ESTIMATING THE PREVALENCE OF BOVINE TUBERCULOSIS (BTB) USING INDIRECT ELISA TEST IN SELECTED COUNTIES OF KENYA

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DECLARATION

This thesis is my original work and has not been presented in any other University.

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DEDICATION

I dedicate this work to my dear wife Rachael Ndune who always supported me in a special way and her words of encouragement kept ringing in my ears. I also dedicate it to my loving parents Samuel and Mary Muasya who were very understanding and supportive.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS AND ACRONYMS

ASDSP - Agricultural Sector Development Support Programme

BCG - Bacillus Calmette Guérin

BTB - Bovine Tuberculosis

CBPP - Contagious Bovine Peuro-Pneumonia

CCT - Comparative Cervical Test

CFT - Caudal Fold Test

CIDT- comparative intradermal tuberculin

CIT - Cervical Intradermal Test

CITT - Comparative Intradermal Tuberculin Test

CVL - Central Veterinary Laboratory

DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen

ELISA - Enzyme Linked Immuno Sorbent Assay

HIV - Human Immunodeficiency Virus

IFN - Gamma-Interferon Assay

MOTT - Mycobacteria Other than Tubercle Bacilli

MRI - Magnetic Resonance Imaging

MTBC - Mycobacterium Tuberculosis Complex

NTM - Non-Tuberculous Mycobacteria

O.I.E - Office International Epizooties

PCR - Polymerase Chain Reaction

PPD - Purified Protein Derivatives

PTB - Paratuberculosis

ROC - Receiver Operating Characteristics

S/P – Sample to Positive ratio

SITT - Single Intradermal Tuberculin Test

TMB - Tetramethylbenzidine

TST - Tuberculin Skin Test

WHO - World Health Organization

ABSTRACT

Bovine tuberculosis (BTB) is an important zoonotic disease whose eradication has proved problematic due to the challenges in effective screening and diagnosis. A study to determine the presence and prevalence of bovine tuberculosis antibodies in seven Counties of Kenya was carried out between August and December 2013.

The study used sera that were collected from seven counties in Kenya between 2011 and August 2013 and stored at the Central Veterinary Laboratories (CVL), Kabete Nairobi. The samples were sorted and only those with well recorded identity selected for testing. The study utilized a newly developed antibody ELISA Kit. A total of 644 bovine serum samples were tested using MPB70 and MPB83 recombinant proteins as capture antigens to detect the presence of BTB antibodies. The prevalence was determined as a proportion of the positive samples out of total samples tested and the data were exported to SPSS 16.0 version for analysis. Descriptive statistical analysis were then done to determine the proportions and distribution across the counties. Graphs and charts were drawn using Microsoft excel.

The study showed a prevalence of 3.57% (23/644) with Wajir County having the highest prevalence of 4.7% (4/85) and Kilifi county having the least at 0% (0/64). On Chi-square and Fishers exact test there was no significant association between BTB infection and the age of animals, categorized as young (below 3 years) and old (above 3 years of age) at (P= 0.05507). Breed and sex indicated no significant association at (P=0.4111) and (P=0.2354) respectively. Kappa statistics for ELISA test and Tuberculin skin test (TST) showed a strong agreement for the two *M. bovis* tests at K= 0.65. This study has documented the prevalence of BTB in cattle for the first time in Kenya utilizing a more specific antibody ELISA. This method of diagnosis presents a quicker and a cheaper way that can be complementary or an alternative to the screening of BTB in live animals. This study also concludes that BTB is present in Kenyan

cattle populations without a significant relation to age sex and breed of animals. On the basis of these findings, this study recommends a survey of all Counties and wildlife reserves to map out prevalence status in the whole country. It also recommends specific policy formulation on the control of BTB in Kenya.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Bovine tuberculosis (BTB) is a chronic infectious disease of cattle caused by *Mycobacterium bovis*. It is usually characterized by development of avascular granulomatous nodules known as tubercles. Most body tissues and organs can be affected, but most frequently lesions are seen in the lymph nodes of the head and thorax, in the intestines, lungs, liver, spleen and peritoneum (Clerke, 1998). *Mycobacterium bovis* is virulent for cattle but can infect other animals and humans causing disease and pathology similar to *Mycobacterium tuberculosis*, which is mainly pathogenic for man (Kaneene and Pfeiffer 2006; Thoen *et al.*, 2006). *Mycobacterium caprae* has been identified as a cause of BTB in some parts of central Europe and its Infection is not substantially different from that caused by *Mycobacterium bovis* with similar tests being used for its diagnosis (Naranjo *et al.*, 2008). Bovine tuberculosis can occasionally assume a more progressive course however in many cases, the course of the infection is chronic and signs may not be seen. In advanced cases when tubercles have formed in many organs clinical signs may be present and varied (World organization for animal Health (O.I.E., 2009). The genus *Mycobacterium* consists of more than 100 species. Most of these are found in the environment and are not normally pathogenic to humans or animals; however a small number of them are pathogenic to various species of animals (Sam *et al.*, 2011).

Bovine tuberculosis is endemic in many areas of the world and poses significant losses directly by reduced cattle productivity, control and eradication programs as well as being a zoonotic infection in man. In the early 1990's the worldwide annual loss due to Bovine

tuberculosis was estimated to be over 3 billion US Dollars (Steele, 1995). In the United States of America (USA) and Great Britain (GB), more than 150 million US Dollars were spent for the eradication of BT including emergency funding in the year 2008 and 2009 (Anon, 2010). There is little known and published data about the cost of BTB in the developing countries. Bovine tuberculosis is endemic in Africa where a great percentage of the population depends on livestock directly or indirectly for a living. In the subtropical African countries particularly, there is very little or no presence of control programmes posing a high risk of human infection and affecting international trade on livestock products (Biet *et al.,* 2005). The prevalence of *M. bovis* has been reported at 2.05% for the first time among slaughter cattle in Kenya (Gathogo *et al.,* 2012).

Test and slaughter policy has been the main method for control and eradication of BTB for many decades in many countries. Different levels of success have been achieved in the countries that have been consistent. However despite the intensive eradication efforts, BTB is still a problem of global proportions. There are several reasons that have been identified as the major causes of difficulty in eradication. These include; the presence of wildlife reservoirs, increased animal movement, pastoral practices and limitation of available diagnostic and screening methods (Schiler *et al.,* 2010). Diagnosis of bovine tuberculosis remains extremely challenging and there is currently no one single test that can fulfil all the criteria necessary to identify all infected animals. Therefore, this has necessitated a combination of approaches to achieve a considerable level of diagnosis. With the possibility of animals remaining infectious without manifesting clinical signs, the identification of infected animals and herds is so crucial. Agent identification through culture, microscopy or nucleic acid recognition is important for confirmation during post mortem and not useful to live animals (Sam *et al.,*

2011). These limitations continually call for research and exploration of novel ways and approaches in diagnosis of BTB in live animals.

1.2 Statement of problem

Bovine tuberculosis has been a persistent infection in many areas of the world despite continued efforts of eradication. In the developing world the situation is uncertain due to very little work in terms of research on prevalence of the infection in herds. Minimum existence of screening and eradication programs in African countries makes it difficult to assess the cost of BTB (Ayele *et al.*, 2004). Meaningful success in the control of this infection can be tagged on a reliable diagnosis that can identify all the individuals transmitting the disease (O.I.E., 2009). While it may be more straightforward to measure a test by its ability to identify all infected animals, this may not be the optimal approach for BTB since it will lead to the rejection of many tests with apparent poor sensitivities. The control and eradication efforts for BTB have been made difficult by the limitations of diagnosis in terms of the cost and reliability of available methods. However the dilemma may not be solved at once, there is need to check the use of new methods either as alternatives or complementary tools (Schiler *et al.*, 2010). The status of BTB in Kenya is not well understood in terms of prevalence, there is need to establish the presence and rate of infection in the country using a more cost effective test.

1.3 Objectives of the study

1.3.1 General objective

To estimate the prevalence of Bovine Tuberculosis by the use of antibody ELISA as a cheaper and a quicker screening method in Kenya and compare seroconversion in the selected regions.

1.3.2 Specific objectives of the study.

i. To estimate the prevalence of Bovine Tuberculosis using the new antibody ELISA in selected samples from different counties of Kenya.

ii. To determine the levels of seroconversion to BTB from the different selected areas of Kenya and the associated risk factors in different counties of Kenya.

1.4 Justification of the study

The limitations of available BTB diagnostic tests to provide satisfactory sensitivity calls for a rethink in the approaches currently employed. There is need to use existing diagnostic tools in combination and to assess the practicability of employing novel methods for the purpose of screening animals for BTB. Tuberculin test has been the accepted test, however it is very involving and time consuming to carry out. There is therefore need to evaluate the feasibility of using the recently approved antibody ELISA utilizing MPB70 and MPB83 antigens as an alternative or a complimentary test to tuberculin skin test in screening of BTB.

1.5 Scope of the study

This study was restricted to the use of a new indirect ELISA test kit to estimate prevalence of Bovine tuberculosis in selected counties of Kenya. It involved the application of IDEXX ELISA kits (IDEXX Technical Services - USA) to check the presence of Bovine Tuberculosis antibodies in serum. This generated data which was used to determine prevalence in the selected areas and to compare antibody reaction levels across the areas and the different risk factors.

CHAPTER TWO

2.0 Literature review

2.1 Definition and presentation of Bovine Tuberculosis

Bovine tuberculosis (BTB) is a chronic respiratory disease, which is generally difficult to diagnose clinically. However, chronic cough, emaciation, inappetance, and other signs of pneumonia such as respiratory distress are observed at relatively late stages of the disease in cattle (Ayele *et al*., 2004). *Mycobacterium bovis*, a member of Mycobacterium tuberculosis complex (MTBC) bacterial strains, is the causative agent of BTB. Mycobacterium Tuberculosis Complex is a big group of closely related pathogens causing tuberculosis disease with similarity of pathology in various mammalian species (Smith *et al.,* 2006; Wirth *et al.*, 2008).

