

	Hits per session					Average hits	Sensitivity (%)
Rat ID	1	2	3	4	5		
Hawking	10	9	10	10	9	9.6	96
Skinner	10	9	9	10	10	9.6	96
Sloth	9	9	10	8	10	9.2	92
Stewart	8	8	8	9	8	8.2	82
Zhang	10	8	10	9	10	9.4	94
Angela	10	9	10	10	10	9.8	98
Aung	10	7	10	9	9	9	90
Jane	10	8	9	9	10	9.2	92
Pippi	10	9	9	8	9	9	90
	9.6	8.4	9.4	9	9.4	9.2	92

Table 5: **Baseline data on specificity (false alarm) of the rats in detection of**
Brucella spp. on five-day sessions prior to testing

Rat ID	False alarm per session					Average false alarm	Specificity (%)
	1	2	3	4	5		
Hawking	0	2	0	2	3	1.4	98
Skinner	0	2	1	1	1	1	99
Sloth	7	4	5	2	2	4	96
Stewart	7	2	0	1	3	2.6	97
Zhang	2	4	1	3	1	2.2	98
Angela	9	2	5	3	2	4.2	95
Aung	2	2	3	1	1	1.8	98
Jane	1	1	1	5	2	2	98
Pippi	0	2	0	2	2	1.2	99
	2.4	2.4	1.4	2.1	1.9	2	98

4.3 Rats Performance during Testing

4.3.1 Performance of the rats when introduced to samples prepared by a new personnel

The average sensitivity during this session was 93% and the average specificity was 96%. Angela was dropped from this session as shown on Table 6 below.

Table 6: **Sensitivity and specificity data when samples were prepared by a**
different person

Rat ID	Hits 1	False alarm 1	Sensitivity (%)	Specificity (%)
Hawking	10	3	100	97
Skinner	9	4	90	96
Sloth	9	8	90	91
Stewart	10	5	100	94
Zhang	9	1	90	99
Angela	4	3	40	97
Aung	8	1	80	99
Jane	9	3	90	97
Pippi	10	5	100	94
	9.3	3.8	93	96

4.3.2 Sensitivity and specificity data of two sessions between Test one and Test two

The average sensitivity and specificity of the rats during the two sessions was 99% and 97% respectively as indicated in Table 7 below. Angela and Stewart failed to complete these sessions.

Table 7: Sensitivity and specificity of rats on two sessions between Test one and Test two using 100 samples

	Hits per session		FA per session		Sensitivity (%)	Specificity (%)
Rat ID	1	2	1	2		
Hawking	10	10	1	1	100	99
Skinner	10	10	0	2	100	98
Sloth	10	9	1	4	90	96
Stewart	8	0	1	0	0	100
Zhang	10	10	0	2	100	98
Angela	8	1	1	0	10	100
Aung	8	10	3	1	100	99
Jane	10	10	2	2	100	98
Pippi	9	10	1	7	100	92
	9.4	8.6	1.1	2.4	99	97

FA=False alarm

4.3.3 Performance of the rats on introduction of new target and non target samples

During this session, sensitivity was 97% and specificity was 93%. Angela and Stewart failed to finish the sessions so their results were not included in this analysis.

Table 8: Sensitivity and specificity data of rats on addition of five day *Brucella* and 18 blood agar

Rat ID	Hits per session	False alarm per session	Sensitivity	Specificity
	1	1	%	%
Hawking	9	7	90	92
Skinner	10	4	100	96

Sloth	10	9	100	90
Stewart	6	5	60	94
Zhang	10	11	100	88
Angela	6	6	60	93
Aung	9	2	90	98
Jane	10	4	100	96
Pippi	10	4	100	96
	9.7	5.9	97	93

During subsequent day, an average sensitivity was 93% and specificity was 89% Table 9.

Table 9:   Sensitivity and specificity data of rats a day after test two using 100 samples per session

Rat ID	Hits per session 1	False alarm per session 1	Sensitivity %	Specificity %
Hawking	10	11	100	88
Skinner	10	10	100	89
Sloth	8	9	80	90
Stewart	10	11	100	88
Zhang	10	13	100	86
Angela	4	8	40	91
Aung	7	7	70	92
Jane	10	5	100	94
Pippi	9	12	90	87
	9.3	9.8	93	89

4.3.4   Performance of the rats on *Brucella* spiked faecal samples

During this session rats showed an average sensitivity and specificity of 76% and 98% respectively, Table 10.

Table 10:   Sensitivity and specificity data of rats on spiked fecal materials

Rat ID	Hit per session 1	False alarm per session 1	Sensitivity %	Specificity %
Hawking	8	1	80	98
Skinner	9	1	90	99
Sloth	7	1	70	99
Stewart	8	0	80	100

Zhang	9	6	90	93
Angela	8	1	80	99
Aung	6	2	60	98
Jane	7	1	70	98
Pippi	7	1	70	99
	7.6	1.6	76	98

CHAPTER FIVE

5.0 DISCUSSION

The present findings suggest that sniffer rats can be of value in detection of *Brucella* infection as it was used in laboratory prepared bovine clinical samples with notably high sensitivity and specificity. The rats showed average baseline sensitivity (target sample) of 92% and equally high specificity (non target samples) of 98%. Thus, on average a rat missed less than one positive sample while committing only two false alarms in a session with 100 samples and 10% target prevalence. Comparing these results with what

happened in early stages with TB detection, Mgode *et al*. (2012) showed the sensitivity and specificity to be 80.4% and 72.4% while the study by Weetjens *et al*. (2009) showed the sensitivity and specificity to be 73.1% and 93.4% respectively. Despite being slight lower as compared to results from this experiment but the sniffer rats went to be applied in TB detection and are to date used as second line in TB detection. Therefore, the sensitivity and specificity values from this experiment suggest that sniffer rats can be of value in *Brucella* detection.

