EVALUATION OF SERUM AGGLUTINATION AND ROSE BENGAL TESTS AGAINST IMMUNOFLOURESCENCE ANTIBODY ASSAY IN DIAGNOSIS OF BRUCELLOSIS IN NAROK AND KIAMBU COUNTY, KENYA

SUSAN NYAWIRA NYAGAH

Reg No 156/10892/07

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (INFECTIOUS DISEASES) IN THE SCHOOL OF MEDICINE KENYATTA UNIVERSITY

May, 2015

DECLARATION

This thesis is my original work and has not been presented for a degree or other awards in any other university.

Susan Nyawira Nyagah

Reg. No.156/10892/07

Signature:..... Date: 27/05/15

We confirm that the work reported in this thesis was carried out by the candidate under our supervision:

Dr. Margaret Muturi
Department of Medical Laboratory Science
School of Medicine
Kenyatta University
Signature:..... Date: 27-05-2015

Prof. Joseph J. N. Ngeranwa
Department of Biochemistry and Biotechnology
School of Pure and Applied Sciences
Kenyatta University
Signature:..... Date: 27th May 2015

DEDICATION

I wish to dedicate this work to my parents, Jane Wanjiku and Moses Nyaga and my brothers and sisters: Charles Wamai, Veronica Muthoni, Esther Njeri, Grace Wanja, John Maina, Anne Mumbi and to my son Amos Nyaga.

ACKNOWLEDGEMENTS

I sincerely thank Dr. Margaret Muturi of Department of Medical Laboratory Sciences, School of Medicine, Kenyatta University and Prof. Joseph Ngeranwa of Department of Biochemistry and Biotechnology, School of Pure and Applied Sciences, Kenyatta University for tirelessly supervising the work that led to successful completion of this thesis. Many thanks to the Director AIC Kijabe mission hospital for allowing me to collect blood samples and hospital information data. I greatly appreciate Mr. Philip Maina and Joseph Kibuchi (laboratory technician, Kijabe mission hospital) for their support. I alsohighly appreciate the Director Veterinary Service (DVS) Dr. Peter Ithondeka, PhD, MBS for allowing me to use the laboratories facilities at the Central Veterinary Laboratories Kabete. I thank Dr. Joseph Macharia, Dr. Peter Mbatha and Dr. Jane Githinji of Central Veterinary Laboratories Kabete for their cooperation and assistance during study period. A lot of gratitude to technicians of Central Veterinary Laboratories Kabete; Mr. Stephen Gacheru, Rosalyne Mbugua, Esther Chege and Nancy Nanjara for their tirelessly support. Special thanks to Anderson wambugu (KARI institute) for his support in production of rabbit-anti human IgGs. I wish to also thank Associate Prof . Samuel Arimi, Nairobi university and Mr. Nganga Muturi, Deputy Karai Secondary School for the words of encouragement during the study. I also thank Mr Crispin Matere (Msc. Nairobi University), Mr. Simon Gachigua (Msc. Kenyatta University) and Lawrence Olaro (Msc. Microbiology Department, Kenyatta University) for their assistance in data analysis. Finally, I thank all those who supported me in one way or another.

ABBREVIATIONS AND ACRONYMS

APCs	Professional antigen presenting cells
B. abortus	*Brucella bortus*
B. melitensis	*Brucella melitensis*
CDC	Centre for disease control and prevention
CF	Correction Factor
CFT	Compliment fixation test
CTL	Cytotoxic T lymphocytes
ELISA	Enzyme linked immunosorbent assay
et. al	et alia (and others)
FAO	Food Agricultural Organisation
FCA	Freud's Complete Adjuvants
FIA	Freud's Incomplete Adjuvants
FITC	Flourescence isothiocyanate
IFA	Immunoflourescenceantibody Assay
IFN	Interferon
Ig	Immunoglobulin IgA
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL- 12	Interleukin -12
LPS	Lipolysaccharide
M	Protein Concentration
MAT	Micro agglutination
MCH class 1	Major histocompatibility complex 1

Mg	Milligram
NPV	Negative predictive value
PCR	Polymerase Chain Reaction Test
PH	Hydrogen ion concentration or Hydroxyl ion concentration
PPV	Positive predictive value
RBT	Rose Bengal Test
SAT	Wright's Serum Agglutination Test
SAS	Statistical Analysis System
SE	Standard error
SPSS	Statistical Packages for Social Sciences
TAT	Tray agglutination Test
Th 1	T helper cell 1
TMP/SMX	Trimeprime –Sulfamethoxazole
WHO	World Health Organisation
χ^2	Chi-square
%	Percentage
<	Less than
>	More than
^{0}Ca	Degree Centigrade
µl	Microliter
Σ	Epsilon

Contents

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Brucellosis in human is characterized by non specific signs and often misdiagnosed as malaria, typhoid, rheumatic fever and pyrexia of unknown origin. Definitive diagnosis of brucellosis requires isolation of *Brucella spp.*but chances of successful isolation decrease as the disease progresses. In Kenya, the current routine diagnostic test, Serum Agglutination Test (SAT) has a major drawback in that its not suitable for patient follow-up, since titers can remain high for a prolonged period. The alternative test, Rose Bengal Test (RBT) is not reproducible as it is very sensitive to vaccinal antibody limiting its use in vaccinated animals. Immunoflourescence antibody assay (IFA) has been described as the gold standard for diagnosis of brucellosis. To date there is no study in Kenya that has examined performance of the two routine tests SAT and RBT in the face of IFA. The objective of this study was to evaluate SAT and RBT against IFA and to estimate the prevalence of brucellosis in Kiambu and Narok counties by the three diagnostic methods. Human blood and unpasteurised milk samples were collected between December 2009 and August2010 in Kiambu and Narok counties. A total of 250 human serum and 250 milk samples were collected from Narok and Kiambu counties. Human serum sample was obtained from Kijabe referral hospital and other neighbouring health facilities. Antibodies to *Brucella spp.* were screened using SAT and RBTin the hospital and later transported to Kabete Veterinary Laboratory for IFA. Milk samples were collected from milk vendors packed in cool boxes and transported to Kabete Veterinary Laboratory where screening was done using one step bovine *Brucella* antibody rapid test. All samples were subjected to IFA to determine the prevalence of brucellosis. The overall prevalence of*brucella* antibodies by IFA as a gold standard was 3.2% and 70.4% and 14.4% and 54.4% in human samples and milk samples in Kiambu and Narok respectively. Prevalence by SAT was 10.4% and 77.6% and 8.3% and 80% by RBT in Kiambu and Narok respectively. Comparison of SAT and IFA gave PPV (63%), NPV (16.4%), sensitivity (75%) and specificity (74%). Comparison of RBT and IFA gave sensitivity (69.56%), specificity (72.78%), PPV(59.81%) and NPV(19.58%). Comparison of One step bovine *brucella* rapid agglutination test and IFA gave sensitivity (16.5%), specificity (53.0%), PPV (19.76%) and NPV (52.43%). Prevalence was higher in females (68%) than males (32%) in Narok particulary 21-30 years. Both SAT and RBT should be scrapped as they lack sensitivity and specificity. IFA which is accurate, sensitive, specific, rapid should be recommended as the diagnostic test of choice. People handling animals and their products are at higher risk of infection.

CHAPTER ONE

INTRODUCTION

1.1 Background

Brucellae are small, gram-negative, nonspore-forming cocobacilli and are able to replicate in the phagosomes of macrophages (Liautard *et al.*, 1996). The genus *Brucella* consists of at least six species, designated on the basis of host preference, antigenic and biochemical characteristics as *Brucella melitensis* (goat and sheep), *Brucella abortus* (cattle), *Brucella suis* (pigs), *Brucella canis* (dogs), *Brucella ovis* (sheep) and *Brucella neotomae* (woodrats). *Brucella abortus*, *B. melitensis* and *B. suis* can infect all humans with similar serious disease consequences. Recently, *Brucella spp.*have been isolated from marine mammals and are infectious for humans. In constrast, human infections with *Brucella ovis* and *Brucella neotomae* have not been reported (Rajashekara *et al.*, 2006).

Brucellosis is a zoonotic disease that affects a variety of livestock and wildlife and results in decreased reproductive efficiency and abortion (Halling *et al.*, 2005).The primary impact of brucellosis stems from losses due to reproductive failure in food animals and loss of human productivity. *Brucella* species are recognized as potential agricultural, civilian and military bioterrorism agents which causes undulant fever in humans (Halling *et al.*, 2005). Brucellosis in domestic animals causes a chronic infectious disease which persists for life (Enright, 1990).

Brucellosis in humans is associated with occupational exposure such as veterinarians, slaughterhouse and laboratory workers for example recent in Korea there was a report of an outbreak of human brucellosis among livestock workers and veterinarians in rural area from

February 2003 to August 2003 (Park *et al.*, 2005). In Spain outbreaks occur frequently on livestock farms which could be due to direct contact with infected animals (Sanchez *et al.*,2005). Laboratory accidents are frequent and significance as biodefence research expands in the academic and biotechnology industries (Fiori *et al.*, 2000).

Brucellosis in human also occur through consumption of unpasteurized raw milk or unheattreated dairy products contaminated with one of the *Brucella* species pathogenic to humans (Palanduz *et al.*, 2000; Zuniga *et al.*, 2005).Human brucellosis is common among pastoralists in many African countries (Muriuki *et al.*, 1997).

In Kenya, 20-90% of the population is at high risk of brucellosis since they rely on livestock production and also live closely with livestock. The sale of unpasteurized milk has increased following liberalization of milk marketing in 1992 (Omore, 1999). Therefore, a large quantity (over 80%) of marketed milk is not pasteurized and the milk is sold through informal market channels (Omore, 1999) putting the consumers at risk of acquiring brucellosis.

However, due to obsecureness of the disease, its often misdiagnosed as malaria, which is prevalent in Kenya (Oomen, 1976; Maichomo *et al.*, 2000). Other diseases which are difficult to distinguish clinically include typhoid, rheumatic fever and pyrexia of unknown origin (PUO) (Maichomo*et al.*, 2000; Baba *et al.*, 2001).