Bovine tuberculosis takes a similar form to many other debilitating and chronic infections like such as Contagious bovine pleural-pneumonia (CBPP), Trypanosomiasis and chronic endoparasitism, which can be difficult to distinguish. The main Pathological syndrome is formation of granulomatous lesions in different tissues and organs of the body. These lesions can either lead to; extensive necrosis, can regress, calcify or liquefy leading to cavity formation. The most common site for tuberculous lesions in the bovine during meat inspection is both upper and lower respiratory tract and the associated lymph nodes (Cassidy, 2006). It can also be found disseminated in other organs when it develops a systemic form (Coetzer and Tustin, 2004)

2.2 Importance of Bovine Tuberculosis

Bovine tuberculosis is a significant zoonosis that can spread from animals to humans, typically by the inhalation of aerosols or the ingestion of unpasteurized milk (Grange, 2001). Bovine tuberculosis is of significant economic importance as it causes considerable loss in milk and meat production and animal reproduction. International trade and other economic sectors may be indirectly affected by the disease through enforced restrictions (Zinsstag *et al.*, 2006). In developed countries, eradication programs have reduced or eliminated tuberculosis in cattle and human where disease is now rare; however, reservoirs in wildlife can make complete eradication difficult (Reviriego and Vermeersch, 2006). Bovine tuberculosis is still common in less developed countries, and economic losses can occur from livestock deaths, chronic disease wastage and trade restrictions. In some situations, this disease may also be a serious threat to endangered species with a risk of unpredictable consequences for entire ecosystems. An example of a threat to an ecosystem is the case of Kruger National Park in South Africa where 38% of buffalos were found to be infected with strains of *M. bovis*, originally introduced from domesticated cattle (Renwick *et al.,* 2006).

BTB is of special importance in many countries of Africa especially the arid and semi-arid regions, where over 50% of all African cattle, sheep and goats are kept with millions of livelihoods dependent on livestock farming (World Health Organization (WHO), 2006; Otte and Chilonda, 2002). A higher susceptibility of HIV-infected persons to *M. bovis* infections has also been observed as a major concern (Lobue, 2006). Human patients with *M. bovis* infections have been reported to be 2.6 times more likely to die during treatment than persons with *M. tuberculosis* infections (Rodwell *et al.,* 2008). In this situation, the poor in developing countries are believed to be at the highest risk of contracting zoonotic tuberculosis and the

adverse consequences (W.H.O., 2006). The above is aggregated by the fact that most of these persons live close to cattle in pastoral systems and consume raw milk

2.3 Aetiology Bovine Tuberculosis

Bovine tuberculosis results from infection by *Mycobacterium bovis*, a gram positive, acid-fast bacterium in the Mycobacterium tuberculosis complex of the family Mycobacteriaceae (Kahn *et al.,* 2003).

2.3.1 Pathogenesis of Bovine tuberculosis

There are several ways in which cattle can become infected with *M. bovis***,** but the main route of infection in cattle is by inhalation (Pollock and Nail 2002). This mode of transmission is dominant where intensive farming is practised. Lesion distribution and pathology in this case predominantly involve the upper and lower respiratory tract and associated lymph nodes**.** (Nail *et al.,*1994). in some situations tuberculin reactors have shown absence of lung lesions; however, lesions within the lung parenchyma can be too small to be easily detected during meat inspection (Mcllroy *et al.,* 1986). Inhalation is considered the most probable and principal mode of transmission for *M. bovis* between cattle (Whipple *et al.,* 1996). Inhalation of tubercle bacilli, possibly a single *M. bovis* bacillus from aerosol droplet lodges within the respiratory tract (Nail *et al.,* 1991). In the alveolar surface of the lung, Bacilli are phagocytosed by macrophages, and subsequently interact with cells involved in innate and acquired immune responses in tissues or draining lymph nodes.

This often results in the formation of a nonvascular nodular granulomatous lesion known as tubercles (Pritchard, 1988). These Characteristic lesions occur mostly in lungs, retropharyngeal lymph nodes, bronchial lymph nodes and mediastinal lymph nodes. Lesions

have also been found in the mesenteric lymph nodes, spleen, liver, serous membranes, pleura and other tissues (Blood and Radostis, 1989). Activated mononuclear macrophages are considered most important in protecting the host against *M. bovi.* Macrophages are involved in processing mycobacterial antigens and presenting them to T-lymphocytes, which are the key recognition cells for immune response to mycobacteria (Theon and Himes, 1986). The granulomatous lesion caused by *M. bovis* in have a characteristic centre of caseous necrosis, with some calcification and a boundary of epithelioid cells (Theon and Steele, 1995). Primary lesions in cattle are rarely contained by the immune response and dissemination occurs through bronchi, lymphatic ducts or spread by haematogenous. The extent to which the lesions spread within the lungs or other tissues determine the presentation of clinical outcome (Theon and Himes, 1986).

2.3.1 Taxonomy of *Mycobacterium*

Mycobacteria belong to the Order Actinomycetales, Family Mycobacteriaceae and Genus *Mycobacterium*. The genus *Mycobacterium* consists 127 species (excluding subspecies) according to the latest approved list of bacterial species (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), 2014). Other previously considered species have been found to be a complex of several closely related species, through biochemical and genetic analyses. An example is *Mycobacterium tuberculosis-complex* (MTBC) which consists: *Mycobacterium tuberculosis*, *Mycobacterium microti*, *Mycobacterium africanum* and *Mycobacterium bovis*. *Mycobacterium bovis* has been further divided into *Mycobacterium bovis*, *Mycobacterium pinnipedii*, *Mycobacterium canetii*, and *Mycobacterium caprae* (Warren *et al.,* 2006). Similarly *M. avium*-complex has been divided into the *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (Butler *et al.,* 1992).

An early technique used to classify mycobacteria was Adansonian taxonomy which was based on the use of both biochemical and cultural properties. However this method is not popular today due to characteristic similarities of subdivided groups. Another criteria of the mid-1950s by Dr. Ernest Runyon grouped *Mycobacterium* excluding those in the *M. tuberculosis* complex and the noncultivable (example, *Mycobacterium leprae*). In this classification *Mycobacteria* were divided into four groups based on pigmentation properties and growth rates.

Over 40 species of mycobacteria were added to the lists of bacterial names approved in the 1980s (Grange, 1996; Shinnick and Good, 1994). Other *Mycobacteria* outside the tuberculosis or leprosy complex have been described by a variety of nomenclature; mycobacteria other than tubercle bacilli (MOTT), atypical mycobacteria, environmental mycobacteria or non-tuberculous mycobacteria (NTM). Most of the NTM species are considered nonpathogenic or opportunistic pathogens and cause disease when host-defenses are compromised (Wolinsky, 1979).

2.3.2 Bacterial characteristics, morphology and Physico-chemical properties

Mycobacterium bovis is a facultative intracellular, aerobic and gram-positive bacterium with slow growing dysgonic colony shapes on Lowenstein-Jensen medium (Kubica *et al.*, 2006). It has an unusual cell wall surface structure characterized by the formation of mycolic acids and a wide array of lipids. This feature gives it a waxy envelop that confers extreme hydrophobicity therefore making the bacteria acid- and alcohol-fast which is exploited to identify mycobacteria via the Ziehl-Neelsen staining technique. The surface lipids also have a potent biologic activity and are believed to contribute a major part in pathogenesis of mycobacterium infection (Glickman and Jacobs 2001).

Specific biochemical and metabolic properties can be used as basis to identify *Mycobacterium bovis.*; *Mycobacterium bovis* requires pyruvate as a growth supplement, it's negative for niacin accumulation and nitrate reduction, shows microaerophilic growth on Lebek medium and resistance to pyrazinamide. *Mycobacterium tuberculosis* is not supplemented by pyruvate on growth, it is positive for nitrate reduction and niacin accumulation, shows aerophilic growth on Lebek medium, and is sensitive to pyrazinamide (Kubica *et al.,* 2006 and Cole 2002). The validity of these mycobacterial characteristics is challenged in other studies (Niemann *et al.,* 2000). There are molecular markers that have been identified and techniques developed that help in the identification and differentiation of *Mycobacterium* spp. especially the members of the MTBC (Huard *et al.,* 2006**).**

2.3.3 History and Evolution of *Mycobacteria bovis*

The MTBC has seven distinct bacterial species named *M. tuberculosis, M. bovis, M. caprae, M. canettii, M. microti, M. africanum* and *M. pinnipedii*. Members of this complex show a certain level of host tropism despite a remarkable sequence similarity of at least 99.9% (DSMZ 2014) with *M. bovis* most commonly affecting cattle, *M. tuberculosis* affecting humans and *M. microti* frequently being found in voles among others. Spill over infection and isolation in different hosts has been observed for most of the bacteria (Rachel *et al.,* 2002). The MTBC have been considered by some researchers as ecotypes, adapted to various hosts. An ecotype can be described as an individual that occupies a particular ecological niche and is usually identified by use of genomic markers **(**Liebana *et al.,* 1996). *Mycobacterium bovis* has a smaller genome size compared to *M. tuberculosis* and due to chromosomal similarity it has been inferred that *M. bovis* originated from *M. tuberculosis* like ancestor. This has led to a

hypothesis that tuberculosis first affected humans before infection jumped to animals (Dick *et al.*, 1997).

2.4 Bovine tuberculosis Epidemiology

2.4.1 Geographical distribution Bovine Tuberculosis

Bovine tuberculosis was once found worldwide; however, control programs have eliminated or markedly reduced this disease from domesticated animals in many countries (OIE, 2009). Nations currently classified as tuberculosis-free include Israel, Iceland, Estonia, Switzerland, Denmark, Australia, Sweden, Norway, Finland, Singapore, Austria, Latvia, Luxembourg, Slovakia, Lithuania, Canada, Barbados, Jamaica and the Czech Republic. This status is mainly confirmed after countries achieving prevalencies of less than 0.1% for a period of six years (OIE, 2012). In other countries active Eradication programs are in progress, these include; some European countries, Japan, New Zealand, the United States, Mexico and some countries of Central and South America (Reviriego *et al.*, 2006; Ryan *et al.*, 2006).

Although bovine tuberculosis has been eradicated from the majority of U.S.A. States, a few infected herds continue to be reported, and a number of states periodically lose their disease-free status. In particular, a focus of infection in wild white-tailed deer has complicated eradication efforts in Michigan. Similar problems exist with infected badgers in the U.K. and Ireland, and infected brush-tailed opossums in New Zealand. Bovine tuberculosis is still widespread in Africa, parts of Asia and some Middle Eastern countries (Ayele *et al.*, 2004).

2.4.2 Host range of Bovine Tuberculosis

Cattle are the primary hosts for *M. bovis*, but other domesticated and wild mammals can also be infected. Species reported to be spillover hosts include sheep, goats, horses, pigs,

dogs, cats, ferrets, camels, llamas, many species of wild ruminants including deer and elk; elephants, rhinoceroses, foxes, coyotes, mink, primates, opossums, otters, seals, sea lions, hares, raccoons, bears, warthogs, large cats (including lions, tigers, leopards, cheetahs and lynx) and several species of rodents. Most mammals may be susceptible (Nishi *et al.*, 2006; O'Brien *et al.*, 2006).

Little is known about the susceptibility of birds to *M. bovis*, although they are generally thought to be resistant. Experimental infections have recently been reported in pigeons after oral, intratracheal or intraperitoneal inoculation. Some avian species, including mallard ducks are resistant to experimental infection (Fitzgerald *et al*, 2003; Fitzgerald *et al*, 2005).

