PREVALENCE AND FACTORS ASSOCIATED WITH BRUCELLOSIS IN LIVESTOCK IN BARINGO COUNTY, KENYA.

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DECLARATION

This thesis is my original work and has not been presented for examination in any other university.

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DEDICATION

I dedicate this research work to my late father Michael Kiplagat.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ACRONYMS AND ABREVIATIONS

µl – Microliters

AGID- Agar Gel Immunodiffusion Test

CFT- Complement Fixation Test

CTAB – Cetyltrimethylammonium bromide

CVIL- Central Veterinary investigation Laboratory-Kabete

DNA -Deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

ELISA- Enzyme Linked Immunosorbent Assay

FAO- Food and Agricultural Organization

IG- Immunoglobulin

KNBS- Kenya National Bureau of Statistics

LPS- Lipopolysaccharide

MET- Mercaptoethanol Test

Ml- Millilitres

MRT- Milk Ring Test

OIE- Office International des Epizooties (World health organization for animals)

PCR- Polymerase chain reaction

RBPT- Rose Bengal plate test

RUFORUM- Regional Universities Forum for capacity building in Agriculture

×g – Gravitational force

SAT- Serum Tube agglutination test

SDS – Sodium Dodecyl Sulphate

SOP- Standard operating procedure

SSA- Sub-Saharan Africa

TE – Tris EDTA

USA- United States of America

UV- Ultraviolet light

WHO- World Health Organization

ABSTRACT

Brucellosis is an important zoonotic disease occurring worldwide. Its importance stems from both public health and economic effects of the disease. The prevalence of brucellosis in livestock from pastoralist herds is usually higher than in settled herds, with human brucellosis highly relating to the disease in animals.

This cross sectional study was carried out in four sub-counties of Baringo County, in which farms were randomly selected from each sub-county. Blood (10ml) from selected cattle (n= 250), sheep (n= 142) and goats (n= 166) was collected in sterile plain vacutainers. Bulk raw cattle milk (n=83) was also collected.

All serum samples were screened for *Brucella* antibodies using Rose Bengal Plate test (RBPT) and by competitive Enzyme Linked Immunosorbent Assay (cELISA). *Brucella* antibodies in milk were assayed using Milk Ring Test (MRT). Polymerase chain reaction (PCR) was carried out on blood clots from all RBPT-positive serum samples as well as on blood clots of 7% of the serum samples that turned negative on RBPT to determine presence of brucella antigens in those samples.

Twenty three (9.2%) of the 250 cattle serum samples reacted positive to RBPT while 17 (6.8%) reacted positive to cELISA with cumulative reactors of 25 (10%). The 166 caprine serum samples had 17 (10.2%) positive reactors to RBPT and 11 (6.6%) by cELISA. Cumulative caprine reactors were 18 (10.8%). Positive ovine serum samples were 10 (7%) and 7 (4.9%) on RBPT and cELISA respectively, yielding positive cumulative reactors of 11 (7.7%). The sensitivity and specificity of RBPT was 88.6% and 96.4% respectively with a predictive value positive of 62% and predictive value negative of 99%. From the 83 milk samples collected, 9 (10.7%) tested positive to Milk Ring Test.

Brucella abortus DNA was extracted from 11 of cattle blood clots and from two goat and one sheep blood clots respectively. *Brucella melitensis* DNA was extracted from one goat blood clot.

Mixed farming was reported by 57% of the respondents, communal grazing reported by 32% of the respondents, use of communal watering points reported by 38% of the interviewees and allowing of animals to calve down on pasture reported by 91% of the respondents. All these were found to be factors associated with brucellosis in the region. However, introduction of a new animal reported by 42% was found not to be a risk factor despite studies elsewhere documenting it as a risk factor.

From the serological results, it is evident that brucellosis occurs in livestock in Baringo, predominantly caused by *B. abortus.* This study also established that there is a huge knowledge gap on its risk factors in the region. It is therefore important to establish an educational campaign in the region on the significance of the disease, and establish possible control measures. This will lower the prevalence of the disease in animals and will go a long way towards minimizing human brucellosis.

CHAPTER ONE

1.0 INTRODUCTION

Brucellosis in livestock in Kenya has been reported to be widely spread and endemic especially among the pastoral communities and many cases have been reported in the annual reports of the Ministry of Agriculture, Livestock and Fisheries; and elsewhere (Kagumba and Nandokha, 1978; Waghela, 1978; McDermott and Arimi, 2002; Kang'ethe, 2000; Mugambi, 2001; Muriuki *et al.,* 1997). The prevalence of brucellosis in cattle from pastoralist herds is usually higher than from settled herds (Hussein *et al.,* 2005) with stock movement, mixing of different animal species and concentration of animals around water points and grazing fields considered important transmission factors (Waghela, 1976; McDermott and Arimi, 2002; Emongor *et al.,* 2000).

Food and Agricultural Organization (FAO), World Health Organization (WHO) and World health organization for animals (OIE) consider brucellosis the most highly spread zoonosis in the world (OIE, 2004; FAO, 2004). It is widely spread within African countries (Chukwu, 1985; Abbas and Agab, 2002) and has previously been considered by the World Health Organization (WHO) as being responsible for more sickness, misery and economic loss than any other zoonotic disease (McDermott and Arimi, 2002). It is also a potential biological weapon (Jovanka *et al*., 2010).

The disease affects mainly domestic animals such as cattle, sheep, goats, pigs and dogs as well as human (Young, 1995). The disease has also been documented in wildlife and marine animals (Cloeckaert *et al.,* 2001). The main route of entry for most brucellae in animals is by ingestion of contaminated pasture and by direct contact with the causative agent (Blood and Radostits, 1989). Domestic carnivorous animals may acquire *B. abortus* and *B. melitensis* by consuming contaminated fetuses, meat, placentae or milk (Prior, 1976; Radostits *et al.,* 1994).

In most of these animal species, manifestation of the disease is remarkably similar, characterised by relapsing bacteraemia that becomes intermittent to chronic in later stages and which may sometimes recur for up to two years in 5-10 % of the infected animals (Waghela *et al.*, 1978). The principal manifestations are reproductive failures and wastage which include abortions, stillbirths or birth of unthrifty neonates, orchitis, seminal vesiculitis, testicular abscesses and epididymitis in males. Abortions usually occur during the second half of gestation. Some neonates are born alive but weak, and may die soon after birth. Retained afterbirths and secondary metritis can occur and lactation may be decreased (Jovanka *et al*., 2010).

Humans are infected by *B. melitensis*, *B. suis*, *B. abortus*, *B. canis*, *B. ovis* and *B. neotomae* in descending order of pathogenicity (Leclerc *et al*., 2002, Glynn *et al.*, 2008). All of these species affecting humans circulate in animals. Statistics show an increased incidence of human brucellosis in persons who are engaged in certain professions such as veterinarians, slaughterhouse employees, dairy farmers and workers, livestock handlers, and laboratory personnel (Glynn *et al.*, 2008).

The World Health Organization (WHO) laboratory biosafety manual classifies *Brucella* in Risk group III. *Brucella abortus* and *B. melitensis* are also important food-borne pathogens that may be acquired by consuming raw milk and milk products such as soft cheese and this is of particular importance in communities consuming raw milk and/or undercooked meat from infected animals (Kadohira *et al.*, 1997; Omore *et al.*, 2005; Kang'ethe *et al.*, 2000).

The infection rate in humans is however markedly lower than the rate in animals (Arnow *et al.*, 1984). It has also been shown that there is a direct relationship between the incidences in animal hosts with the disease pattern in human beings; this is because infection in humans is highly influenced by animal husbandry practices, food customs and standards of hygiene (Arnow *et al.*, 1984; McDermott and Arimi, 2002).

Brucellosis in humans occurs in all age groups (Mantur *et al.,* 2007, Kiambi, 2012) and is characterized by influenza-like clinical disease with undulating fever, sweats, malaise, arthralgia, weakness, anorexia, headache, myalgia and back pain (Maichomo *et al.*,2000; Fallatah *et al.,* 2005; Haddadi *et al.,* 2006; Kokoglu *et al.,* 2006). The infection could sometimes persist and result in various complications as described by Nicholas *et al.,* (2001); Wang *et al.,* (1999); Abhay *et al.,* (2007)*;* Cem *et al.,* (2009) and Dalal *et al.,* (2009). It resembles other febrile conditions such as malaria, Q- fever, typhoid and tuberculosis among others, and is thus usually wrongly treated as such (Muriuki *et al.,* 1997; Maichomo *et al.,* 2000; Kiambi, 2012).

Kenya is incapacitated by limited data and knowledge of brucellosis so that many cases go unrecognized and unreported. However, human brucellosis is more common where extensive cattle production systems predominate with almost a prevalence of 14% to 21% being documented (Muriuki *et al.,* 1997, Richards *et al.,* 2010, Kiambi, 2012).

Despite the growing recognition of the importance of zoonotic diseases, most of these diseases particularly brucellosis are among the most neglected, poorly understood and/or less controlled (Abbas and Agab, 2002). Brucellosis may be controlled and/or prevented through vaccination of animals. However, Kenya and many countries in sub-Saharan Africa have no vaccination policy on the control of brucellosis in either animals or humans. Some highly commercialized farms in Kenya still opt to have their cattle vaccinated with *B. abortus* strain 19 (s19), but the practice is declining due to the high vaccine costs (McDermott and Arimi, 2002). The vaccine remains useful at research and learning institutions and rarely for control of brucellosis in animals. Decision-making is therefore urgently required to determine the importance of brucellosis control relative to other public health and animal health concerns particularly among the pastoral communities (McDermott and Arimi, 2002). It is also important to constantly determine the level of the disease especially in animals in pastoral and

agro-pastoral areas, as brucellosis has been shown to be high among livestock belonging to these communities (Kagumba and Nandokha, 1978; McDermott and Arimi, 2002), and to establish potential control strategies that can be used in these regions to effectively minimize the disease among the livestock. This will minimize economic losses resulting from animal brucellosis, especially the reproductive wastages. It will also lower the incidences in the humans.

Baringo County, located in mid-western Kenya is inhabited largely by Tugen community who practice both pastoral and agro-pastoral farming. Most parts of the County are largely semi-arid favouring pastoralism. A quick retrospective analysis of data in health facilities in this area showed high human positive reactions to brucellosis rapid diagnostic kits used and since human infections are almost always from animals, this study endeavoured to establish the disease situation in livestock in Baringo County with the following objectives:-

1.1 Overall objective

To estimate the prevalence and determine factors associated with brucellosis in livestock in Baringo County, Kenya.

1.2 Specific objectives

i. To determine sero-prevalence of brucellosis in cattle, sheep and goat herds in Baringo County, Kenya.

ii. To genetically characterize the *Brucella* species affecting cattle, sheep and goats in Baringo county, Kenya.

iii. To assess the risk factors associated with brucellosis in Baringo County, Kenya.

1.3 Null hypothesis

Brucellosis does not occur in livestock in Baringo County, Kenya.

1.4 Justification:

Brucellosis is of great social and economic importance, requiring multifaceted control strategies and thus continuous surveillance in livestock populations is important. There is limited awareness of infection control practices by most of the livestock owners and therefore there is increased potential for uncontrolled spread of brucellosis to susceptible livestock. There are pastoralist and agro-pastoralist farmers in Baringo county, Kenya, who prefer consumption of unpasteurized milk and are involved in unhygienic animal management practices that can lead to the spread of the disease in livestock and ultimately to humans. There is also little information on the epidemiology of brucellosis in Baringo region particularly in animals; whose daily contact with humans puts the humans at risk of contracting the disease mainly through handling and/or consumption of infected animal products. Densities of animal populations, herd size and management, as well as environmental factors are also thought to be important determinants of the infection dynamics within and between herds. In order to come up with tangible control measures for the area, the current situation of the disease, in terms of prevalence and establishment of the factors associated with spread of the disease must be established. This study was conducted to establish this data which subsequently will be used by respective authorities for establishment of control measures for the disease.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background information

Brucellosis, is a worldwide zoonosis (Memish, 2004; Smits *et al.,* 2004; Robert *et al.,* 2010) named after Sir David Bruce, who in 1887 was the first to isolate the causative agent of the disease belonging to the genus *Brucella* (*B. melitensis*) from the liver of a soldier dying of a febrile disease in Malta; that had caused considerable morbidity and mortality among British military personnel. Its source was found to have a link to sheep and goats' milk (Radostits *et al.,* 1994). Subsequently in 1897, Fredrick Bang, a Danish Veterinary professor isolated *B. abortus* from aborted cattle fetus (McMahan, 1944). In 1914, Traum isolated *B. suis* from fetuses of infected pigs (Radostits *et al.,* 1994).

Since then, the disease caused by members of genus *Brucella* has been referred to by various names. For example in cattle, the disease goes by the names: Bang's disease, Enzootic abortion and Infectious abortion among others; whilst in man, the names include: Malta fever and Mediterranean fever (Radostits *et al.,* 1994). Other names used in man include: Undulant fever, Gastric fever, Mediterranean gastric fever, Gibraltar-Rock fever, Cyprus fever, Neapolitan fever, Intermittent gastric fever, Intermittent typhoid fever and Pseudotyphus among others (Radostits *et al.,* 1994).

The infectious agents are bacteria of the genus *Brucella* which belongs to family *Brucellaceae*, order *Rhizobiales*, class *Alphaproteobacteria* and phylum *Proteobacteria* (Alton *et al.,* 1988). There are nine distinct *Brucella* species, which are *Brucella abortus*, *Brucella ovis, Brucella melitensis, Brucella suis, Brucella canis, Brucella neotomae, Brucella microti, Brucella ceti* and *Brucella pinnipedialis* (Garritty *et al.,* 2005; Scholz *et al.,* 2008).

2.2 Characteristics of *Brucella* organisms

Brucella organisms are small Gram-negative aerobic bacteria that appear as short rods or coccobacilli, measuring about 0.6 to 1.5μm by 0.5 to 0.7μm in size (Walker, 1999). They are non-spore-formers, non-motile, non-flagellated and have no true capsules (Holt *et al.,* 1994). However, *B. abortus* and *B. melitensis* have been shown to possess a rudimentary capsule-like envelope on electron microscopy (Walker, 1999). Each of these species has biotypes that differ on the basis of biochemical activity, resistance to aniline and phage (Holt *et al.,* 1994).

Brucella cultures exist either as smooth, non-smooth variants of smooth cells or rough variants, but cultures are generally designated either as smooth or rough on original identification on basal or selective media (Alton *et al.,* 1988).

Although the structure of their cell wall is not completely understood, gross analyses have indicated the presence of proteins, lipids, muramic acid, carbohydrates, and 2-keto-3-deoxyoctulsonic acid between the smooth and non-smooth species (Garritty *et al.,* 2005). The external layer of the outer membrane of the cell wall comprises mainly of lipopolysaccharides (LPS) interspersed with a variety of lipids and proteins (Garritty *et al.,* 2005). Like other Gram-negative bacteria, dominant surface antigens in both smooth and rough strains are linked to the lipopolysaccharides (Walker, 1999). The smooth species; *B. abortus*, *B. melitensis* and *B. suis* possess two important surface antigens (A and M) which are present on these lipopolysaccharides, although in varying proportions among the species (Walker, 1999; Garritty *et al.,* 2005). The cultures of these *Brucella* species agglutinate with absorbed monospecific A or M antiserum samples (Alton *et al.,* 1988). Only the permanently rough species (*B. ovis* and *B. canis)* agglutinate with the R, anti-rough, monospecific serum (Quinn *et al.,* 1999).

Under the cell wall is the periplasmic space, which is believed to be the site of a variety of hydrolytic enzymes (Garritty *et al.,* 2005).

The periplasmic space is underlined by cytoplasmic membrane whose typical triple-layered lipoprotein structure resembles that of other Gram-negative bacteria and encloses the cytoplasm (Corbel and Brinley-Morgan, 1984). The *Brucella* cytoplasm, where the osmiophobic nuclear material is located, is homogenous and is interspersed with small vacuoles and polysaccharide-containing granules (Garritty *et al.,* 2005). These organisms have aerobic respiratory type of metabolism and a cytochrome based electron transport system with oxygen or nitrate as the final electron acceptor (Corbel and Brinley-Morgan, 1984).

2.3 Resistance and survival of *Brucella* organisms

Brucella organisms may be killed at a temperature of sixty degrees Celsius (60 ^{0}C) for ten minutes, but dense suspensions, such as laboratory cultures, require more drastic heat treatment to ensure their inactivation (Quinn *et al.,* 1999). They are also destroyed by phenol in fifteen minutes (Alton *et al.,* 1988). Infected milk is rendered safe by efficient pasteurization (Alton *et al.,* 1988). *Brucella* organisms are very sensitive to direct sunlight and moderately sensitive to acid, so that they tend to die out in sour milk and in cheese that has undergone lactic acid fermentation (Alton *et al.,* 1988). The organisms, particularly *B. melitensis* can survive in soil for up to ten (10) weeks, manure and dust for six to eight (6-8) weeks, and remain viable in dead foetal material for even longer (Corbel and Morgan, 1984). They have been isolated from butter, cheese and ice-cream prepared from infected milk (David *et al.,* 2002). They may survive in carcass meat, pork and ham for several weeks under refrigeration (Alton *et al.,* 1988). However, they are destroyed at low temperatures (0 ^{0}C) if refrigerated for a month (Acha and Szyfres, 1986). Pickling and smoking reduce survival of *Brucella* organisms. They are also susceptible to common disinfectants if used at appropriate concentration and temperature (David *et al.,* 2002). They are sensitive *in vitro* to a wide range of antibiotics; but only a few are effective therapeutically (David *et al.,* 2002).

2.4 Pathogenicity of *Brucella* organisms

Brucella organisms are obligate intracellular parasites and each of the distinct species tends to have a preferred natural host (Quinn *et al.,* 1999), but will infect a wide range of other animals and man (Garritty *et al.,* 2005).

Brucella abortus has cattle as the preferred natural host, *B. melitensis* prefers goats and sometimes sheep, *B. canis* for canines, *B. suis* for swine, *B. ovis* for ovine, *B. neotomae* for desert wood rat (*Neotoma lepida*), *B. pinnipedialis* for pinnipeds which include seals, sea lions and walruses, *B. ceti* and *B. microti* for cetaceans **(**Odontoceti) comprising of whales, dolphins, porpoises and voles (*Microtus arvalis*) (Quinn *et al.,* 1999).