There are a few hospital and health centres in most parts of Kenya that carry out diagnosis for brucellosis due to scanty resource allocation. A study in Narok, Kenya by Muriuki *et al.*(1997) confirmed diagnosis of 55% of flu-like cases as malaria and 21.2 % as brucellosis in seven health

units routinely testing for malaria and brucellosis using the Rose Bengal Test (RBPT) from among a total of 60 health units in a pastoral area.

Culture method has a sensitivity of 60%. Subcultures are advised to be kept for at least 4 weeks which is not done routinely for most bacteria cultures.Bone marrow culture sensitivity is 80-90 % (Gotuzzo *et al.*, 1986). However, harvesting bone marrow for culture is an invasive and painful technique and the results have not been universally reproducible (Pappas *et al.*, 2006). Nevertheless, isolation of *Brucella sp.* requires high security laboratory facilities (biological containment level 3), highly skilled personnel, an extendend turnround time for results and it is considered a hazardious procedure.

The current diagnostic test, serum agglutination test has a major problem in that its not suitable for patient follow-up, since titres can remain high for a prolonged period (Pappas *et al.*, 2006). Poor reproducibility has been demonstrated with a frequently used serological screening test, the Rose Bengal Test (Maichomo*et al.*, 1998). Rose Bengal Testis very sensitive to vaccinal antibody, seriously limiting its use in vaccinated animals (Alton *et al.*, 1990)

The immunofluorescence antibodies assay (IFA) could be performed outside the diagnostic laboratory, allowing for rapid and accurate diagnosis. The IFAcan distinguish vaccinal antibody in most vaccinated animals and it eliminate cross reactivity by some antibodies (Lucero *et al.*, 2003).

1.2 Problem Statement

Symptoms of brucellosis are broad and nonspecific making clinical diagnosis difficult. The misleading clinical picture makes it difficult to give the correct treatment and patient suffer with the disease for a long period of time. Brucellosis is largely misdiagnosed and the prevalence is underestimated wordwide.

Some of the health facilities where the diagnosis is possible, are far from reach and have few trained personnel. The method of diagnosis also pose a problem. The current diagnostic method are serum agglutination test (SAT)and Rose Bengal Test (RBT). SAT pose drawback in that titre can remain high in the serum for a prolonged period of time while Rose Bengal Test (RBT) is not reproducible. Another drawback with the SAT is inability to diagnose *B. canis* due to lack of cross reaction. The SAT detects IgG less efficiently. False negative in SAT may be caused by the presence of blocking antibodies (the prozone phenomenon) in the α2-globulin (IgA) and the α-globulin (IgG) fractions. Overall blood culture are positive in 53.4% to 90% of patients with brucellosis but the chances of successful isolation of the organism decrease as the disease progresses, performance of repeated subcultures is labour intensive. Blood cultures are routinely discarded after 5-7 days incubation period, and therefore isolation of slow growing *Brucella* may be missed.

The Immunoflourescence antibody assay (IFA) is rugged, relatively inexpensive, simple and very rapid. It is important to validate the serum agglutination test and Rose Bengal Test to determine their effectiveness in diagnosing of brucellosis. It also important to use IFA to determine the prevalence of brucellosis. New diagnostic methods are required. This study evaluated the SAT and RBT and estimated the prevalence of brucellosis in the selected population.

1.3 Justification

Adequate information on the occurrence of brucellosis in man and cattle is not available in Kenya. This is because of lack of facilities for diagnosis and reporting (Corbel, 1989). Therefore laboratory testing is an absolute prerequisite for a proper diagnosis of human brucellosis. Poor reproducibility has been demonstrated with a frequently used serological screening test (Rose Bengal Test), when performed at different study sites (Maichomo *et al.,* 1998). Serum agglutination tests have a major drawback in that they are not suitable for patient follow-up, since titers can remain high for a prolonged period once exposed (Pappas *et al.,* 2006).Confirmation of brucellosis using clinical and laboratory diagnosis is required to minimize cases of misdiagnosis and ensure correct treatment of disease is administered.This study was based in Kiambu and Narok counties becauseextensivenomadic pastoralist and intensive zero grazing system in Narok and Kiambu counties respectively enhancing contrast information. Human serum was collected from hospital benches in Kijabe referral hospital which borders the two counties. Human serum was also sampled from other few hospital. Milk was consumed in both counties by both adults and children. Both counties have well established infrastructure facilitating easy movement.

1.4 Research Questions

(i)What is the prevalence of human brucellosis in Narok and Kiambu counties?

(ii)Is serum agglutination test and Rose Bengal test valid in diagnosis of humanbrucellosis when compared to Immunoflourescence microscopy ?

(iii)What occupationsare mostly associated with exposure to brucellosis in Kiambu and Narok counties?

1.5 Hypotheses

1.5.1 Null Hypotheses

i) There is no statistically significant difference in prevalence of brucellosis between Narok and Kiambu counties.

ii) Demographic characteristic (age, gender, occupation and ingestion of unpasteurised milk) do not predispose human beings to *Brucella* infection.

iii) The SAT and RBT are not valid in diagnosing human brucellosis.

1.6 Objectives

1.6.1 Broad Objective

To evaluate serum agglutination test (SAT) and Rose Bengal test against the Immunoflourescence antibody assay in diagnosis of brucellosis in Narok and Kiambu counties.

1.6.2 Specific Objectives

(i)To determine the prevalence of brucellosis in Narok and Kiambu counties by Serum agglutination test, Rose Bengal test and Immunoflourescence antibody assay.

(ii)To validate Serum agglutination test and Rose Bengal test against Immunoflourescence antibody assay as a gold standard.

(iii)To determine association between occupations and exposure tohuman brucellosis in Narok and Kiambu counties.

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of Brucellosis

The genus *Brucella* comprises gram negative, facultative, intracellular pathogens (Alton *et al.,* 1975). Which they occur singly, in pairs, short chains or small clusters (Corbel *et al.,* 1989). They are non-sporulating and considered to be non-capsulated flagella(Corbel *et al.*, 1989). Moreover matrix and porin proteins penetrate the peptidoglycan layer at irregular intervals and are partially exposed at the cell surface (Corbel, 1989). Polysaccharide granules and small vacuoles are fairly evenly dispersed throughout cytoplasm (Corbel, 1989). They are categorized as α - group 2 subdivisions of the proteobacteria along with agrobacterium, bartonella, rickettsia, ochrobactrum, rhizobium, rhizobacteria and rhodobacter (Pappas *et al.,* 2006).

Currently, there are six species of *Brucella spp.* based on phenotypic characteristics, antigenic variation and prevalence of infection in different animals hosts. They include *Brucellae abortus* isolated from cattle, *Brucella canis* isolated from dogs, *Brucellae neotomae* isolated from desert wood rats, *Brucella ovis* commonly found in sheep, *Brucella suis* isolated from pigs, reindeer, and hare (Corbel *et al.,*1997) and *Brucella malitensis* isolated from sheep. Two new species were isolated from marine animals *Brucella pinnipedialis* and *Brucella ceti* in mid 1990s (Foster *et al.,* 2007) while more recently a novel species, *Brucella microti* was detected and isolated from common vole (Scholz *et al.*, 2008).

2.2 Epidemiology of Brucellosis

Brucellosis continues to be a major public health concern worldwide and is the most common zoonotic infection (Pappas *et al.*, 2006). While some countries have eliminated or substantially reduced it by extensive eradication programs it remains endemic in many areas of the world (Whatmore *et al.*, 2007). Areas currently considered to have high brucellosis prevalence are Middle East, the Mediterranean Basin (Portugal, Spain, Italy, Greece, Turkey, Near East, North Africa), South and Central America, South Eastern Europe, Asia, Africa and the Caribbean. In the USA, the only known focus of *Brucella abortus* infection left is in bison (50% of the animals tested positive) and elk in the Greater Yellowstone Area(Godfroid *et al.*, 2005).

The ultimate source of infection are infected animals especially the major food-producing animals: catte, sheep, goats and pigs. Others including bison, buffalo, camels, dogs, horses, reindeer and yaksara are significant source of infection in some regions. Brucellosis has also been identified in marine mammals , including dolphins, porpoises and seals and these may present an emerging harzard to persons occupationally exposed to infected tissues from them. Indeed, spill over from wildlife to cattle is regularly reported around these natural parks (Godfroid *et al.*, 2005). Brucellosis is also found in wild animal that exist in herds for example biston in North America and wild boar in Germany (Al Dahouk *et al.*, 2005). Human experience only a limited risk from wild animal because of lack of contact with these animals and infrequent use of meat and milk produce from wild animals. However, this raises concern in developing countries like Kenya where wild animals interact with domesticated ones leading to infection of agricultural animals for example in arid areas like Narok county. Human beings get the infection from animal reservoir directly via three routes: (1) direct contact through the conjunctivae, or through abraded contacts skinwith excretions, secretions, or tissues of infected animals or contaminated fomites. (2) Inhalation of infectious aerosols with invasion occurring through the mucosa of the upper

respiratory tract or the lung and (3) ingestion of tissues, foodstuff, or fluids containing organisms (Nicoletti, 1989).

Brucellosis occurs throughout sub-Saharan Africa, but its prevalence is unknown (Pappas *et al.*, 2005). However, seroprevalence of 3.8% has been reported in nomadic pastoralists from Chad (Schelling *et al.*, 2003). Certain occupations are associated with high risk of infection with brucellosis including farmers, farm labourers, stockmen, shepherds, sheep shearers, goatherds, pig keepers, veterinarians and inseminators. Occupational contacts, including butchers, slaughterhouse workers, milkman, and cow attendants in one state in eastern Sudan revealed 1% were infected (El-Ansary *et al.*, 2001). Moreover, in contrast, slaughter house workers in Djibouti gave 6.5% positive (Chantal *et al.*, 1996) and at high risk groups from Eritrea showed a seroprevalence between 3.0% and 7.1% (Omer *et al.*, 2002).