2.4.3 The role of wildlife in transmission of Bovine Tuberculosis

Mycobacterium bovis infections in wildlife can affect the ecosystem and the disease constitutes a threat to endangered species and can hamper BTB eradication and control programmes in domestic cattle. Tuberculosis in wildlife has posed serious difficulties for BTB control in the UK and Ireland where the badger represents an important disease reservoir (Van de Vosse *et al*., 2003). In the New Zealand, the brush-tailed possum (*Trichosurus vulpecula*) is a maintenance host of *M. bovis* (Tortli *et al.*, 2005). White-tailed deer (*Odocoileus virginianus*) has been identified as a reservoir for *M. bovis* in Michigan, USA (Wolinsky, 1995) and in central and Eastern Europe, *Mycobacterium bovis* has been isolated from wild boars (*Sus scrofa*) (Van de Vosse *et al*., 2005). There have been an increasing amount of published reports describing *M. bovis* isolation on a large variety of mammalian hosts in different regions throughout the world (Bartralot *et al*., 2005). Relatively little is known about the importance of *M. bovis* infections in wildlife on the African continent. However, available

data from southern Africa suggests that the prevalence of wildlife tuberculosis has reached a dramatic dimension with the African buffalo (*Syncerus caffer*) being considered the most important reservoir of *M. bovis*. Others that have been thought to be reservoirs are the Kafue lechwe (*Kobus leche*) and greater kudu (*Tragelaphus strepsiceros*). Bovine tuberculosis in Africa has been described as a multi species host pathogen due to the many hosts (Marchetti *et al*., 2004).

In the Kruger National Park in South Africa, a gradient of infection from South to North has been noted with 38% of the buffalos being infected in the Southern region, 16% in the Central region and 2% in the Northern region (Marchetti *et al*., 2004). Spillover to carnivores such as Lions (*Panthera leo*), Leopards (*Panthera pardus*), Cheetahs (*Acinonyx jubatus*) and Hyenas (*Crocuta crocuta*) was also expected due to their position in the food chain (Renwick *et al.,* 2006). Other animal species like the Chacma baboon (*Papio ursinus*), and warthog (*Pharcovhoerus aethiopicus*) have also been reported to be infected with Bovine tuberculosis (Bartralot *et al*., 2005). *Mycobacterium bovis* has also been isolated from buffalo and warthog in the Ruwenzori National Park in Uganda (Johnson *et al.,* 2006).

2.4.4 Mode of transmission of Bovine tuberculosis

Mycobacterium bovis can be transmitted by the inhalation of aerosols, ingestion, or through breaks in the skin. The importance of these routes varies between species. Bovine tuberculosis is usually maintained in cattle populations, but a few other species can become reservoir hosts (Menzies and Neil, 2000). Populations of spillover hosts do not maintain *M. bovis* indefinitely in the absence of maintenance hosts, but may transmit the infection between their members (or to other species) for a time. Some spillover hosts can become maintenance hosts if their population density is high (Lyashchenko *et al.,* 2008).

Cattle shed *M. bovis* in respiratory secretions, feces and milk, and sometimes in the urine, vaginal secretions or semen. Large numbers of organisms may be shed in the late stages of infection, however asymptomatic and anergic carriers also occur. In most cases, *M. bovis* is transmitted between cattle in aerosols during close contact (Menzies and Neil, 2000). Some animals become infected when they ingest the organism; this route may be particularly important in calves that nurse from infected cows. All infected cattle may not transmit the disease. Non-human primates are usually infected by inhalation. Badgers with advanced disease can shed *M. bovis* in the urine, and organisms have been found in the feces thus infecting cattle as they feed on contaminated fields (AHD, 1986)

Mycobacterium bovis can infect humans, primarily by the ingestion of unpasteurized dairy products but also in aerosols and through breaks in the skin. Raw or undercooked meat can also be a source of the organism. Person-to-person transmission is rare in immunocompetent individuals, but *M. bovis* has occasionally been transmitted within small clusters of people, particularly alcoholics or HIV-infected individuals. *Mycobacterium bovis* can survive for several months in the environment, particularly in cold, dark and moist conditions. At 12-24°C (54-75°F), the survival time varies from 18 to 332 days, depending on the exposure to sunlight. This organism is infrequently isolated from soil or pastures grazed by infected cattle (Jackson *et al.*, 1995). *Mycobacterium bovis* can survive in natural pastures for, at most, a few weeks (Williams and Hoy, 1930).

2.5 Diagnosis of Bovine Tuberculosis

Diagnosis of BTB by clinical examination is very limited since most animals infected with *M. bovis* do not show clinical signs of the disease and that there are no pathognomonic signs in cattle (OIE, 2009). Direct identification of the organism following post mortem

examination and sampling in conjunction with ante-mortem immunological tests where positive animals are slaughtered or through passive surveillance as part of routine meat inspection protocols is commonly practised. Broadly, there are two approaches to the direct diagnosis of Mycobacterium bovis in cattle. They are based upon the detection of the organism either through direct culture or using molecular methods. In contrast, the direct diagnosis of TB in humans is largely based upon bacteriology and microscopy by use of a sputum sample. Imaging such as radiography and magnetic resonance imaging (MRI) is also used when available. The above tests are not practically applicable in testing of animals (Ayele *et al*., 2004). Tuberculin skin tests and interferon-gamma test are mostly employed to live animals. Polymerase Chain Reaction (PCR) and culture of *Mycobacteria* are used to confirm diagnosis mainly on post mortem samples after slaughter (Sam *et al.,* 2011).

2.5.1 Ante-Mortem Laboratory diagnosis

These diagnostic methods can be subjected to live animals through delayed hypersensitivity testing or blood sampling for laboratory assays. Tuberculin skin test (TST) represents the OIE prescribed test for international trade and constitutes a delayed type hypersensitivity test (Anon, 2008a). It measures dermal swelling due to cell-mediated immune response (CMI) 72 hours after intradermal injection of purified protein derivative (PPD). It is usually done in the skin of the caudal fold (CFT) or neck (CIT). The skin of the neck is regarded to be more sensitive to a tuberculin-related hypersensitivity reaction than the skin of the caudal fold. (Aranaz *et al.,* 2006)

Because animals are frequently exposed to or infected with various non-tuberculous mycobacteria, cross-reactive responses to PPD may occur. Many antigens within the PPD are shared between non-tuberculous and tuberculous mycobacteria. The comparative cervical test

(CCT) is used to differentiate between animals infected with *M. bovis* and those sensitized to PPD as a result of exposure to other mycobacteria (Anon, 2008b). Advantages of the TST and reasons for its wide use are; low costs, availability, long time of use experience and lack of alternative ante mortem diagnostic methods (De la Rua-Domenech *at al.,* 2006). Purified protein derivative (PPD) are prepared from heat killed cultures of respective mycobacterium species. This antigen has got high sensitivity and low specifity due to cross reactivity caused by the common epitopes in many *Mycobacterium* species (Smith *et al.,* 2006). Evidence has shown considerable variation in the quality of PPD tuberculin that is produced in different areas of the world, with some exhibiting very poor potency and likely to affect sensitivity (Sam *et al.,* 2011; Schiller *et al*., 2010b).

Gamma-interferon assay is an alternative test for international trade in which the release of a lymphokine gamma interferon (IFN-γ) is measured in whole-blood culture. The assay is based on the release of IFN-γ from sensitised lymphocytes during a 16 to 24 hour incubation period with PPD (Wood *et al.,* 1990). The test makes use of the comparison of IFN-γ production following stimulation with avian PPD and bovine PPD. The detection of bovine IFN-γ is carried out with a sandwich ELISA that uses two monoclonal antibodies to bovine IFN-γ. The blood samples should be transported to the laboratory and the assay protocol set up not later than 24 hours after blood collection (Coad *et al.,* 2007; Ryan *et al.,* 2000). Concerns about the accuracy have been expressed in some areas where non-specificity is prevalent. The use of defined mycobacterial antigens such as ESAT 6 and CFP-10 shows promise for improved specificity and these antigens have been employed in a number of countries such as the United Kingdom and New Zealand for serial testing (Buddle *et al*., 2001).

Lymphocyte proliferation assay is an in-vitro assay compares the reactivity of peripheral blood lymphocytes to *M. bovis* (PPD-B) and *M. avium* (PPD-A). The test can be performed on whole blood (Buddle *et al*. 2001) or purified lymphocytes from peripheral blood samples (Griffin *et al.,* 1994). These assays aim at increasing the specificity of the test by removing the response of lymphocytes to cross-reactive antigens associated with non-pathogenic species of *Mycobacteria* to which the animal may have been exposed. Results are analysed as the difference in value obtained response to PPD-B minus PPD-A response (Coad *et al.*, 2007). The test has not been used for routine diagnosis because it is time consuming and the complexity of laboratory execution requiring long incubation times and the use of radio-active nucleotides. Like the IFN-γ test, the lymphocyte proliferation assay should be performed shortly after blood is collected (Griffin *et al.*, 1994).

2.5.2 Post mortem *Mycobacterium bovis* Identification

These are considered very sensitive and include; Polymerase Chain Reaction (PCR), Microscopic examination and mycobacterial culture (Olea-Popelka *et al.*, 2008). Microscopic examination of *Mycobacterium bovis* can be demonstrated on direct smears from clinical samples and on prepared tissue materials (Whipple *et al.*, 1996). The acid fastness of *M. bovis* is normally demonstrated with the classic Ziehl–Neelsen stain and fluorescent acid-fast stain. For culture and isolation, the suspect sample sediment is inoculated on to egg-based media, such as Lowenstein–Jensen, Coletsos base or Stonebrinks. These media contains either pyruvate or glycerol. An agar-based medium such as Middlebrook 7H10 or 7H11 or blood based agar medium (Cousins *et al,* 1989) may also be used. Cultures are incubated for a minimum of 8 weeks to 12 weeks at 37°C with or without CO2. The media should be in tightly closed tubes to avoid desiccation. Slopes are examined for macroscopic growth at

intervals during the incubation period (Cousins *et al.,* 1989). When growth is visible, smears are prepared and stained by the Ziehl–Neelsen technique. Growth of *M. bovis* generally depends on the media used and occurs within 3 to 6 weeks of incubation. Liquid culture can also be used where growth is measured by radiometry or fluorometry means (Sam *et al.,* 2011)

The polymerase chain reaction (PCR) is nucleic acid recognition methods for identifying the presence of bacteria specific sequences of DNA. There has been a lot of work on developing robust tuberculosis DNA detection methods in both human and veterinary medicine (Miller *et al.,* 2002). A study utilizing optimized PCR test on bovine post mortem tissues resulted in test sensitivity of 65% compared to conventional pathology and culture (Parra *et al.,* 2008). Another study on budgers' demonstrated unreliability of PCR to diagnose BTB (Anon, 2010). A new 2 hour PCR automated test protocol the Cepheid GeneXpert System (Sunnyvale California) has been developed, for the diagnosis of M. tuberculosis complex infections in humans using sputum samples (Helb *et al.,* 2010). The method has shown promising results with up to 98.2% sensitivity in smear positive patients (Boehme *et al.,* 2010) whether this can be useful for *M. bovis* remains to be determined. These forms of diagnosis have been used in slaughter house surveys to give estimates of animals infected. The main challenge with this category of diagnosis is that sampling may not be representative of the herd structure (Pollock *et al.,* 2005).