Cross infections are common, for example cattle can be infected by *B. melitensis* and *B. suis* besides *B. abortus*. Pigs can be infected by both *B. suis* and *B. abortus* while horses get infected by *B. abortus, B. suis, B. melitensis* and *B. ovis* (Jubb *et al.,* 1993). Goats and sheep are also commonly infected by *B. abortus* (Leal-Klevezas *et al.,* 2000; Kabagambe *et al.,* 2001).

These microorganisms are responsible for the several infectious conditions that result in placentitis and abortion in pregnant female animals, epididymitis and orchitis in male animals as well as localised chronic conditions such as hygroma, arthritis and bursitis (Blood and Radostits, 1989).

Six of these species (*B. melitensis, B. suis, B. abortus, B. canis, B. ovis* and *B. Neotomae*) infect humans, and are recognized as important zoonotic pathogens of public health concern (Walker, 1999). They cause a disease syndrome that is characterised by the occurrence of recurrent fever (Malta or Mediterranean fever), generalized aches and non-specific focal conditions such as arthritis, orchitis and diskospondylitis (Quinn *et al.,* 1999).

2.5 Identification of *Brucella* species by culture and isolation

2.5.1 Suitable samples for culture

For the diagnosis of brucellosis, the organism may be recovered from a variety of materials which usually depends on the presenting clinical signs (OIE, 2004). In animals, the placenta is the most infective and contains the greatest concentration of bacteria; this is followed by the lymph nodes, blood and milk (OIE, 2004). Furthermore, other materials rich in the organism include: stomach contents, spleen and lungs from aborted fetuses, vaginal swabs, semen, and arthritis or hygroma fluids from adult animals. From animal carcasses, the preferred tissues for culture are the mammary gland, supramammary, medial and internal iliac, retropharyngeal, parotid and prescapular lymph nodes and spleen (OIE, 2004; Ahmed *et al.,* 2010). *Brucella* organisms are best extracted from blood in humans (Poiester *et al.*, 2010).

All specimens must be packed separately, cooled and transported immediately to the laboratory in leak proof containers (OIE, 2004). Much as for humans, blood is the material of choice, specimens need to be obtained early in the disease when there is peak bacteraemia (Poiester *et al.,* 2010). The samples should be frozen until required for culture (OIE, 2004).

2.5.2 Culture material suitable for *Brucella* isolation

Direct isolation and culture of *Brucella* are usually performed on basal solid selective media (Alton *et al.*, 1988), in a highly safe laboratory, usually in a biosafety level three laboratory. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants which are usually in high numbers especially in samples obtained at post-mortem (Alton *et al.,* 1988).

Brucella species are chemo-organotrophic microorganisms requiring complex media containing several amino acids, thiamine, biotin, nicotinamide and magnesium salts, while X (haemin) and V (nicotinamide adenine dinucleotide [NAD]) factors are not required (Alton *et*

al., 1988; Holt *et al.,* 1994). Growth is inhibited on media containing bile salts, tellurite or selenite (Alton *et al.,* 1988).

Poor growth of *Brucella* organisms is seen in simple nutrient liquid media unless these media are supplemented with blood, serum or tissue extracts (Moyer and Holocomb, 2005). Such liquid media require vigorous agitation to improve aeration because *Brucella* are highly fastidious than any other aerobic bacteria (Alton *et al.,* 1988).

Most of the *Brucella* organisms can be isolated in unsupplemented, enriched peptone based media, or blood agar (Quinn *et al.,* 1999). Good growth is obtained on *Brucella* medium base (Oxoid), sucrose dextrose agar (Oxoid), tryptone soy agar or glycerol dextrose agar (Oxoid) supplemented with 5% bovine or horse serum (OIE, 2004; Moyer and Holocomb, 2005). A non-selective biphasic Castaneda's medium is recommended for the isolation of *Brucella* organisms from blood or other body fluids or milk where enrichment culture is highly advisable (OIE, 2004). Since they are slow growing, the use of selective media is recommended for primary isolation from most clinical specimens because of the high numbers of overgrowing contaminants (Marin *et al.,* 1996). Such selective media are prepared by incorporating antibiotics and bacteriostatic dyes into basic enriched media such as *Brucella* medium base (Oxoid). An example of such medium is Farrell's medium (Oxoid), prepared by adding six antibiotics; bacitracin, vancomycin, nalidixic acid, polymyxin B, nystatin and cycloheximide into sucrose dextrose agar for the isolation of *B. abortus* from contaminated milk samples (Farrell, 1974). Farrell's medium was found not to be an ideal medium for the isolation of *B. melitensis*, because the concentrations of nalidixic acid and vancomycin in this medium have inhibitory effects on some strains (Marin *et al.,* 1996). Therefore, the use of modified Thayer-Martin' medium supplemented with haemoglobin (10g/l), colistin methanesulphonate, vancomycin, nitrofurantoin, nystatin and amphotericin B in tandem with Farrell's medium is believed to enhance the chances of isolating *B. melitensis* (OIE, 2004).

However, due to its carcinogenicity, cycloheximide has been removed from the *Brucella* selective supplements used in the Farrell's medium (Anon, 2005).

These antibiotic supplements of the Farrell's medium are commonly added, in different combinations and proportions into any one of the basal media such as *Brucella* medium base (Oxoid), Tryptone soya agar (Oxoid), Serum dextrose agar (Oxoid), Columbia blood agar (BioMerieux) and other media bases, for the formulation of selective media for isolation of *Brucella* species (OIE, 2004). Selective BCYE (polymyxin, anisomycin, cefamandole) is commercially available (Raad *et al.,* 1990). Moyer and Holocomb (2005) reported the use of chocolate agar containing selective supplements (BBL Laboratories) for the isolation of *Brucella* organisms. Terzolo *et al.,* (Terzolo *et al.,* 1991) used Skirrow's agar to isolate *B. abortus, B. suis, B. melitensis, B. canis* and *B. ovis* from contaminated vaginal exudates and milk. Hornsby *et al*., (Hornsby *et al.,* 2000) also found Skirrow's agar, together with Modified Kuzdas medium and Tryptone soya agar (TSA) suitable for the recovery of the vaccine strain *B. abortus* RB51. Similarly, the use of new media such as rifampin *Brucella* medium and malachite *Brucella* medium (MBM), together with TSA, has been found to enhance the recovery of *B. abortus* RB 51 (Hornsby *et al.,* 2000). For the isolation of *Brucella* species from milk samples, although solid media have been used successfully (Farrell, 1974), the use of enrichment media such as serum dextrose, tryptone soy or *Brucella* broth containing selective supplements of at least amphotericin B and vancomycin are advisable since *Brucella* organisms are usually present in very low numbers in milk to be detected on solid media (OIE, 2004).

2.5.3 Growth requirements and colonial morphology

Brucella organisms are slow growing fastidious organisms with complex growth requirements (Alton *et al.,* 1988). Most of the *Brucella* strains require several amino acids such as thiamine,

nicotinamide, calcium pantothionate and Magnesium ions for growth on primary culture (Holt *et al.,* 1994). They require anaerobic environment growth and metabolism (Holt *et al.,* 1994). On primary isolation, many *Brucella* organisms require supplementation with 5-10% CO_2 (Alton *et al.,* 1988). Although the growth of *Brucella* organisms may occur between 20 ºC and 40 ºC, growth occurs optimally at 37 ºC and a pH of 6.6 to 7.4 (Corbel and Morgan, 1984; Anon, 2005).

Some species, particularly *B. abortus* metabolize sugar alcohol (erythritol) instead of glucose. Erythritol is found abundantly in uteri of pregnant animals and is thought to improve *in-vivo* growth (Jubb *et al.,* 1993). *In vitro* growth is improved by addition of serum, tissue extracts or blood (Holt *et al.,* 1994). Iron and magnesium are also thought to catalyse growth (Alton *et al.,* 1975).

In static broth medium, when *Brucella* organisms are incubated at 37 ºC for seven days, the smooth strains are seen to produce moderate uniform turbidity with pale powdery deposits (Alton *et al.,* 1988). Only vigorous strains of *B. abortus, B. melitensis* and *B. suis* grow on MacConkey agar in a few days producing small, and non-lactose fermenting colonies (Wilson *et al.,* 1990).

In semi-solid media, carbondioxide-dependent strains produce a disc of growth a few millimetres below the surface, whereas carbondioxide- independent strains produce uniform turbidity from the surface down to a depth of a few millimetres (Alton *et al.,* 1988). Growth on selective media may be delayed by several days and some strains may not produce discernible colonies until about 14 days of growth (Walker, 1999).

The colonial forms when viewed in reflected light are round, glistening, pin-point, 1-2 millimetres in diameter, with smooth margins intermediate, or mucoid. Smooth colonies can be easily emulsified to stable saline solution while the rough forms are granular (Alton *et al.,*

1988; Quinn *et al.,* 1999). These (both smooth and rough) colonies tend to become larger and darker later as they age but remain clear (Alton *et al.,* 1988). On serum dextrose agar, or any other clear medium, when examined on a microscope under low power magnification, *Brucella* colonies have a raised surface, translucent with entire margins, displaying a characteristic pale "honey drop-like" appearance. This is also seen when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white (Corbel and Brinley-Morgan, 1984). Changes in the colonial morphology are generally associated with changes in virulence, serological properties and/or phage sensitivity. Typical colonial morphology and positive agglutination with a *Brucella* antiserum provide presumptive identification of the isolate as *Brucella* (Wilson *et al.,* 1990).

2.5.4 Microscopic appearance and staining characteristics of *Brucella* organisms

On Gram stain, *Brucella* organisms are observed as Gram negative coccobacilli or short rods measuring from 0.6 to 1.5 μm long and from 0.5 to 0.7 μm wide (Alton *et al.,* 1988). They are usually arranged singly and less frequently in pairs, short chains or small groups and do not usually exhibit bipolar staining (Holt *et al.,* 1994). The morphology of *Brucella* organisms is fairly constant, except in old cultures where pleomorphic forms may be evident (Garritty *et al.,* 2005).

Brucella melitensis is considered to be more coccal than *B. abortus* with the latter being more capable of changing to bacillary forms especially in rich media like blood agar (Corbel and Brinley-Morgan, 1984; Garritty, 2005).

Marin *et al.* (1996) reported that a presumptive bacteriological diagnosis of *Brucella* can be made by means of the microscopic examination of smears from vaginal swabs, placentae or aborted fetuses, stained with the Stamp modification of the Ziehl-Neelsen staining method. However, morphologically-similar microorganisms, such as *Chlamydophila abortus,*

Chlamydia psittaci and *Coxiella burnetti* can mislead the diagnosis because of their superficial similarity (Marin *et al.,* 1996; Poiester *et al.,* 2010).

Brucella organisms are not true acid fast, but are resistant to decolourisation by weak acids and thus stain red by the Machiavelli's and Stamp's modification of the Ziehl–Nielsen's staining (Poiester *et al.,* 2010). This is the usual procedure for the examination of smears of organs or biological fluids that have been previously fixed with heat or ethanol, and by this method, *Brucella* organisms stain red against a blue background (Alton *et al.,* 1988; Poiester *et al.,* 2010). The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. The cell wall is responsible for the Gram negativity and antigenic composition (Alton *et al.,* 1988).

2.5.5 Biochemical characteristics of *Brucella* organisms

Brucella organisms yield energy by an oxidative process through the pentose-phosphate pathway, in which the rate of oxidation differs among species (Corbel and Brinley-Morgan, 1984).

On the basis of biochemical tests, members of the genus *Brucella* are defined as catalase positive, oxidase positive (except *B. ovis*), and urease positive (except *B. ovis* and *B. neotomae*) although the rate of hydrolysis varies. *Brucella suis* is known to spilt urea faster than all the other species changing the colour of Christensen's medium almost immediately on inoculation while *B. abortus* and *B. melitensis* taking longer durations (Holt *et al.,* 1994). They also reduce nitrates to nitrites and do not exhibit motility in semi-solid media (Quinn *et al.,* 1999). *Brucella* species, with the exception of *B. neotomae,* do not produce acid from carbohydrates in conventional peptone media and have been shown (Meyer and Cameron, 1961) that each *Brucella* species has specific pattern of oxygen utilization on selected amino acids and carbohydrates when measured by Warburg apparatus as expressed as oxygen

coefficient (Holt *et al.,* 1994). In addition, they do not produce indole, gelatinases, haemolysins, acetyl methyl carbinol (Voges Proskauer test), formic and acetic acids from glucose (Methyl red test) (Holt *et al.,* 1994). They are non-haemolytic but may turn blood agar medium greenish (Alton *et al.,* 1988)

2.5.6 Molecular typing of *Brucella* organisms

The genus *Brucella* currently consist nine species with validly published names (Quinn *et al.,* 1999). Within most species, further differentiation into biovars exists (Alton *et al.,* 1988). Genetically, all *Brucella* species are highly related to each other, exhibiting sequence similarity values of 98% to 100% in aligned regions (core genome) (Hoyer and McCullough, 1968; Cloeckaert *et al.,* 2001). The population structure is clonal (Foster *et al.,* 2009). Despite this close genetic relatedness, the various species can be clearly distinguished from each other by application of high resolution molecular typing tools, in addition to assessment of phenotype and host preference (Cloeckaert *et al.,* 2001).

Molecular typing of *Brucella* organisms has been attempted using DNA-DNA or DNA-RNA hybridisation methods; polymerase chain reaction (PCR) based methods such as the repetitive extragenic palindromic PCR (REP-PCR) and the enterobacterial intergenic consensus sequences PCR (ERIC-PCR) (Mercier *et al.,* 1996), the arbitrarily primed PCR (AP-PCR) (Fekete *et al.,* 2007) and the restriction fragment length polymorphism PCR (RFLP-PCR) (Cloeckaert *et al.,* 2001). These PCR based methods have been reviewed in detail by Bricker (Bricker and Halling 1994; Bricker, 2002; Bricker *et al.,* 2003). Highly discriminatory multilocus variable number of tandem repeats (VNTR) analysis (MLVA) has been shown to allow both species delineation and differentiation of individual isolates and thus represents a perfect first-line tool for molecular epidemiological studies within outbreak investigations (Cloeckaert *et al.,* 2001). Bricker *et al.* (Bricker *et al.,* 2003) found the technique to be the most discriminatory for *Brucella* species. However, using this technique, *Brucella* species

have been found to be highly homogenous and based on DNA-DNA hybridisation, a single species of *B. melitensis* has been proposed, with the other species being biovars (e.g. *B. melitensis* biovar *abortus*) (Verger *et al.,* 1985; Cloeckaert *et al.*, 2001). This genomic similarity makes the differentiation of *Brucella* species a bit complicated, and often a study of biological and physiological characteristics is required (Alton *et al.,* 1988). The debate of whether *Brucella* organisms should comprise a single genospecies or multiple species has been a source of much controversy and debate (Cutler *et al.,* 2005). But the recent reappraisal of the *Brucella* species by review of their population structure and analysis of their genetic diversity by methods other than DNA-DNA hybridisation (Moreno *et al.,* 2002) has reasserted the return to the pre-1986 taxonomy where the multiple species and biovars concept is used (Osterman and Moriyon, 2003; Foster *et al.,* 2007).

For typing of *Brucella* species, the multiplex AMOS PCR, named for its applicability to "*abortus, melitensis, ovis, suis"* species, is often used (Bricker *et al.,* 2003). This PCR and PCR protocols derived from it allow discrimination between *Brucella* species and between vaccine and wild-type strains (Bricker and Halling, 1994). Advancement of the AMOS PCR has also been done to a level that the technique can identify variants within *Brucella* species (Bricker *et al.,* 2003).

Other techniques that have been developed include the single nucleotide polymorphism (SNP) analysis and multilocus sequence typing (MLST) or multilocus sequence analysis (MLSA). The latter is also suitable for phylogenetic reconstructions, owing to the highly clonal evolution of the different species. More recently, whole genome sequencing (WGS) and the resulting global genome wide SNP analysis have become available (Bricker *et al.,* 2003).

2.6 Brucellosis in domestic animals

2.6.1 Introduction

Brucellosis in animals is generally characterised by epizootic abortions, chronic endometritis, infertility, arthritis, orchitis or chronic infections (Cutler, *et al.,* 2005). However, different animal species may present with other non-specific clinical signs for example *Brucella ovis* causes epididymitis and infertility in rams and a rare cause of abortion in ewes (Van Tonder *et al.,* 1994). The infection with *B. sui* in pigs causes an acute or chronic disease that is characterised by abortions, stillbirths, heavy mortality in piglets, sterility in sows, and orchitis in boars (Blood and Radostits, 1989; Bishop *et al.,* 1994).

Canine brucellosis is characterised by abortion storms in females and testicular atrophy, epididymitis and infertility in males and generalised lymphadenitis in both males and females (Oncel, 2005).

2.6.2 Aetiology

Bovine brucellosis is primarily caused by biovars of *B. abortus* and occasionally by *B. melitensis* in cattle kept closely together with goats and sheep (OIE, 2004). Although *B. suis* has been reported to cause mammary gland infection, it has not been associated with abortion in cattle (Ewalt *et al.,* 1997). In most parts of the world, cattle brucella infections are due to *B. abortus* biovar 1; type 2 being less frequent (Mohan *et al.,* 1996; Quinn *et al.,* 1999). *Brucella abortus* biotype 3 has been reported from East Africa, Egypt and India; *B. abortus* biotype 5 has been isolated in Germany and Britain; while the other biotypes are infrequently isolated (Quinn *et al.,* 1999). **Caprine and ovine** brucellosis is caused majorly by *B. melitensis* biovars 1, 2 or 3 (OIE, 2004). Recently, *B. abortus* has been reported to be common among sheep and goats (Leal-Klevezas *et al.,* 2000; Kabagambe *et al.,* 2001; OIE, 2004). *Brucella melitensis* is morphologically similar to *B. abortus* and sometimes it is difficult to distinguish the two but can be identified using molecular techniques such as polymerase chain reaction (PCR) (Alton *et al.,* 1988; Bricker, 2003).

Ovine epididymitis is caused primarily by *B. ovis* (Radostits *et al.,* 1994). *Brucella ovis* stains blue with modified Koster's stain, in contrast to the other *Brucella* species which stain pink (Garritty *et al.,* 2005). *Brucella ovis* are agglutinated by antiserum samples for the rough (R) surface antigen but do not agglutinate with monospecific antiserum samples for A and M surface antigens (Garritty *et al.,* 2005). **Porcine** brucellosis is caused primarily by biotypes of *B. suis* which are morphologically similar to other *Brucella* species (Garritty *et al.,* 2005). **Canine** brucellosis is caused by *B. canis* (Carmichael, 1966). The morphological characteristics of *B. canis* are similar to the other members of the genus, *Brucella*. However, just like *B. ovis*, its cultures exist in rough colonial phase and are agglutinated only by antiserum samples for the R surface antigen (Garritty *et al.,* 2005). Canines can also be infected by *B. abortus*, *B. melitensis* and *B. suis* (Shin and Carmichael, 1999; Wanke, 2004). *Brucella abortus* and *B. suis* are reported to commonly infect equines (Quinn *et al.,* 1999, Walker, 1999). Summary of common *Brucella* species and preferred hosts is given in Table 1.