Despite high average performance of the rats which has been shown with their high sensitivity and specificity, two rats (22%) did not complete their sessions after failing to complete their 30 minutes' sessions while the rest seven (78%) completed. This is, however, a major objection to using animals for operational disease detection. Their failure may be due to many factors including their behavior which is variable. It has been suggested that, they cannot automatically be assumed that different animals will react in the same fashion to the same scent, or that the same animal will react in the same way to that scent on different occasions (Elliker *et al.*, 2014). When a different person in test one prepared samples, sniffer rats did not show an appreciable change (t (7) = -0.31, p = .767) in sensitivity (92% during baseline and 93% during test one session) but showed a slight, albeit significant (t (7) = 2.48, p = .042) decrease of specificity 96% versus 98% in the five preceding sessions. During this test (test one), the average rat continued to miss less than one positive sample but increased false alarms to nearly four in a session with 100 samples and 10% target prevalence. This was done so because of an experience learnt previous at APOPO that sniffer rats have a tendency of picking up small details through their sniffing ability which is said to be highly developed, if something is prepared for them daily in the same way by the same person they may pick any parameter that may help them getting their target so that they are being rewarded even if the target is wrong

one. Using a different person in preparation was meant to be certain with this rat's behaviour (Poling *et al.,* 2011).

After test one, sniffer rats returned to normal sessions which was conducted in two consecutive days. Their performance returned to baseline levels during these two sessions. Thus on average nearly every rat was able to finds all 10 *Brucella* samples while committing fewer than three false alarms among 90 non-target (*Brucella* negative) samples.

During test two, every rat correctly indicated the presence of the three -five day *Brucella* cultures, including one which was blind sample. However, overall false alarm rates increased to nearly six out of 90 non-target samples (93% specificity compared to 98% on baseline) with the majority of these occurring to the 18 blood agar (roughly 30% of these samples). It declined further to 89% in the subsequent session as it was used one more after test two. The decline in specificity is probably attributed to the use of blood agar in the first two initiation sessions which were then omitted. At the beginning of this experiment it was suggested that the background or media to be used in carrying the target and non target samples were nutrient agar and blood agar. But later it was suggested to use only nutrient agar alone because it was convenient to prepare one medium. Also using blood agar would mean rats have to deal with three media that is Ferrell's medium which was used in culture, blood agar and nutrient agar.

With spiked faecal samples, the overall sensitivity decreased to an average 76% in comparison to the baseline sensitivity of 93%. Here at least five of the nine rats trained, found at least one of the three fecal samples spiked with the cultured *Brucella* and some rats finding two of these samples, including the blinded trials. Notably, specificity

improved from the previous sessions (test two) that included the blood agar non-targets, returning to baseline levels averaging 98% per rat. This suggests that the rats were willing to hold their noses over the fecal samples (reducing the contribution of this factor in the missed spiked samples) and were not motivated to indicate samples based on novelty alone (as could be the case for the 5 - day culture/blood agar sessions).

In TB detection from which the idea of using sniffer rats was generated, the rats targeted a blend of specific volatile organic compounds produced by *Mycobacterium tuberculosis* (Mgode *et al.,* 2012). This was also adopted with *Brucella* samples which were collected on different days to try to figure out if at these different days there is any emission of odour that sniffer rats are detecting. So, samples were collected on day five, seven, ten and 12 because when culturing, *Brucella* may start to grow on day three up to 30 days (Kaltungo *et al.,* 2014) and those that did not show any growth after 14 days were discarded. During training, rats did not show any difference between samples collected at different days.

CHAPTER SIX

6.0   CONCLUSION AND RECOMMENDATIONS

6.1   Conclusion

The results from this study have pinpointed that sniffer rats can detect *Brucella* with high accuracy (sensitivity and specificity) in both inactivated culture materials and spiked faecal samples. This is an indication that if validated, sniffer rats could be used as one of diagnostic method for brucellosis which translates to positive impact on treatment as well as planning and delivering effective disease control programs.

6.2 Recommendations

Based on the above conclusions the following are the recommendations;

i.   Sniffer rats have shown ability in detecting *Brucella* species in a laboratory prepared samples, therefore, if they are to be used either as first or second line diagnostic tool in human and animals, more research are required to ascertain its applicability in clinical samples.

ii.   It is recommended that economic feasibility study to be done to compare the cost of using sniffer rats against the available tests in the market.

iii. In this study only *Brucella abortus* was used, it is worthy evaluating the detection of other *Brucella* spp. as the disease is caused by several species of *Brucella*.

iv.   In TB detection, sniffer rats targeted a blend of specific volatile organic compounds produced by *Mycobacterium tuberculosis*. It is also import to check the component(s) of *Brucella abortus* that the rats are sniffing in brucellosis detection.