2.5.3 Limitations for *Mycobacterium bovis* diagnosis

Despite being the recommended and most popular method Tuberculin skin test (TST) has many known limitations including difficulties in administration and interpretation of results, its need for a second-step visit and its low degree of standardization (De la Rua-Domenech *et al.,* 2006). An alternative novel ante mortem test is the IDEXX indirect ELISA

which utilizes more specific capture antigens for detection of *M. bovis* antibodies. There are several advantages to using serological methods, e.g. ELISA, for the diagnosis of BTB. Sampling can be repeated as often as necessary without affecting the immune status of the animal. The tests require only one handling of the animals and only one visit of the veterinarian to the farm. The interpretation is based on numerical S/P values that makes it more objective than the TST (Lilenbaum *et al.,* 2001). In the indirect ELISA technique, binding of specific antibodies to recombinant antigens is measures (Lilenbaum and Fonseca, 2006). The MPB70 protein, represents approximately 10% of the PPD (Wiker and Harboe 1992) and has been identified as a B-cell target in bovine tuberculous and a very specific protein (Lightbody *et al.*, 2001).

The use of IDDEX antibody ELISA method has not been documented in Kenya before and presents a good opportunity for its application. This will be a good chance to estimate Prevalence in the selected counties and to compare its performance with Tuberculin Skin Test. There are very little publications available in Kenya about the prevalence of bovine tuberculosis compared to other countries in the region. With many zoonotic cases and several deaths from BTB reported in Kenya between the years 2007 and 2009 (WAHID, 2013) there is need to survey the animal prevalence status. This was a good start in developing Data in the country.

CHAPTER THREE

3.0 MATERIAL AND METHODS

3.1 Study Area

The study was carried out in seven Counties of Kenya. Kenya has 47 counties all of which practice livestock farming at varying management systems. In the arid North there is mainly pastoral nomadic form of livestock keeping while in the Central, part of central and south Rift Valley and Western counties, there is more intensive forms of cattle husbandry. Samples used in the study were obtained from Seven Counties based on availability. These counties included: Likipia, Kilifi, Taita Taveta Wajir, Kajiado, West Pokot and Kwale (Figure 3.1).

Laikipia County is located 260Km north of Nairobi in the along the equator and experiences a cool temperate climate with average of between 400mm and 750mm rainfall annually. There are many private wildlife conservation area and ranches with most of the residents in Laikipia North being pastoralists. Kilifi County is located 520Km South east of Nairobi and 30 Km North of Mombasa in the Coast region**.** The annual temperatures range between 21°C - 34°C and receives an average precipitation of 1300mm annually. Subsistent farming is practiced Livestock farming mainly being indigenous breeds. Taita Taveta County is 320Km to the South east of Nairobi in the Coastal region of Kenya. The county receives an average precipitation of 650 mm while the annual temperature varies from 18 to 25^0C. Livestock rearing system is mainly beef ranching and subsistence mixed farming.

Kwale County is located 520Km South east of Nairobi and 10Km south of Mombasa in the Coast region**.** Agriculture is mainly subsistent mixed farming with the county experiencing an average temperature of 24.2 ^{0}c and an average annual rainfall of 750mm.

Kajiado County extends 30 Km south of Nairobi to the Tanzanian border. Most parts of the county are Arid and Semi-Arid (ASAL) with livestock rearing being the predominant economic activity. Wajir County is 700Km to the north east of Nairobi. The county is mainly Arid and Semi-Arid (ASAL) receiving an average annually rainfall of 240mm. Livestock keeping is the main economic activity in the form of nomadic pastoralism. West Pokot County is located 500Km to the North west of Nairobi. Rainfall varies from 400mm to 1,500 mm per annum while temperatures range from 10 °C to 30 °C. Livestock farming is mainly nomadic pastoralism and mixed farming (ASDSP, 2014).

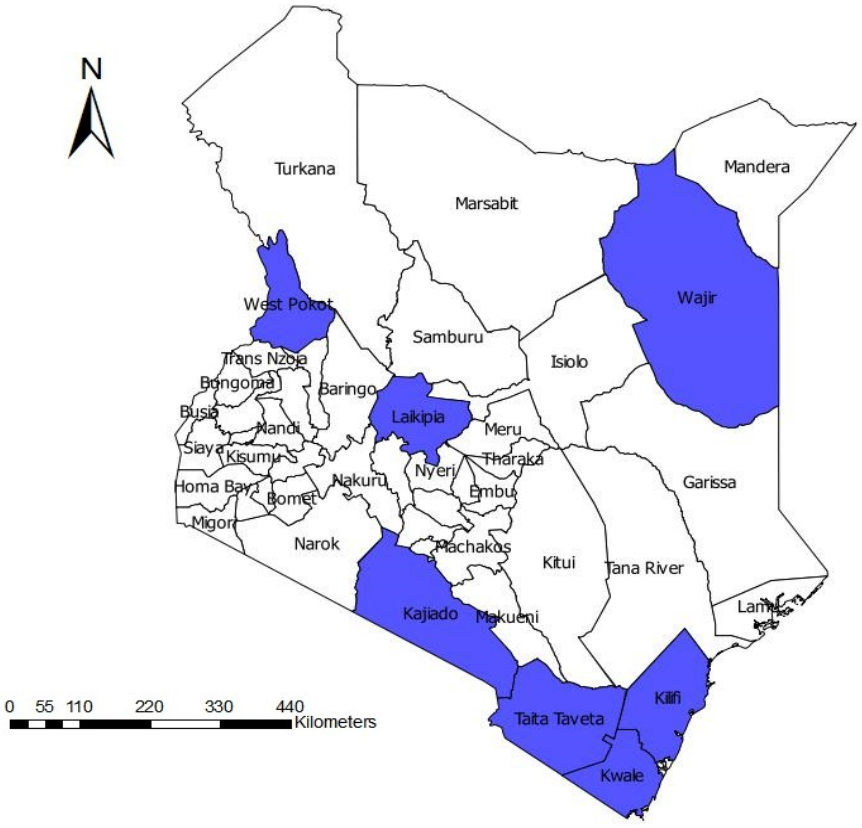

Figure 3.1 Map of Kenya showing the seven study counties used for the study

3.2 Study design

This was a cross sectional study to estimate the prevalence of Bovine Tuberculosis (BTB) using a new indirect ELISA test on serum samples. The ELISA test used the MPB70 and MPB83 recombinant proteins as capture antigens which is a complementary or alternative routine screening test. The serum samples were obtained from the Central Veterinary Laboratories (CVL) Nairobi to estimate prevalence of BTB in the seven counties of Kenya. The serum samples were collected from different localities/areas in the seven counties during routine Department of Veterinary Services livestock surveillance programmes. The details of interest recorded from the samples before testing were; the age of animal, County of origin, village code or ranch name, sex and animal identity. For Laikipia County there was extra information on breed of the animal, District of origin and BTB result from tuberculin skin test conducted before a serum sample was collected.

3.3 Sample size determination

The serum samples were obtained from the serum bank at the Central Veterinary Laboratories (CVL), Kabete Nairobi. The samples were earlier collected for a brucellosis surveillance project. The samples were collected throughout the country, with the unit of sampling being the randomly selected villages in every district from the year 2011 to August 2013.

The minimum sample size for this cross-sectional study was determined using the formula by Dohoo *et al.*, (2003) and were 87 as shown below:

$$n = \frac{(1.96)^2 p(1-p)}{L^2},$$

- Where L is the required precision, was assumed at 95%

- p is the anticipated prevalence. Assumed to be 6%

- $\frac{(1.96)^2 \times 0.06(1-0.06)}{(0.05)^2} = \frac{3.8416 \times 0.0564}{0.0025} = 86.66$

The sample size was however increased to 644 due to availability of the test kits. The samples were sorted and those that were clearly labeled and from cattle more than one year of age were selected for the study. P was estimated at six percent which was drawn from the average of the two previously published prevalencies in Kenya (Gathogo *et al.*, 2012; Kang'ethe *et al.*, 2007).

3.4. Sample analysis using the Indirect ELISA test

The test used was an indirect ELISA that detects presence of antibody to *M. bovis* in bovine serum. The test kits manufactured by IDEXX Technical Services (USA), were validated and certified for use in May 2012 under the registration number 20120107. Present findings indicate that the IDEXX *M. bovis* ELISA provides a test sensitivity of 63% and specificity of 98% with samples from cattle naturally infected with *M. bovis* (Waters *et al.*, 2011). This test has a two hour procedure protocol and utilizes recombinant MPB70 and MPB83 proteins as capture antigen in the 96 wells micro-titer plates. The reagents (plate 3.1) were stored between 2-8 degrees centigrade and were allowed to attain 18-26 degrees centigrade before the start of the test. Both reagents and samples were diluted with 49 parts diluents to make 50 parts.

Plate 3.1: Showing all the reagents after unpacking the ELISA Kit

3.4.1 Test procedure

Antigen coated plates sufficient for planned samples were tested at a time and withdrawn from the desiccated bag. 100 micro liters of the diluted negative control, positive control and serum samples were dispensed in the wells (plate 3:2). The wells were then covered and incubated in 18-26 degrees centigrade for 60 minutes. The liquid was then aspirated and the wells washed 4 times with 300 micro liter of wash solution avoiding drying in between washes (Plate 3:3). 100 micro liters of the conjugate (monoclonal anti-bovine IgG-horseradish peroxidase) was then dispensed into the wells, covered and incubated for 30 minutes. The liquid was aspirated and the wells washed 4 times as described previously. TMB (Tetramethylbenzidine) substrate was then dispensed into the wells at 100 micro liters, covered and incubated for 15 minutes. The stop solution was then dispensed into the wells and absorbance read at 450nm on a micro-titer plate reader using air or water blank (plate 3:4) then results calculated.