Table 1: Summary of common *Brucella* species and preferred hosts

HOST	*B. abortus*	*B. melitensis*	*B. suis*	*B. canis*	*B. ovis*
Cattle	+	+	+ (rare)	-	-
Buffaloes	+	+	-	-	-
Bison	+	-	-	-	-
Sheep	+	+	+ (possible)	-	+
Goats	+	+	-	-	-
Swine	+(rare)	+(rare)	+	-	-
Dogs	+	+	+(rare)	+	-
Camels	+(rare)	+	-	-	-
Caribou/Reindeer	-	-	+	-	-
Elk	+	-	-	-	-
Horses	+	+ (rare)	+(rare)	-	-
Rodents	+ (rare)	+(rare)	+	-	-

2.6.3 Epidemiology

Brucellosis occurs worldwide, except in countries where it has been eradicated, including Britain, Norway, Sweden, Finland, Denmark, Germany, Belgium, the Netherlands, Switzerland, Austria, Czech Republic, Slovakia, New Zealand, Canada, France and Italy (Pappas *et al*., 2009). In most *Brucella*- free countries, eradication was done through

implementation of stringent disease control strategies that included test and slaughter policies (Pappas *et al.,* 2009). However, the disease is important in developing countries, with *Brucella abortus* strains being the most common occurring particularly in the tropical countries (OIE, 2004; Kunda *et al.,* 2007). Bovine brucellosis is reported to occur in most countries in Africa (Chukwu, 1985; Faye *et al.,* 2005). The prevalence of the disease varies between countries, regions and farming sectors due to vast differences in terrain, climate, social customs, resources, livestock management and attitude towards disease control (Nicoletti, 1984; McDermott and Arimi, 2002; Bishop *et al.,* 1994). Caprine and ovine brucellosis are common in Mediterranean and Middle East region and other parts of the world such as Africa, Central America and Mexico where the incidence is very high and the disease is known to be enzootic (Herr, 1994; Banai *et al.,* 2002; OIE, 2004; Leyla *et al.,* 2003).

There is substantial amount of information on brucellosis in most parts of Africa particularly for ruminants and wildlife (Bishop *et al.,* 1994; Mugambi, 2001; McDermott and Arimi, 2002; Gous *et al.,* 2005; Muma *et al.,* 2006; Muriuki *et al.,* 1997). However, the extent of distribution of equine brucellosis is not really known (Gous *et al.,* 2005). It is believed that the distribution of equine brucellosis follows that of cattle and to some extent swine brucellosis (Radostits *et al.,* 1994). Horses kept together with infected cattle are at a higher risk of exposure to *Brucella* infections (Quinn *et al.,* 1999).

2.6.4 Transmission

Densities of animal populations, herd size and management, as well as environmental factors are thought to be important risk factors that determine the dynamics of brucellosis within and between herds (Omer *et al.,* 2000b, McDermott and Arimi, 2005).

Transmission of *Brucella* organisms is mainly by direct or indirect contact of the serosal surfaces with infective organisms (Quinn *et al.,* 1999). Although cattle have been infected experimentally by conjunctival, vaginal and intramammary routes, the main route of infection

in the field is by ingestion of the pathogenic *Brucella* organisms especially through contaminated feed and water (Cunningham, 1977). Most animals acquire infection by licking infected material such as infected new-borns or placenta, grazing on infected pasture or consuming other feedstuffs and drinking water contaminated by aborted material or uterine discharges from an infected animal (Blood and Radostits, 1989). Although not common, infection may occur via conjunctiva or by inhalation (Quinn *et al.*, 1999). While new-borns of infected dams may get infected *in utero*, majority of such infections will clear a few months after birth while a few may remain latently infected (Blood and Radostits, 1989). Although calves, piglets, kids and lambs suckle and ingest large numbers of viable organisms from colostrum, it is unlikely that they will be infected by this route as antibodies in colostrum seem to be protective (Cunningham, 1977). There are also direct relationships between dam infections and neonatal mortality (Domenech *et al*., 1982a). Transmission by coitus is unlikely, but semen from infected bulls that is used for artificial insemination could be a source of infection (Franklin, 1965). This is because during artificial insemination, semen is usually deposited at the cervico-uterine junction and the uterine body, and it has been shown that the cervical epithelial lining as well as the animal vaginal acidity plays protective roles in the transmission of brucellosis in cattle (Lambert *et al.,* 1963; Radostits *et al.,* 1994).

In sheep, infection spreads from the infected rams to ewes and *vice versa* during mating (Burgess *et al.,* 1985; Radostits *et al.,* 1994) at which time, *B. ovis* may be excreted from infected rams even before the development of lesions (Burgess *et al.,* 1985; Radostits *et al.,* 1994). Ram to ram transmission is a common occurrence through homosexual contact (Baggley *et al.,* 1985) but ewe to ewe transmission is unlikely (Van Tonder *et al.,* 1994).

In pigs and canines, transmission is believed to be majorly by coitus and also by ingestion route (Blood and Radostits, 1989; Shin and Carmichael, 1999; Wanke, 2004).

2.6.5 Pathogenesis

The pathogenesis of brucellosis in all animal species is poorly understood and has intrigued researchers for a long time virtually due to the ability of brucella organisms to survive within cells (Radostits *et al.,* 1994). Although there have been preliminary reports of involvement of toxins, fimbriae and plasmids, none of these has been demonstrated as main determinants and like other Gram-negative bacteria, lipopolysaccharides (LPS) are presumed to play an important role (Ficht, 2003; Delrue *et al.,* 2004). It is known that *B. abortus* and *B. melitensis* have predilection for the pregnant uterus, udder, testicle and accessory male sex glands, lymph nodes, joint capsules and bursae (Blood and Radostits, 1989). Hence, brucellosis is usually a disease of the sexually mature animals (Quinn *et al.,* 1999).

Primary bacteraemia is preceded by multiplication of the micro-organisms at the site of entry followed by localisation in the lymph nodes, the udder and the uterus and mild systemic reaction (Collier and Molello, 1964; Radostits *et al.,* 1994). It is believed that soon after entry in the host *Brucella* organisms are engulfed by phagocytic cells in which they multiply and get transported to regional lymph nodes where initial localisation and proliferation occur (Walker, 1999) with subsequent development of hyperplasia and infiltration of inflammatory cells leading to granulomatous lesions (Bishop and Bosman, 1994; Anon, 2006). They subsequently enter the circulatory system via the thoracic duct for dissemination to parenchymatous organs and other sites (Quinn *et al.,* 1999). Colonisation of the fetus and placenta occurs rapidly and the factors that control this tropism are speculated to be allantoic fluid factors such as erythritol and steroid hormones (Walker, 1999). The hormones are secreted by the pregnant uterus and stimulate luxuriant growth of *Brucella* organisms (Quinn *et al.,* 1999). The rapid multiplication of *Brucella* organisms results in the development of severe ulcerative placentitis endometritis of the intercotyledonary spaces with development of yellowish gelatinous fluid in pregnant animals (Walker, 1999). The allantochorion, foetal fluids, and placental cotyledons are then destroyed (Cunningham, 1977). The cotyledons

become necrotic (Walker, 1999). However, there is a considerable variation in the nature of the uterine and placental lesions in both experimental and natural *Brucella* infections (Bishop *et al.,* 1994). On the other hand, in pregnant ewes the colonisation of the uterus and the placenta by *B. ovis* is unlikely to occur and the organism rarely causes placentitis and abortion (Frank *et al.,* 1974). The most plausible reason for this is that the growth of *B. ovis* is inhibited by erythritol, present in the gravid uterus (Quinn *et al.,* 1999) although low grade pyogenic infection and subsequent foetal death in spite of little or no foetal invasion has been reported in pregnant ewes (Collier and Molello, 1964).

Depending on the severity of the placentitis, abortion, premature birth or still births may occur (Bishop *et al.,* 1994). The exact mechanism under which abortion occur is not known, but believed to be due to the interference with foetal circulation due to placentitis, or the direct effect of endotoxins, or directly from foetal stress due to inflammation of foetal tissues and organs (Walker, 1999). Endotoxins of *Brucella* organisms may induce the production of stress hormone called cortisol that leads to decreased progesterone production and an increase in the oestrogen production (Enright *et al.,* 1984). Decreases in progesterone levels and increase in oestrogen levels are known to induce abortions and premature parturitions (Anon, 2006).

Chronic infections are characterized by granulomatous foci in the lymphatic tissues, liver, spleen, bone marrow and other locations (Carter and Chengappa, 1991; Van Tonder *et al.,* 1994; Walker, 1999).

2.6.6 Intracellular survival

The genetic basis of virulence of *Brucella* organisms is not fully understood (Kohler *et al.*, 2003). One of the mechanisms through which *Brucella* organisms are able to cause persistent infection in the host is through their ability to survive inside macrophages, which would normally kill and destroy other bacteria (Ficht, 2003). They are adapted to surviving in the phagosome as their natural living niche believed to be associated with the rough endoplasmic reticulum (ER) (Celli and Gorvel, 2004). Other studies have indicated that this phagosome contains acidic environments, is low in nutrients, contains cholesterol, looks different from any existing organelle, and the name "brucellosome" has been proposed for this structure (Kohler *et al.,* 2003). In these brucellosomes, *Brucella* organisms are able to produce virulence genes (*VirB*) which promote multiplication of the organisms in such environments (Kohler *et al.,* 2003).

Experimental results suggest that the early phagosome is very acidic and poor in nutrients, resulting in induction of *VirB* and genes which encode stress proteins. *VirB* participates in the creation of the 'brucellosome' characterised by absence of fusion with lysosomes, neutral pH, and absence of certain nutrient components (Kohler *et al.,* 2003).

2.6.7 Clinical signs

The incubation period of brucellosis in animals varies markedly depending on the size of the infective dose, age, sex, stage of gestation and immunity of the affected animals (Bishop *et al.,* 1994).

Brucellosis highly reproduces in milk glands and uterus of pregnant and lactating animals. These organisms reside in chorionic epithelial cells, which cause necrosis in placental cotyledons (Cunningham, 1977; Walker, 1999). This usually leads to abortion of fetuses in pregnant animals due to intrauterine infection (Quinn *et al.,* 1999). Although infected animals usually recover on their own, they discharge pathogenic organisms through their uterine, urine

and milk secretions for a variable period and cause infection in other animals or humans (Radostits *et al.,* 1994). On the other hand, cattle and goats may remain infected during their entire life in which they suffer chronic brucellosis which may not result in abortion (Wanke, 2004). Moreover, they can transmit the disease to other animals and may be an important source of human infection through their milk and meat products. Occurrence of clinical signs of disease in livestock is dependent on level of safety of the herds. In non- vaccinated flocks, abortion is the most important symptom of the disease (Walker, 1999). Abortion occurs in cattle after the fifth month of pregnancy while it occurs in the last two months of pregnancy in sheep and goats (Blood and Radostits, 1989). Although incidence of abortion is observed in the second and on-going months of pregnancy in a number of infected animals, most animals do not miscarry in the second and subsequent pregnancies (Wanke, 2004). Retained placenta, metritis, hygroma, orchitis, epididymitis, decreased milk production, permanent or temporary infertility, delay in reproductive seasons and increased lactation intervals can be cited as other symptoms of this disease (Bishop *et al.,* 1994). Symptoms such as fever, respiratory impairment, weight loss, diarrhea and limping may be observed in acute form of the disease (Shin and Carmichael, 1999; Megid *et al.,* 2010). In all sexes, severe lymphadenitis involving the retropharyngeal and inguinal lymph nodes are often present, although other lymph nodes may be affected (Wanke, 2004).

2.6.8 Impact of brucellosis and bioterrorism

Due to its effects on multiple animal species as well as humans, the impact of brucellosis is great in Kenya and other Sub-Saharan countries mainly due to morbidity, mortality and indirect losses due to treatment costs (Perry *et al.,* 2002). In infected cattle populations, brucellosis leads to lower calving rate due to temporary infertility and/or abortion, resulting in decreased milk production in cows, increased replacement costs as well as lowered sale value of infected cows (Chukwu, 1987). General economic losses, however, go far beyond the financial losses suffered by cattle producers alone (Chukwu, 1987) because humans are at risk

as long as animals remain infected. McDermott *et al.* (1987 b) found out that RBPT-positive cows had a higher abortion rate, 34.9%, compared to 15.7% in RBPT-negative cow. Milk production also goes down with a potential *Brucella* mastitis and contamination of milk (Radostits *et al.,* 1994). It also leads to reduced work capacity in infected humans through sickness of the affected people; government costs on research and eradication schemes as well as losses of financial investments through culling and condemnation of infected animals due to breeding failure (Domenech *et al.,* 1982a).

Brucellosis is not only a major zoonotic problem but is also linked with bioterrorism and belongs to category B (Yagupsky and Baron, 2005). The severity of this disease, lack of vaccines suitable for use in man and frequent failure of clinical laboratories to correctly identify isolates has led to the investigation of *Brucella* as an agent for bioterrorism. Before 1954, when Britain was focusing on anthrax, brucellosis was the first microorganism chosen by the United States to develop as a weapon. This microorganism could be effectively disseminated in four pound bombs (Yagupsky and Baron, 2005). *Brucella melitensis* and *B. suis* have been developed experimentally as biological weapons by state sponsored programmes (OIE, 2004). Their relative stability in aerosol form combined with low infectious dose make them suitable agents for this purpose. *Brucella* could be used to attack human and/or animal populations.

The impact is likely to be greatest in those areas in which the disease is not endemic. The organism can be obtained from natural sources in many parts of the world. Health and veterinary authorities should be aware of this potential source of infection.

2.6.9 Latency

Some heifer calves that acquire infection in early life, test negative to serological tests conducted at six months of age and yet abort during first pregnancy (Cunningham, 1977). There is growing evidence that in some calves born of infected dams, hidden and localised foci of viable organisms remain even though they test serologically negative (Lapraik, 1982; Radostits *et al.,* 1994). This condition is referred to as "latency" or hidden infection in which an animal exhibits no signs of infection. Pregnancy reactivates infection due to the production of erythritol which stimulates the proliferation of *Brucella* organisms (Quinn *et al.,* 1999). Such heifers could spread infections if they are moved to new susceptible herds (Lapraik, 1982).

2.7 Immunity

2.7.1 Humoral immunity

When cattle are vaccinated with *B. abortus* strain 19, a live attenuated vaccine, immunoglobulin M (IgM) develops earlier than IgG, being first detected at about 5-7 days and reaching a peak at 13-21 days (Brinley-Morgan, 1967). On the other hand, IgG is first detected 14-21 days post vaccination, reaching a peak after 28-42 days (Brinley-Morgan, 1967). Two IgG isotypes, IgG1 and IgG2 are produced; the latter in small amounts (Nielsen *et al.,* 1998). When cattle are challenged with virulent strains of *B. abortus* a similar pattern is observed except that the IgG reaches a higher maximum level and persists for much longer periods (Brinley-Morgan, 1967; Nielsen *et al.,* 2009). In chronic brucellosis, some animals may have high levels of IgG1 that agglutinates poorly and can mask the normally efficient agglutinating properties of any IgM present (Quinn *et al.,* 1999). Immunoglobulin A may be produced, but the concentration is very low and these are only important in secretions such as milk in which it is a major component (Duncan *et al.,* 1972). Most cross-reacting antibodies, from exposure to micro-organisms other than *Brucella* species consist mainly of IgM (Nielsen, 2002).

Although antibodies remain for the entire animals' life, the level of such antibodies cannot offer immunity against *Brucella* infection and thus annual boosters are preferentially given (Radostits *et al.*, 1994).

2.7.2 Cell-mediated immunity

Advances in the field of immunology have clearly demonstrated that the level of immunity to intracellular pathogens cannot be assessed only on the basis of the level of circulating antibodies (Nelson, 1977). There is growing evidence that cell-mediated immunity against *Brucella abortus* involves antigen-specific T-cell activation, CD4+, CD8+, T cells, in addition to humoral responses (Golding *et al.,* 2001; Oliveira *et al.,* 2002). Host protection against *Brucella abortus* is believed to be mediated by Th1 immune response (Zhan *et al.,* 1993). *Brucella abortus* triggers the host antigen presenting cells (APC) to secrete interlukin-12 (IL-12), which in turn causes Th0 cells to differentiate into Th1 cells that secrete gamma interferon (IFN-γ) that up-regulates macrophage killing mechanisms (Zhan *et al.,* 1993). In addition, IL-12 produced by APC triggers natural killer (NK) cells to become killer cells and secrete IFN-γ (Golding *et al.,* 2001). Cytokines secreted by CD4+ help to activate CD8+ T-cells and B-cells, stimulating their differentiation into cytotoxic T-cells and plasma cells respectively (Golding *et al.,* 2001). The cytotoxic T-cells that secrete IFN-γ are able to kill *B. abortus* infected macrophages (Oliveira *et al.,* 2002). However, there is limited knowledge on the nature of antigens involved in the stimulation of the protective cellular immunity against brucellosis (Oliveira *et al.,* 2002).

2.8 Post-mortem lesions

Brucella infections cause relatively similar lesions in infected hosts (Kahn and Line, 2003). Some aborted fetuses appear normal; others are autolysed or have variable amounts of subcutaneous oedema and blood-stained fluid in their body cavities (Kahn and Line, 2003). In ruminant fetuses, the spleen and/or liver may be enlarged, and the lungs may exhibit pneumonia and fibrous pleuritis (Lucero, 2005). Abortions caused by *Brucella* species are typically accompanied by placentitis (Sohn *et al.,* 2003). The cotyledons may be red, yellow, normal or necrotic. In cattle and small ruminants, the intercotyledonary region is typically leathery, with a wet appearance and focal thickening. There may be exudate on the surface (Smith, 1963).