REFERENCES

Abdoel, T., Dias, I. T., Cardoso, R. and Smits, H. (2008). Simple and rapid field tests for Brucellosis in livestock. *Veterinary Microbiology* 10: 1 - 8.

Abubakar, M., Mansoor, M. and Arshed, M. J. (2012). Bovine brucellosis: old and new concepts with Pakistan perspective. *Pakistan Veterinary Journal* 32(2): 147-155.

Ahmad, M. and Majali, A. (2005). Sero epidemiology of caprine brucellosis in Jordan. *Small Ruminant Research* 58: 13–18.

Ahmed, M. O., Elmeshri, S. E., Abuzweda, A. R., Blauo, M., Abouzeed, Y. M., Ibrahim, A. H., Salem, A.F., Abid, S., Elfahem, S. and Elrais, A. (2010). Seroprevalence of brucellosis in animals and human populations in the western mountains region in Libya. *Euro surveillance* 15: 1 - 30.

Al-Garadia, M. A., Khairani-Bejo, S., Zunita, Z. and Omar, A. R. (2011). Detection of *Brucella melitensis* in blood samples collected from goats. *Journal of Animal and Veterinary Advances* 10(11): 1437 - 1444.

Al-Mariri, A. and Haj-Mahmoud, N. (2010). Detection of *Brucella abortus* in Bovine Milk by Polymerase Chain Reaction. *Journal of the University of Veterinary and Pharmaceutical Sciences in Brno, Czech Republic* 79: 277 - 280.

Asfaw, G. M. and Mamo, K. G. (2016). A Review on Diagnostic Methods of Brucellosis. *Journal of Veterinary Science and Technology* 7(3): 8 - 34.

Assenga, J. A., Matemba, L. E., Muller, S. K., Malakalinga, J. J. and Kazwala, R. R. (2015). Epidemiology of *Brucella* infection in the human, livestock and wildlife interface in the Katavi-Rukwa ecosystem, Tanzania. *Biomedical Central Veterinary Research* 11: 303-309.

Baily, G. G., Krahn, J. B., Drasar, B. S. and Stoker, N. G. (1992). Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *The American Journal of Tropical Medicine and Hygiene* 95: 271 - 275.

Bedard, B. G., Martin, S. W. and Chinombo, D. (1993). A prevalence study of bovine Tuberculosis and brucellosis in Malawi. *Preventive Veterinary Medicine* 16: 193 - 205.

Bishop, G. C., Bosman, P. P. and Herr, S. (1994). Bovine Brucellosis: In: *Infectious Disease of Livestock with Special Reference to Southern African.* (Edited by Coetzer, J. A. W., Thompson, G. R. and Tustin, R. C). Oxford University Press, UK. pp. 1053 - 1066.

Blood, D. C., Radostits, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D. (2007). Veterinary Medicine: A textbook of the diseases of cattle, horses, sheep, pigs and goats. 10th Edition, Saunders Limited. 2065pp.

Bret, P. K., Hoover, D. K. and Friedlander, A. M. (2008). *Brucellosis. In: Medical Aspects of Chemical and Biological Warfare.* (Edited by Sidell, F. R., Takafuji, E. T. and Franz, D. R). Textbook of military medicine, Office of the Surgeon General, USA. pp. 185 - 198.

Cadmus, S. I. B., Adesokan, H. K. and Stack, J. (2008). The use of the milk ring test and Rose Bengal test in brucellosis control and eradication in Nigeria. *Journal of the South African Veterinary Association* 79: 113 - 115.

Cadmus, S. I. B., Adesokan, H. K., Adedokun, B. O. and Stack, J. A. (2010). Seroprevalence of bovine brucellosis in trade cattle slaughtered in Ibadan, Nigeria, from 2004 – 2006. *Journal of the South African Veterinary Association* 81: 50 - 53.

Carter, G. R., Chengappa, M. M. and Roberts, A. W. (1995). *Essential of Veterinary Microbiology,* 5th edition, Williams and Wilkins. pp. 199 - 204.

Chitupila, G. Y., Komba, E. V. G. and Mtui-Malamsha, N. J. (2015). Epidemiological study of bovine brucellosis in indigenous cattle population in Kibondo and Kakonko Districts, Western Tanzania. *Livestock Research for Rural Development* 6: 1-12.

Corbel, M. J. (1997). Brucellosis: An overview. *Emerging Infectious Diseases* 3(2): 1 - 165.

Cutler, S. J., Whatmore, A. M. and Commander, N. J. (2005). Brucellosis new aspects of an old disease. A Review. *Journal of Applied Microbiology* 98: 1270 – 1281.

David, L. H. and Arthur, M. F. (1998). *Brucellosis. In: Medical Aspects of Chemical and Biological Warfare Chapter 25.* (Edited by Sidell, F. R., Takafuji, E.T. and Franz, D. R.), Textbook of Military Medicine, Office of the Surgeon General, USA. pp. 513 – 521.

Davis, D. S., Templeton, J. W., Ficht, T. A., Williams, J. D., Kopec, J. D. and Adams, L. G. (1990). *Brucella abortus* in captive bison. I. Serology, bacteriology, pathogenesis and to cattle. *Journal Wildlife Disease* 26: 360 - 371.

De Figueiredo, P., Ficht, T. A., Rice-Ficht, A., Rossetti, C. A. and Adams, L. G. (2015). Pathogenesis and immunobiology of brucellosis: Review of Brucella-host interactions. *American Journal of Pathology* 185(6): 1505 - 1517.