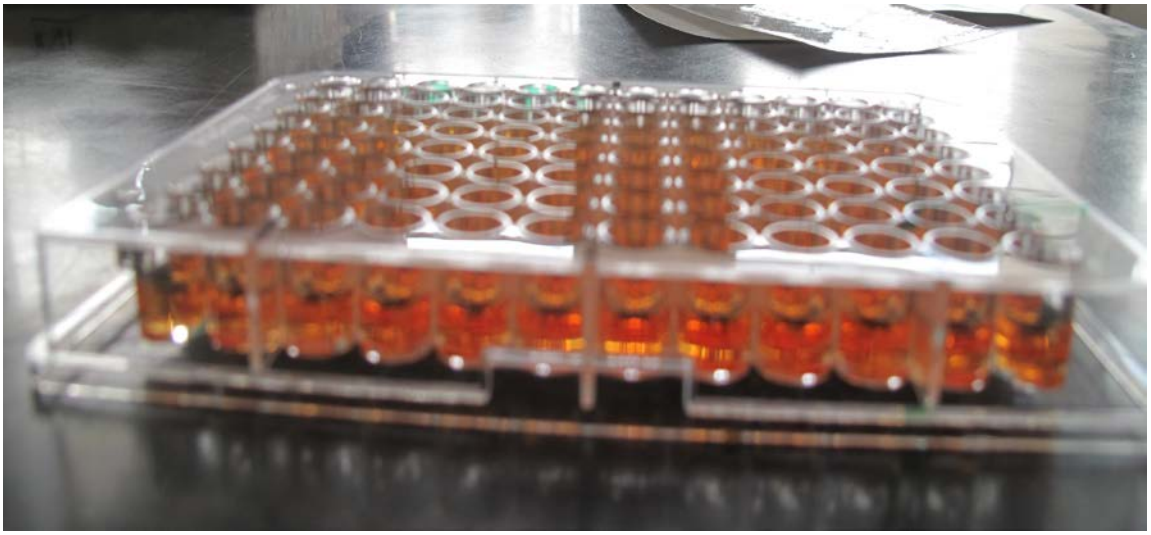

Plate 3.2: Microtiter plate during the test procedure

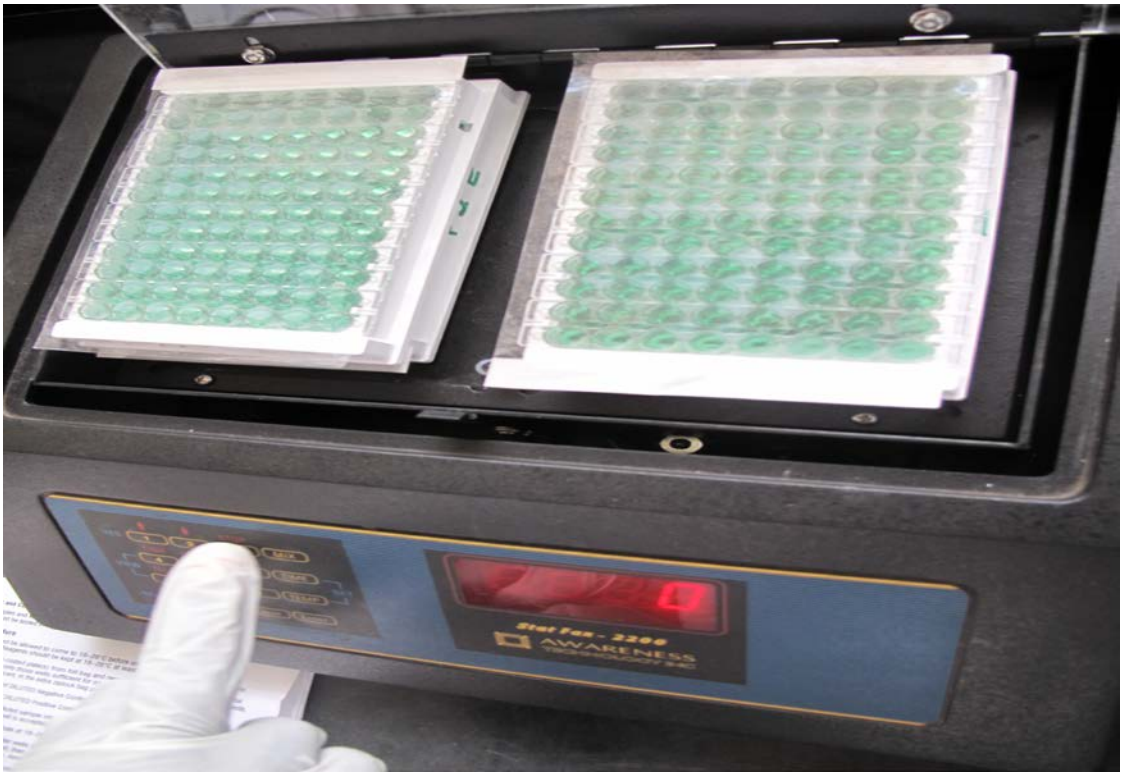

Plate 3.3: Microtiter plate in incubator shaker

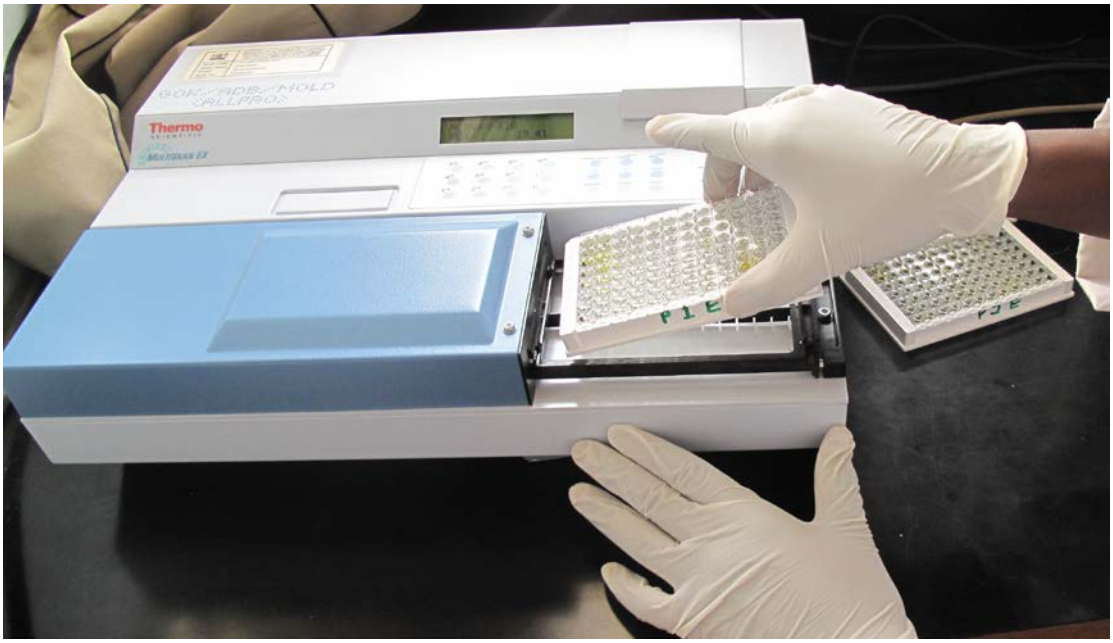

Plate 3.4: ELISA reading during the procedure

3.4.2 Interpretation of the ELISA results

The sample to positive (S/P) ratio outcome for the positive control mean must be greater than or equal to 0.3 while the negative control mean must be less or equal to 0.2 as instructed by the Manufacturer. The presence or absence of antibody to *M. bovis* was determined by calculating the sample to positive (S/P) ratio for each sample.

The S/P greater or equal to 0.30 were considered positive for *M. bovis* antibodies while samples with less than 0.30 were considered negative for *M. bovis* antibodies.

3.4.3 Disposal of used test materials

The sharps and plastic materials including microtiter plates and pipette tips were collected in special covered disposal containers for incineration. The remaining reagents in their containers and wrappings were also incinerated. Fluids and serum washed from micro

titer plates were collected in covered containers and decontaminated using 0.5% Sodium hypochlorite then discarded.

3.5 Statistical analysis

Data from ELISA results was initially entered into Excel 2007 (Microsoft Corporation, USA) with the following information; sampling location, age, sex, test results and the optical density reading. The prevalence of BTB was determined as a proportion of the positive cases out of total samples tested. The data were then exported to SPSS 16.0 version for statistical analysis. Descriptive statistical analysis was done to determine the proportion of positive samples and reactivity distribution across the counties and graphs displaying the proportions were prepared using Microsoft Excel. The association between seropositivity versus age, breed and sex was determined using the Chi-square test and Fisher's exact test.

CHAPTER FOUR

4.0 RESULTS

There were in total 644 bovine samples analysed in the laboratory from 7 Counties of Kenya. Each sample had on its record the laboratory number assigned specifically for the ELISA test, OD reading, the calculated S/P ratio, cattle identity as recorded in the specific sampling units, sampling location either by ranch, owner or by the village sample was taken from and sex and age. For Laikipia County there was additional recording of breed and the Tuberculin skin test (TST) result.

4.1 Description Analysis

4.1.1 Descriptive analysis across the Counties.

The range of samples tested across the counties was 45 (6.99%) to 276 (42.9%). Table 4.1 shows the distribution of samples tested for BTB in the seven Counties. Laikipia County had the most number of samples tested at 276 while Kwale county had the least at 45 samples.

Table 4.1: The distribution of 644 bovine samples tested for Bovine tuberculosis across the seven counties**.**

County	Total samples
Laikipia	276
Wajir	85
Taita Taveta	60
pokot	68
Kwale	45
Kilifi	64
Kajiado	46
Total	644

4.1.2 Sex and age distribution

There were 112 (17.4%) male samples and 532(82.6%) females' samples. Kwale County had the biggest proportion of males at 22/45 (48.9%) while Wajir County had the highest absolute number of males compared to other counties at 31/85. All the samples tested from Pokot and Kajiado Counties were from female animals (Table 4.2).

Table 4.2: Distribution of sex of the animals tested for Bovine tuberculosis across the seven Counties

	Laikipia	Wajir	Pokot	Kajiado	Kwale	Taita T.	Kilifi	Total
Male	14	31	0	0	22	22	23	112
	5.1%	36.5%	0%	0%	48.9%	36.7%	35.9%	17.1%
Female	262	54	68	46	23	38	41	532
	94.9%	63.5%	100%	100%	51.1%	63.3%	64.1%	82.9%
Total	276	85	68	46	45	60	64	644

Laikipia and Pokot Counties had the widest distribution of samples across all the age categories while Kajiado and Wajir had the least distribution (Table 4.3). The age category with the highest number of samples was in category 4 (those over 3 years and below six years of age) at 336/644 (51.2%), the biggest proportion in the category being from Laikipia County 242/336 (72%). The least number of samples was from age category 5 (those over six years of age).

Table 4.3: Age distribution of the animals tested for bovine tuberculosis from the seven counties**.**

County	Age categories					Total
	1	2	3	4	5	
Laikipia	15	1	7	242	11	276
Wajir	0	0	54	31	0	85
Pokot	28	13	5	19	3	68
Kajiado	0	0	4	42	0	46
Kwale	10	18	17	0	0	45
Taita Taveta	12	29	19	0	0	60
Kilifi	8	27	27	2	0	64
Total	73	88	133	336	14	644

Key: **1 -** One year and those below one year

2 - Over 1 year and below 2 years of age

3 - Over 2 years and below 3 years of age

4 - Over 3 years and below six years of age

5 - Over six years of age.