In adults, granulomatous to purulent lesions may be found in the male and female reproductive tract, mammary gland, supramammary lymph nodes, other lymphoid tissues, bones, joints and other tissues and organs (Sohn *et al.,* 2003). Mild to severe endometritis may be seen after an abortion, and males can have unilateral or bilateral epididymitis and/or orchitis. In *B. abortus*-infected cattle, hygromas may be found on the knees, stifles, hock, angle of the haunch, and between the nuchal ligament and the primary thoracic spines especially in tropical countries (Giannacopulos *et al.,* 2002). Fistulous withers are common among *Brucella* infected equines (Kahn and Line, 2003).

2.9 Diagnosis of brucellosis

2.9.1 Clinical diagnosis

The diagnosis of brucellosis on the basis of clinical signs is usually difficult in all animal species since signs are non-pathognomonic (Saunders, 1958; Van Tonder *et al.,* 1994; Kahn and Line, 2003). Abortions are suggestive of brucellosis, although other causes of abortion should be ruled out. Several infectious diseases in cattle for example: Rift Valley fever

(RVF), Salmonellosis, Leptospirosis and Listeriosis could cause abortion “storms” in cattle (Blood and Radostits, 1989).

2.9.2 Laboratory diagnosis

2.9.2.1 Culture and isolation

The isolation and identification of *Brucella* offers a definitive diagnosis of brucellosis and may be useful for epidemiological purposes and to monitor the progress of a vaccination programme (Bishop *et al.*, 1994).

Under field conditions, obtaining aborted fetuses or placenta may be difficult. Often, the aborted fetuses or aborted materials are autolysed when they are located, and in most cases they may not be located. If the aborted fetus is located and still fresh, suitable specimens for the culture of *Brucella* species include: the stomach contents, pieces of the foetal liver, spleen and lung or the placental cotyledons (Alton *et al.*, 1988; Bishop *et al.*, 1994).

When aborted material cannot be utilized, culture and isolation of *Brucella* organisms is usually performed on vaginal discharges, milk or blood (Bishop *et al.*, 1994). A major disadvantage of relying on culture and isolation is that some cases are misdiagnosed as negative because *Brucella* organisms are slow growing and fastidious micro-organisms that are easily overgrown by contaminating bacteria. Hence, for practical reasons, the only method relied upon for herd diagnosis is demonstration of antibodies in animals previously exposed to antigens of *Brucella* organisms. In recent years, molecular methods such as the PCR have been suggested as alternative gold standard tests to confirm brucellosis (Bricker, 2002). However, such methods may be too expensive to be relied upon in routine diagnosis of bovine brucellosis, especially in resource-poor countries, and the question always remains if the PCR detects viable bacteria or not.

2.9.2.2 Staining of organisms

Smears of placental cotyledon, blood, vaginal discharge or foetal stomach contents may be stained using modified Ziehl-Nielsen (Stamp) or Koster's' methods (Alton *et al.,* 1988; Marin *et al.,* 1996). The presence of large aggregates of intracellular, weakly acid-fast organisms with *Brucella* morphology is presumptive evidence of brucellosis (Herr, 1994). However, morphologically-related microorganisms, such as *Chlamydophila abortus, Chlamydia psittaci* and *Coxiella burnetti* can mislead the diagnosis because of their superficial similarity (Marin *et al.,* 1996; Poiester *et al.,* 2010).

2.9.2.3 Serological tests

Body fluids such as serum, uterine discharge, vaginal mucus, milk, or semen plasma from a suspected animal may contain different quantities of antibodies of the M, G1, G2, and A isotypes directed against *Brucella* organisms (Beh, 1974). Several serological tests have been evaluated for the diagnosis of brucellosis in livestock (Blasco *et al.,* 1994; Jacques, 1998; Minas *et al.,* 2005; Ramirez- Pfeiffer *et al.,* 2006). Infected animals may not always produce all antibody isotypes in detectable quantities; therefore, results from several serological tests should be used as a presumptive evidence of infection (FAO, 2005). In addition, depending on the sensitivity and specificity, serological tests can be used to screen for, or confirm brucellosis.

Detection of specific antibody particularly in serum or milk remains the most practical means of diagnosis of brucellosis (Kahn and Line, 2003). The most efficient and cost-effective method is usually screening all samples using a cheap and rapid test which is sensitive enough to detect a high proportion of infected animals (Anon, 2000). However, latent infections occur in some animals which are serologically negative and vaccinated animals may be serologically positive, and these can interfere with interpretation of results (Anon, 1986). Presumptive diagnosis may be made on presence of antibodies in serum, milk, vaginal mucus

or seminal plasma (Kahn and Line, 2003). Test for the detection of specific immunoglobulin includes: Milk Ring Test (MRT) used in lactating cattle, Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), Mercaptoethanol Test (MET), Complement Fixation Test (CFT), and Enzyme Linked Immunosorbent Assays (ELISA) (Alton *et al*, 1988; Anon, 1986; Anon, 2000).

2.9.2.3.1 Milk Ring Test (MRT)

Milk Ring Test (MRT) is a simple and effective method. It can be used on cow's and camels' milk (OIE, 2004; Wanjohi, 2009). This test is used to detect antibodies in milk. Development of a positive reaction is dependent on two reactions (i) fat globules in the milk which are aggregated by milk antibodies (fat-globule agglutinins) and (ii) Stained *Brucella* cells (haematoxylin-stained antigen), added to the milk which are agglutinated by the *Brucella* antibody/fat globule. The complex then rises to form a coloured cream layer at the top (Alton *et al*, 1988; Anon, 1986). This is a sensitive screening test used on bulk milk samples either to detect infected animals on a herd basis or to monitor clean herds.

Factors that may cause false positive results include: a high prevalence of mastitis, a high proportion of cows in early or late lactation, recent (within three to four months) vaccination with strain 19 vaccine, and souring of milk (Coetzer and Tustin, 2004). Milk samples may be preserved for testing by adding 0.5ml of a formalin solution (prepared by mixing 7.5ml of 37% formaldehyde with one litre of distilled water) to a 10ml milk sample. The duration and temperature at which milk samples are stored (45^0 C > for more than 5 minutes) may cause false negative. Pasteurized milk cannot be used to carry out MRT (Alton *et al*, 1988).

2.9.2.3.2 Rose Bengal Plate Test (RBPT)

Rose Bengal Plate Test is one of a group of tests known as the buffered *Brucella* antigen tests which rely on the principle that the ability of IgM antibodies to bind to antigen is markedly reduced at a low pH (Alton *et al.,* 1988). It is a qualitative screening test with a high sensitivity in which a positive reaction is an indication of the disease, not only in the individual animal but in the whole herd. It has been reported to be up to 98.3% sensitive (Acha and Szyfres, 1986).

The test misses very few infected animals, thus it is very good for use in the field for screening purposes (OIE, 2004). It is simple to perform and is a rapid, homogeneous and sensitive standardized assay (up to 98.3% sensitive) (Acha and Szyfres, 1986). In addition the sample (serum) required is easily accessible, consumables are cheap and require less complicated equipment (OIE, 2004). On the negative side, interpretation may be subjective, has no multiplex capability, it is prone to false positive serological reactions and false negative results may occur due to a prozone effect (Brinley-Morgan, 1997). As a test, it has limited mobility as it cannot be performed on whole blood or plasma samples. Despite a high sensitivity, the specificity can be disappointingly low and as a consequence the positive predictive value of the test is low and a positive test result requires confirmation by a more specific test (OIE, 2004).

Generally, it is a simple spot agglutination test, modification of the plate agglutination test. The antigen, which has been stained with Rose Bengal stain, is buffered at a pH of 3.65 (Alton *et al*, 1988; Anon, 1986). At this level of activity, non-specific agglutinins are destroyed and immunoglobulin G (IgG), normally the most abundant antibody in the serum of infected animals, agglutinates strongly (Anon, 1986; Brinley-Morgan, 1997). Equal volumes (30µl) of test serum and antigen are mixed, shaken for four minutes and viewed over an X-ray

viewer or naked eye and any degree of agglutination is recorded as positive (Alton *et al,* 1988).

Accordingly, RBPT is considered as a satisfactory screening test in cattle, sheep and goats (Nicoletti, 1980; OIE, 2004). This test is prescribed for international trade in cattle and small ruminants by the OIE (Office International des Epizooties) (Anon, 2000).

2.9.2.3.3 Serum Agglutination Test (SAT)

This has been used extensively for brucellosis diagnosis and, although simple and cheap to perform, its low sensitivity and specificity mean that it should only be used in the absence of alternative techniques (OIE, 2004; Quinn *et al.,* 1999). This test is positive 7 - 10 days after infection (Godfroid *et al.,* 2002). During this stage of the disease the level of agglutinins associated with both immunoglobulins M (IgM) and IgG continue to rise. Sensitivity is rather low ranging from 61– 69%. High titre serum samples may not cause agglutination in low dilution (the prozone effect) (Quinn *et al.,* 1999). Therefore a range of serum dilutions from 1 to 10 to over 1000 should be made (Herr *et al*, 1991; Herr, 1994).

2.9.2.3.4 Mercaptoethanol (2-MET) Test

This is an adaptation of the SAT titre. There are two forms of this test, which uses either 2-mercaptoethanol (Rose and Roepke, 1964) or dithiothreitol (Klein and Behan, 1981). Dithiothreitol is preferable because of the toxicity of 2-mercaptoethanol. The test measures mainly IgG, because the disulphide bridges of IgM are broken, reducing it to monometric molecules, and therefore, unable to agglutinate. However, IgG can also be reduced in the process, giving false negative results; though in general, reduction of IgM increases specificity (Poiester *et al.,* 2010). The test does not eliminate vaccine generated antibodies, therefore is not recommended for international trade. The 2-MET test is, however, used extensively for national control and/or eradication programmes (Nielsen, 2002).

Low titre agglutinins due to residual IgM may persist for several months after the infection has cleared. The agglutinating ability of IgM and IgA is destroyed by 2-MET; therefore agglutination in this test is indicative of presence of IgG and likelihood of persisting infection (Holt *et al,* 1994).

2.9.2.3.5 Complement Fixation Test (CFT)

This test is regarded throughout the world as being the confirmatory test for the serological detection of infected animals (Rogers *et al.,* 1989). It has been modified, standardized and adapted to a microtiter system (Alton *et al,* 1988; Anon, 2000). Some researchers reported its superiority to the other mentioned tests (Mohammed *et al,* 1981; Gameel *et al,* 1983; Asfaw *et al,* 1998). Complement Fixation test detects predominately IgG antibodies as most of IgM ones are destroyed during serum deactivation; it is thus so used as a confirmatory test (FAO, 2003). The test distinguishes reaction caused by other factors like vaccines and other bacterial infections. *Escherichia coli* O157, *Yersinia enterocolitica O:9, Vibrio cholerae, Pseudomonas mallophilia and Salmonella* serotypes which share common chain of lipopolysaccharide (LPS) antigen with smooth *Brucella* strains and therefore cross react. *Francisella tularensis* also cross reacts for unknown reason (Wrathall *et al.,* 1983). Rough *Brucella* strains also cross-react with *Actinobacilus equuli, Pasteurella multocida* and *Pseudomonas aeruginosa* (Corbel, 1990; Cloeckaert *et al,* 1992; Garin-Bastuji *et al,* 1999). These organisms contribute to false positive reactors for brucellosis in animal herds. Thus, the use of highly specific test such as monoclonal antibody-based competitive - Enzyme linked Immunosorbent Assay (c-ELISA) and CFT minimizes the risk of cross-serological reactions between *Brucella* and these groups of bacteria (Vizcaino *et al,* 1991; OIE, 2004).

Drawbacks include: false negative results with the IgG2 type antibodies and the fact that it is technically challenging to perform as a large number of reagents, controls and reagent titrations are required (Sanogo *et al.,* 2013). This test requires good laboratory facilities and

trained staff. Prozone formations, cross reactions anti-complementary activities are problems encountered with CFT (OIE, 2004).

Complement Fixation test is important in distinguishing calf-hood vaccination from those due to infection. The CFT titres do not wane as the disease becomes chronic and often CFT reaches diagnostic levels sooner than the SAT following natural infection (Seagerman *et al*, 1999).

2.9.2.3.6 Enzyme linked Immunosorbent Assay (ELISA)

The ELISA tests offer excellent sensitivity and specificity whilst being robust, fairly simple to perform with a minimum of equipment and readily available from a number of commercial sources in kit form (FAO, 2003). Competitive ELISA (c-ELISA) and Indirect ELISA (i-ELISA) tests can be used as supplementary tests to CFT (McGiven *et al.*, 2013). They have an advantage over other serological tests of being more sensitive (Baldi *et al.*, 1994; Anon, 2000). The ELISA test is also capable of differentiating acute from chronic infections (Lee *et al.*, 1985). Recently, ELISA has been used not only for detecting *Brucella* antibodies in serum samples but also in camel milk (Straten *et al*, 1997; Azwai *et al*, 2001). Besides its higher sensitivity than other conventional tests, ELISA is found to detect serum samples as positive about 2 to 4 weeks earlier (Gameel, 1983). It can also be used both for screening and confirmatory tests (FAO, 2003). These tests are prescribed for international trade in livestock by the OIE (Anon, 2000).

Competitive ELISA was developed to differentiate vaccine antibodies of *B. abortus* S19 from natural infection antibodies (Minas *et al.*, 2005). It is simpler to perform than the CFT and may be readily standardized by the use of purified S-LPS antigen and monoclonal antibody for competition. However, the cELISA cannot completely eliminate cross reactions from other bacteria like *Y. enterocolitica O: 9* (OIE, 2004).

2.9.2.4 Molecular detection of *Brucella* organisms

The *Brucella* genome is encoded on two circular chromosomes with sizes close to 2.05 Mb and 1.15 Mb for each species (Michaux-Charachon *et al.,* 1997). Only the small chromosomes of *B. suis*, *B. canis* and *B. neotomae* are 50 kb longer. The guanine/cytosine (G + C) contents in the DNA of various members of the genus *Brucella* are very similar, 55 to 58% (Hoyer and McCullough, 1968; Verger *et al.,* 1995). Both chromosomes contain almost identical proportions of potential coding regions (1028 and 1035, respectively). Housekeeping genes are evenly distributed all over the genome, which makes a long coexistence highly probable (Moreno and Moriyon, 2002). Chromosomal mapping revealed a high conservation of restricted sites and gene order. Variability is localized to certain regions, most often on the small chromosome. The nucleotide sequence similarity between all *Brucella* species is also high and DNA-DNA homology exceeds 90%. The six species are so closely related that a monospecies genus has been suggested (Verger *et al.,* 1995). This hypothesis was also confirmed by 16S rRNA gene sequence analysis and was reflected in the biochemical characteristics of the organisms (Moreno *et al.,* 1990).

Nevertheless, remarkable differences are found in host specificity and pathogenic properties of all the *Brucella* species with each being genetically isolated and different (Verger *et al.,* 1995).

Virulence is restricted to a small number of specific hosts, active multiplication is not possible in the environment and genetic exchange, e.g. through plasmid, temperate bacteriophages or transformation, does not occur naturally in *Brucella* (Michaux-Charachon *et al.,* 1997).

Culturing has advantage of detecting the organisms directly but it is time consuming as it takes about 10 days or longer for identification of the causative agents and has reduced sensitivity in chronic infection. Besides, the culture materials must be handled carefully, as *Brucella* organisms are class III pathogens (Alton *et al.,* 1988). Amplification of DNA by

PCR has currently been used for the diagnosis of several infectious diseases caused by fastidious or slowly growing bacteria. Different target genes, primer pairs, PCR techniques and extraction procedures have been used by different scientists for detection of *Brucella* DNA (Bricker *et al.,* 2000; Bricker *et al.,* 2003).

Various regions of the *Brucella* genome have been identified and used in PCR assays; for example, the IS711-genetic element, also known as IS6501 (Bricker and Halling, 1994; Ouahrani-Bettache *et al.,* 1996), 16S rRNA (Romero *et al.,* 1995), 31 kDa outer membrane protein (Baily *et al.,* 1992; Gallien *et al.,* 1998; Sreevatsan *et al.,* 2000), *bcsp*31 (Guarino *et al.,* 2000), 43 kDa outer membrane protein (Fekete *et al.,* 1990) and *omp*2 gene (Leal-Klevezas *et al.,* 2000), using crude cell lysates and DNA extracted from cell lysates of *Brucella* species. The method has been optimized for a number of *Brucella* species using tissues, blood or milk samples (Bricker *et al.,* 2003).

Polymerase chain reaction (PCR) is an *in vitro* technique for the nucleic acid amplification, which is commonly used to diagnose infectious diseases (Bricker *et al.,* 2000). The assay can be used to detect *Brucella* DNA in pure cultures and in clinical specimens, that is, serum, whole-blood and urine samples, various tissues, cerebrospinal, synovial or pleural fluids, and pus (Colmenero *et al.,* 2010). Direct detection of *Brucella* DNA in brucellosis animals and/ or patients is a challenge because of the small number of bacteria present in clinical samples and inhibitory effects arising from matrix components (Scholz *et al.,* 2007). Basic sample preparation methods should diminish inhibitory effects and concentrate the bacterial DNA template. Residual PCR inhibition in complex matrices can be unmasked by the use of an internal amplification control (Scholz *et al.,* 2007). The QIAamp™ DNA Mini Kit (Qiagen Inc., Valencia, California, USA) and the UltraClean™ DNA BloodSpin Kit (MO BIO Laboratories Inc., Carlsbad, California, USA) are among the many commercial kits that have

been successfully used to extract *Brucella* DNA from whole-blood, serum and tissue samples (Pappas *et al.,* 2009).

Diagnosis of brucellosis by PCR is relatively simple and accurate. It has been used to not only diagnose brucellosis in almost all domestic and wild animals but also to characterize *Brucella* species infecting such animals (Bricker *et al.,* 1994; Fayazi *et al.,* 2002; Bricker *et al.,* 2003). Sensitivity and specificity of PCR provides a valuable and quick tool for diagnosis of brucellosis (Bricker *et al.,* 1994) and danger to staff exposure is minimal such that, requirement for level three laboratories for containment is not mandatory and therefore cost is also reasonable (Wei, *et al.,* 2010). The advantages of PCR are numerous. Independent of the disease stage, it is more sensitive than blood cultures and more specific than serological tests (Wei *et al.,* 2010). Real time PCR using the IS711-based insertion element assay has been shown to be the most sensitive, specific, efficient, and reproducible method to detect *Brucella* species (Wei *et al.,* 2010). Moreover, real-time PCR enables high-throughput screening of clinical samples and delivers results within a few hours. False negatives in PCR assays are rare and mainly occur due to amplification of the present polymerase inhibitors like haemoglobin, urine, heparin, phenol, and sodium dodecyl sulphate (Fayazi *et al.,* 2002) hence accurate sampling techniques that minimize contamination are critical.