Donev, D. (2010). Brucellosis control and eradication in the South Eastern European Countries: Current status and perspective strategies. *Macedonian Journal of Medical Sciences* 3(3): 221 - 228.

Donev, D., Karadzovski, Z., Kasapinov, B. and Lazarevik, V. (2010). Epidemiological and Public Health Aspects of Brucellosis in the Republic of Macedonia. Contributions. *Journal of Biological Medical Science* 1: 33 - 54.

Ducrotoy, M., Bertu, W. J., Matope, G., Cadmus, S., Conde-Álvarez, R., Gusi, A. M.,Welburn, S., Ocholi, R., Blasco, J. and Moriyón, I. (2017). Brucellosis in Sub-Saharan Africa: Current challenges for management, diagnosis and control. *Acta Tropical* 165: 179 - 193.

Edwards, T. L., Browne, C. M., Schoon, A., Cox, C. and Poling, A. (2017). Animal Olfactory Detection of Human Diseases: Guidelines and Systematic Review, *Journal of Veterinary Behaviour* 2(9): 45 – 78.

Elliker, K. R., Sommerville, B. A., Broom, D. M., Neal, D. E. and Armstrong, S. (2014). Key considerations for the experimental training and evaluation of cancer odour detection dogs : lessons learnt from a double-blind, controlled trial of prostate cancer detection. *Biomedical Centre Urology* 14(1): 1–9.

Ellis, H., Mulder, C., Valverde, E., Poling, A. and Edwards, T. (2017). Reproducibility of African giant pouched rats detecting Mycobacterium tuberculosis. *Biomedical Centre Infectious Diseases* 17(1): 4 – 9.

England, T., Kelly, L., Jones, R. D., MacMillan, A. and Wooldridge, M. (2004). A simulation model of brucellosis spread in British cattle under several testing regimes. *Preventive Veterinary Medicine* 63: 63 - 73.

Enström, S., Nthiwa, D., Bett, B., Karlsson, A., Alonso, S. and Lindahl, J. F. (2017). Brucella seroprevalence in cattle near a wildlife reserve in Kenya. *Biomedical Centre Research Notes* 3: 1–5.

Fensterbank, R. (1986). Brucellosis in cattle, sheep and goats diagnosis, control and vaccination. *Preventive Veterinary Medicine* 5(5): 605 - 618.

Ferreira, A. C., Cardoso, R., Dias, I.T., Mariano, I. and Belo, A. (2003). Evaluation of a modified Rose Bengal test and an indirect enzyme-linked immunosorbent assay for the diagnosis of *Brucella melitensis* infection in sheep. *Journal of Veterinary Research* 34: 297 - 305.

Foster, J. T., Okinaka, R. T., Svensson, R., Shaw, K.B., De, R.A., Robison, W.S., Probert, L.J., Kenefic, W. D. and Keim, P. (2008). Real-time PCR assays of single-nucleotide polymorphisms defining the major *Brucella* clades. *Journal of Clinical Microbiology* 46: 296 - 301.

Franc, K. A., Krecek, R. C., Häsler, B. N. and Arenas-Gamboa, A. M. (2018). Brucellosis remains a neglected disease in the developing world: a call for interdisciplinary action. *BMC Public Health* 18(1): 125.

Garin-Bastuji, B., Blasco, J. M., Marín, C. and Albert, D. (2006). The diagnosis of

brucellosis in sheep and goats, old and new tools. *Small Ruminant Research* 62: 63 - 70.

Glenn, R., Markenson, K., Rodger, M., Tillotson-Criss, M., Katherine, S. F., Robert, S. Stewart, Jr., Michael, Y. M., Honolulu, H. and Tacoma. (1997). The use of the polymerase chain reaction to detect bacteria in amniotic fluid in pregnancies complicated by pre term labour. pp. 1471 - 1477.

Godfroid, J. (2002). Brucellosis in wildlife. *Revue Scientifique et Technique De Office International Des Epizooties* 21: 277 - 286.

Godfroid, J., Cloeckaert, A., Liautard, J. P., Kohler, S., Fretin, D., Walravens, K., Garin, B. B. and Letesson, J .J. (2005). From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a reemerging Zoonosis. *Veterinary Research* 36: 313 - 326.

Godfroid, J., Nielsen, K. and Saegerman, C. (2010). Diagnosis of Brucellosis in Livestock and Wildlife. *Croatian Medical Journal* 51(4): 296 - 305.

Godfroid, J., Saegerman, C., Wellemans, V., Walravens, K., Letesson, J.J., Tibor, A., Mcmillan, A., Spencer, S., Sanna, M., Bakker, D., Pouillot, R. and Garin-Bastuji, B. (2002). How to substantiate eradication of bovine brucellosis when a specific serological Reactions occur in the course of brucellosis testing. *Veterinary Microbiology* 90: 461 - 477.

Golshani, M. and Buozari, S. (2017). *A Review of Brucellosis in Iran: Epidemiology, Risk*

Factors, Diagnosis, Control and Prevention 21: 349 - 359.

Huber, B., Scholz, C. H., Lucero, N. and Busee, H. (2009). Development of a PCR assay for typing and subtyping of *Brucella* species. *International Journal of Medical Microbiology* 28: 210 - 215.