Laboratory staff involved in culturing *Brucella* are also at risk. Nevertheless, the performance of diagnostic procedures on patients with suspected disease may lead to culture of organisms which are not correctly identified until laboratory acquired infection raises the level of suspicion. The use of rapid identification gallery test systems has caused *Brucella* strains to be misidentified as *Moxella spp.*with various consequences for the staff. Inhalation of aerosols generated by manipulation of cultures present the greatest hazard especially if breakage of containers occurs during such processes as centrifuging. The preparation and use of live vaccines is also hazardous through accidental injection and is rifampicin resistant. Therefore, the use of virulent strains to prepare diagnostic antigens should also be avoided where possible. Provision of improved diagnosis is crucial to enable prevalence investigations to be undertaken (Muriuki *et al.*, 1997).

A study in Narok, Kenya, patients presenting with flu-like symptoms were tested for brucellosis (RBPT), Malaria (blood smear test) and typhoid (Widal test) (Maichomo, 1997; Maichomo *et al.*, 1998, 2000). However, results showed that brucellosis (12%) and typhoid cases were (40%) higher than expected. None the less, local clinics can conduct the RBPT (Muriuki *et al.*, 1997; Maichomo *et al.*, 1998).

Brucellosis in the Mediterranean, chiefly due to *Brucella malitensis*, has the highest age and sex related incidence in males in their mid-20s. A report from Northern Saudi Arabia found that 60% cases of brucellosis occurred in individuals aged 13-40 years whereas 21% occurred in those younger than 13 years, 16% in those aged 40-60 years and 2.5 % in those older than 60 years. For unknown reasons, men aged 13-40 years are particularly vulnerable to the manifestation of illness due to *Brucella malitensis*. Possible explanations include engaging in activities that increase exposure to *Brucella* and less deligent personal hygiene. The predilection is not universal given that 60% of cases in Jordan occur in individuals younger than 24 years. The published data suggest that brucellosis is common in children in developing countries because milk is not usually pasteurized. Transmision to infant occur through breastfeeding or ingestion of raw milk. Prepubertal infection in children account for less than 2% of the cases.Worldwide, brucellosis is more common in males particularly young adults than females(Wafa Al- Nassir *et al.*, 2013). A report from northern Saudi Arabia found a male to female ratio of 1.7 -1 for age 13-40 years(Wafa Al- Nassir *et al.*, 2013).

Foodborne brucellosis is not limited to age or sex (Wafa Al- Nassir *et al.*, 2013). Interest in brucellosis has been increasing because of the growing phenomena of international tourism and

migration, in addition to the potential use of *Brucella* as a biological weapon (Wafa Al- Nassir *et al.*, 2013).

2.3 Pathogenesisof Brucellosis Disease

2.3.1 Transmission of Disease

Brucellosis is transmitted from animal reservoirs via inhalation of infectious aerosols with invasion occurring through the mucous of the upper respiratory tract or the lung and ingestion of tissues, foodstuff or fluids containing organisms (Nicoletti, 1989). However, contamination of skin wounds may be a problem for persons working in slaughter houses or meat packing plants or for veterinarians (Godfroid *et al.*, 2005). *Brucella spp.*persist for several days in milk (even when it turns sour). Therefore, these products have to be made from pasteurized milk (Godfroid *et al.*, 2005). Furthermore, sale of improperly prepared cheese and other dairy products by commercial vendors results in additional cases in urban populations in many countries and in traders who visit areas where brucellosis is endemic (Nicollet, 1989). Nevertheless, human to human transmission by tissue transplantation or sexual contact has occasionally been reported but are in epidemiological terms, insignificant (Godfroid *et al.*, 2005). Infection in man is a dead end process and further transmission to other persons or to other host species occurs so rarely that it can make no contribution to the natural history of the disease (Corbel, 1989).

2.3.2 Pathology

Brucellae possess a unique ability to invade both phagocytic and nonphagocytic cells and able to survive in the intracellular environment by avoiding the immune system. Following penetration of the mucosal epithelium, the bacteria aretransported, either free or within phagocytic cells, to the regional lymph nodes. The spread and multiplication of *Brucella* in lymph nodes, spleen, liver, bone marrow, mammary glands, and sex organs occurs via macrophages.*Brucella* not only resist

killing by neutrophils following phagocytosis but also replicate inside macrophages and nonprofessional phagocytes(Ko *et al.,* 2003).The organism reside and multiplyusing parts of the cytoskeleton in specialized acidic environments compartmentwithout interrupting cell cycleand function. They are apoptosis inhibitors, thus creating a frame for external survival and replication (Pappas *et al.,* 2006). After replication, *brucella* are released with the help of hemolysins and induced cell necrosis (Pappas *et al.,* 2006). Critical aspects in this response include secretion of IL-12 and IFN- alpa involving APCs and Th 1 cells. MCH class 1-restricted CTLs are generated and are required to clear the infection (Golding *et al.,* 2001). Interferon-gamma has a central role in the pathogenesis of brucellosis by activating macrophages, producing reactive oxygen species and nitrogen intermediates; by inducing apoptosis, enhancing cell differentiation and cytokine production; by converting immunoglobulin G to immunoglobulin G2a; and increasing the expression of antigen presenting molecules (Pappas *et al.,* 2006). In general, rough strains, containing less or no O polysaccharides (OPS), are less virulent than smooth strains and less resistant to complement attack (Ko *et al.,* 2003). Although *Brucella*infection is primarily controlled through cell-mediated immunity rather than antibody activity, some immunity to infection is provided by serum immunoglobulin (Ig). Intially, IgM levels rise, followed by IgG titers. IgM may remain in serum in low levels for several months, whereas IgG eventually decline. Persistently elevated IgG titers or second rises in IgG usually indicate chronic or relapsed infection. IgA antibodies are elevated levels and also may persist for very periods of time(Wafa Al-Nassir, 2013).

2.4 Diagnostic Methods for Brucellosis

2.4.1 Clinical Diagnosis

The symptoms and signs most commonly reported are fever, fatigue, malaise, chills, sweats, headaches, and weight loss (Kochar *et al.*, 2007; Mantur *et al.*, 2007). Given that symptoms and signs of brucellosis are non-specific, laboratory diagnosis is vital, which has serious challenges. However, the definitive diagnosis of brucellosis is based on culture, serologic techniques or both accompanied by correct clinical history information such asthe profession, food ingested, contact with infected animals and travel to endemic areas.

2.4.2 Laboratory Diagnosis

Brucella agent may be present in blood, cerebrospinal fluid, semen and occasionally urine (CDC). Biosafety level 2 practices are recommended for activities with clinical specimens of human or animal origin containing or potentially containing pathogenic *Brucella spp* (CDC).

2.4.2.1 Culture

Specimens for culture include blood, bone marrow, solid tissues samples or excludate such as pus (Greenwood *et al.*, 2000). Serum dextrose agar is a suitable solid medium which is equivalent liquid medium (Corbel, 1989). However, *Brucella*broth contains tryptone 10 g, peptaminen 10 g, dextrose 1 g, yeast 2 g, sodium chloride 5 g, sodium bisulfate 0.1, supplement (cycloheximide, bacitracin, circulin and polymyxin B), and 0.25 ml of 4 % sodium citrate as anticoagulant (Ozkurt *et al.*, 2002). On primary isolation growth is usually less evident in less than 24 hours on a suitable medium. Laboratory- adapted strains grow vigorously and produce visible colonies after 24 –h incubation (Corbel, 1989). After 48 h growth at 37 oC, most *Brucella* strains will produce colonies 0.5 to 1.0 mm in diameter which are raised, convex and have circular outline. Their color and

texture depend upon the colonial phase of the organisn whereby smoothstrains colonies are transparent and pale yellow (droplet of honey'' appearance) with a shiny surface when viewed in transmitted light.

The most often used and simplest bacterialogical procedure is blood broth in 10% carbon dioxide. However, the relative proportion of successful blood culture reported varies between 85.4 %and 17% but the chances of successful isolation of the organism decrease with time. Convectional blood cultures are positive by the 4^{th} day of incubation, majority are positive between the 7^{th} and 21^{st} days and 2% are positive after the 27^{th} day. Therefore, culture should be maintained for 45 days before they are declared negative (Diaz and Moriyon, 1998). Culture method sensitivity is 60%. Subcultures are advised to be kept for at least 4 weeks which is not done routinely for most bacteria cultures (Wafa Al- Nassir *et al.*, 2013).

Bone marrow cultures are the gold standard for the diagnosis of brucellosis because the relatively high concentration of *Brucella* in the reticuloendothelial system makes it easier to detect the organism. Also *Brucella*survive the intracellular killing by phagocytes and polymorphonuclear leukocytes. Bone marrow culture sensitivity is 80-90 % (Gotuzzo *et al.*, 1986). Furthermore, bacterial elimination from the bone marrow is equivalent to microbial eradication. However, harvesting bone marrow for culture is invasive, painful technique, and results have not been universally reproducible (Pappas *et al.*, 2006). Nevertheless, isolation of *Brucella sp.* requires high security laboratory facilities (biological containment level 3), highly skilled personnel, an extendend turnround time for results and it is considered a hazardious procedure(Nielson and Yu, 2010).

2.4.2.2 Serological diagnosis

Serological testing is the most commonly used method of brucellosis diagnosis:

Standard agglutination test (SAT) was developed by Bruce, measures antibodies against smooth lipopolysaccharide and it remains the most popular test tool for the diagnosis of brucellosis(Wafa Al- Nassir *et al.*, 2013).

Tray agglutination test is also popular. Titers of more than 1:160 in conjuction with compatible clinical presentation is considered to be highly suggestive of infection(Wafa Al- Nassir *et al.*, 2013). The challenges of this test include potential cross-reactivity with IgM of other organism such as *Francisella tularensis, salmonella urbana, Yersinia enterocolitica, Vibrio cholera* and otherbacteria(Wafa Al- Nassir *et al.*, 2013). Prozone phenomenon may occur which result in false-negative results therefore, routine dilution of the serum beyond 1:320 wouldhelp to prevent such a problem (WHO, 2006). Serum agglutination test have a major drawback in that they are not suitable for patient follow-up, since antibody titers can remain high for a prolonged period (Pappas *et al.*, 2006). The agglutination test is also very sensitive to antibody resulting from vaccination (Alton, 1990). The SAT detects IgG less efficiently, especially IgG1 resulting in low assay specificity (Nielson and Yu, 2010).