2.9.2.5 Skin test

Skin test is an allergic test that detects the specific cellular immune response induced by *Brucella* infection. The injection of brucellergen, a protein extract of a rough strain of *Brucella* species, is followed by a local inflammatory response in a sensitized animal. This delayed type hypersensitivity reaction is measured by the increase in skin thickness at the site of inoculation (Alton *et al.,* 1988). This test is highly efficient in discriminating between true brucellosis cases and false positive serological reactions. The skin test is highly specific but

its weak sensitivity makes it a good test for herds but not for individual certification. It cannot discriminate between infection and vaccination (Quinn *et al.,* 1999).

2.9.2.6 Laboratory animal inoculation

Mice have been reported to be the animal model most frequently used in brucellosis research (Mense *et al.,* 2001; Silva *et al.,* 2011). Nevertheless, it has been reported that guinea pigs are also susceptible and can be used (Avong, 2000; Ocholi, 2005; OIE, 2004). Animal inoculation may be either subcutaneously or through abraded skin in guinea-pigs or, preferably, intravenously, intraperitoneally, or through the digestive tract or nasal (aerosol) routes in mice (OIE, 2004; Silva *et al.*, 2011). The spleen of mice is cultured 7 days after inoculation, while serum samples of guinea pigs are subjected to specific tests 3 and 6 weeks after inoculation (OIE, 2004). It is noteworthy however, that gastric acid can interfere with the infectivity of *Brucella* in laboratory animals (Silva *et al.,* 2011).

2.9.2.7 Other tests for brucellosis

Urinalysis may likely demonstrate a sterile pyuria similar to tuberculosis while arthrocentesis can be performed for septic arthritis. The joint aspirate can demonstrate an exudative fluid with low cell count and predominance of mononuclear cells (Radostits *et al.,* 1994). Radiographic evaluations in infected animals may reveal evidence of acute or chronic *Brucella* leptomeningitis, subarachnoid haemorrhage or cerebral abscess following cranial radiography (Blood and Radostits, 1989).

Similarly, echocardiography can also be used to evaluate possible endocarditis. Mycotic aneurysms of the aorta or carotids may be observed on duplex arteriography. Furthermore, bone marrow biopsy and liver biopsy may also be performed to obtain specimen for diagnosis, especially during the acute phase of the disease (Maloney and Fraser, 2006).

2.10 Treatment, control and prevention of brucellosis

2.10.1 Treatment

Treatment of brucellosis in animals is usually futile and normally not undertaken due to the possibility of exposure to humans from handling infected animals and also due to less effectiveness of available drugs and the cost implications (Timoney *et al.,* 1988; Quinn *et al.,* 1999). Different drugs and agents such as trace elements, vitamin mixtures, and antimicrobial agents such as phenol, azo and flavine dyes, have been shown to be lethal to *Brucella* organisms *in vitro*, but all have yielded mixed results when used *in vivo* (Quinn *et al.,* 1999). Under *in vitro* conditions, *B. abortus* have been found to be sensitive to gentamicin, kanamycin, tetracyclines and rifampin (Timoney *et al.,* 1988; Wanke, 2004). A combination of oxytetracycline and streptomycin was found to successfully treat 71.4% of the infected rams, while sulphonamides and penicillin were found to be less effective (Wanke, 2004).

A four week continuous treatment using a combination of tetracycline and streptomycin or dihydrostreptomycin, administered within the first three months of infection have been found to give successful therapy (Shin and Carmichael, 1999; Wanke, 2004). However, recrudescence of infection after the cessation of antibiotic treatment is not uncommon (Wanke, 2004).

2.10.2 Control and prevention

Control and prevention of brucellosis in farm animals depend on the animal species involved, *Brucella* species involved, management practices and availability and efficacy of vaccines (Radostits *et al.,* 1994). The options to control the disease include immunization, testing and culling of positive reactors and improving management practices and movement control (Hunter, 1994).

Brucellosis has been controlled and successfully eradicated in some countries through vaccination, coupled with test and slaughter policies. In many countries, the practice of

purchasing animals to improve genetics and intensive management systems often makes the control of brucellosis difficult due to exposure to infection of many highly susceptible animals (Nicoletti, 1984). Similarly, in developing countries in the subtropics, control of the disease is complicated by such practices as communal grazing, pastoralism and non-controlled livestock trade (Timoney *et al.,* 1988; McDermott and Arimi, 2002). Under such management, hygienic measures as segregation of purchased animals or keeping parturition animals separated from the herd is difficult and mostly impractical.

2.10.2.1 Control by vaccination

Several vaccines have been developed and are licensed and available for use in some countries. In 1906, Bang observed that cattle could be protected from infection by immunising them with live virulent cultures of *Brucella* organisms (Bishop *et al.,* 1994); it is, however, safe to use live attenuated vaccine, *B. abortus*, S19 which is safe and effective in controlling bovine brucellosis (Nelson, 1977). Vaccination with *B. abortus* S19 by itself will not eradicate bovine brucellosis, but it raises the level of immunity for individual animals such that undesirable consequences of brucellosis are minimised following exposure to virulent strains of *B. abortus* (Nelson, 1977). The use of *B. abortus* S19 vaccine should only be recommended where the prevalence of the disease is high and cessation of vaccination should be considered when the prevalence is reduced to 0.2% or less (Alton *et al.,* 1988). Nevertheless, *B. abortus* S19 vaccine has been the most widely used vaccine in the control of bovine brucellosis (Schurig *et al.,* 1991).

The normal practice of using a standard dose of $5x10^{10}$ viable organisms per dose (Bishop *et al.,* 1994), to vaccinate calves between 3 to 6 months of age has been reported to give long term immunity and benefits of re-vaccination has not been firmly demonstrated (Berman and Irwin, 1952), contrary to what has been reported (Nicoletti *et al.,* 1978). Moreover, antibody titres would decline to a point where 6-8 months after vaccination it is rare to find IgG in the

serum (Nelson, 1977). This will be an added advantage in countries where test and slaughter is practiced since occurrence of *B. abortus* S19 cross-reacting antibodies will be minimised. Although some studies have advocated for the use of a reduced dose (2x 10^8 to 3x 10^9 organisms/dose) (Bishop *et al.*, 1994) to vaccinate adult animals to control bovine brucellosis (Alton and Corner, 1981), the benefits of this practice are debatable (Nelson, 1977). A major set-back of using *B. abortus* S19 vaccine in adult cattle is that significantly more animals will have persistent antibody titres than those vaccinated as calves (Nelson, 1977; Beckett and MacDiarmid, 1985). This will interfere with serological tests in herds where test and slaughter is being practiced. In addition, the use of *B. abortus* S19 has been associated with abortions in cows vaccinated during pregnancy (Beckett and MacDiarmid, 1985), sterility problems in males, occasionally with low levels of protection (Nelson, 1977) and arthropathy (Corbel *et al.*, 1989).

A variety of vaccines prepared from killed cells of *Brucella* organisms have been tried and tested (Schurig *et al.*, 1991), but with the exception of *B. abortus* strain 45/20 (McEwen and Priestley, 1938), the practical use of these preparations has been very limited. *Brucella abortus* 45/20 was found to offer protection comparable to that of *B. abortus* S19 if administered as double doses in adjuvant (McEwen and Priestley, 1940). The need for a booster and the irritant nature of the adjuvant might make this vaccine more expensive to use and less desirable than *B. abortus* S19. Moreover, like any other killed vaccine, the use of *B. abortus* 45/20 may be associated with low level of cell-mediated immunity which is critical in protection against infection with *Brucella* species (Oliveira *et al.*, 2002). A potential vaccine candidate, *B. abortus* M-strain, was discontinued from trials because the strain offered low protection (Huddleston, 1946).

A rough mutant *B. abortus* RB51 has been a promising vaccine candidate, lacking the antibody inducing antigens but still giving a similar cellular protection as *B. abortus* S19

(Schurig *et al.,* 1991). However, its efficiency over *B. abortus* S19 remains a subject of debate (OIE, 2004). Similar to *B. abortus* S19, the *B. abortus* RB51 vaccine has been reported to cause placental infection and placentitis, and abortion in vaccinated cattle (Palmer *et al.,* 1996; OIE, 2004) as well as infections in humans (OIE, 2004). The use of DNA vaccines in farm livestock is not commonly used (Schurig *et al.,* 1991; Davis and Elzer, 2002).

In small ruminants, vaccination is recommended using Elbeg's *B. melitensis*, Rev. 1, a live attenuated vaccine (Elber, 1981; Banai *et al.,* 2002). Although *B. suis* strain 2 vaccine has been advocated for vaccinating sheep against *B. melitensis* infection, it has been demonstrated that *B. melitensis* Rev 1 gives a better protection (Verger *et al.,* 1995). The use of a killed vaccine, H38, prepared from *B. melitensis* biovar 1 has been reported, but this vaccine has been associated with protection failures (Alton, 1987).

2.10.2.2 Control programme on herd basis

The strategies to control brucellosis may differ from herd to herd depending on such factors as the level of infection present, resources available and general immune status of the herd. During an abortion storm, the test and disposal of reactors may be unsatisfactory because the spread of infection occurs faster than disposal is possible (Nicoletti, 1984). Hence, isolation of infected animals, isolation of females at parturition; proper disposal of aborted fetus, placental tissue and uterine discharge, and subsequent disinfection of the contaminated areas is recommended (Blood and Radostits, 1989).

In heavily infected herds, all calves should be vaccinated using the recommended vaccines such as *B. abortus* S19 or *B abortus* RB51. In serologically positive herds, positive reactors should be culled (Blood and Radostits, 1989). In herds where infection is light, vaccination of calves may be optional, but herds should be monitored regularly using milk ring tests (Blood and Radostits, 1989). Maintenance of closed herds would provide beneficial results if eradication of the disease is the ultimate goal.

2.11 HUMAN BRUCELLOSIS

2.11.1 Introduction

Brucellosis is an important zoonosis that has serious implications on the human health. It remains the most common zoonotic disease worldwide with more than 500 000 new cases annually (Pappas *et al.,* 2009) caused by six *Brucella* species: *B. melitensis*, *B. suis*, *B. abortus, B. canis, B. ovis* and *B. Neotomae* affect humans in descending order of pathogenicity (Quinn *et al.,* 1999). *Brucella melitensis* is highly pathogenic for humans and accounts for the majority of cases and all the three biovars are equally involved (Doganay and Aygen, 2003; Pappas *et al.,* 2009). Occasional cases of infection due to *B. abortus* S19 vaccine strain have been reported in vaccination accidents (Nelson, 1977). Of the *B. suis* biovars, only 1 and 3 are commonly associated with human brucellosis (OIE, 2004). *Brucella suis* biotype 2 is generally considered to be non-pathogenic for humans (Garritty *et al.,* 2005). Cases due to *B. canis* are infrequent, but important especially in laboratory workers (Doganay and Aygen, 2003; Wanke, 2004).

2.11.2 Epidemiology of human brucellosis

Diagnosis of brucellosis is often difficult to establish due to similarity of clinical presentations with other febrile infections prevalent in sub-Saharan Africa and limited laboratory capacity to adequately confirm the disease (Maichomo *et al.,* 1998). Over the years, brucellosis has been controlled and almost eradicated in most developed countries mainly due to various sanitary socio-economic, political reasons and the evolution of international travel (Pappas *et al.,* 2009).

Although the true incidence of human brucellosis is unknown globally, (Corbel, 2006), several areas traditionally considered to be endemic like France, Israel, and most of Latin America have achieved control of the disease (Pappas *et al*., 2009).

Endemicity of animal brucellosis in Africa and particularly in Sub-Saharan Africa which has approximately 16% prevalence continues to serve as constant source of infection to humans (Corbel, 2006). A brucellosis prevalence of 13.3% in Uganda (Mutanda, 1998) and 6.2% in Tanzania (Kunda *et al.,* 2007) has been recorded.

Kenya has very limited data and poor knowledge of brucellosis particularly among humans in pastoralist communities where the disease in animals is believed to be high; thus many cases go unrecognized and unreported. However, a few studies done indicate human brucellosis is more common where there are extensive cattle production systems, recording prevalence of between 14% to 21% (Muriuki *et al.,* 1997, Mugambi, 2001; Richards *et al.,* 2010; Kiambi, 2012).

2.11.3 Transmission of brucellosis to humans

Inhalation brucellosis may result from exposure to contaminated dust and dried dung by infected aborting animals. Contact infection may also result from contamination of skin or conjunctivae from soiled surfaces (Pappas *et al.,* 2009). Water sources, such as wells, may also be contaminated by recently aborted animals or by run-off of rain water from contaminated areas (Quinn *et al.,* 1999, Taleski *et al.,* 2002).

Brucellosis is not usually transmitted from person to person (Pappas *et al.,* 2009). Rarely, *Brucella* organisms have been transmitted by bone marrow transplantation, blood transfusion or sexual intercourse (Amalia, 2001, Meltzer *et al.,* 2010). In some cases, infants have appeared to be infected through the placenta and in others by the ingestion of breast milk (Amalia, 2001). Brucellosis was reported in an obstetrician who swallowed secretions while trying to clear a congenitally infected infant's respiratory tract at birth (Amalia, 2001).

Certain occupations are associated with a high risk of infection with brucellosis (Meltzer *et al.,* 2010). These include people who work with farm animals, especially cattle, sheep, goats

and pigs: farmers, farm labourers, animal attendants, stockmen, shepherds, sheep shearers, goat herders, pig keepers, veterinarians and inseminators are at risk through direct contact with infected animals or through exposure to a heavily contaminated environment (Chukwu, 1987; Pappas *et al.,* 2009).

The main source of human brucellosis is ingestion of contaminated fresh milk or dairy products prepared from unpasteurized milk as well as undercooked infected meat (Kang'ethe *et al.,* 2000; OIE, 2004).

2.11.4 Clinical manifestation of human brucellosis

Brucellosis in humans is a multisystemic infection that varies considerably and may last for three days to six months and occasionally for longer than a year; and signs are often non-specific (Hugh, 2000). Patients may present with an acute systemic, febrile insidious chronic infection or a localized inflammatory process (Sisirak *et al.,* 2009, Madkour *et al*, 2005; Kiambi, 2011). Patients present with non-specific signs such as recurrent fever, weakness, depression, low libido, sweating, arthralgia, lethargy, enlarged lymph glands, joint pains, anorexia, nausea, vomiting and weight loss (Corbel, 2006; Doganay and Aygen, 2003; Lucero *et al.,* 2005). Abortion in pregnant women is not a common feature of human brucellosis, most probably due to the absence of growth stimulants (erythritol) for *Brucella* species in the gravid uterus (Isaias *et al.,* 2008).

Other forms of presentations of the disease include respiratory system involvement (Georgios *et al.,* 2003*),* ocular complications (Isaias *et al.,* 2008) and epididymo-orchitis in males (Amalia, 2001). In-utero infection of the fetus, visceral abscesses (Nicholas *et al.,* 2001*),* cardiovascular complications with implantable cardioverter defibrillator replacement (Abhay *et al.,*2007*,* Wang *et al.,* 1999*)* and osteoarticular and gastrointestinal system complications (Ali *et al.,* 2003).

Mortality due to brucellosis in humans is low, usually in less than 5% of the infections (Wafa *et al.*, 2009) of which 80% of the fatalities is due to cardiac infections leading to endocarditis and subsequent cardiac failure; and sometimes due to hyperpyrexia, severe toxaemia or meningo-encephalitis **(**Wang *et al.*, 1999).

2.11.5 Diagnosis, treatment and prophylaxis

2.11.5.1 Diagnosis

Because of variable symptoms and non-pathognomonic clinical signs, clinical diagnosis of brucellosis in humans is usually difficult and usually is misdiagnosed as malaria or typhoid fever (Young, 1995; Lucero *et al.*, 2005). The most specific diagnostic test for human brucellosis is the culture and isolation of the causative microorganism (Alton *et al.,* 1988). The blood broth culture in 10% CO_2 is the simplest and most often utilised bacteriologic procedure (Diaz and Moriyon, 1989). Although the success rate is considered to be variable, three blood cultures drawn over a 24 hour period, particularly from febrile patients are generally sufficient (Diaz and Moriyon, 1989). In the case of local complications, culture material, if possible should be collected from the affected places such as liver, lymph node, abscess, synovial fluid or cerebrospinal fluid (Doganay and Aygen, 2003).

Although the only definitive diagnostic test is bacteriologic isolation of the causative microorganism, cultures are not always positive, and serological methods must be used as indication of disease (Diaz and Moriyon, 1989). Several serological tests such as the SAT, MET, RBPT, the anti-*Brucella* Coombs test, CFT and ELISA have been used successfully to detect antibodies against *Brucella* organisms in humans (Diaz and Moriyon, 1989). Serological cross reactions occur due to other non-*Brucella* bacteria such as *Yersinia enterocolitica* O: 9 infections since they share epitopes (Doganay and Aygen, 2003). A c-ELISA test offers advantage because it has higher sensitivity and is highly specific and superior to the conventional tests (Lucero *et al.*, 2003).

2.11.5.2 Treatment

The fact that *Brucella* organisms are localized within the cells (obligate intracellular micro-organisms) of the reticuloendothelial system presents a treatment difficulty since many antibiotics cannot cross the cell walls causing intermittent bacteraemia (Alton *et al.*, 1988) calling for prolonged continuous treatment protocols (Quinn *et al.*, 1999).

Different treatment programmes have been previously proposed and used with varying success (Ariza *et al.*, 1985; Akova *et al.*, 1993; Corbel, 2006), but the treatment recommended by WHO is rifampin 600 to 900 mg and doxycycline 200 mg daily for a minimum of six weeks (Doganay and Aygen, 2003). However, a combination of intramuscular streptomycin and oral tetracycline gives fewer relapses than the rifampin-doxycycline combination (Ariza *et al.*, 1985; Shin and Carmichael, 1999).

Furthermore, quinolones in combination of rifampicin have been found to be as effective as the streptomycin-tetracycline combination (Akova *et al.*, 1993). Infections with complications such as neuro-brucellosis or endocarditis are treated with a combination therapy with rifampin, doxycycline and ceftriaxone for 2-3 weeks and yield satisfactory results (Doganay and Aygen, 2003).