Ibironke, A. A., McCrindle, C. M. E., Fasina, F. O. and Godfroid, J. (2008). Evaluation of problems and possible solutions linked to the surveillance and control of bovine brucellosis in sub-Saharan Africa, with special emphasis on Nigeria. *Veterinaria Italiana* 44(3): 549 – 556.

Jiwa, S. F. H., Kazwala, R. R., Tungaraza, R., Kimera, S. I. and Kalaye, W. J. (1996). Bovine brucellosis serum agglutination test prevalence and breed predisposition according to prevalent management systems in the lake zone of Tanzania. *Preventive Veterinary Medicine.* pp. 341 - 346.

Kabagambe, E. K., Elzer, P. H., Geaghan, J. P., Opuda-Asibo, J., Scholl, D. T. and Miller, J. E. (2001). Risk factors for Brucella seropositivity in goat herds in eastern and western Uganda. *Preventive Veterinary Medicine* 52: 91-108.

Kaltungo, B. Y., Saidu, S.N.A., Musa, I.W. and Baba, A.Y. (2014). Brucellosis: A Neglected Zoonosis. *African Journal of Medicine and Medical Sciences* 4(12): 1551–1574.

Kaoud, H. A., Zaki, M. M., El-Dahshan, A. R. and Nasr, S. A. (2010). Epidemiology of brucellosis among farm animals. *Nature and Science* 8(5): 2 – 9.

Karimuribo, E. D., Ngowi, H. A., Swai, E. S. and Kambarage, D. M. (2007). Prevalence of brucellosis in crossbred and indigenous cattle in Tanzania. *Livestock Research for Rural Development* 19(10): 1 - 58.

Khan, S. T. G. A. (2007). Epidemiology and Epizootology of Brucellosis: a Review. *Pakistan Veterinary Journal* 27(3): 145 – 151.

Kitaly, J. (1984). Bovine brucellosis in Government parastatal and Ujamaa village dairy farms in Central Zone of Tanzania: Assessment of Control measures in some farms In: *Proceedings of the 2nd Tanzania Veterinary Association Scientific Conference,* 4th-6th December. Arusha, Tanzania.

Kunda, J., Fitzpatrick, J., French, N., Kazwala, R., Kambarage, D., Mfinanga, G. S. Alastair macmillan, A. and Cleaveland, S. (2010). Quantifying risk factors for human brucellosis in rural Northern Tanzania. *PLoS ONE* 5(4): e9968.

Lyimo, B. (2013). Prevalence of bovine brucellosis in smallholder dairy farms in Morogoro municipality, Tanzania.Sokoine University of Agriculture, Morogoro, Tanzania. pp. 1-57.

Mahoney, A., Weetjens, B. J., Cox, C., Beyene, N., Reither, K., Makingi, G. and Poling, A. (2012). *Pouched Rats' Detection of Tuberculosis in Human Sputum: Comparison to Culturing and Polymerase Chain Reaction. 2012.* [https://doi.org/10.1155/2012/716989]. Site visited on 2/6/2019.

Mariana, N. X., Tatiane, A. P., Andréas, B. H., Renée, M. T. and Renato, L. S. (2010). Pathogenesis of *Brucella spp*. *Open Veterinary Science Journal* 4: 109 – 118.

Mathew, C., Stokstad, M., Johansen, T. B., Klevar, S., Mdegela, R. H., Mwamengele, G. and Godfroid, J. (2015). First isolation, identification, phenotypic and genotypic characterization of Brucella abortus biovar 3 from dairy cattle in Tanzania. *BMC Veterinary Research* 11(1): 1–9.

Matope, G. (2009). The impact of restocking communal cattle on the spread of brucellosis in Zimbabwe. PhD thesis for award degree of Doctorate of Harare University, Zimbabwe. 261pp.

Matope, G., Bhebhe, E., Muma, J. B., Lund, A. and Skjerve, E. (2010). Herd-level Factors for *Brucella* seropositivity in cattle reared in smallholder dairy farms of Zimbabwe. *Preventive Veterinary Medicine* 94: 213–221.

McGiven, J. A., Tucker, J. D., Perrett, L. L., Stack, J. A., Brew, S. D. and MacMillan, A. P. (2003). Validation of FPA and cELISA for the detection of antibodies to *Brucella abortus* in cattle sera and comparison to SAT, CFT and iELISA. *Journal of Immunological Methods* 278: 171–178.

Mellau, L. S. B., Kuya, S. L. and Wambura, P. N. (2009). Seroprevalence of brucellosis in domestic ruminants in livestock-wildlife interface: A case study of Ngorongoro Conservation Area, Arusha. Tanzania. *Tanzania Veterinary Journal* 26 (1): 44 - 50.

Mgode, G. F., Weetjens, B. J., Nawrath, T., Cox, C., Jubitana, M., Bedotto, M. and

Kaufmann, S. H. E. (2012). *Diagnosis of Tuberculosis by Trained African Giant Pouched Rats and Confounding Impact of Pathogens and Microflora.* pp. 274 – 280.

Michel, A. L., Coetzee, M. L., Keet, I. D. F., Mare, L., Warren, R., Cooper, D., Bengis, R. G., Kremer, K. and van Helden, P. (2009). Molecular epidemiology of *Mycobacterium bovis* isolates from free ranging wildlife in South African game reserves. *Veterinary Microbiology* 133: 335- 343.