Therefore, the SAT is generally not used as a single test but rather in combination with other tests (Nielson and Yu, 2010). The production of IgM in response to cross- reacting antigens often induces significant levels of agglutinating antibody which causes specificity problems in the SAT (Nielson and Yu, 2010).

The potential usefulness of the Rose Bengal Test (RBT) for diagnosing human brucellosis was suggested by the Food and Agriculture Organisation and World Health Organisation Committee(Diaz and Moriyon, 1989). This method has replaced the rapid slide test of Huddleson which was prone to false –negative results (Diaz and Moriyon, 1989). However, the RBT is very sensitive to vaccinal antibody, seriously limiting its use in vaccinated animals (Alton, 1990). Interpretations of RBT results can also be affected by personal experience (Cho *et al.*, 2010).

The complement fixation test (CFT) requires many of reagents and is technically challenging(Nielson and Yu, 2010).The basic test consist of*B. abortus* whole cell antigen incubated with dilutions of heat inactivated serum (heated to destroy indigenious complement) and a titrated source of complement usually guinea pig serum(Nielson and Yu, 2010).After a suitable time a pretitrated amount of sheep erythrocytes coated with rabbit antibody is added.However, because a number of reagent must be titrated daily and a number of controls for all the reagents and reactions are required, the test is time consuming and technically challenging. It is also an expensive test because of the number of reagents used(Nielson and Yu, 2010).Other problems include the subjectivity of the interpretation of results, occasional direct activation of complement by serum (anticomplementary activity), prozoning resulting in false negative result and inability of the test for use with haemolysed serum samples(Nielson and Yu, 2010).

The competitive enzyme immunoassay for the detection of serum antibody to *Brucella* is a multispecies assay which appears to be capable of differentiating vaccinal and cross-reacting antibodies elicited by field infection in cattle (Lucero*et al.*, 1999). The test detect IgG antibody, which is useful for evaluating treatment effectiveness, for monitoring clinical conditions, and prognosis. It is relatively easier to perform than complement fixation test and somewhat faster than

TAT. Fewer cross-reactions with antigens of other microorganism (or antibodies) occur, and the use of Mab detection reagents enables standardization. ELISA tests are relatively costlier test in comparison to agglutination test that require equipment and experience(Lucero*et al.*, 1999). The indirect enzyme immunoassay generally have very high sensitivity but because they are largely unable to distinguish *B.abortus* S19 vaccinal antibody and cross reacting antibody, the specificity can be slightly lower(Nielson and Yu, 2010). The specificity of the competitive enzyme immunoassay is relatively high although it is slightly less sensitive than the indirect enzyme immunoassay(Nielson and Yu, 2010).

The Immunoflourescence microscopy (IFA) was developed as a test that could be performed outside the diagnostic laboratory, allowing for rapid and accurate diagnosis (Lucero *et al.*, 2013). The IFA test is rugged, relatively inexpensive, simple and very rapid (Nielson, 2002). The IFA is a homogenious assay requiring no washing steps or removal of unreacted component. It can be performed in a 96 well format or a tube formatfor rapid diagnosis. The IFA is very accurate and sensitivity and specificity can be manipulated by altering the cut-off value between positive and negative reactions to provide a highly sensitive screening test as well as a highly specific confirmatory test (Nielson, 2002). The IFA can distinguish vaccinal antibody in most vaccinated animals and it eliminate reactivity by some cross-reacting antibodies as well (Nielson, 2002).

The first published PCR-based diagnostic assay was reported by (Fekete *et al.*, 1990). This technique which produces the multiplication of predermined specific nucleotide sequences offer a potential for the microbiological diagnosis of brucellosis because the organism is difficult to isolate by standard culture method and the laboratory risk of handling organisms are considerable. The method has been applied successfully using a range of primers that allow gene specific species

recognition. As yet they are not routinely used because they are expensive and require sophisticated apparatus which are rare in many health laboratories (Cook and Zumla, 2003).

In a study conducted in Kuwaiti (Bannatyne *et al.,* 1997) large scale evalution of single –nested PCR for the laboratory diagnosis of human brucellosis. A single tube nested PCR assay identified a 52 bp fragment from the genus specific *Brucella* IS711 was used prospectively in clinical practice for the diagnosis of human brucellosis. Out of 263 suspected cases of brucellosis, serology was positive in 199 and culture in 89, while the *Brucella* PCR was positive in 193 patients (sensitivity 96.98%). The study recorded two false negativePCR results. Both had been on long-term antibiotics for previously diagnosed brucellosis but their adherence werequestionable allowing relapse. PCR gave good results similar to serology testing with added advantage of identifying the presence of the organism and was superior to culture.

2.5 Treatment of Brucelllosis

The treatment recommended by the World Health Organization for acute brucellosis in adults is rifampicin 600 to 900mg and doxycycline 100mg twice daily for a minimum of six weeks (FAO and WHO 1986). Some still claim that the combination of intramuscular streptomycin (1 g/day for 2-3 weeks) with an oral tetracycline (2 g/day for 6 weeks) gives fewer relapses (Mantur *et al.*, 2006). Trimeprime –Sulfamethoxazole (TMP/SMX) is a popular compound in many areas, usually used in triple regimens. Furthermore, various combinations that incorporate ciprofloxacin have been tried clinically, yielding similar efficacy to that of classic regimens (Karabay *et al.,* 2004).

Childhood brucellosis can be successfully treated with a combination of two drugs; Doxycycline 4 mg/kg/day and rifampicin 10 mg/kg/day orally for six weeks. Moreover, some authors advise that gentamicin (5 mg/kg/day intramuscularly) be administered concomitantly for the initials 5-7 days

of therapy in order to prevent relapses (Mantur *et al.*, 2004). TMP/SMX 8 mg/40mg/kg/day can be used for children less than 6 years of age. Tetracycline is not recommended for use in children younger than 8 years of ages unless other appropriate drugs are ineffective or are contraindicated (AHFS Drug information, 2006). Additionally, tetracycline may result in overgrowth of non susceptible organism, including fungi (AHFS Drug Information, 2006). Rifampicin is administered by month and is preferred over tetracycline for children and pregnant women with brucellosis (Young, 1989). For neurobrucellosis, combination therapy with two or three drugs- that is doxycycline, rifampicin, and TMP/SMX that penetrate central nervous system is recommended (McLean *et al.*, 1992). The combination of doxycycline with rifampicin and trimethoprim-sulfamethoxazole has been used successfully for the treatment of endocarditis. However, it is generally believed that surgical intervention (valve replacement) combined with antibiotic therapy is the best approach.

2.6 Management and Control

As a general rule, prevention of human brucellosis depends predominantly on the control of the disease in animals (Godfroid *et al.*, 2005). In addition, personal hygiene, environmental sanitation, pasteurization of dairy products, and health education controls the illness (Nicollet, 1989). The transmission and spread of brucellosis is affected by a variety of factors and good knowledge of these is essential to the success of a control policy (Reviriego *et al.*, 2000; Bikas *et al.*, 2003; Minas *et al.*, 2004).

Effective reduction of disease prevalence in livestock through mass vaccination eventually will also lead to a reduction of brucellosis in the human population. However vaccination alone is not sufficient and should be accompanied by other measures such as restriction of animal's movement

and trade, culling of animals and improved farm sanitation to reduce further spread of the disease. In addition, a surveillance system is essential to control the efficacy of control measures and identify outbreaks at an early stage. Attempt at vaccinating people at risk have proved ineffective (Schuring *et al.*, 2002). As a result it is necessary to take protective measures such as protective clothing, barriers while handling still births, products of conception and cultures can reduce occupation related brucellosis (Young, 1995). Consequently, laboratory workers should culture the organism only with appropriate biosafety level 2 or 3 containment (Hoover, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was conducted in urban and rural areas around Kiambu and Narok counties respectively. Blood samples were collected in Kijabe referral hospital and other health institutions that includedOldonyo Muka health centre, Ololtoto, Kojonga dispensary,Sakutek dispensary in Narok and Ndeiya health centre, Lusigetti health centre, Githunguri dispensary, Kamiti health centre in Kiambu (appendix vii and viii).

3.2 Study Design

This study was cross sectional in design in which the samples were arrived at in a convenient sampling in Kiambu and Narok counties. A total of 250 human samples were obtained from kijabe referral hospital benches which had been screened for other infections. Accesiblecentrifuged samples were collected, put in sterile vacutainers and packed in cool boxes. Samples were stored at -20^{0}C. A total of 250 milk samples were collected from milk vendors from Narok and Kiambu counties. Available and willing milk vendors collected milk from 250 individual farmers from both counties and kept the milk in bus stages where it was collected and transported to Kabete Central Veterinary Laboratory in a cool box. Milk was put in 10 ml sterile specimen bottle using a pasteurpipette. Milk samples were stored at -20^{0}C.

3.3 Sample Size

Population sample size was calculated according to (Thrustfield, 2005).Thus

$$n = \frac{1.96^2 \cdot P_{exp}(1-P_{exp})}{d^2}$$

Where n = sample size required, P exp is expected prevalence, d^2 is desired absolute precision.

d^2= standard normal deviate (1.96 which correspond to 95% confidence interval).

P= proportion of the target population estimated to have particular characteristic prevalence is 21% (Muriuki *et al.*, 1997) was used, where;

d=degree of accuracy =0.05.

Therefore, n= $(1.96^2 \times 0.21 \times 0.79)/0.05^2$ andn= $(1.96^2 \times 0.1 \times 0.9)/0.05^2$

=250 blood samples and 250 milk samples. A total of 500 samples were collected.