2.11.5.3 Prophylaxis

It is generally recognised that the prevention of human brucellosis is best achieved by control or eradication of the disease in animals, combined with adequate heat treatment of potentially contaminated food products (Schurig *et al.*, 1991). In addition, a lot is achieved through education campaigns (Kang'ethe *et al.*, 2000). Pasteurisation of milk and adequate cooking of animal food products removes the risk of spread through milk, milk products, meat and meat products (Kuplulu and Sarimehmetoglu, 2004).

There are no safe and effective vaccines for use in preventing human brucellosis. A derivative of *B. abortus* S19, 19-BA, given intradermally by scarification was tried, but gave limited protection for a relatively short duration (Schurig *et al.,* 1991).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Ethical Approval

This study entailed collection of milk and blood samples from farmers' livestock. Ethical clearance was obtained from the Ethical Review Committee of the Faculty of Veterinary Medicine, University of Nairobi and from the Directorate of Veterinary services (DVS). The farmers were informed of the study and their verbal consent was sought prior to commencement of data collection.

3.2 Study area

This was a cross sectional study done in Baringo County Kenya. The location of Baringo County is shown in figure 1.

Baringo County is one of the counties in mid-western Kenya. Its capital and largest town is Kabarnet. It borders Turkana to the North, Samburu and Laikipia to the East, Koibatek to the South, Keiyo Marakwet and West Pokot to the West. It covers an area of 11,075.3 square km, of which about 140.5 square kilometres is covered by water surface. Baringo County lies between Latitudes 00 degrees 13" South and 1 degree 40" north and Longitudes 35 degrees 36" and 36" degrees 30" east.

According to the Kenya National Bureau of Statistics (KNBS, 2009) national census, Baringo has a population of 555,561 people (110,649 households) mainly comprising the Tugen community which is a subset of the Kalenjin tribe. The County has an estimated 307,000 cattle; 497,000 goats; 203,000 sheep and 301 pigs. Most of these livestock (80-90%) are in the smallholder farming sector, usually in small herd sizes of less than 15 animals per herd (KNBS, 2009).

Agriculture is the major economic activity of Baringo people, comprising mainly dairy farming and growing of maize (particularly in the highlands), groundnuts and cotton. The remainder of the county is mainly rangelands with the rearing of goats, sheep, cattle and camels as well as bee keeping forming the major agricultural activities.

Baringo County is largely characterized by desert shrubs with dry thorny trees and thorny bushes with small patches of grassland, with temperate forests and evergreen forests composed of semi deciduous bushes and wooded grassland towards the south. The mean annual zonal rainfall averages between 450 mm to 900 mm in the semi-arid areas of Mogotio, Marigat (study areas) and Tiatty sub-counties, and 800-1400 mm in the semi-humid areas of Koibatek, Baringo central (also study areas) and Kabartonjo sub-counties. Generally, the temperatures range from 35-38 ^{o}C in the lowlands and 15-20 ^{o}C in the highlands (softkenya.com/Baringo County accessed on 2nd March, 2015).

This region was selected for study because of the following reasons:

a) Baringo County is inhabited by people with different agricultural activities depending on climatic, environmental and cultural issues. Pastoral farming account for 34%, Agro-pastoral (9%) and marginal mixed farming (39%). Mixed farming and irrigated cropping account for 14% and 4% respectively (Sequence Read Data-SRA, 2013). Studies elsewhere have shown that the prevalence of brucellosis is high among the pastoralists (McDermott and Arimi, 2002; Mugambi, 2001; Muriuki *et al.*, 1997, Acha and Szyfres, 1986).
b) A quick retrospective study of data in health facilities in the County showed that a large percentage of patients with febrile clinical presentation were diagnosed with brucellosis.

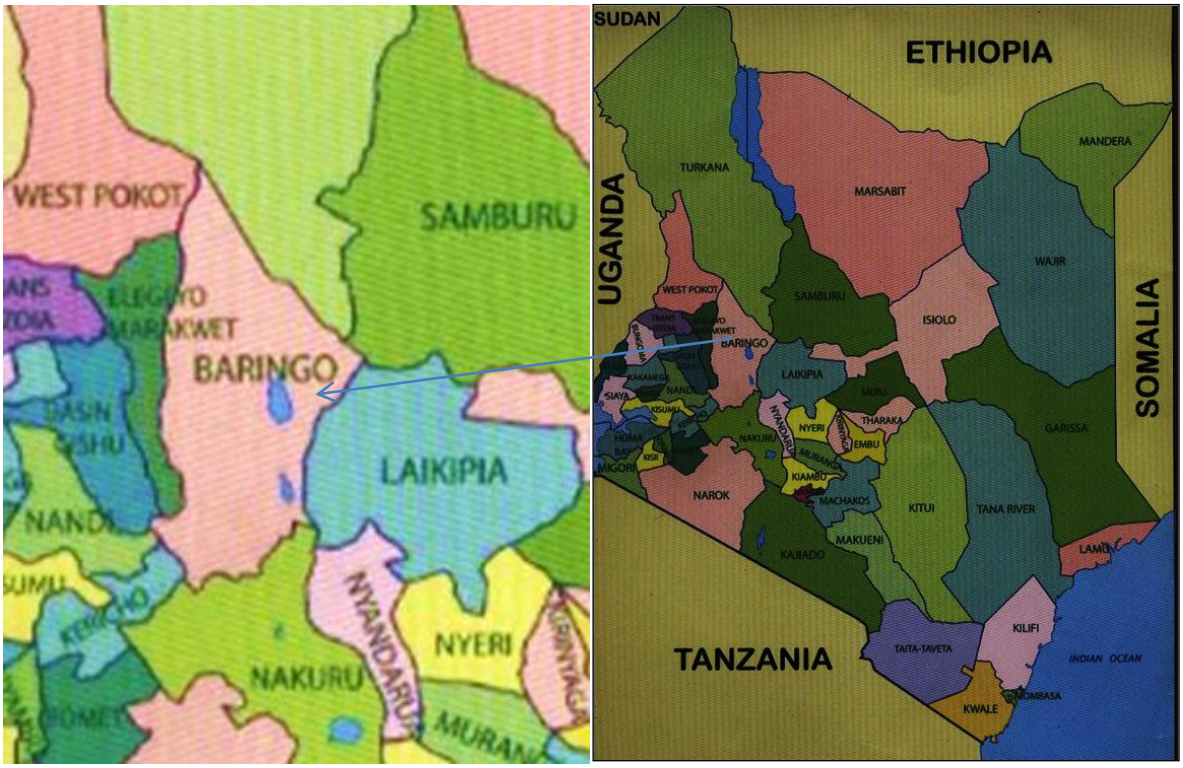

Figure 1: Map of Kenya showing the location of Baringo County and its bordering counties.

Source*:* *Independent Electoral and Boundaries Commission of Kenya, 2009.*

3.3 Sample size determination

Dahoo *et al* formula, (Dahoo *et al.,* 2003) was used to determine the sample size for this study as shown below:

$$n = \frac{Z\alpha^2 pq}{L2}$$

Where:

n= required sample size;

Z = Confidence level at 95% (standard value of 1.96),

P = Prevalent estimate,

q= 1-p,

L= Precision error (0.05).

Assumptions

- p=13.7% for cattle (McDermott and Arimi, 2002),
- p=8.1% for sheep (Mugambi, 2001),
- p=8.4% for goats (Mugambi, 2001),
- Confidence interval at 95%,
- precision of 0.05,
- that samples are independent and randomly selected.

For cattle, target sample size was calculated as: $(n) = \frac{1.96 \times 1.96 \times 0.137 \times 0.863}{0.0025} = 182$

Using the same formula for sheep and goats, the target sample sizes were 115 and 118 respectively. However, 250 cattle serum samples were finally collected with 142 and 166 sheep and goat serum samples respectively.

3.4 Sampling

Biosafety measures were ensured during sample collection by having personal protective equipment in place and disinfecting any surfaces with spills using 70% alcohol. Test samples were taken from livestock (cattle, sheep and goats) from the randomly selected herds/flocks.

Four sub-counties out of the six in the county were randomly selected (2 pastoral and 2 agro-pastoral). Two of these were in the pastoral areas (of the three in the lowlands) and two were in the highland agro-pastoral areas (of the three in the highlands). These were Baringo central and Koibatek (agro-pastoral in the highlands), Marigat and Mogotio sub-counties (agro-pastoral in the lowlands). Within these sub-counties, divisions were then sampled randomly. Farms were then randomly selected from a list of herds obtained from the veterinary and agricultural offices. In each herd/flock, five animals of each species were sampled by systematic random method (by picking every second mature animal that entered the crush) since KNBS (2009) found out that the average herd size in Baringo County is five in each homestead. For farms with less than five animals, either of the species, all the animals in that herd/flock were sampled.

3.5 Sample collection

Ten millilitres of blood was aseptically drawn from coccygeal or jugular veins of the selected animals after applying pressure onto the jugular fallow into plain vacutainers that were clearly labelled with a permanent marker. Blood samples in the tubes were allowed to stand in a rack for about one hour before being stored at 4 ^{0}C. Sampling was done 3-4 days before the specimens were delivered to the laboratory for processing, owing to the fact that raw milk was also collected for analysis while still fresh. Blood samples were then transported in a cool box

with ice packs to Central Veterinary Investigation Laboratories (CVIL- Kabete) for further analysis. At CVIL, the blood samples were centrifuged at 4000×g for 5 minutes so as to separate blood clot from serum. Serum was stored in sterile serum vials while the clots were kept in the vacutainers at -20^{0}C.

Bulk raw milk samples were also collected from each herd visited. Twenty millilitres (20ml) of the bulk raw milk was drawn into sterile clearly labeled universal bottles containing 1ml formalin (prepared by mixing 7.5ml of 37% formaldehyde with one litre of distilled water) for preservation. The milk samples were then stored in a refrigerator at 4^{o}C and transported to CVIL for analysis.

3.6 Determination of sero-prevalence

3.6.1 Laboratory procedures

Biosafety measures were ensured during sample analysis. Personal protective equipment including disposable gloves and laboratory coats were put on and assays done in biosafety cabinets where applicable. All surfaces were cleaned and disinfected using 10% sodium hypochloride and 70% alcohol.

At Central Veterinary Investigation Laboratories (CVIL- Kabete), milk was tested using Milk Ring Test (MRT) while serum samples were assayed using Rose Bengal Plate test. Serum samples were further assayed using competitive ELISA at Faculty of Veterinary medicine, University of Nairobi. Polymerase chain reaction (PCR) was done on blood clots at Department of Biochemistry, University of Nairobi. The respective procedures are described below.

3.6.1.1. Milk Ring Test technique

This test was conducted by pipetting 1 ml of the raw unpasteurized milk into a 1.2 ml Skatron tubes (Skatronas, Lier, Norway). One drop of stained *B. abortus* antigen was then added and mixed thoroughly. The tubes containing the milk samples and antigen were thereafter incubated at 37^0C for one hour and results read. Positive and negative controls were included with each set of tests. The test results were further left at 4^0C overnight and results re-read and recorded. A positive test was expected to have a blue ring at the cream and white milk column while a negative result was expected to have a white cream layer and a blue milk column as indicated in table 2. Figure 2 shows the investigator setting up the test.

Table 2: Interpretation of Milk Ring Test.

Colour of the top cream ring	**Colour of the milk column**	**Milk ring test reading**
Blue	White	Positive
White	Blue	Negative

Plate 1: **Investigator setting up Milk Ring Test at Central Veterinary Investigation Laboratories (CVIL) Kabete, Kenya. Watching is laboratory technician, Miss Eunice Ng'ang'a.**

3.6.1.2 Procedure for Rose Bengal Plate Test

Antigen, control serum samples (obtained from Central Veterinary Investigation Laboratory-Kabete Kenya) and test serum samples were removed from the refrigerator one hour before the test was done to attain room temperature. Only enough antigens for the day's test were removed from the refrigerator. In every test plate, positive and negative control tests were set (Alton *et al.,* 1975). The positive control was derived from stabilized diluted rabbit serum containing antibodies to *Brucella* antigen while the negative controls were diluted rabbit serum samples nonreactive to *Brucella* antigen.

Test serum (30µl) was placed on a white tile. The antigen bottle was shaken well but gently and 30µl of the antigen placed near the serum spot using a pipette with sterile tips.

Immediately after the last drop of antigen had been added to the plate, serum samples were mixed thoroughly with the antigen using clean applicator sticks, to produce a circular zone of approximately two centimetres (2cm) in diameter. The mixtures were then agitated for four minutes at ambient temperature on a rocker.

Reading of the test: Agglutination on the test zone was taken as positive with respect to the positive and negative controls on that test plates. No agglutination was taken as negative.

3.6.1.3 Competitive ELISA (c-ELISA) technique

Principle behind the test

The kit procedure is based on a solid phase competitive ELISA where samples together with a monoclonal antibody (mAB) specific for an epitope on the O- polysaccharide portion of the smooth lipopolysaccharide (S-LPS) antigen, are exposed to *B. abortus* S-LPS coated wells on microtiter plates. It detects specific antibodies to *B. abortus*, *B. melitensis* and *B. suis* and minimizes the cross reactions with other gram negative bacteria. In cattle, it is also capable of discriminating between infected animals and animals vaccinated with *Brucella* strain 19.

This test was carried out using the Svanovir^{TM} Brucella-Ab c- ELISA test kits (Svanova Biotech, Uppsala, Sweden), used according to the manufacturer's instructions. All serum samples were tested. Briefly, the test was carried out in 96 well polystyrene plates (Nalge Nunc, Denmark) that were pre-coated with non-infectious Smooth lipopolysaccharide (S-LPS) *Brucella abortus* antigen. All reagents and samples were first left to equilibrate to room temperature before use.

Serum diluted 1:10 (by adding 45µl of sample dilution buffer into each well that was to be used for the serum samples, serum controls and conjugate controls and using 5µl of the test sample serum) was added to each well, and immediately followed by equal volumes (50µl) of pre-diluted mouse monoclonal antibodies specific for a common epitope of the O-polysaccharide (OPS) of the smooth LPS molecule.

The plates were then sealed and reagents mixed thoroughly for five minutes by tapping the sides of the plate and incubated at room temperature for 30 minutes. The plates were then rinsed four (4) times with PBS- Tween Buffer. The reactivity of the mouse monoclonal antibody was detected using goat antibody to mouse IgG that was conjugated to horseradish peroxidase incubated for 30 minutes at room temperature. Substrate solution (Tetramethylbenzidine in substrate containing hydrogen peroxide) was then added at the rate of 100µl per well and left to stand at room temperature for 10 minutes. The reaction was stopped using 50 µl of 2M sulphuric acid. Optical densities were read at 450nm. Antibody titres were calculated as percentage inhibition (PI) defined by the ELISA kit manufacturer as;

$$PI = 100 - \frac{(\text{Mean OD}_{\text{value of sample or control}})}{(\text{Mean OD}_{\text{value of conjugate control, cc}})} \times 100$$

Serum samples were classified as positive and negative according to the manufacturer's recommendations. Thus all serum samples with PI values of ≥30% were classified as positive while those <30% were classified as negative.

3.7 Molecular characterization of *Brucella* species using Polymerase Chain Reaction (PCR)

3.7.1 Genomic DNA extraction

De-oxyribonucleic acid (DNA) was extracted from all the samples that reacted positive on RBPT and from 7% of the samples that were negative on RBPT. It was extracted from corresponding blood clots following the procedure described by Chachaty and Saulnier (2000). Positive Control sample was extracted from S19 vaccine (live attenuated *B. abortus*). The procedure was carried out in a class II biosafety cabinet in a biosafety level 2 laboratory. Appendix 1 shows concentrations of the reagents used.

First, erythrocyte lysis was done by adding 1ml of the erythrocyte lysis solution (155mM NH_4Cl, 10mM $NaHCO_3$, 100mM disodium EDTA, pH 7.4) to 400µl of blood clot and procedure repeated until the white blood cells lost the reddish colouring. Each time this was done, centrifugation at 4,000 ×g for 3 minutes was carried out and the supernatant was discarded. The pellets were then re-suspended in 567 µl Tris EDTA (TE) buffer (10mM Tris-HCL, 1mM EDTA at pH of 8.0). This was followed by addition of 30 µl SDS (10% in water), 3 µl of 0.2mg/ml proteinase K and 10µl of lysozyme. The result was then mixed thoroughly and incubated for 1hour at 37^0C.

Addition of 100 µl of 5M NaCl was done and mixed thoroughly followed by addition of 80 µl CTAB (hexadecytrimethylammonium bromide, 2%) solution, mixing and incubation for 10 minutes at 65^0C. An approximately equal volume (500 µl) of phenol/chloroform/isoamyl alcohol (25:24:1) was then added and mixed thoroughly, then centrifuged for 10 minutes at high speed (13,800×g) at room temperature. The viscous aqueous supernatant (top layer) was carefully recovered into a sterile labeled micro-centrifuge tube and the interface left behind.

Seven hundred and fifty microliters (750 µl) of chloroform/isoamyl alcohol was then added and mixed. Spinning at 13, 800×g for 10 minutes at room temperature was then done. The aqueous supernatant was then carefully collected into fresh microcentrifuge tubes. This was followed by addition of 0.6 volume isopropanol to precipitate the nucleic acids. Mixing by inversion was then done and the mixture left overnight at -20 ^{O}C; then centrifuged at 10 minutes at 13,800 ×g in a microfuge at room temperature and supernatant eliminated. The pellet (containing the DNA) was then washed with 500µl 70% ethanol, mixed thoroughly, and centrifuged at 13,800 ×g at room temperature. Careful removal of the supernatant with a pipette was then done and residual ethanol removal achieved by air drying for 30 minutes in a biosafety cabinet. De-oxyribonucleic acid pellet was then re-suspended in 20 µl double distilled sterile water.

The resultant DNA was then stored at 4^0C for a short period (24-48 hours) or at -20^0C for longer periods in small aliquots until use.

3.7.2 De-oxyribonucleic acid quality and purity control

Quality and purity of DNA were checked by submarine agarose gel electrophoresis using 0.8% agarose in 0.5X TBE (pH 8.0) buffer. Ethidium bromide (1%) was added at rate of 5µl/100ml. The wells were loaded with 5µl of DNA preparations mixed with 1µl of 6X gel loading buffer dye. Electrophoresis was carried out at 100V for one hour at room temperature and the DNA visualized under UV transilluminator and photographed.

3.7.3 Conventional polymerase chain reaction (PCR)

Each target DNA was amplified in a 20-µl volume consisting of 2× TopTaq®) PCR buffer (20 mM Tris, 3 mM $MgCl_2$, 100 mM KCl, 50 mM $(NH4)_2SO4$, pH 9.0) and 0.2 mM each of dNTPs) 0.2 mM of each of the oligonucleotide primers as a cocktail (Table 3). Table 3 shows PCR mixture.