Minga, U. M. and Balemba, O. B. (1990). ELISA, A new sero-diagnosis test for brucellosis in Tanzania. *Proceedings of the 8th Tanzania Veterinary Association Scientific Conferenc*e, Arusha Tanzania. 98pp.

Minja, K. S. G. (2002). Prevalence of Brucellosis in Indigenous cattle: Implication for Human Occupation Groups in Hanang and Babati districts of Tanzania. Dissertation for Award degree of Master's degree of Sokoine University of Agriculture, Tanzania. pp. 1 - 67.

Mitka, S., Anetakis, C., Souliou, E., Diza, E. and Kansouzidou, A. (2007). Evaluation of different PCR assays for early detection of acute and relapsing brucellosis in humans in comparison with conventional methods. *Journal of Clinical Microbiology* 45(4): 1211–1218.

Mohan, K., Makaya, P. V., Muvavarirwa, P., Matope, G., Mahembe, E. and Pawandiwa, A. (1996). Brucellosis surveillance and control in Zimbabwe: bacteriological and

serological investigation in dairy herds. *Journal of Veterinary Research* 63: 47–51.

Morta, P., Queipo-Ortuno, J. M., Reguera, M. J., Garcia-Ordonez, A. M., Cardenas, A. and Colmenero, D.J. (2003). Development and evaluation of a PCR-ELISA assay for diagnosis of human brucellosis. *Journal of Clinical Microbiology* 41: 144-148.

Msanga, J. F., Mukanga, D. J. A. and Tungaraza, R. (1986). Bovine brucellosis in the lake zone of Tanzania: the present situation. *Bulletin of Animal Health and Production in Africa* 34(4): 2 -23.

Mtui-Malamsha, N. (2001). Epidemiology study of brucellosis in humans and animals in Babati and Hanang district of Tanzania. Dissertation for Award of MSc. Degree at Sokoine University of Agriculture, Morogoro, Tanzania. pp 48.

Muchowski, J. K., Koylass, M. S., Dainty, A. C. and Stack, J. A. (2015). Using molecular tools to identify the geographical origin of a case of human brucellosis. *Epidemiology and Infection* 3(14): 143.

Muma, J. B., Munyeme, M., Matope, G., Siamudaala, V. M., Munang'andu, H. M., Matandiko, W. J., Godfroid, E. and Tryland, M. (2011). *Brucella* seroprevalence of the Kafue lechwe (*Kobus leche kafuensis*) and Black lechwe (*Kobus leche smithemani*): Exposure associated to contact with cattle. *Preventive Veterinary Medicine* 100: 256 - 260.

Muma, J. B., Munyeme, M., Samui, K. L., Skejerve, E. and Oloya, B. C. (2007). Risk factors for brucellosis in indigenous cattle reared in livestock–wildlife interface areas of Zambia. *Preventive Veterinary Medicine* 80: 306–317.

Muma, J. B., Samui, K. L., Siamudaala, V. M., Oloya, J., Matope, G., Omer, M. K., Munyeme, M., Mubita, C. and Skjerve, E. (2006). Prevalence of antibodies to *Brucella* spp. and individual risk factors of infection in traditional cattle, goats and sheep reared in livestock-wildlife interface areas of Zambia. *Tropical Animal Health and Production* 38: 195-206.

Navarro, E., Casao, M. A. and Solera, J. (2004). Diagnosis of human brucellosis using PCR. *Expert Review of Molecular Diagnostics* 4: 115−123.

Neta, A. V. C., Mol, J. P. S., Xavier, T. A., Paixao, T. A., Lage, A. P. and Santos, R. L. (2010). Pathogenesis of bovine brucellosis. *Veterinary Journal* 184: 146 – 155.

Nielsen, K. (2002). Diagnosis of brucellosis by serology. *Veterinary Microbiology* 90: 447- 459.

Nielsen, K., Gall, D., Smith, P., Balsevicius, S. and Garrido, F. (2004). Comparison of serological tests for the detection of ovine and caprine antibody to *Brucella melitensis*. *Review Science Technique Journal* 23: 979 - 987.

OIE, (2000). An update on zoonoses (*P. Pastoreted*.). *Review Science Technique Journal* 19: 1 − 32.

OIE, (2008). Manual of the diagnostic tests and vaccines for terrestrial animals, Volume 15th Edition. *Office International Des Epizooties,* Paris, France. pp. 409 - 438.

OIE, (2009). Porcine Brucellosis. Version adapted by the World Assembly of Delegates of the Office International des epizooties, Paris, France. pp. 67-89.

OIE, (2010). Training manual on wildlife diseases and surveillance. *Workshop for OIE National Focal Points for Wildlife World Organisation for Animal Health,* Paris, France. 46pp.

Otaru, M. M. M. (1985). Potential zoonotic diseases in the southern zone of Tanzania. In: Proceedings of the 3rd Tanzania Veterinary Association Scientific Conference. Arusha Tanzania. *Parasitology* 160(2): 34–42.

Poester, F. P., Nielsen, K., Samartino, L. E., and Yu, W. L. (2010). Diagnosis of Brucellosis, *The Open Veterinary Science Journal* 3: 46 – 60.

Poling, A., Weetjens, B. J., Cox, C., Mgode, G., Jubitana, M., Kazwala, R. and Huis, D. (2010). Short Report : Using Giant African Pouched Rats to Detect Tuberculosis in Human Sputum Samples : 2009 Findings 83(6): 1308–1310.