3.4 Sampling Technique

This study was conducted using convenient and purposive technique for both blood and milk samples. The blood samples were obtained from various hospitals as follows Kijabe (50),Ndeiya health centre, Lusigetti health centre, Githunguri dispensary, Kamiti health centre and other major health facilities in Kiambucounty (75) while 125 samples were obtained from Oldonyo Muka health centre, Ololtoto, Kojonga dispensary, Sakutek dispensary and other major health facilities in Narok county. The blood samples were collected from hospital laboratories which had been previously screened for other infections from December 2009 to August 2010. Blood samples which had been centrifuged in the hospital was put in sterile tubes using pasteur pipette and some were centrifuged at 1000×g per minute. The samples were packed in cool box and kept in a refrigerator below -20^{0}C.

Milk samples were collected from intensive system in Kiambu and extensive systems in Narok. Milk samples were collected from milk vendors on foot and mobile trader on bicycles and motorbikes. Accesible and willing milk vendors submitted 25 ml from 250 individual farmers from Narok and Kiambu counties. Each sample was put in 10 ml sterile specimen bottle and packed in

cool box. The cool box containing milk was transported to Kabete Central Veterinary Laboratory where they were refrigerated at -20^{0}C.

3.5 Collection of Samples

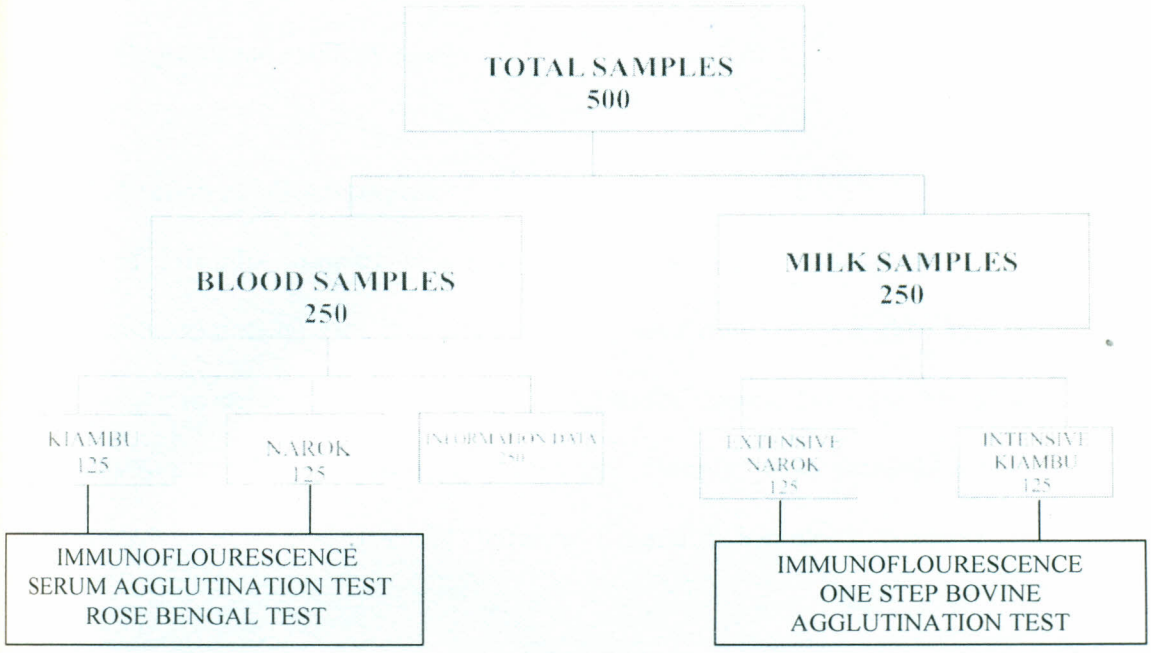


Figure: 3.5 sample size determination

3.5.1 Clinical Information Data

A total of 250 clinical symptoms data was collected from patients files both inpatients and out patient files from December 2009 and August 2010. Each blood sample had patient's name and the number which was used to retrieve the patients record. The symptoms were mostly fever, headaches and loss of weight. The data was compiled according to the diseases they were diagnosed with malaria, typhoid, TB, brucellosis, pneumonia, menengitis, cholera and syphilis.

3.5.2 Collection of Blood Samples

A total of 250 blood samples were collected from Kijabe referral health facilities laboratories and other major health facilities in Kiambu and Narok counties. Accesible centrifuged samples were

put in 10 mls sterile tubes using a pasteur pipette and packed in cool boxes. Some samples were centrifuged at1000 ×g per minute and kept in refrigeration at -20^{0}C. Blood samples were screened for brucellosis using SAT and RBT at the Kijabe referral hospital laboratories. Screened blood samples were later transported to Kabete Veterinary Laboratory for confirmation test using Immunoflourescence antibody assay.

3.5.3 Collection of Milk Samples

A total of 250 milk samples were collected from individual farmers by accessible and willing mobile milk vendors on foot, bicycles and motor bikes from the areas they buy milk. The 10 ml of milk was put in sterile specimen tubes provided to the traders. The milk was packed in cool boxes and conveyed to Kabete Veterinary Laboratory. Samples were screened using one step bovine *Brucella* Ab rapid antibody test and confirmed using IFA. Samples in cool boxes were refrigerated at-20^{0}C.

3.6 Serological Tests

3.6.1 Wright's Serum Agglutination Test (SAT)

The antigen for the sero-agglutination in tubes was a concentration suspension of *Brucella abortus* (Weybridge 99 strain) inactivated by heat and phenol which allows the serological diagnostic of *Brucella melitensis* and*abortus, suis.* This antigen is supplied at 10× working concentration. After dilution the antigen was standardised to give 50% of agglutination with a final dilution of 1/650 of the International *Brucella abortus* standard serum. The principle of Wright's sero-agglutination technique is slow agglutination in tubes. Successive dilutions of serum to be titrated were brought into contact with constant concentrations of brucellosis antigen. The reagent used included sera to be tested, positive and negative sera, concentration antigen (10×), distilled water and phenoled salt water.

3.6.1.2 Procedure for SAT

The samples to be analysed were diluted and the antigen added. A serial dilution was used and the tube with highest dilution showing agglutination was considered as the end point and was the antibody titre. A total of 8 test tubes were placed on test tube rack and labeled 1 to 8. To tube number one, 1.9 ml freshly prepared isotonic saline and 0.1 ml of sample serum were added and mixed thoroughly. A total of 1 ml of the diluted serum from tube number 1 was transferred to tube number 2 and the mixture thoroughly shaken. The serial dilution was continued till tube number 7. A total of 1 ml of diluted serum was discarded from tube number 7. A total of I ml of isotonic saline was put to test tube number 8 to serve as control.

To all the tubes 50µl of *Brucella* antigen suspension (*Brucella abortus* preserved with 0.95% sodium azide) was added and the mixture thoroughly shaken to ensure uniformity. The tubes were covered and incubated at 37^0Cfor 24 hours. Presence of agglutination was observed macroscopically in each tube of the dilution series and the antibody titre recorded. Where there was no agglutination the results were recorded as negative. In this study the dilutions ranged from 1:20 up to 1:1280. However for determining the disease status the cut off was set at 1:160 and was considered together with the appropriate clinical manifestations. The antigen was stored at $+4^0$C without freezing (Institut Pourquier-326 rue de la Galera -34090 Montpellier –France).

3.6.2Improved Rose Bengal Test(RBT)

The Rose Bengal was used for the qualitative detection of anti-*Brucella* antibodies in human serum (Cho *et al.*, 2010). The bacterial suspension of *Brucella abortus*stained with Rose Bengal dye was used for detection of *Brucella*specific IgG and IgM antibodies.

All the reagents were brought to room temperature. One drop (20µl) of each control was placed into separate circles on the glass slide. The antigen vials were shaken gently before use.One drop (20 µl) of suspension was added to each circle to the next sample drop. The two were mixed with disposable stirrer and spread over the entire area enclosed by a ring. A new stirrer was used for each sample. The tile was slowly rotated and read for agglutination after one minute.

3.6.3 Generation of *Brucella* Antigen

Production of *Brucella* antigen was carried out using Hemoline diphasic performance medium (bioMerieuxMarcy- l'Etoile, France). Castenoda bottle was kept at room temperature. Repositionable protective cap was removed and stopper disinfected with alcohol or iodine solution. The sample was collected using a syringe.Castenoda bottle was filled with blood between the 1st and 2nd graduations printed on thebottle label. Stopper was disinfected again. Protective cap was replaced. Bottle was tilted several times from sides to sides to mix the blood and the broth.

The blood sample was sent to the laboratory one hour after collection. Bottle was vented with the ventilating system (Hemoline TM venting needle). Agar surface was flooded by placing the bottle in a horizontal position for 5-10 minutes to inoculate the agar. Pre-incubation of broth was done to encourage the growth of the colonies. Incubation was done at 37 in an upright position. Appropriate temperatures were choosen for the intended use, in accordance with current standards.

The bottle was examined every day for 19 days. Re -inoculation of the agar was done every 48 hours by flooding. The agar and broth were observed. Subculturing was done on the appropriate media. Identification of the organism(s) isolated was performed using biochemical tests such as,Zeal Nelson, urea, catalase and oxidase. The *Brucella* antigen was stored at -20^{0}C.

3.6.4 Immunoflourescence Antibody Assay (IFA)

The IFA slides in this kit utilized fixed *Brucellae* within a matrix of egg yolk sac sonicate (SmarTest Diagnostics Orgenics Ltd., part of the Inverness Medical Innovations Group, Israel). The procedure used was adopted from manufacture's procedure based on canine patient and control sera.Dilution of sera was done using PBS (Orgenics Ltd, 2006).

3.6.4.1 Procedure for IFA

The main reagents provided in IFA kit included *Brucella* antigen, egg yolk, PBS, detergents (sonicate), sera to be tested, positive and negative control sera and conjugate (rabbit anti-human IgG). The *Brucella* antigen was generated as described under section 3.6.3.