Table 3: Contents of each PCR tube

Reagent	Volume for one reaction
2×PCR buffer (TopTaq®)	10µl
PCR grade water	6µl
Forward primer (cocktail) (*B. abortus, B. melitensis, B. suis* and *B. ovis*)	1µl
Reverse primer	1µl
Template DNA	2µl
TOTAL	20µl

The cocktail forward primer contained *B. abortus* (forward), *B. melitensis* (forward), *B. ovis* (forward) and *B. suis* (forward), each 25µM. The reverse primer was universal, 1S711, 25 µM which anneals to all *Brucella* DNA irrespective of the *Brucella* species. Table 4 shows the sequences of the primers used.

Table 4: Polymerase chain reaction primer sequences

Primer	Sequence
B. abortus (Forward)	GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC
B. melitensis (Forward)	AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA
B. ovis (Forward)	CGG-GTT-CTG-GCA-CCA-TCG-TCG
B. suis (Forward)	GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG
IS711 (Universal reverse)	TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT

The cycling conditions were 95^0C for 5 minutes (initial denaturation), followed by 40 cycles of 95 ^{0}C for 15 seconds (template denaturation), 52 ^{0}C for 30 seconds (primer annealing), 72 ^{0}C for 90 seconds (primer extension) and final extension for 5 minutes at 72^0C (Bricker *et al.*, 2003). The reactions were stored at 4 ^{0}C until the amplified products were separated by gel electrophoresis (100V for one hour) on a 1% agarose gel in TAE (44.6 mM Tris, 44.5 mM boric acid, 1 Mm ethylenediaminetetraacetic acid; pH 8.3). The gel was then stained with 1% Ethidium bromide as previously described and the DNA bands observed by UV fluorescence and photographed.

Identification was based on the size and number of DNA products that are amplified from each DNA sample. The size of the amplicon/band was determined against a maker (100 base pair (bp) ladder).

The expected sizes of amplicons and interpretations were as follows: All *Brucella* species have 178 base pair (bp) PCR products except vaccine S19. *Brucella abortus* amplifies a 498 bp product; vaccine RB51 has an extra 364 bp band. *Brucella melitensis* (all biovars) primers

amplify a 731 bp product; *B. ovis* primers amplify a 976 bp product while *B. suis* primers amplify a 285 bp product (Bricker, 2003).

3.8. Determination of factors associated with brucellosis: Data collection by questionnaires

A semi-structured questionnaire (appendix 2) was used to collect data on possible factors that could contribute to transmission of brucellosis in livestock, and/or to humans in the region and assess the knowledge, attitudes and practices of the respective communities in Baringo County. A number of herd-level predictor variables that included: herd size, farm size, stocking density, type of grazing (communal or own pasture), source of drinking water, method of tick control (communal or own dip tank), presence of a purchased animal, sale of animals in the past few years, keeping cattle together with sheep and goats, animals calving on pasture, keeping cattle in confined pens at night, hiring animals from neighbours for use, method of breeding (natural or artificial), source of bull for breeding (own or hired), keeping records and knowledge of bovine brucellosis were included on the questionnaire.

Data on possible risk factors for humans contracting the disease such as drinking raw milk, milking of animals, assisting animals during parturition and/or abortion, removing retained after-birth without personal protective equipment and disposing aborted or placenta material with bare hands was also collected.

3.9 Data handling and analysis

All data collected was entered and stored in a computer data base, excel, 2010. Descriptive statistics, frequencies and proportions were done to ascertain the different variables in the structured questionnaire using SPSS (Statistical Package for the Social Sciences) statistical package, version 20. Risk estimates were calculated using SPPS by determining the odds ratios to test for association and determine risk factors of the disease in Baringo County. Specificity and sensitivity of RBPT was also determined, using cELISA as the gold standard.

CHAPTER 4

4.0 RESULTS

4.1 Serological survey

The target sample size was 182 cattle, 118 goats and 115 sheep from 50 herds distributed equally among the four sub-counties; however, a total of 558 animal blood samples were collected and assayed (250 cattle, 166 goats and 142 sheep) from 84 herds. All serum samples were analysed using RBPT and c-ELISA while milk samples (n= 84) were analysed using MRT to determine *Brucella* antibodies in the respective samples.

4.1.1 Rose Bengal Plate Test results

Table 5 shows the proportions of the positive samples on RBPT in relation to the species in the study. Seropositivity was recorded across the species sampled (cattle, sheep and goats) with goat samples giving higher prevalence of the three animal species.

Rose Bengal Plate test was based on visible agglutination with respect to the positive and negative controls. Figure 3 illustrates results of positive samples, negative samples and both negative and negative controls.

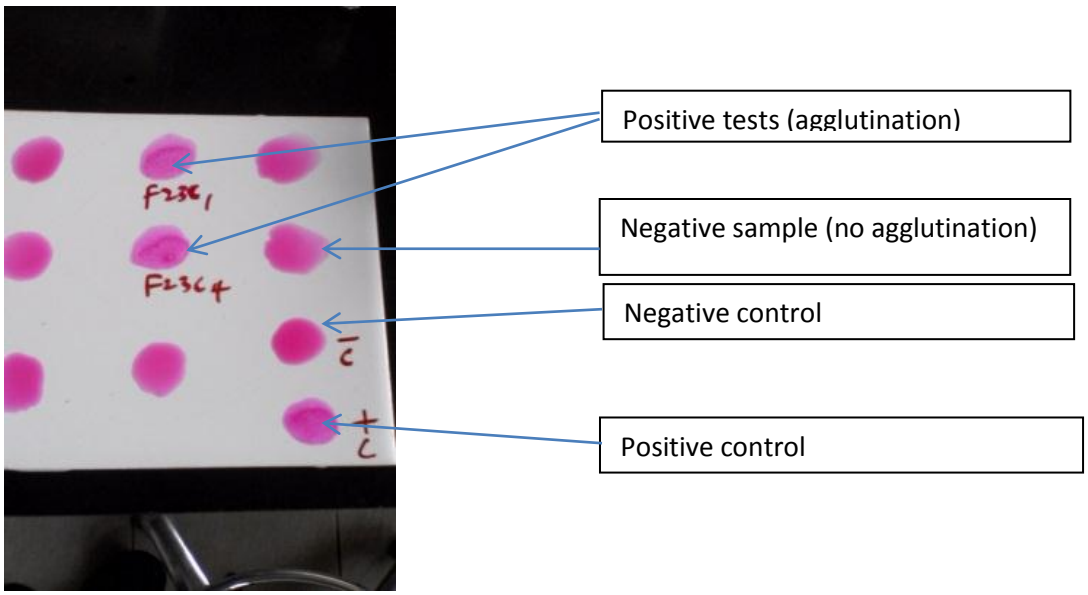

Plate 2: **Rose Bengal Plate Test positive and negative presentations.**
The sample labeled F23CI and F23C4 are samples from one farm (Farm 23, cow number 1 and 4) showing agglutination on RBPT. Sample C- is a negative control while C+ is the positive control with agglutination. The other unlabeled samples show no agglutination, thus recorded as negatives.

Table 5: Rose Bengal Plate test results for each species and proportions of the positive reactors

ANIMAL SPECIES	SERUM SAMPLES		
	No. tested	**No. positive**	**Proportion positive (%)**
Cattle	250	23	9.2
Goats	166	17	10.24
Sheep	142	10	7.04
Total	558	50	8.96

Rose Bengal Plate test positive results were distributed throughout the county in the four sub-counties sampled in different proportions as indicated in Table 6. More positives were detected in the lowland areas (Mogotio and Marigat) than in the highland areas (Koibatek and Baringo central). This was seen across the three animal species sampled. However, goats from all the sub-counties had almost similar prevalence.

Table 6: Number of positive samples for RBPT in relation to the four sub-counties and animal species sampled in Baringo County

NUMBER POSITIVE PER SUB-COUNTY	ANIMAL SPECIES		
	Cattle number tested (%) positive	Goats number tested (%)positive	Sheep number tested (%)positive
Marigat	9/68 (13.2%)	6/51(11.7%)	5/39 (12.8%)
Mogotio	6/53 (11.3%)	4/38 (10.5%)	2/36 (5.6%)
Koibatek	4/48 (8.3%)	4/36 (11.1%)	1/33 (3.0%)
Baringo Central	4/81 (4.9%)	3/41 (7.3%)	2/34 (5.9%)
TOTAL	23 (9.2%)	17 (10.2%)	10 (7.0%)

4.1.2 Milk Ring Test results

All the milk samples (n=84) were subjected to MRT. A positive reaction was as described in Table 2. Figure 4 shows result of a positive and a negative test while Table 7 shows the proportion of positive samples tested in relation to the four sub-counties of Baringo County sampled. More positive samples were recorded from the samples collected from the sub-counties in the lowlands (Marigat and Mogotio) than from the samples collected from the highland areas (Koibatek and Baringo Central).

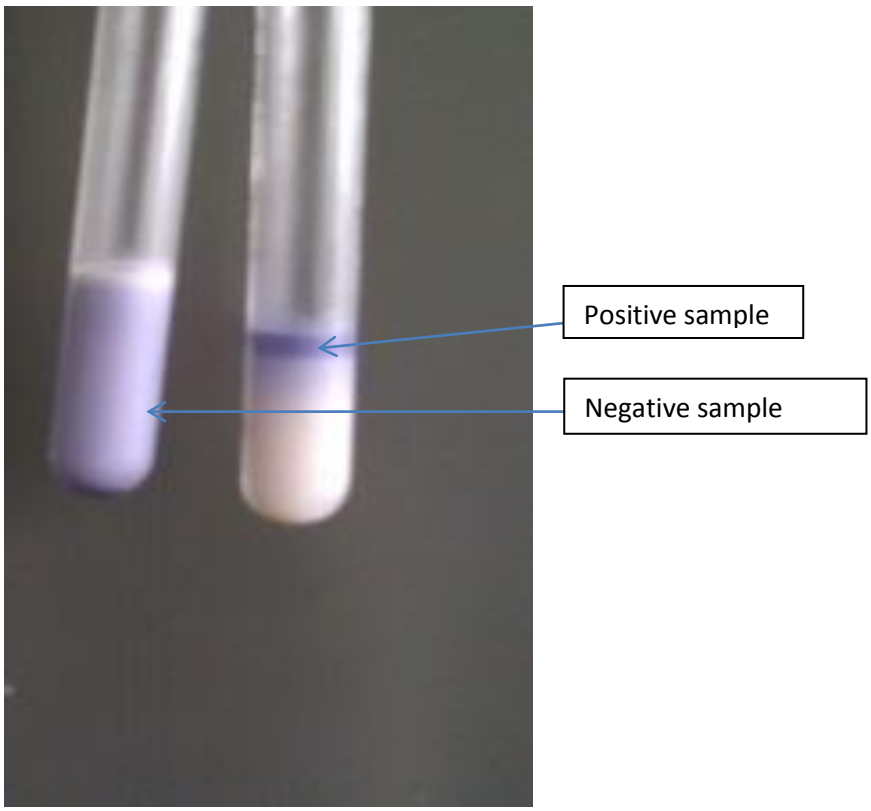

Plate 3: **Positive and negative milk samples on MRT**

The sample with a blue ring at the cream layer illustrates a positive result while the sample with blue milk column and white cream layer indicate a negative result.

Table 7: Serological results for MRT, with respect to each sub-county

SUB-COUNTY	NUMBER SAMPLED	NUMBER/PROPORTION POSITIVE
Baringo Central	28	2 (7.14%)
Marigat	24	4 (16.67%)
Koibatek	15	1 (6.67%)
Mogotio	17	3 (11.76%)
Total	84	9 (10.71%)

Each of the serum samples was further assayed using competitive ELISA. Of all the samples (n=558), 35 (6.3%) reacted positive on cELISA. These included 17 (3.0%) from cattle, 11 (2.0%) from goats and 7 (1.3%) from sheep. Table 8 shows c-ELISA positive results in respect to each species and sub-county; while Table 9 shows the breakdown of RBPT in relation to cELISA in the specific species. The cumulative reactor column represents samples that reacted positive on either of the two tests (RBPT and cELISA). Higher overall positive reactors were recorded among the caprine although the seropositivity was also recorded across all the species. The positive proportions recorded on cELISA kit were lower than the positives recorded by RBPT across the species.

Table 8: Number of positive samples on c-ELISA in relation to the four sub-counties and animal species sampled in Baringo County

NUMBER POSITIVE PER SUB-COUNTY	ANIMAL SPECIES		
	Cattle number tested (%) positive	Goats number tested (%)positive	Sheep number tested (%)positive
Baringo Central	3/81 (3.7%)	1/41 (2.4%)	2/34 (5.6%)
Marigat	8/68 (11.8%)	5/51 (9.8%)	3/39 (7.7%)
Mogotio	5/53 (9.4%)	4/38(10.5%)	2/36 (5.6%)
Koibatek	1/48 (2.1%)	1/36 (2.8%)	0 (0%)
TOTAL	17 (6.8%)	11 (6.6%)	7 (4.9%)

Table 9: A breakdown of serological reactions with respect to different tests and animal species

SPECIES	RBPT(% positive)	c-ELISA (% positive)	Cumulative positives (% positive)
Cattle	23 (9.2%)	17 (6.8%)	25 (10.0%)
Goats	17 (10.2%)	11 (6.6%)	18 (10.8%)
Sheep	10 (7.0%)	7 (4.9%)	11 (7.7%)
Total	50 (8.96%)	35 (6.3%)	54 (9.7%)

When compared to cELISA, (taking the ELISA to be the gold standard in this study), the sensitivity of the RBPT was 88.6% with a specificity of 96.4%. Rose Bengal Plate test diagnosed 4 false negatives and 19 false positives as indicated in Table 9 below.

Table 10: Comparison of RBPT and cELISA

RBPT	**cELISA**		
	Positive	Negative	Total
Positive	31	19	50
Negative	4	504	508
Total	35	523	558 (n)
	Sensitivity= 88.6%	Specificity= 96.4%	
	RBPT positive predictive value = 62%	RBPT negative predictive value = 99%	

Overall agreement = 95.9%

4.2. Polymerase chain reaction (PCR) results

All serum samples which reacted positive on RBPT were assayed using conventional PCR as described by Chachaty and Saulnier, 2000. Furthermore, 7% of the serum samples that gave negative results on RBPT were also assayed using PCR. The expected amplicon sizes were based against a 100 base pair maker as shown in Figure 5.

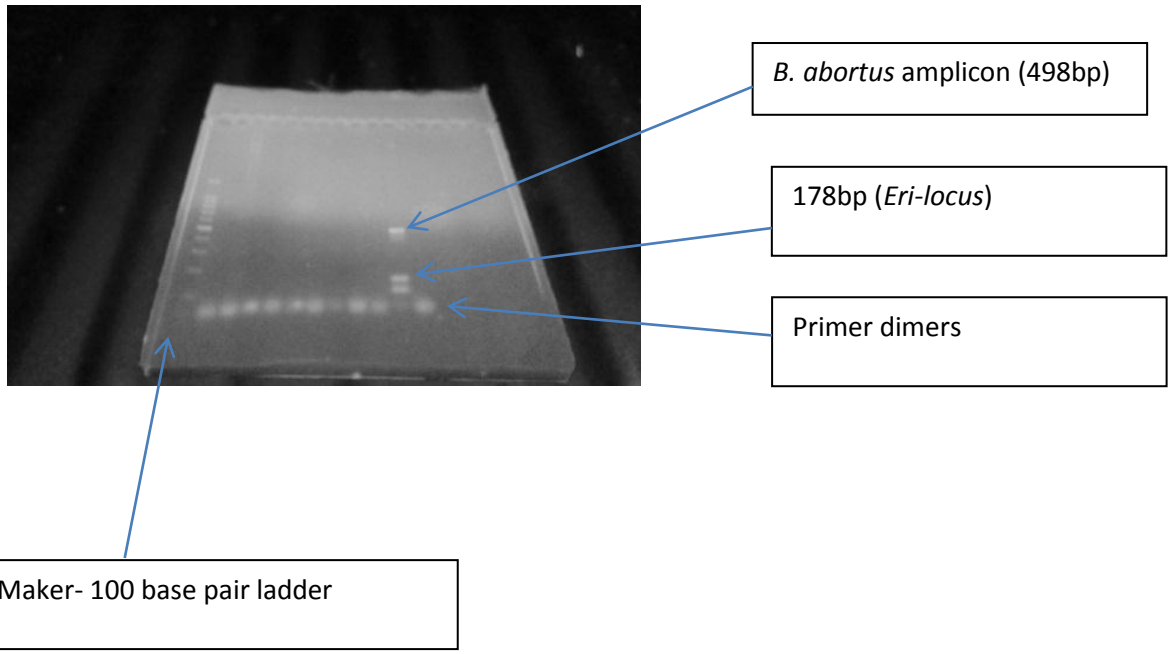

Plate 4: **A photograph of agarose gel electrophoresed PCR products**
The first lane (from left) shows different band sizes of 100 base pair ladder (maker) while the arrows indicate specific amplicon sizes in relation to the maker from positive samples.

In total, the PCR assay detected *B. abortus* from 14 of the RBPT positive samples (28%), and only one sample had *B. melitensis* (2%). Of the *B. abortus* positive samples, 11 were from cattle, two from goats and one from a sheep. *Brucella melitensis* was detected from one goat sample. From the RBPT-negative samples, no *Brucella* DNA was detected. Table 10 gives a summary of the animal samples that tested positive for respective *Brucella* species detected using PCR.

Table 11: *Brucella* species detected by PCR

Animal species	***B. abortus***	***B. melitensis***	***B. ovis***	***B. suis***	***Total***
Cattle	11	0	0	0	11
Goats	2	1*	0	0	3
Sheep	1	0	0	0	1
TOTAL	14	1*	0	0	15

***** *Samples with faint band on gel electrophoresis.*

4.3 Determination of factors associated with brucellosis (KAP survey)

Tables 11, 12, 13 and 14 gives summaries of risk estimates for factors found associated with brucellosis in livestock in Baringo County. Most respondents, (57.6%; 49/85) practiced mixed farming, kept cattle, sheep and goats in the same herd. Approximately a third of them, (31.8%; 27/85) used communal grazing; while 37.6% (32/85) used communal water points to water their livestock. Recent introduction of new animals was reported by 42.4% (36/85) of the persons interviewed; while 90.6% (77/85) of the livestock owners interviewed said their animals calved down in pasture, where other livestock grazed.