4**.2 Prevalence of *bovine tuberculosis* in the seven counties**

A total of 23/644 (3.57%) samples were positive to *Mycobacterium bovis* antibodies using the INDEXX ELISA kit.

4.2.1 Prevalence of *Mycobacterium bovis* by County

The overall prevalence rate for BTB based on the ELISA test was 3.75%. The seven Counties had varying prevalence rate based on the ELISA antibody test (Table 4.4). Four counties had a higher prevalence than the average prevalence from all samples tested and these included Wajir with the highest at 4.71 % (4/85), followed by Pokot county with 4.41% (3/68), Kajiado county with 4.35% (2/46) and Laikipia County with 3.99% (11/276).Taita Taveta and Kwale Counties had prevalence rates below the average rate at 3.33%and 2.22% respectively. Kilifi County had the lowest prevalence of 0% with no positive sample out of a total of 64 tested.

Table 4.4: Prevalence of *Mycobacterium bovis* in the seven counties

County	Total Samples	Number Positive	Prevalence (%)
Laikipia	276	11	**3.99**
Wajir	85	4	**4.71**
Taita Taveta	60	2	**3.33**
Pokot	68	3	**4.41**
Kwale	45	1	**2.22**
Kilifi	64	0	**0.00**
Kajiado	46	2	**4.35**
All	**644**	**23**	**3.57**

4.2.2 Prevalence of *Mycobacterium bovis* by units of sampling

There were a total of fifty nine sampling units across the seven counties from which the samples were obtained. Laikipia and Kilifi had the highest at twelve followed by Kwale with eight unit. These units were ranches for Laikipia while in other counties they were villages from different locations across each county. Apart from Kajiado where locations were not recorded, Laikipia had the highest unit prevalence of 58.63% (7/12) followed by Pokot County which had 40% (2/5) (Table 4.5).

Table 4.5: Prevalence of *Mycobacterium bovis* by units of sampling

County	Total Units	Positive Units	Negative Units	Prevalence (%)
Laikipia	12	7	5	58.33
Pokot	5	2	3	40
Wajir	11	4	7	36.36
Kajiado	1	1	0	100
Kwale	9	1	8	11.11
Kilifi	12	0	12	0
Taita Taveta	9	2	7	22.22
All counties	59	17	42	30.5

4.3 Prevalence of *Mycobacterium bovis* in Laikipia County

Laikipia County had the highest number of samples tested in this study and also had more information recorded alongside the samples. The samples were from three districts as shown in Table 4.6. Laikipia East had the highest number of samples 171/276 (70%) with 9/171 (5.3%) being positive for Mycobacterium bovis antibodies. Laikipia North and West

districts had only one positive sample each with the latter contributing the least number of samples.

Table 4.6: Number of Bovine tuberculosis positive samples from Laikipia County by districts

District	Number positive	Total
Laikipia West	1 (1.09%)	92
Laikipia East	9 (5.26)	171
Laikipia North	1 (7.67%)	13
Total	11 (3.99%)	276

Serum samples were obtained from 12 farms/ ranches in Laikipia County (Table 4.7). Mutara ranch had the highest number of samples 33.7% (93/276) with the least being from a farm belonging to a Mr. J. Kabugi at 0.4% (1/276). Ol-pajeta ranch had the highest number of positive samples 4.5% (3/67). Seven (7) ranches/farms had at least one positive sample while the other five (5) ranches/farms did not have any animal showing antibodies against *Mycobacterium bovis.*

Table 4.7: Samples from Laikipia County farms, indicating numbers of animals positive to Bovine tuberculosis

Ranch/farm	Number positive	Total
Al Pajeta	3 (4.48%)	67
J. G. Kingori	0 (0%)	17
J. Kabugi	0 (0%)	1
K. Mbuthia	0 (0%)	6
Loruku Ltd	2 (6.67)	30
Major Mwiti	0 (0%)	4
Mrs. Mwangi	1 (20%)	5
MutaaraRranch	1 (4.35%)	93
Nanyuki Ranching	2 (5.71%)	35
Olekaparo Farm	1 (16.67%)	6
P. Maina	1 (20%)	5
S. Kilusu	0 (0%)	7
Total	**11**	**276**

Samples from Laikipia County were from six recorded breeds totaling to 250 while the remaining 26 samples did not have the breeds indicated. The distribution of the positive samples by breeds are as shown in Table 4.8 with Ayshire showing the highest factor at 7.1% (1/28) and Friesian second with 6.8% (3/44) while Brown swiss, Ankole and the non-specified did not have any positive reactors.

Table 4.8: Prevalence of *Mycobacterium bovis* by Breeds in Laikipia County

Breed	Number positive (%)	Total
Ayrshire	2 (7.1%)	28
Friesian	3 (6.8%)	44
Boran	5 (3.5%)	143
Zebu	1(3.1%)	32
Ankole	0	1
Brown swiss	0	2
Non-specified	0	26
Total	**11**	**276**

4.4 Sample to Positive Ratio (S/P) distribution of the Bovine tuberculosis ELISA results

The results below in table 4.9 p show that the calculated ratio which was used to determine the positive serum samples. Ratios above 0.3 were considered positive while those below were negative. Table 4.9 shows the S/P Results distribution for comparison of seroconversion across the seven counties. The highest concentration of results at 414/644 was between S/P value -0.1 and 0.1.

Table 4.9: Sample to Positive (S/P) ratio distribution from the Bovine tuberculosis ELISA results across the seven counties

S/P Ratio Range	Counties							
	LK	WJR	PKT	KJD	KWL	KLF	TVT	ALL
<-0.3	1	1	0	0	0	13	0	15
-0.299 to -0.2	14	17	0	0	0	21	0	52
-0.199 to -0.1	45	4	0	0	0	19	0	68
-0.099 to 0	157	19	23	19	24	5	29	**276**
.001 to 0.1	26	25	31	17	12	2	25	**138**
0.111to 0.2	11	8	10	5	7	3	4	48
0.211 to 0.03	11	7	1	3	1	1	0	24
0.0311 to 0.4	8	4	2	2	1	0	1	18
>0.411	3	0	1	0	0	0	1	5
Totals	276	85	68	46	45	64	60	644

LK- Laikipia, WJR- Wajir, PKT- Pokot, KJD-Kajiado, KWL Kwale KLF- Kilifi and TVT Taita Taveta.

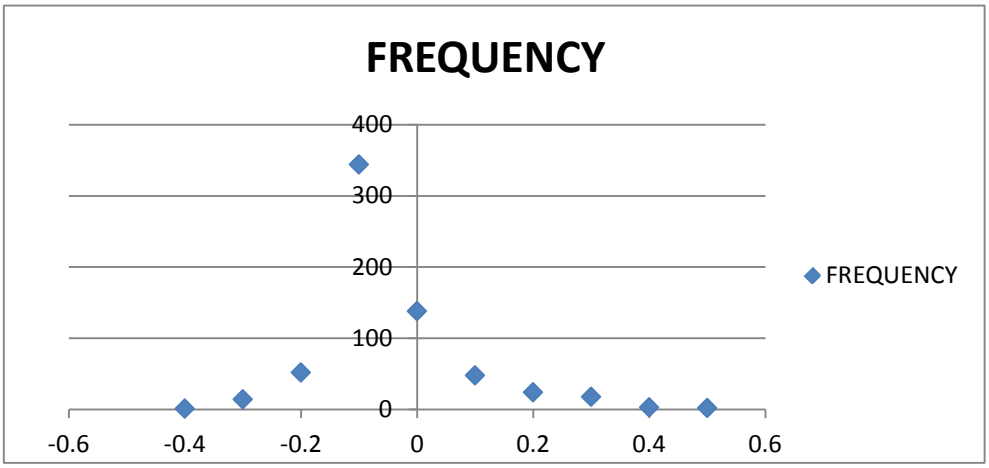

Figure 4.1: the total Frequency distribution of the S/P Ratios Range from the Bovine tuberculosis ELISA results in the seven Counties

Figure 4.1 shows graphical distribution of the S/P ratios, depicting more values below 0 in a normal distribution pattern. The highest frequency of S/P Ratio was between 0 and -0.01 at 42.9% (276/644) followed by 0.0 to 0.1 at 21.4% (138/644) making a total of 64% (412/644). There were 3.57% (23/ 644) above the positive threshold of 0.3 (positive for *M. bovis* antibodies). Kajiado County had the highest S/P ratio mean with Laikipia having the lowest as shown in Figure 4.2 below.

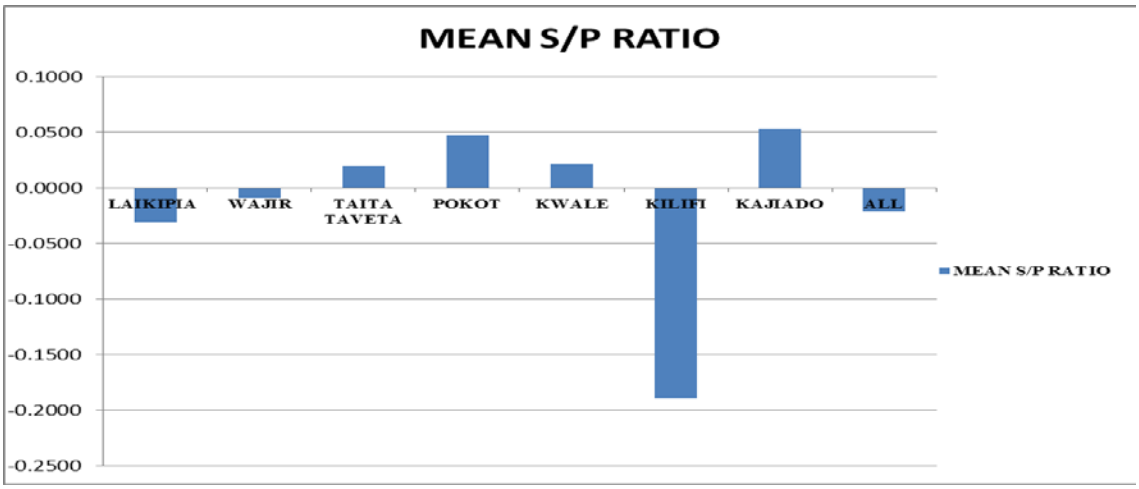

Figure 4.2; Means of the S/P Ratio from the ELISA results across the Counties

4.5 Association between prevalence of *Mycobacterium bovis* and various factors

4.5.1 Association between *Mycobacterium bovis* and sex of the animal

Out of the 644 samples tested, 82.6% (532/644) were from female animals while 27.4% (112/644) were from male animals (Table 4.10). About 1.8% (2/112) of serum samples from male animals tested positive for antibodies against *Mycobacterium bovis* while 3.9 % (21/532) of the serum samples from females tested positive. On Fisher exact test, there was no significant association between sex of the animal and ELISA outcome (p= 0.4111).