All the reagents were brought to room temperature.A 1:50 v/v screening dilutions (1 part patient serum with 49 parts PBS) was prepared for all untested serum specimens. For sera found positive on a previous assay run, two serial fold dilutions in PBS starting with 1:50 was prepared. Acute convescent pairs wascompared by assaying all dilutions in parallel. Dilutions of the positive control was prepared in PBS to include one dilution above the stated endpoint and one dilution below the stated endpoint (1:200-1:800). For each serum or control dilutions to be tested, 10 µl was added to one slide well. For each assay, the Negative Control, Positive Control and (step 2) was prepared. The stained substrate slide was read at 400 X magnification. Slide werestored at 2-8^{0}C in the dark for up to 24 hours. Slides were placed into a humidity chamber and incubated in water bath or incubator for 30 minutes at 37 ^{0}C$\pm$ 0.5 ^{0}C. The slide wells were rinsed well in gentle

stream of PBS from the wash bottle three times shaking PBS from the slide into a sink between each wash. The next step followed without allowing slide wells to dry. To each slide well, 10 µl rabbit anti human IgG conjugate was added, then slide returned to the humidity chambers for 30 minutes incubation in the water bath or incubator at $37^0C \pm 0.5\ ^0C$. Incubation was done in the dark to protect photosensitive conjugate. Slide were washed 3 times as before. Then 2-3 drops of mounting medium was then added to each slide and covered with slips. Stained substrate slide were read at 400 X magnification. Slide werestored at 2-8 ^0C in the dark for up to 24 hours.The resulting reactions were visualized using standard fluorescence microscopy, where a positive reaction was seen as sharply defined apple- green flourescent *Brucella* (coccobacilli) against a constrasting red background of the yolk sac material. A negative reaction was seen as either red-counterstained yolk sac alone or fluorescence. Slide werestored at 2-8 ^0C in the dark for up to 24 hours.

3.6.4.2 Quality Control for IFA

The Negative Control and dilutions of the Positive Control was assayed with each daily run. The Negative Control well was an example of a non reactive serum, with either uniform red counterstain or slight (less than 1+), but uniform, greenish staining. The positive Control wells gave the end point titer from 1:100 to 1:800. The fluorescence intensity at 1:400 was used as cut-off level required for a patient reaction to be called positive. If either of the controls does not react as specified, the assay run was considered void. Reagent components and procedural steps was rechecked, and the assay repeated from beginning. The negative control well was an example of fluorescence patterns that were considered negative. If bright staining was seen in this well, similar to that seen in the positive control wells, there was a breakdown in technique and the assay was repeated.

3.6.4.3 Interpretation of Results for IFA

A positive reaction appeared as brightly fluorescent (at least 1+) sharp, regular stained coccobacili evenly distributed in a matrix of red –counterstained egg yolk sac sonicate, while the negative control well was an example of fluorescence patterns that were to be considered negative. The positive control wells gave an endpoint titer of 1:00 to 1:800. The fluorescence intensity at 1:400 however, was used as a cut-off level required for a patient reaction to be called positive.The size, appearance and density of the *Brucellae* wascompared with the positive and negative controls reactions. Patterns of reactivity different from those seen in the positive control was considered non-specific(Philip*et al.*, 1978).

3.6.3.4 Isolation of Rabbit Anti-human

The rabbit antihuman IgG conjugate was produced according to (EnCor Biotechnology Inc. Gainesville, Florida, 2015).Protein preparation was done by mixing with ammonium sulfate solution to make the desired percentage concentration. Saturated solution of ammonium sulfate was prepared using for example 550 g made up to 1L (i.e 10 ml of blood per 3.14 g salt and 5.7 ml distilled water). The solution was mixed gently to dissolve all the ammonium sulphate and was allowed to cool at RT to working temperature on a magnetic stirrer. Crystals of ammonium sulfate formed to inform that the solution was fully saturated. Appropriate volume of saturate solution of ammonium sulfate was added to the sample to get the desired concentration. Mixture was stirred for 1 hour to fully equilibrate. Solution was centrifuged at 1000g for 15 minutes to pellet out protein. Pellets were dissolved in PBS for subsequent protein analysis and dialysis (Wiley, 1998).

3.6.4.5 Dialysis

The protein (rabbit anti-human igG) in solution (ammonium salt solution) was separated from ions, water and other small molecules by filtration through a semipermeable membrane. For dialysis the small molecules were simply changed in sample volume. During dialysis, the tubing were selected

in size to hold the sample in a 10-30 cm length. Before use, the tubings were dipped in distilled water to soften and decrease the pore sizes. Tubings were closed at both ends with two knots. Protein in ammonium salt solution was put inside the tubing.Dialysis was done against four changes of 500 ml of PBS. Increasing the number of changes of buffer improves the exchange more than increasing the volume of each change. Minimum time of dialysis against each change was4 h (Alan and Robin, 1987).

3.6.4.5.1 Zeba TM Desalt Spin Columns, 0.5 ml

The Zeba Desalt spin Columns contains a high -performance resin that offers exceptional desalting and protein recovery characteristics. Sample volumes (30-130µl) containing as low as 20µg/ml of protein can be processed providing exceptional protein recovery and more than 95% retention of salts and other small molecules (<1000 Da). These columns required no chromatography system or cumbersome column preparation or equilibration. Additionally, the spin-colunm method eliminated the need to wait for samples to emerge by gravity flow, allowing multiple sample processing in ~6 minutes.

3.6.4.5.2 Desalting Procedure for Dialysis

Additional materials required for desalting were variable speed bench top microcentrifuge and 1.5 microcentrifuge collection tubes. Spin colunm preparation was by removing colunm's closure and cap loosened. The column was placed in a 1.5-2.0 ml microcentrifuge collection tube. The column was centrifuged at 1,500 ×g for 1 minute to remove storage solution. Excess fluid was removed through blot bottom of the column. A mark was placed on the side of the column where the compacted resin slanted upward. The column was placed in the microcentrifuge with the mark facing in all subsequent centrifuge steps. Improperorientation resulted in reduced desalting efficiency.

In sample loading, the column was placed in a new collection tube, the cap was removed and slowly 30-130µl of sample was applied to the center of the compact resin bed. (Optional) for sample volumes <70 µl a 15 µl stacker of ultrapure water or buffer was applied to the top of the resin bed after the sample had fully absorbed to ensure maximal protein recovery. The column was centrifuged at 1,500 ×g for 2 minutes to collect desalted sample. Desalting column was discardedafter use.

3.6.4.6 Inoculation of Health Human IgG Sample in a Rabbit

Polyclonal antibody production was performed on rabbits and involved the use of Freud's Complete and Incomplete Adjuvant's (FCA, FIA). Immunization sites were shaved using scapel. A concentrated antigen solution was added to the adjuvants to reduce quantity of emulsion injected. Multiple injection sites were used (up to 6) with limitation volume at each site (1 ml). FCA was used for initial and boosted with FIA. A total of 14 days was required between injections. Rabbits were bred through ear vein.

3.6.4.6.1 Immunization schedule for Rabbits

Day 0: Pre-immunization bleed to serve as blank. It was stored frozen. A total of 1 ml immunogen/adjuvant mix was injected into each 6 subcutaneous sites on the back of the rabbits.

Day 14:Boosting was done with an equivalent amount of adjuvant.

Day 21: Test breed and assay antibody response was done. A total 5-10 ml of blood was collected which was adequate for measuring antibody response.

Day 28: Similar schedule of alternating boosts and test breeds until a satisfactory response was observed. Rabbits were executed for blood collection (Pierce Biotechnology Inc., 2005).

3.6.4.6.2 To Test for Response for Hyper Immunity /Immune Response

The main materials provided for testing hyper immune included agarlevelling table, boiling water bath and glass plates or microscopic slides (26 ×76 mm). Glass plates and microscope slides were precoated to provide supports for noble agar gels which acted as an adhesive surface for supporting the analytical gel and prevented it from floating away during washing and staining procedures. The noble agar on the plate was allowed to dry onto the glass surface.A 0.5 g noble agar was boiled in 100 ml water until it dissolved (15-20 min). Agar solution was allowed to cool to 50^0C. For plates 0.2 ml/cm2) was pipetted on thelevelling table. Agar was allowed to gel and left overnight at room temperature, or (4-6 hat 40^0C) until completely dry. Precoated slides were protected from moisture.

3.6.4.6.3 Staining Precipitation Lines

The Materials provided for staining precipitation lines included noble agar gel containing immunoprecipitate, Phosphate buffered saline containing 0.02% w/v sodium azide (PBS- azide), Whatman 3 MM filter paper Stain: 0.5 % w/v, Coomassie brilliant blue R in ethanol: water:acetic acid 50:45:5 by volume and destain: water:acetic acid:methanol 87:8:5 by volume. The gel was washed in 5 changes of 100 ml PBS-azide over 48 h, dried by covering with a sheet of filter paper and leaving for 16 h at room temperature or 2-6 h at 40^0C. The gel was immersed in stain for 5-10 min untilstained bands were visible. It was destained in 3-4 changes of destain solution until background was clear. The plate was dried in air at room temperature.

3.6.4.6.3 Diffusion techniques for Precipitation Lines

Materials provided for double immunodiffusion included precoated microscopic slide, noble agar, leveling table, humid chamber, phosphate buffered saline containg 0.02% w/v sodium azide, gel punches, Whatman no. 1 filter paper, vacuum line, rabbit antihuman IgG globulin,control pre-immune serum and boiling water bath.

Antibody was allowed to encounter antigen by diffusion alone. Double immunodiffusion was done by preparing a buffered noble agar gel and antigen antibody loaded into separate wells bored in the agar about 0.5-2 cm apart. After incubation for 48 hours antibody- antigen recognition was detected by formation of an immunoprecipotation line between the wells. Concentration gradients of antigen and antibody was formed automatically by diffusion process alone. Immunoprecipitation occurred between the wells providing equivalenceconcentration was obtained at some point where antigen and antibody concentrationoverlapped.