Poling, A., Weetjens, B. J., Cox, C. and Beyene, N. (2011). Tuberculosis detection by giant African pouched rats (Cricetomys gambianus). *The Behavior Analyst* 34 (1): 47-54.

Radositits, O. M., Gay, C. C., Hinchcliff, K. W. and Constable, P. D. (2006). *Veterinary Medicine*. 10th edition. A textbook of the diseases of cattle sheep, goats, pigs and horses, Saunders, Edinburgh. pp. 96 – 104.

Rajala, E. (2016). *Brucellain Tajikistan - Zoonotic Risks of Urbanized Livestock in a Low-income Country*. Swedish University of Agricultural Sciences. pp. 39 - 40.

Refai, M. (2002). Incidence and control of brucellosis in the Near East region. *Veterinary Microbiology* 90: 81 - 110.

Roberto, F. F., Newby, D. T. and Hadfield, T. L. (2003). Real-Time PCR detection of *Brucella abortus*: A Comparative study of SYBR Green I, 5, Exonuclease and Hybridization Probe Assays. *Applied and Environmental Microbiology* 3: 4753 – 4759.

Sachs, R., Staak, C. and Groocock, C. M. (1968). Serological investigation of brucellosis in game animals in Tanzania. *Bulletin of Epizootic Disease in Africa* 16: 93 -100.

Schiemann, B. and Staak, C. (1971). *Brucella melitensis* in impala (*Aepyceros melampus*).*Veterinary Record* 88 (13): 344 -349.

Scholz, H. C. (2016*). Brucella vulpis* sp. nov., isolated from mandibular lymph nodes of red foxes (Vulpes vulpes). *International Journal Systematic Evolutionary Microbiology* 66: 2090 - 2098.

Scholz, H. C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H. and Cloeckaert, A. (2010). *Brucella microti* spp. Nov., isolated from the common vole *Microtus arvalis*. *International Journal Systematic Evolutionary Microbiology* 58(2): 375 – 382.

Seleem, N. M., Boyle, S. M. and Sriranganathan, N. (2010). Brucellosis: A re-emerging Zoonosis. *Veterinary Microbiology* 140: 392 – 398.

Shang, D., Xiao, D. and Yin, J. (2002). Epidemiology and control of brucellosis in China. *Veterinary Microbiology* 90: 165 – 182.

Sharma, R., Chisnall, C. and Cooke, R.P.D. (2008). Evaluation of in-house and commercial immunoassays for the sero-diagnosis of brucellosis in a non-endemic low Prevalence population. *Journal of Infection* 56: 108-113.

Shirima, G. M. (2005). The epidemiology of brucellosis in animals and humans in Arusha and Manyara regions of Tanzania. Thesis for Award of PhD. Degree at Glascow University UK. 316pp.

Smirnova, E. A., Vasin, A. V., Sandybaev, N. T., Klotchenko, S. A., Plotnikova, M. A., Chervyakova, O. V. and Kiselev, O. I. (2013). Current Methods of Human and Animal Brucellosis Diagnostics. *Advances in Infectious Diseases* 3(3): 177–184.

Smits, H. L. and Cutler, S. J. (2004). Contributions of biotechnology to the control and prevention of brucellosis in Africa. *African Journal of Biotechnology* 12(3): 631 – 636.

Soler-Lloréns, P., Gil-Ramírez, Y., Zabalza-Baranguá, A., Iriarte, M., Conde-Álvarez, R., Zúñiga-Ripa, A. and López-Goñi, I. (2014). Mutants in the lipopolysaccharide of Brucella ovis are attenuated and protect against B. ovis infection in mice. *Veterinary Research* 45(1): 1–11.

Staak, C. and Protz, D. (1973). Brucellosis survey in Masailand and Mbulu districts of Tanzania. *Bulletin of Epizootic Diseases in Africa* 21: 67-74.

Stack, J. A., Harrison, M. and Perrett, L. L. (2002). Evaluation of a selective medium for *Brucella* isolation using natamycin. *Journal of Applied Microbiology* 92: 724-728.

Swai, E. S. and Schoonman, L. (2010). The Use of Rose Bengal Plate Test to assess cattle exposure to *Brucella* infection in traditional and smallholder dairy production systems of Tanga region of Tanzania. *Veterinary Medicine International* 837950: 1 – 8.

Swai, E. S., Karimuribo, E. D., Kambarage, D. M. and Moshy, W. E. (2009). A longitudinal study on morbidity and mortality in young stock smallholder dairy cattle with special reference to tick borne infections in Tanga region, Tanzania. *Veterinary Parasitology*. 60 (2):34-42

Swai, E. S., Mshanga, D., Sanka, N. P. and Marandu, N. H. (2005). Prevalence of bovine brucellosis in smallholder dairy farming area, Moshi, Tanzania. *Bulletin of Animal Health and Production in Africa* 53: 97 – 105.

Swai, E. M. (1997). Studies on the prevalence of bovine brucellosis and reproductive performance in small-scale dairy cattle herds in Dar es Salaam and Morogoro regions. Dissertation for Award Master's Degree of Sokoine University of Agriculture, Morogoro, Tanzania. pp. 67.