Table 4.10; Association between seroconversion to *Mycobacterium bovis* and sex of the animals

	Female	Male	Totals
Positive	21	2	**23**
Negative	511	110	**621**
Totals	**532**	**112**	**644**

4.5.2 Association between *Mycobacterium bovis* and age

The age of the animals was categorized into five groups designated numbers 1-5 as shown in Table 4.4. Category four (3 to 6 years) had the highest frequency of positive samples recorded at 65% (15/23). The other categories had two samples each testing positive for *Mycobacterium bovis*. On Chi-square test, there was a very marginal significant association between age of the animal and ELISA outcome (p= 0.05507). The latter showed that the probability of testing positive for *M. bovis* was not affected by the age of animal (Table 4.11).

Table 4.11: Association between *Mycobacterium bovis* and age of animals

	Age categories					Total
	1	2	3	4	5	
Positive	2	2	2	15	2	23
Negative	71	86	131	321	12	621
Total	73	88	133	336	14	644

Stratifying the age categories into two groups by Chi square statistics showed marginal significant association between the two groups (P= 0.05507) when those below three years (category 1) were compared with those above three years (Category 2) (Table 4.12).

Table 4.12: Old (Over six years category) and below six years against Test outcome

	Positive	Negative	Total
1 (< 3)	6	288	304
2 (≥3)	17	333	350
Total	23	631	644

4.6 Association between prevalence of *Mycobacterium bovis* and various factors in Laikipia County

4.6.1 Comparison between *Mycobacterium bovis* Tuberculin skin test (TST) and ELISA results

Out of the 276 samples tested from Laikipia County 254 had additional results from a tuberculin skin test (TST). Eight (8) samples tested positive on both TST and ELISA; 239

testing negative for both with an observed proportional agreement of 0.97 (97%). Four (4) samples testing positive for TST tested negative for ELISA while Three (3) testing positive for ELISA tested negative for TST. There was an apparent prevalence of 0.04 (4%) for ELISA and 0.047 (4.7%) for TST. Kappa statistics for the two tests showed a strong agreement between the two *M. bovis* tests at K=0.65 (Table 4.13).

Table 4.13: Kappa agreement test between Tuberculin skin test and indirect ELISA results

	ELISA Positive	ELISA Negative	Total	Apparent prevalence (TST)
TST Positive	8	4	12	0.047
TST Negative	3	239	242	
Total	11	243	254	
Apparent prevalence (ELISA)	0.04			
Observed proportional agreement			0.97	
Chance Apparent prevalence (both +)			0.0018	
Chance Apparent prevalence (both -)			0.91	
Chance proportional agreement			0.9118	
Observed minus Chance agreement			0.058	
Maximum possible agreement beyond chance level			0.088	
Kappa			0.659	

4.6.2 Association between *Mycobacterium bovis* prevalence and sex of animal in Laikipia County

Out of the 276 samples tested using ELISA94.6% (261/276) were from female animals while 5.4% (15/276) were from male animals. About 6.7% (1/15) of serum samples from male

animals tested positive for antibodies against *M. bovis* while 3.8 % (10/261) of the serum samples from females tested positive. On Fisher exact test, there was no significant association between sex of the animal and ELISA outcome (p= 0.9310) (Table 4.14).

Table 4.14: Association between Bovine tuberculosis ELISA results and sex in Laikipia County

	Positive	Negative	Total
Male	1	14	15
Female	10	251	261
Total	11	265	276

4.6.3 Association between *Mycobacterium bovis* Prevalence and age of animals in Laikipia County

Age category 4 (3 to 6 years) had the highest proportion of positive samples recorded at 81.8% (9/11). Those above six years of age had two positive reactors while categories one, two and three did not have any sample testing positive for *Mycobacterium bovis*. On Fisher exact test, there was no significant association between age of the animal and ELISA outcome (p = 0.1306) (Table 4.15).

Table 4.15: Association between Bovine tuberculosis ELISA results and age for Laikipia County

	1 (Below Six years)	2 (Above six Years)	Total
Positive	9	2	11
Negative	256	9	265
Total	265	11	276

4.6.4 Association between *Mycobacterium bovis* prevalence and breed of animals in Laikipia County

The Boran breed had the highest number of samples that were tested at 51.8% (143/276) with Ankole having the least number at only one sample. About 9.4% (26/ 276) did not have the breed recorded during sampling. The highest breed prevalence is Ayrshire at 7.1% (2/28) followed by Friesian with 6.85% (3/44), Boran with 3.4% (5/143) and Zebu Breed with 3.1% (1/32). Ankole, Brown Swiss and the 26 samples without breed record did not have a positive reactor (Figure 4.3).

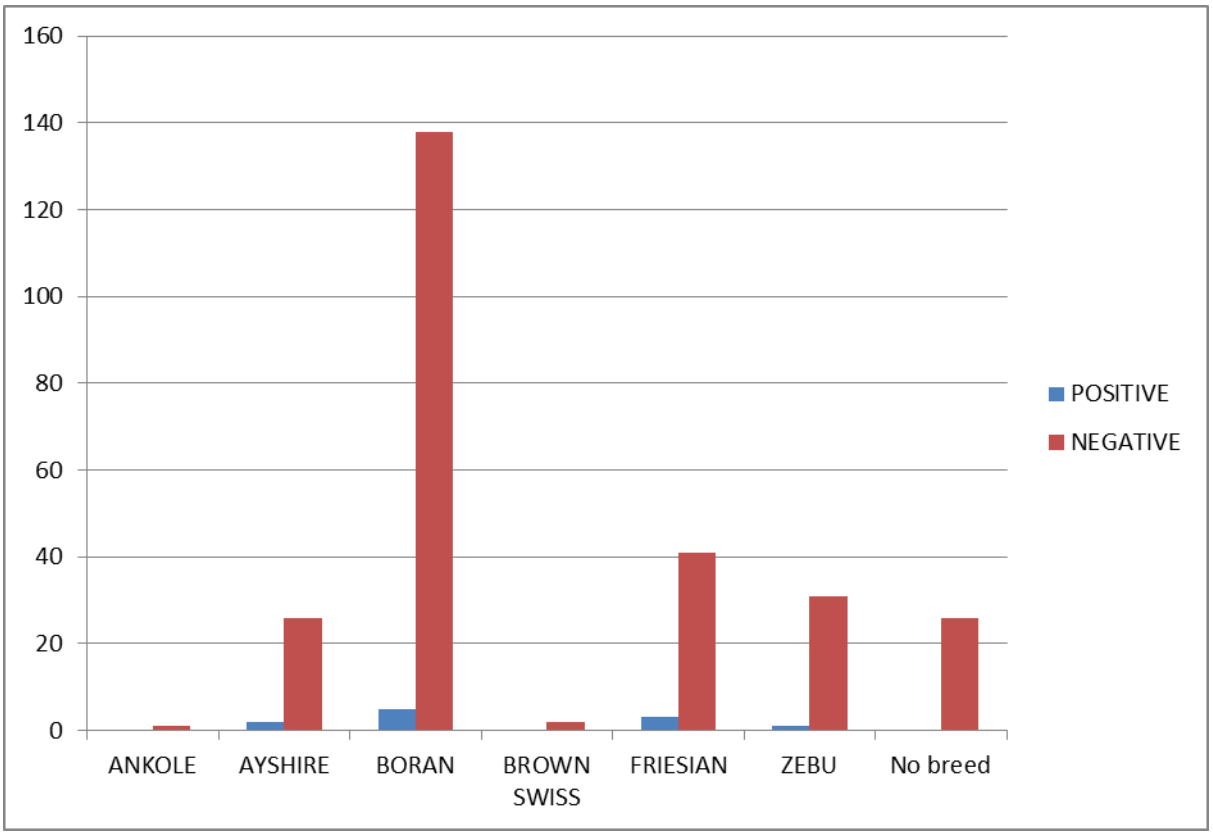

Figure 4.3: Proportions of positive and negative *Mycobacterium bovis* ELISA results by breed in Laikipia County

When the animals were classified by breeds to two groups; exotic (*Bos taurus*) and indigenous (*Bos indicus*) (Table 4.16), there was no statistical association on Chi square, (P=1.4116)

Table 4.16: Association between bovine tuberculosis ELISA results and breeds for Laikipia County

	Positive	Negative	Total
Exotic breeds	5	69	74
Indigenous	6	171	177
Total	11	240	251

CHAPTER FIVE

5.0 DISCUSSION

The use of indirect ELISA in the diagnosis of BTB has not been widely applied in many parts of the world. Tuberculin skin test has been the most common methods of ante mortem screening of cattle for a long period and in many areas of the world. This study was performed on stored bovine serum samples to estimate and establish the status of BTB in Kenya using antibody ELISA. It is the first time this method is employed in Kenya for diagnosis of BTB. IDEXX *M. bovis* antibody ELISA's diagnostic performance has been evaluated in various studies and reports published in the past three years. A cross-sectional study conducted on 459 dairy and slaughter cattle in Ethiopia showed a sensitivity of 50% in comparison with bacterial culture as gold standard (Eyob *et al*., 2014). Other reports have shown sensitivities of; 63% (Waters *et al*., 2011) and 64.2% (Lawrence *et al*., 2012). Studies on Irish cattle have indicated that IDEXX BTB test sensitivities increased markedly with the severity of the disease (Waters *et. al*., 2011).

Bovine tuberculosis in cattle progresses slowly and provokes cellular immunity within the host initially. As the infectious load increases, there is a reduction in cellular response, while the humoral response tends to increase and is more evident and detectable (Medeiros *et al*., 2010). *In vivo* skin test and the *in vitro* Gamma-Interferon (IFN) Assay are based on cellular response to infection. Depending on the particular stage of the disease, these two tests may be negative since cellular response may no longer be detectable. To identify BTB in all stages of infection, studies focusing on the recognition of *M. bovis* antigens have been conducted to demonstrate a humoral response therefore improving diagnosis (Aagard *et al.*, 2006). Capture antigens especially recombinant MPB70 and MPB83 have been used in

antibody ELISA as alternative and or complementary diagnostic tools to improve detection of cattle that do not react to tuberculin skin test (Smith *et al*., 2006).

This study utilized the indirect ELISA on the 644 bovine serum samples among which 276 had been tested using tuberculin test. This test uses two proteins which have been shown to elicit strong cell mediated immunity (CMI) in the early stages of the tuberculosis infection. The MPB83 and MPB70 are major antigens highly expressed by *M. bovis* and considerably less abundantly expressed by *M. tuberculosis* (Juarez *et al*., 2001). They have also been shown to be useful antigens in differentiating animals infected with *M. bovis* from animals infected with Paratuberculosis (PTB) or BCG-vaccinated as well as from animals vaccinated against PTB (Wiker *et al.*, 1998). The IDEXX test detects antibodies to MPB70 and MPB83, and the serum antibody responses to MPB83 have been detected in cattle infected with *Mycobacterium kansasii*, making it the only known *Mycobacterium* species cross react with the antigen (Waters *et al.*, 2006). Use of this test was therefore expected to give a better estimation in terms of the prevalence status and sensitivity in relation to tuberculin test which is the only other ante-mortem means of diagnosis commonly used. The possibility of a previous tuberculin test being used would not have compromised or affected results in study.