Glass tubes coated with agar were placed on the leveling bench and holes were punched in the agar using the cork borer or gel punch of precision pattern. Plugs of agar were removed from the wells using a pasteur pipette attached to the vacuum line. Pressure was adjusted so that the plugs were quickly and evenly removed. Dilutions of antiserum, control serum, and antigen in PBS –azide was prepared. Appropriate dilutions were neat and ½, ¼, 1/8, 1/16, 1/32. Twenty microlitre (20 µl) neat antiserum was prepared and pipetted into the centre well of the pattern and dilutions of antigen (20 µl /well) was pipette in order of concentration in the surrounding wells. It was repeated with control serum. A 20 µl neat (1 mg/ml) antigen was put in the centre of the well of another set of well bored in the same pattern and (20 µl /well) at the centre well of another set of wells bored in the same pattern and dilutions of antiserum (20 µl /well) was pipette in order of dilutions in the surrounding wells. Set up was incubated overnight at room temperature in the humid chamber. Glass plates were examined for immunoprecipitation lines (Alan & Robin, 1987).

3.6.4.7 Labeling of Rabbit Anti-human IgG Conjugate with FITC

Pierce FITC Antibody Labelling Kit, contained sufficient reagents to label and purify 3×1 mg (2 mg/ml) of IgG or similar amounts of other proteins(F& S Scientific Ltd.). The kit contents included FITC (flourescence isothiocyanate), 3 vials, Molecular mass:389.2, extinction

coefficient:70,000 M-1 cm-1 (at 594 nm in aqueous buffer, pH 8), Ex/Em wavelength:494/517 nm, borate buffer (0.67 M), 1 ml, purification resin, 5ml, spin columns, 6 each and microcentrifuge collection tubes, 12 each, phosphate –buffered saline (PBS; for measuring the FITC – to protein ratio).

Upon receipt FITC was stored at -20^{0}C. All other components were stored at 4^{0}C.The Thermo Scientific Pierce FITC Antibody Labelling Kit contained all the necessary components for three protein labeling reactions and subsequent excess dye removal. Flourescein isothiocyanate (FITC) crosslinks to amino, sulfhydryl, imidazoyl, tyrosyl and carbonyl groups on proteins; however, only derivatives of primary and secondary amines yield stable products. Reactions were most efficient at pH 8-9 and must be performed in an amine –free buffer such as borate or carbonate/ bicarbonate. Generally, the protein wasreacted with a 15- to20-fold molar excess of FITC, which resulted in several FITC molecule. The purification resin and spin columns eliminated equilibration steps and theneeded to be collected and monitored gravity-flow fractions. This system enabled efficient removal of excess FITC and, therefore, accurate determination of the dye- to- protein ratio and exceptional protein recovery.

3.6.4.7. 1 Protein Preparation for FITC

The materials provided for protein preparation was optimal labeling buffer, 50 mM sodium borate and pH 8.5. Just before use, the labeling buffer was prepared by diluting borate buffer (0.67M) to 0.05 M in PBS or ultrapure water for protein lyophilized in phosphate buffered saline (PBS). Only enough labeling buffer required for the reaction was prepared (for example, to prepare 1 ml, 75µl of Borate Buffer (0.67M) was added to 925µl of ultrapure water or PBS).One milligram (1 mg) of protein was reconstituted with 0.5 ml labeling buffer. For protein in phosphate buffered saline , a 40 µl of Borate Buffer (0.67 M) was added to 0.5 ml of 2 mg/ml protein in PBS. If the protein was more than 2mg/ml, concentration was adjusted to 2 mg/ml with labeling buffer.

3.6.4.7.2Protein labeling for FITC

The materials provided for FITC labeling included spin columns, fluorescence isothiocyanate and purified rabbit antihuman IgG conjugate. All reagents were brought to room temperature. A 0.5 ml of the prepared protein was added to the vial of FITC reagent and pipetted up- and - down 10 times until all the dyes dissolved. Mixture was vortexed briefly. The reagent was completely dissolved for effective labeling. The vial was briefly centrifuged to collect the sample in the bottom of the tube. The reaction mixture was incubated for 60 minutes at room temperature protected from light.

The labeled protein was stored protected from light at 4^0C for up to one month. Alternatively, the labeled protein was stored in single – use aliquots of 0.5 ml at -20^0C. Repeated freeze /thaw cycles was avoided. The final concentration of conjugate was, 1 mg/ml, a stabilizing agent, such as bovine serum albumin was added at 1-10 mg/ml.

For FITC- to –protein ratio estimation, a small amount of labeled purified proteins was diluted in PBS. One (1) cm path length cuvette was used to measure absorbance at 280 nm and 495 nm (i.e., the A max of FITC).

$$\text{Protein comcentration (M)} = \sum \frac{A280 - AMaxXCF}{\text{Pr}otein}$$

ΣProtein = protein molar extinction coefficient (molar extinction coefficient of IgG was ~ 210,000 M-1 cm-1).

$$\text{CF} = \text{Correction factor} = \frac{A280}{AMax}$$

The degree of labeling was calculated as follows:

Moles FITC per mole protein =Amax of the labeled protein ×dilution factor

Σ_{fluor} × protein concentration (M)

= 70,000 (FITC molar extinction coefficient) (Thermo Fisher Scientific, 2008).

3.7 One step Bovine Brucella Antibody Rapid Test for Milk Samples

The materials provided to perform the assay included thirty antigen rapid B. Ab test kits, assay diluents, 1 bottle, instructions for use and capillary tube (20 µl/drop).The kit is a chromatography immunoassay for the qualitative detection of *Brucella abortus* antibody in whole blood, plasma, serum and milk. The kit has a letter T and C as ''Test line'' and ''control line'' on the surface of the kit which are not visible before applying any samples. A purple Test line was visible in the result window if *Brucella abortus* antibodies in the specimen was enough. The specially selected *Brucella abortus* antigens were used in the test as both capture and detector materials which enable the Antigen Rapid B. *Brucella* Ab kit to identify the *Brucella abortus* antibodies in specimens, with a high degree of accuracy.

3.7.1 Procedure of the Test Antigen Rapid B. *Brucella* Ab kit

The procedure of the Test Antigen Rapid B. *Brucella* Ab kit involved slowly adding one drop (20 µl) by capillary tube (with dark color score line for indicating a volume of 20 µl) of serum, plasma, whole blood or raw milk was added to the sample well and then 4 drops of assay diluents added. If the migration had not appeared after 1 minute, more drop of assay diluents was added to the sample well. A test result was seen as a purple band in the result window of the kit. The test result was interpreted at 20 minutes. No interpretation was done after 20 minutes. Interpreting result was based on reading the test results at room temperature of 15 ~ 30^0C.

The presence of only one purple color band within the result window indicated a negative result. The presence of two color bands (''T'' band and ''C'' band) within the result window, no matter which band appeared first, indicated a positive result. If the purple color band was not visible within the result window after perfoming the test, the result was considered invalid hence

recommended that the specimen be retested. The Antigen Rapid B. *Brucella* Ab kit has been compared with the Rose Bengal Test. The overall accuracy was greater or equal to 97.0% (BioNote, Inc. 2005 Korea).

3.8 Laboratory Data Recording

Data for the 250 serum samples was recorded in terms of prevalence, sensitivity, specificity, positive predictive value and negative predictive value. IFA was used as gold standard.Table 3.1 shows the components of formula used to compute prevalence, sensitivity, specificity, positive and negative predictive values.

Table: 3.1 General plan for determining components of formula

True disease status		Positive	Negative	Total
Test result	Positive	A	B	a +b
	Negative	C	D	c +d
	Total	a +c	b +d	a +b +c +d = 250

The study parameters were computed as follows;

Prevalence = Number of positive samples / total number of samples tested expressed as a percentage.

Sensitivity = a /a +c.

Specificity= d/ b +d.

Positive predictive value= a/ a+b

Negative predictive value= d/c +d (Sunkon and Kerlikowske, 2008).

3.9 Data Analysis

The data recorded was analysed using statistical analysis system (SAS) and was used to analyse the data. Prevalence of blood and milk from Narok and Kiambu samples were tested using frequencies, percentageand statistical significance tests computed using correlation coeficient.Associations in various occupational groups was calculated using chi-square test (χ^2)and paired t test. The test results were presented in form of tables and figures.

CHAPTER FOUR

RESULTS

4.1 Study subjects

Two hundred and fifty patient's blood were randomly selected from Kijabe mission hospital and other health institution in the region, 125 from Kiambu and 125 from Narok counties. Also data was obtained from the same patient's files at Kijabe hospital and other health institutions from December 2009 and August 2010. One hundred and fourty seven (147) were females and 103 were males.

4.2 Prevalence of Brucellosis in Kiambu and Narok by SAT, RBT and IFA

Out of 125 serum samples analysed from Narok, 88 (70.4%) were positive for brucellosis consisting of 28 males (32%) and 60 females (68 %) of the total positive samples by Immunoflourescence antibody assay. The difference was statistically significance ($P< 0.05$). The results of Kiambu sample analysis gave 4 (3.2 %) positive of which 2 were males and 2 females by IFA (Table 4.1).

Table:4.1 Occurrence of brucellosis by IFA in human samples titers

Antibody titre	1:00	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	Total
IFA Positive	4	3	9	7	28	22	13	6	92
IFA Negative	40	32	24	21	24	8	5	4	158

Out of 125 serum samples analysed from Narok, 97 (77.6 %) were positive by Serum agglutination test. The results of Kiambu analysis by SAT was 13 (10.4 %) (Table: 4.2)

Table:4.2 Occurrence of brucellosis by serum agglutination test in human samples titers

	Antibody titre	**1:00**	**1:20**	**1:40**	**1:80**	**1:160**	**1:320**	**1:640**	**1:1280**
Kiambu	Frequency	29	30	28	25	8	2	2	1
Narok	Frequency	20	9	16	13	29	17	14	7

Out of 125 serum samples analysed from Narok, 100 (80 %) were positive by Rose Bengal Test. The results of Kiambu sample analysis gave 7(5.6 %) by RBT(Table 4.3).