Temba, P. B. (2012). Seroprevalence of a *Brucella abortus* and other *Brucella* species in Wildlife-Livestock interface: a case study of Mikumi-Selous Ecosystem. A Dissertation for Award Degree of Master of Science in Public Health and Food Safety of Sokoine University of Agriculture Morogoro, Tanzania. 130pp.

Tenover, F. C. (1998). Diagnostic deoxyribonucleic acid probes for infectious diseases. *Clinical Microbiology Reviews* 1: 82 – 101.

Theegarten, D., Albrecht, S., Tötsch, M., Teschler, H., Neubauer, H. and AlDahouk, S. (2008). *Brucellosis of the Lung: Case Report and Review of the Literature* 452: 97 – 101.

Weetjens, B. J., Mgode, G. F., Machangu, R. S., Kazwala, R., Mfinanga, G. and Lwilla, F. (2009). African pouched rats for the detection of pulmonary tuberculosis in sputum samples. *International Journal of Tuberculosis and Lung Disease* 13: 737–743.

Weinhäup, I., Schöpf, K. C., Khaschabi, D., Kapaga, A. M. and Msami, H. M. (2000). Investigations on the prevalence of bovine tuberculosis and brucellosis in dairy

cattle in Dar es Salaam Region and in zebu cattle in Lugoba area, Tanzania. *Tropical Animal Health and Production* 32: 147 – 154.

Whatmore, A. M. (2009). Current understanding of the genetic diversity of *Brucella,* an Expanding genus of zoonotic pathogens. FAO/WHO Collaborating Centre for Brucellosis, OIE Brucellosis Reference Centre, Veterinary Laboratories Agency, Addlestone, Surrey KT15 3NB, United Kingdom. pp. 1168-1184.

Whatmore, A. M., Dawson, C., Groussaud, P., Koylass, M., King, A., Shankster, S., Sohn, A., Probert, W. and McDonald, W. (2008). A marine mammal *Brucella* genotype associated with zoonotic infection Emerging Infection disease. *Journal of Clinical Microbiology* 14: 517-518.

Whatmore, A. M., Murphy, T. J., Shankster, S., Young, E., Cutler, S. J. and MacMil- lan, A. P. (2005). Use of Amplified Fragment Length Polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. *Journal of Clinical Microbiology* 43: 761-769.

Whatmore, A. M., Davison, N., Cloeckaert, A., AlDahouk, S., Zygmunt, M. S. and Brew, S. D. (2014). *Brucella papioni s*sp. nov., isolated from baboons (*Papio* spp.). *International Journal of Systematic and Evolutionary Microbiology* 12: 4120 - 4128.

Whatmore, A. M., Perrett, L. L. and MacMillan, A. P. (2007). Characterisation of the genetic diversity of *Brucella* by multilocus sequencing. *Biomedical Central* 7: 34. doi:10.1186/1471-2180-7-34

WHO, (2006). *Brucellosis in Humans and Animals.* World Health Organisation, Geneva. 65pp.

WHO, (2009). Integrated control of neglected zoonotic diseases in *WHO/EU/ILRI/DBL/FAO/OIE/AU/WHO meeting*, ILRI headquarters, Nairobi. pp. 1-70.

Xavier, M. N., Costa, E. A., França, A. S. and Silva, A. P. C. (2014). Indirect ELISA for diagnosis of *Brucella ovis* infection in rams. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia* 66: 1695 – 1702.

Xavier, M. N., Costa, E. A., Paixão, T. A. and Santos, R. L. (2009). Genus *Brucella* and clinical manifestations. *Ciência Rural* 39(7): 2252 – 2260.

Yingst, S. L., Huzella, L. M., Chuvala, L. and Wolcott, M. (2010). A rhesus macaque (*Macaca mulatta*) model of aerosol-exposure brucellosis (*Brucella suis*): pathology and diagnostic implications. *Journal of Medical Microbiology* 59: 724 – 730.

Zerva, L., Bourantas, I. K., Mitka, S., Kansouzidou, A. and Legakis, N. J. (2001). Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. *Journal of Clinical Microbiology* 39(4): 1661- 1664.

APPENDIX

Appendix 1: A research permit provided by Vice Chancellor of Sokoine

University of Agriculture (SUA)

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;

Date: 3rd December, 2018

Our Ref. SUA/ADM/R.1/8/311

Chief Executive Officer,
APOPO,
P.O. Box 3078,
MOROGORO.

Re: UNIVERSITY STAFF, STUDENTS AND RESEARCHERS CLEARANCE

The Sokoine University of Agriculture was established by University Act No. 7 of 2005 and SUA Charter, 2007 which became operational on 1st January 2007 repealing Act No. 6 of 1984. One of the mission objectives of the university 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 staff, students, research associate and researchers of SUA on behalf of the Tanzania Commission for Science and Technology.
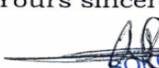
The purpose of this letter is to introduce to you **Mr. Raphael R. Mwampashi** a bonafide **MSc. (Public Health and Food Safety)** student with registration number **MPH/D/2017/0003** of SUA. By this letter **Mr. Raphael R. Mwampashi** has been granted clearance to conduct research in the country. The title of the research in question is "**To investigate the potential of sniffer rats in detection of *brucella* in cattle**".

The period for which this permission has been granted is from **December, 2018** to **June, 2019**. The research will be conducted in **Morogoro.**

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. Peter R. Gillah
FOR: VICE-CHANCELLOR

Copy to: Student – **Mr. Raphael R. Mwampashi**