Of the 644 samples tested, females had contributed the highest numbers in all the counties. This can be attributed to the fact that farmers keep more females for the purposes of reproducing their herds. Ages appeared to be normally distributed from one year old to nine years with majority being between 3 years and 6 years. This is similar to what has been recorded for arid and semi-arid region production systems in sub Saharan Africa (Otte and Chilonda, 2002). The overall prevalence recorded in this study was 3.57% in the seven counties with a range of 4.74% in the highest prevalent county to 0% in the lowest prevalent county. Like in many other countries in the region Kenya lacks enough data and reference

record of BTB status. The counties involved in the study ranged from pastoral systems of cattle keeping to subsistent small holder farms. All the seven counties have some level of wildlife presence or border a wildlife park or reserve. Published material in Kenya indicates 2.0% *M. bovis* prevalence among slaughter cattle in a study conducted in two abattoirs in the outskirts of Nairobi (Gathogo *et al.,* 2012). The origin of cattle in that study was 18 counties of Kenya covering 77% landmass. This revealed that diagnosis of tuberculosis in slaughter cattle was very low. A study by Kang'ethe *et al.,* (2007) indicated a prevalence of 10% in dairy and non-dairy herds around Nairobi area.

Another study in Kenya investigating the epidemiology of bovine tuberculosis in the wildlife-livestock interphase in Maasai Mara and Amboseli ecosystems showed a prevalence of 14.8% in wildlife compared to an overall prevalence of 2.3% in livestock (Lekolool, 2011). An earlier report showed isolation of *M. Bovis* in baboons feeding on abattoir offal in Kenya (Sapolsky and Else, 1987). In Tanzania a study conducted in the Dar-es-salaam area showed a less than 1% prevalence using Single Intradermal Tuberculin Test (SITT) with 6.8% being doubtful **(**Weinhaupl *et al.,* 2000). A Prevalence of 2% and a herd prevalence of 51% has been reported in cattle pastoral herds of Uganda's Karamoja region (Oloya *et al.,* 2006; Oloya e*t al.,* 2007). In the West and Central Africa varied prevalence of above 10% have been reported in the periurban areas as well as the countryside (Zinsstag *et al.,* 2006; Delafosse *et al.,* 1995; Muller *et al*., 2008). Most of these studies are based on intra-dermal tuberculin test or slaughter sampling.

The different counties showed different prevalence irrespective of the different number sampled per county. The prevalence per county varied from 4.71 % in Wajir with the highest rate followed by Pokot County with 4.41% to 0% in Kilifi County. There was also a marked variation in the mean OD readings and mean S/P ratio across the counties. This has also been

observed and reported in other regions. The distribution of the domestic ruminant population and dairy production varies within the ecological zones. Different factors and variations in production systems of livestock management contribute to these observations. In South America for example reports show the highest levels of BTB occurrence in the surrounding areas of larger cities where intensive dairy production is most common (Szyfres, 1972). Large variations in BTB occurrence within different regions of the same country have also been reported in Africa (Cosivi *et al*., 1995). The variation in distribution and transmission of BTB is most likely to be influenced by macro- and micro-climates affecting the stability of the agent in the environment and probable vulnerability of the animals due to management practices. The different management systems like pastoralism, mixed farming and intensive dairy farming, may have a significant influence on the distribution of animal tuberculosis (Cosivi *et al*., 1995).

There was also a variation in herd prevalence between the counties considering the sampling units that were recorded. There were a total of fifty nine sampling units across the seven counties from which the samples were obtained. Laikipia and Kilifi had the highest at 12 with Kajiado having no recorded units. The units were ranches for Laikipia while in other counties they were villages from different locations across each county. A similar occurrence is reported in other areas where herd prevalence varies from region to region. The herd prevalence is mostly higher than the population prevalence in most of the reported cases. A study in Northern Ethiopia between 2007 and 2008 utilizing comparative intradermal tuberculin (CIDT) indicated that a herd prevalence of BTB in outdoor and indoor production systems at 12.9% and 26.1%, respectively. In the outdoor and indoor management systems, individual animal prevalence of BTB were 4.5% and 8.1% respectively with the overall prevalence of bovine tuberculosis being 7.1% (Mohammed *et al.,* 2012). Another study

conducted in dairy farms of the State of Himachal Pradesh, India using tuberculin skin testing (TST) to determine the prevalence of bovine tuberculosis, an overall animal prevalence of 14.3% and a farm prevalence of 16.7% were reported (Aneesh *et al.,* 2010).

The highest herd prevalence from the study was in Laikipia where management practices are more organized in ranches and with a more likely potential of wildlife interaction. A study in Zambia reported a higher risk for BTB in animals kept close to wildlife contact than those kept in villages far away from wildlife. Cattle grazing in sustained close contact with wildlife had a prevalence of 11.6%, followed by the intermediate zone with irregular wildlife contact at 5.1% and those grazing away from wildlife contact had prevalence of 2%. This can explain the high prevalence in Ol-pajeta ranch and Laikipia County where animals have a high chance of wildlife contact than the other areas in the study (Munyemea *et al.,* 2008). On the other hand prevalence in Wajir (36.4%) and Pokot (40%) Counties were lower despite having mainly pastoral forms of cattle husbandry. This agrees with other published reports of a higher prevalence in area of intensive animal management (Cosivi *et al*., 1995; Mohammed *et al.,* 2012). The coastal areas of Taita Taveta (22.2%) and Kwale (11.1%) Counties had lower herd prevalence with Kilifi showing 0% despite these areas being close to wildlife ecosystems. The form of management is mainly subsistent mixed farming in the areas where the samples were collected. For the coastal Counties, sampling was not done from the ranches.

Out of the total 644 samples tested, 82.6% were from female animals while 27.4% were from male animals. About 1.8% of serum samples from male animals tested positive for antibodies against *M. bovis* while 3.9 % of the serum samples from females tested positive. There was no significant association between the sex of the animal and the ELISA outcome. Gender has been mentioned as a risk factor in studies carried out in few places especially

Africa and opinion is diverge regarding its influence to *M. bovis* infection susceptibility (Marrie *et al.,* 2009). A cross-sectional study conducted in Tanzania from 1994 to 1997, which included over 5000 indigenous and exotic cattle, revealed that male cattle were significantly more affected by BTB than female animals (Kazwala *et al.,* 2001). Another cross-sectional study from 2006 to 2007, in Uganda revealed significantly more females positive to the skin test than males (Inangolet *et al.,* 2008).

There was no significant association between age of the animal and ELISA outcome. This meant that the chances of an animal testing positive for BTB antibodies did not change with age. The duration of exposure increases with age; older animals are more likely to have been exposed at one point in their life than calves or younger cows. The above has also been observed in epidemiological studies carried out in Zambia and Tanzania (Cook *et al.,* 1996; Cleaveland *et al.,* 2007). Irish workers observed that calves were less likely to be positive reactors to both ELISA and tuberculin skin test than older animals (Griffin *et al.,* 1996). Animals have been reported to get infected at a young age, but only express the disease clinically when they are adults. Mycobacterium organisms have the ability to remain in a latent state for a long period before reactivation at an older age when immunity is less or compromised (Pollock & Neil, 2002). This finding was unique since many other studies have found age to be a key risk factor in BTB epidemiology. Despite this, researchers have not been able to demonstrate that a true dormant state exists in cattle (Van Rhijn *et al.,* 2008).

In Laikipia County the tuberculin skin test (TST) results were recorded alongside the serum samples. These results and the indirect ELISA results were subjected to Kappa statistics for a test of agreement. Kappa statistics showed a strong association agreement for the two *M. bovis* tests at K=0.65. A similar kappa test comparison of TST and ELISA test utilizing MPB70/MPB83 capture antigens in Brazil demonstrated adequate agreement with a kappa

index of 0.688 (Souza *et al.,* 2012). In this study the sensitivity and specificity of IDEXX *M. bovis* ELISA was determined at the cut-off values established by the manufacturer. It has been shown that the condition can vary in different regions due to differences in prevailing host, pathogen and environmental factors. With the tests to determine the cut-off point at S/P 0.3 by the manufacturer done in America and Europe, the situation in Kenya may be different. For example a study done in Ethiopia indicated a marked difference in sensitivities between the manufacturer's given Cut-off (.0.3) and a lower cut-off of (0.136) at 50% and 80% respectively without affecting the specificity significantly. The lower cut-off was determined using receiver operating characteristics (ROC) analysis using culture as a gold standard (Eyob *et. al*., 2014). A similar situation could be possible for Kenya, therefore there is need to assess this method in comparison with a gold standard and determine the suitable cut-off S/P ratio by a ROC curve.

This study being antibody testing has not been used widely before in determining the prevalence of BTB. It will present an alternative or a complementary ante-mortem test to determine BTB status in Kenya and other regions where little is known. Being a two hour laboratory protocol and also having the capacity to detect early infections, it will be a feasible and a quick method to use for large surveys on both stored and freshly collected serum samples. As it has been shown that Cell mediated immunity (CMI) reduced later in infection, it will also be reliable to use the humoral immunity with more reliability at any stage of infection (Medeiros *et al*., 2010). The cost and time required to run the indirect ELISA test is much less compared to tuberculin skin test which requires at least two field visits with a possible risk of having withdrawals before completion of the test.

CHAPTER SIX

6.1 Conclusions

I. This study showed that BTB is present in Kenyan cattle populations in six out of the seven counties.

II. Sex and breed were not significantly associated with BTB infection in the seven counties while Age was marginally significant.

III. The study showed that the use of antibody ELISA is feasible, reliable, cheaper and less time consuming.

IV. The performance of the ELISA test showed a strong agreement when compared to the tuberculin test which is used as the gold standard for screening BTB on live animals.

6.2 Recommendations

I. There is an urgent need to survey all the counties for BTB status in order to advise on control policy and minimize the risk of human transmission.

II. Awareness should be created to both professionals and farmers about BTB control and its reporting.

III. The need to conduct a study to determine a suitable IDEXX ELISA cut-off S/P Ratio for the Kenyan condition. By the use of a gold standard test like culture or PCR a more specific cut-off S/P ratio can be determined to improve sensitivity without affecting the specificity.

IV. Since there is a deficiency in the available data on the prevalence of BTB in domestic animals. With the high numbers of human cases recorded in Kenya for the years between 2007 and 2009, there is need to investigate if these infections are related to livestock sources and the risks involved.

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