Cases of retained afterbirths in the region were high, reported by 20% (17/85) of the farms visited; while 27% (23/85) of the farms reported to have had a case of abortion in at least one of their livestock in the previous two years. Of the farms that had had abortion cases, 2/27 (7.4%) reported to have left the aborted material in pasture; and 44.4% (12/27) of those farm owners handled the aborted fetus with bare hands.

All the respondents, (100%; 85/85) in the study consumed fresh milk; most of them, (97.6%; 83/85) boiled fresh milk before consumption while 2.4% (2/85) said they consumed raw milk. A few people (12.9%; 11/85) reported to flash-boil their milk (bringing milk to just near boiling).

Some people in Baringo, (28.2%; 24/85) prepared sour milk (‘mursik’) from raw milk. This study also revealed that, of the livestock owners interviewed, 11.8% (10/85) had not heard of brucellosis; while 7% (6/85) did not know that humans get infected with the disease. However, of those aware of brucellosis, 58.2% (46/79) believed that people get infected with brucellosis by consuming raw/ under-boiled milk and/or raw/under-cooked meat; while 12.7% (10/79) did not know how humans got infected. It was also noted that 20% (17/85) of the homesteads visited had had at least one the family members diagnosed of brucellosis.

Table 12: Risk Estimate for farms practicing mixed farming

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for FACTOR (Reported mixed farming / Not reported mixed farming)	18.778	6.737	52.336
For cohort Brucellosis = Positive	4.333	2.368	7.930
For cohort Brucellosis = Negative	0.231	0.126	0.422

The risk of brucellosis for farmers who reported mixed farming was four times more than that of farms not practicing mixed farming.

Table 13: Risk Estimate for farms using communal grazing fields

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for FACTOR (Using communal grazing / Not using communal grazing)	5.641	1.754	18.142
For cohort Brucellosis = Positive	2.375	1.264	4.463
For cohort Brucellosis = Negative	0.421	0.224	0.791

The risk of brucellosis for farms using communal grazing was found to be twice that of farms that did not use communal grazing.

Table 14: Risk Estimate for farms using communal watering points

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for FACTOR (Using communal watering points / Not using communal watering points)	49.000	11.136	215.598
For cohort Brucellosis = Positive	7.000	2.773	17.671
For cohort Brucellosis = Negative	0.143	0.057	0.361

The risk of brucellosis for farms which reported to be using communal watering points was seven fold that of farms that reported not to be using communal watering points.

Table 15: Risk Estimate for farms that allowed animals to calve down on pasture/grazing fields

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for FACTOR (Calving on pasture / Not calving on pasture)	72.250	25.639	203.600
For cohort Brucellosis = Positive	8.500	4.393	16.446
For cohort Brucellosis = Negative	0.118	0.061	0.228

The risk of brucellosis for farms that allowed their animals to calve down on pasture was nine more than that of farms that reported not to have allowed their animals to calve down on pasture.

Although introduction of a new animal into the herd has been documented to be a risk factor of brucellosis (McDermott and Arimi, 2002), this study found out that introduction of a new animal into the herd in Baringo County was not a major risk to brucellosis.

CHAPTER 5

5.0. DISCUSION

Brucellosis is a major constraint to ruminant production systems in most parts of the world. This disease has been eradicated in most industrialized nations, but its occurrence is still on the increase in developing countries such as in sub-Saharan Africa with a prevalence of about 16%. In these regions where the disease remains endemic, it is usually a serious zoonotic risk (Falade *et al.,* 1980; Brisibe *et al.,* 1996; Seifert, 1996; FAO, 2004; Robert *et al.,* 2010).

This study highlights presence of brucellosis in cattle, sheep and goats in Baringo County, Kenya. The sero-prevalence established in this study (9.7% overall) is nearly similar to that reported by others who researched on the same work in animals in pastoral areas (McDermott and Arimi, 2002 [13.7%]; Mugambi, 2001 [8.4%]). Since pastoralists are known to be practicing communal animal grazing and watering, this could be a common factor among these communities.

Brucella infection in farm animals is a great problem in most countries of the world, and particularly in sub-Saharan Africa. In this study, 10% of the herds were found positive for *Brucella* antibodies by MRT, therefore there was potential of transmission of brucellosis to humans through such contaminated unpasteurized milk. A similar study in Uganda reported that 12% of milk marketed was contaminated with *Brucella* antibodies and its direct effects on those who consumed the contaminated milk are remarkable (Makita *et al.,* 2010).

Rose Bengal Plate Test is considered as 'satisfactory screening test' (Nicoletti, 1980; OIE, 2004; Quinn *et al.,* 1999); and the highest specificity and sensitivity of ELISA has led it to be used as confirmatory test in serial testing with RBPT (OIE, 2004). Several factors may affect the results of serological findings of a screening test, particularly RBPT and ELISA (Quinn *et al.,* 1999). Higher sero-prevalence of brucellosis has been recorded when multiple serological tests are used in parallel (Waghela *et al,* 1978; Al-Khalaf and El-Khaladi, 1989; Mugambi,

2001) because of sensitivity variations among the tests (Andreani *et al*, 1982). Majid *et al* (1999) reported higher seroprevalence rate (ranging from 14% to 43.9%) using RBPT alone (highly sensitive test). Reported lower prevalence rates by some authors could be a result of using tests with low diagnostic sensitivity or as a consequence of serial multiple tests. This study recorded a prevalence of 9% by RBPT and 6.3% by cELISA (overall prevalence of 9.7%). However, such serological results (particularly from the less specific RBPT) could be affected by cross-reacting bacteria such as *Escherichia coli, Yersinia enterocolitica* and *Salmonella* serotypes (Cloeckaert *et al,* 1992; Garin-Bastuji *et al,* 1999; Mugambi, 2001). *Brucella abortus* may cross-react serologically with *Escherichia coli* sero-group O:157, *Yersinia enterocolitica* serovar O:9, *Salmonella* serotypes of the Kaufmann-white group N, *Francisella tularensis, Pseudomonas maltophilia,* and *Vibrio cholerae* (Nielsen *et al.,* 2009). This is because the immunodominant O-chain of the smooth lipopolysaccharide (S- LPS) of these bacteria contains antigenic motives called epitopes that may be detected in brucellosis serological tests that use whole *Brucella abortus* cells or S-LPS extracts.

This study established that more herds are infected in the lowlands (Marigat and Mogotio) than in the highlands. This may be because the lowland areas of Baringo are inhabited largely by pastoralists who mainly rely on communal grazing and watering points, leading to extensive mixing of various animal species from different farms. This is a common factor that leads to brucellosis transmission in livestock as it has been previously determined by other research work in pastoral areas (McDermott and Arimi, 2002), as well as in this study.

The molecular findings in this study indicate that *B. abortus* is the common species of *Brucella* affecting livestock in Baringo County. Presence of *Brucella* DNA confirms that there was an active disease in the region at the time of sampling. However, the low rate of *Brucella* DNA isolation could be due to the DNA extraction method used. Using commercial kits such as QIAamp™ DNA Mini Kit (Qiagen Inc., Valencia, California, USA) and the

UltraClean™ DNA BloodSpin Kit (MO BIO Laboratories Inc., Carlsbad, California, USA) for DNA extraction combined with real time PCR might have given higher positive samples. Failure to isolate DNA from initially seropositive samples could be attributed to either a chronic infection, where there are only antibodies in the serum samples and no active disease (antigen) or could have been a false seropositive reaction by the test used.

Although traditionally *B. abortus* is known to have cattle as the preferred host, cross infections occur, as it was found out in this study where small ruminants were infected. Infection by *B. abortus* in sheep and goats has been reported in several countries, mainly in developing ones (Leal-Klevezas *et al.,* 2000). Kabagambe *et al.,* (2001) reported that even mixed infections by both *B. abortus* and *B. melitensis* may occur, while Ocholi *et al.,* (2004) reported the recovery of *B. abortus* from livestock, including goats and sheep, in Nigeria.

Small ruminant infections by *B. abortus* are said to be spill-over infections from infected cattle when kept in close proximity, particularly if they are sharing common grazing and/or watering points (McDermott and Arimi, 2002). This study has demonstrated that livestock (cattle, sheep and goats) in Baringo County harbour *B. abortus*; they, therefore, remain a potential threat and risk to humans in the region and potentially lead to reduced livestock productivity. This could possibly explain why RBPT, which is primarily used to detect *B. abortus* antibodies mainly in cattle, gave positive reactions also on sheep and goat serum samples tested

In this study, animal movement and concentration of livestock near watering points were found to be common practices; the environment favours disease transmission in such setups. These factors have also been previously incriminated to be important risk factors to brucellosis (McDermott and Arimi, 2002). Most of the livestock owners in Baringo, particularly in the lowlands (pastoralists) practiced a high degree of ruminant diversification. Keeping a mixture of animals is also common in other areas and has economic and ecological

advantages (Ayan, 1984; Wilson *et al*, 1990). While this may have economic importance, in the event that one or more animals are infected with brucellosis, such mixing increases the chances of transmission of the disease among the livestock in the whole herd.

Although this study did not establish prevalence of disease in humans, it can be presumed that the disease could also be high among the human population, particularly due to *B. abortus* infection considering that most of the persons interviewed in this study come in contact with animal or animal derived products in one way or the other. Other studies have a positive correlation between the prevalence of brucellosis in animals and in humans (Mugambi, 2001). Since human infections are almost always from infected animals, the level of the disease in humans could be similar or even higher to the one seen in animals (Corbel, 2006; Nicoletti, 2002).

From the information gathered from the respondents, there is a huge gap in knowledge on brucellosis. Most people in Baringo County do not understand risk factors, control and preventive measures that can be employed both for animal and human *Brucella* infections. It is therefore important to establish an educational campaign in the region to enlighten the communities on the disease, risk factors as well as control strategies particularly in livestock; an exercise which will go a long way in lowering human brucellosis.

CHAPTER SIX

6.0 CONCLUSIONS AND RECCOMENDATIONS

6.1 CONCLUSION

i. A substantial proportion of livestock in Baringo County are *Brucella* seropositive.

ii. Brucellosis in livestock in Baringo County, Kenya is largely caused by *B. abortus*.

iii. There is a huge knowledge gap on the risk factors of brucellosis, with communal grazing and watering of animals, animal movement and allowing animals calve down on pasture being the main risk factors to brucellosis in livestock in the region. These practices can be attributed to the scarcity of knowledge on brucellosis, its effects and control strategies in livestock herds.

6.2 RECCOMENDATIONS

i. Establishment of brucellosis control measures in livestock in Baringo County should be put in place.

ii. Livestock in Baringo County need be vaccinated with *B. abortus* vaccine (s19 vaccine).

iii. It is important to urgently establish an educational campaign on brucellosis in the region to disseminate information to all stakeholders particularly regarding risk factors in order to impart knowledge and change their attitudes and practices; an exercise which will help in reduction of the disease in animals.

iv. Another study to establish the disease situation in humans in the area is recommended so as to compare it with the level of the disease in livestock; this will also provide some guidance on the control measures that need to be put in place.

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APPENDICES

APPENDIX 1

Concentrations of reagents used for isolation of DNA

- Sodium dodecyl sulphate (SDS): 10% in water (autoclaved and stored at room temperature).
- Tris-EDTA (TE) buffer: 10mM Tris-HCL,1mM EDTA (pH 8.0)
- Proteinase K: 20mg/ml in TE buffer (stored in small, single-use aliquots at -20^0c).
- NaCl: 5M (stored at room temperature)
- CTAB/NaCl solution: CTAB (Cetyltrimethylammonium bromide) in 0.7M NaCl.
- Chloroform/isoamyl alcohol ratio: 24:1.
- Phenol/Chloroform/isoamyl alcohol: 25:24:1, pH of 7.8
- Isopropanol.
- Ethanol: 70%

Erythrocyte lysis solution

- 155mM NH_4Cl
- 10mM $NaHCO_3$
- 100mM disodium EDTA (pH 7.4)

APPENDIX 2

A SURVEY TO DETERMINE THE FACTORS ASSOCIATED WITH BRUCELLOSIS IN SMALLHOLDER CATTLE, SHEEP AND GOATS IN BARINGO COUNTY, KENYA.

A) FARM IDENTIFICATION ***Questionnaire***

No……

1. Date of visit and interview: …….

2. Farm Sampling No…….

4. Name of owner: ……..

6. District ……Division……Location……..Village……

B): FARM STRUCTURE

8. Type of farm, e.g. dairy or mixed dairy and beef…….

9. Herd size……

11. Are cattle kept together with small stock (sheep and goats)?

0. No……..

1. Yes……

12. Animal census

Beef cattle	Dairy cattle	Sheep	goats	pigs	Others

13. A breakdown of herd structure

Cattle

Cows Bulls Heifers>1 yr Calves< 1 yr Steers/oxen

Small ruminants

Does Ewes Bucks Rams Kids<6months Lambs<6months

C): FARM MANAGEMENT

GENERAL MANAGEMENT

14. What type of grazing does the farm use?

0. Communal……..

1. Own pasture……..…

3. Zero grazing…….

15. What type of feeding management do you use?

0. Pasture only……..…

1. Supplementary feeding and pasture……..…

16. Water source?

0. Communal……...

1. Own supply…….

17. If own water supply, do different animal species drink from the same watering vessel?

0. Yes…….

1. No……..

17. Who is responsible for feeding the animals?

0. Self……

1. Hired caretaker……..

3. Other family members…….If other family, who in the family?.....

18. Who is responsible for milking the animals?

0. Wife……..

1. Husband……..

3. Hired person …….

4. Anyone in the family can milk……

18. Do animals come in contact with wild animals?

0. No……

1. Yes……..If yes, which wild animals……..

BREEDING

19. What kind of breeding methods do you use on your farm?

0. Artificial insemination: ……..

1. Natural methods……..

20. If natural breeding is used, where do you get the bulls?

0. Own…….

1. Borrowed……

3. Hired……..

21. If artificial insemination (AI), who provides the AI service?

0. Government…….

1. Other provider …… ….. Specify…….

22. Where do cows calve?

0. Calving pens…….

1. On pasture……

23. How do dispose the afterbirth

(placenta)?.....

24. Who assists the cows when calving?

0. Husband…….1. Wife…….

3. Children……..4. Hired caretaker……..

5. Veterinary officer…….

25. Who disposes the afterbirth?

0. Husband…….1. Wife……..

2. Children……..3. Hired caretaker……..

4. Veterinary officer……..

26. Have any of your animals aborted in the last few years?

0. No……..

1. Yes: ……..If yes, which (species) aborted?

0. Cow……..….1. Ewe…….2. Doe……..

[For farms that have had abortion before]:

27. Was a vet informed?

0. No……. Why……..

1. Yes…… What was the diagnosis if any…….

28. How was the aborted foetus disposed?

0. Given to dog or other animal……

1. Buried

2. Burned

3. Left in pasture

4. Other ……..Specify……

28. Who disposed the foetus?

0. Husband…….

1. Wife…….

2. Children……..

3. Hired caretaker……..

4. Veterinary officer

29. How did they handle the aborted foetus?

0. With bare hands……

1. Wore gloves…….

2. Other ……..Specify…….

HOUSING

30. Where are animals kept overnight?

0. In pens……..…

1. On pasture……..

31. How is the slurry removed?

0. Taken to the fields……1. Left in pens……..…

D) MARKETING

32. Do you sell milk?

0. Yes……..

1. No……..

33. If yes, where do you sell milk?

0. Local community…….

1. Commercial processor……

34. Do you boil milk before consuming?

0. Yes……..if yes, for about how long do you leave the milk boiling…….
1. No……

35.Do you prepare sour milk (mala or musik)

0. Yes……..

1. No……..

36. Do you boil milk before making sour milk (‘mala or mursik’)?

0. Yes…….

1. No…….

35. Did you buy animals in the last three years?

0. No…….

1. Yes…….

36. What is the source of your stock?

0. Animal market……

1. Neighbours……

2. Others specify……

37. Did you sell any animals in locality during the last two years?

0. No…….

1. Yes…….

DISEASE CONTROL

19. Do you carry out any tick control in your animals?

0. Yes……

1. No…….

20. If you control ticks in your farm, which methods do use?

0. Communal dip tank……..

1. Own spray…….

20. Where do you normally receive veterinary support?

0. Veterinary Department……..

1. CBAHW (Community Based Animal Health Workers/Private Animal Health providers (AHAs)……..

2. None of the above……

21. Do you keep any written records?

0. No……

1. Yes…… Which ones…….

22. Have you heard of brucellosis?

0. No…….. 1. Yes……..

23. If yes, have you ever vaccinated your animals against brucellosis?

0. No……..If no, why?.....

1. Yes……

24. If yes, how do livestock become infected by brucellosis?.....

25. Can brucellosis be transmitted from a pregnant animal to its calf/kid?

0.Yes……..

1. No……..

2. I do not know……..

26. Can brucellosis be transmitted from an infected animal to an uninfected animal during mating?

0. Yes……

1. No……

2. I do not know…….

27. How do you protect your animals from being infected with brucellosis?

……

……28. Have you ever heard about a vaccine against brucellosis?

0. Yes……..

1. No…….

29. If yes, have your livestock been vaccinated against brucellosis?

0. Yes ……. 1. No …….

If the farmer knows about brucellosis vaccination and has not vaccinated his livestock ask the following question

30. Why have you not vaccinated the animals?

0. Vaccines not available…….
1. Vaccines are expensive…..
2. Others (specify)……

D) MARKETING

31. Do you sell milk?

32. If yes, where do you sell milk?

0. Local community…….

1. Commercial processor……

33. Do you boil milk before consuming?

0. Yes……..

1. No……

34. Do you prepare sour milk (mala or musik)

0. Yes…….

1. No……

35. Do you boil milk before making sour milk (‘mala or musik’)?

0. Yes…….

1. No…….

36. Did you buy animals in the last three years?

0. No…….

1. Yes…….

37. What is the source of your stock?

0. Animal market……

1. Neighbors……

2. Others specify……

38. Did you sell any animals in locality during the last two years?

0. No…….

1. Yes…….

39. Do you hire animals from neighbours for use?

0. No……

1. Yes…….

40. Has any member of your family been sick of brucellosis?

0. Yes……..

1. No……

41: If yes, who in the family?

0. Child……
1. Mother…….
2. Father……
3. Care taker

42. Did the person get medical attention?

0. Yes…….

1. No…….

2. Used herbal drugs……...

43. What is your general perception on brucellosis?

……

Thank you.