Table 4:3 Prevalence of brucellosis in Kiambu and Narok by SAT, RBT and IFA

Counties	SAT		RBT		IFA	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Positive (%)
Kiambu (n=125)	13 (10.4%)	112	7 (5.6%)	118	4 (3.2%)	121
Narok (n=125)	97(77.6%)	28	100 (80%)	25	88(70.4%)	37
(n=250)	110 (44%)	140	107 (43%)	143	92(36.8%)	158

4.2.2 Prevalence of Brucellosis in Narok and Kiambu Counties by Sex by IFA

Out of 92 tested positive for brucellosis, 28 (32%) were males and 60(68%) were females from Narok county. Out of 92 tested positive for brucellosis, 2 (50%) were males and 2(50%) were females from Kiambu by IFA (Table 4.4).

Table: 4.4 Prevalence of brucellosis in Narok and Kiambu counties bysex by IFA

	Male	Female
Narok	32	68
Kiambu	50	50
Total	82	118

The prevalence of brucellosis was significantly affected by gender where the rate of detection was significantly higher among females (68%) than males(32%) in Narok as detected by Immunoflourescence microscopy ($P<0.05$), df = 1, $\chi^2 = 6.697$, P value = 0.010. The prevalence of brucellosis was non significantly affected by gender in Kiambu county ($P>0.05$ by chi-square test) as detected by Immunoflourescence antibody assay.

4.2.3 Prevalence of Brucellosis by Ageby IFA

Out of 92 tested positive for brucellosis, 60 females and 28 males were from Narok while 2 females of age group 21-30 and 31-40 years and 2 males age group 21-30 and 41-50 years were from Kiambu county by IFA (Figure: 4.1).

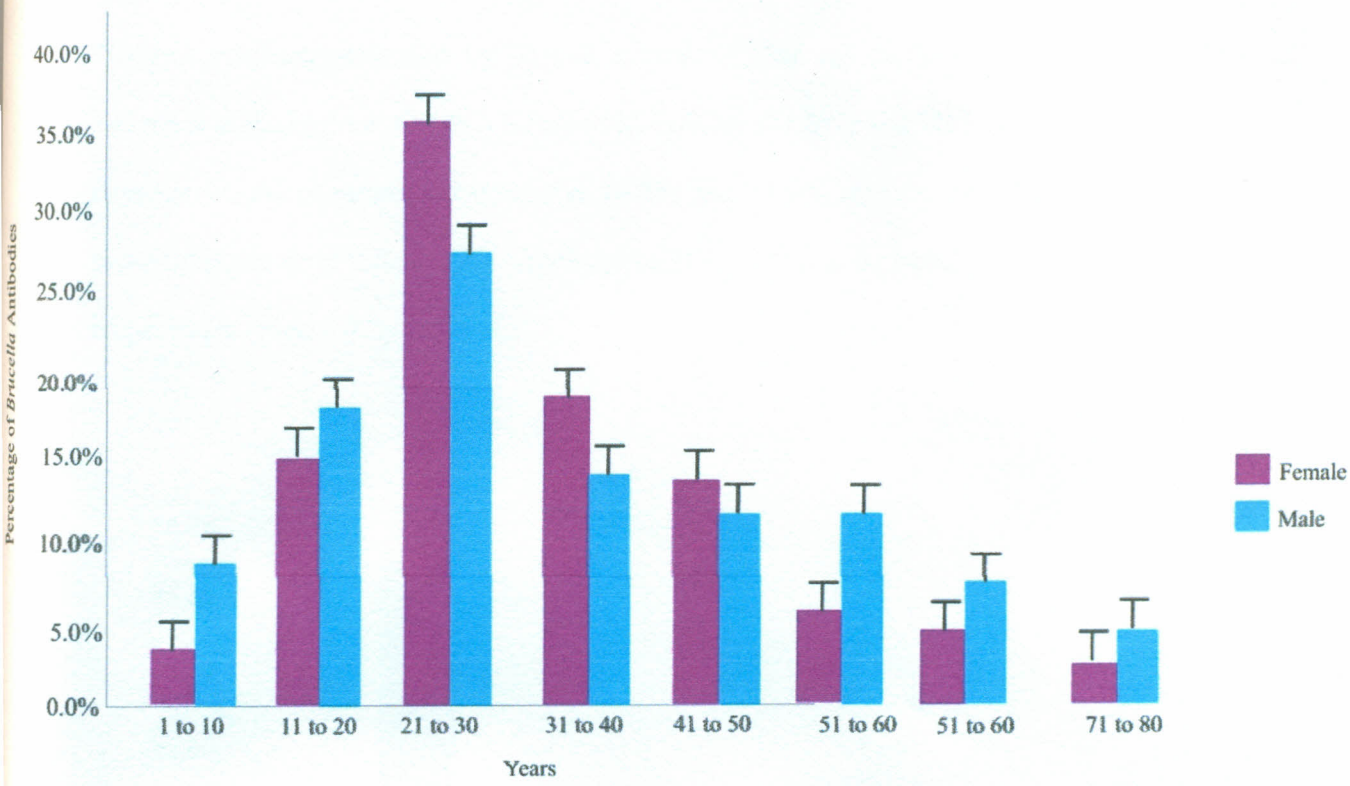


Figure 4.1: Prevalence of brucellosis by age by IFA from Narok county

The prevalence of brucellosis among different age groups showed a statistically significant difference ($P \leq 0.001$)n=8, df=7 in both females and males in Narok. *Brucella* antibodies were highest in Narok county among females in age group 21-30years (36.7%), followed by 31-40years (16.7%) and 11-20 (15%) while the lowest was 41-50 (13.3%), 51-60 (6.7%), 61-70 (5%) followed by age group 1-10(3.3%) and 71-80 (3.3%) as detected by Immunoflourescence antibody assay. In males *Brucella* antibodies was highest in age group 21-30(28.6%), followed by 11-20(17.9%), 31-40 (14.3%) while the lowest was 41-50(10.7%) and 51-60(10.7%) followed by 1-10(7.1%), 61-70(7.1%) and 71-80(3.5%) as detected by Immunoflourescence antibody assay.

4.3Diseases Diagnosed at Kijabe Hospital

Diseases diagnosed in the hospital for 250 participants were retrieved from their files by different tests supplemented by clinical symptoms. Among diseases diagnosed at the hospital, malaria was the highest at (28%) followed by typhoid (21.6%) and TB (17.2%). Brucellosis and pneumonia followed at 13.2% and 11.6% respectively. The leastprevalent was menengitis, cholera, followed by syphilis at (4.1%), (2.8%) and (1.6%) respectively (Figure 4.2).

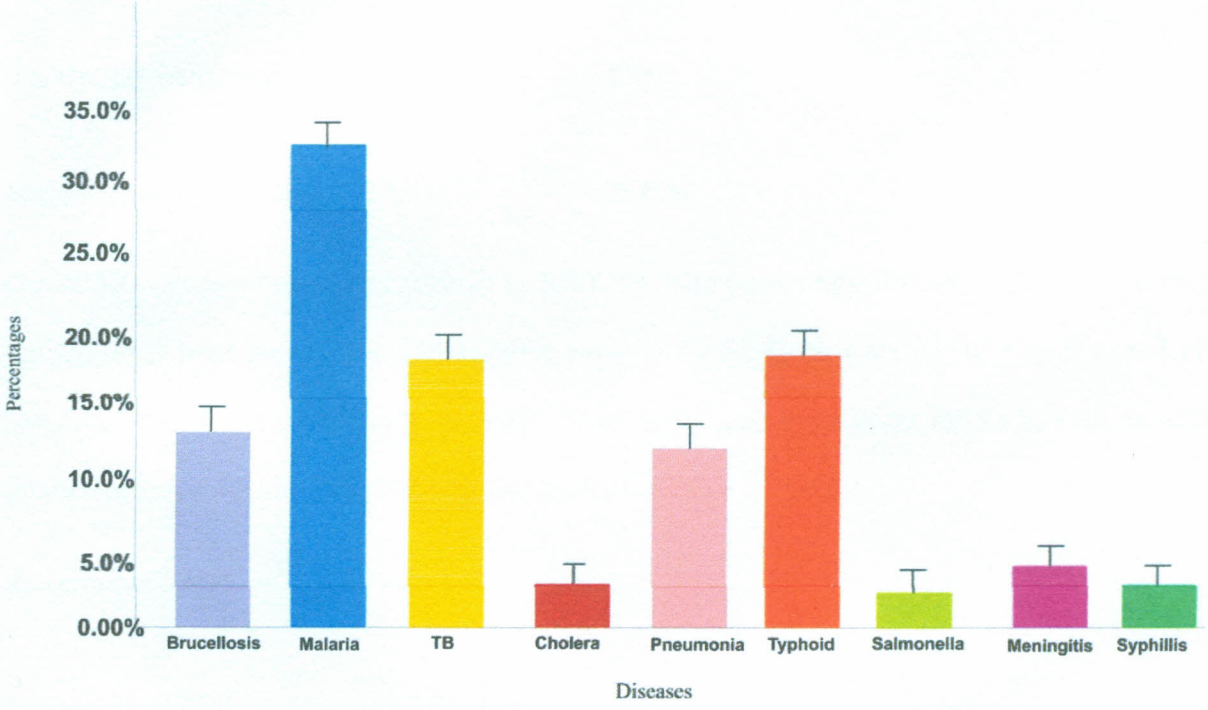


Figure 4.2: Diseases diagnosed in Kijabe hospital by different tests supplemented Byclinical symptoms

4.4 Validation of SAT and RBT using IFA in Human Serum

The IFA was used as a gold standard to determine the actual disease status in the participants. Out of 110 samples considered positive by SAT, 69 were positive by IFA

while 41 were negative. On the other hand out of the 140 negative samples by SAT,23 were positive by IFA and 117 negative. This put the sensitivity of SAT at 75%, specificity at 74% while the positive predictive value was 63% and negative predictive value 16.4% (Figure 4.3).

$$\text{Sensitivity of SAT} = \frac{69}{92\,x\,100} = 75\%$$

$$\text{Specificity of SAT} = \frac{117}{158} X100 = 74\%$$

$$\text{Positive predictive value of SAT} = \frac{69\,X\,100}{110} = 63\%$$

$$\text{Negative predictive value of SAT} = \frac{23\,X100}{140} = 16.4\%$$

Out of 107 samples considered positive by RBT, 64 were positive by IFA while 43 were negative. On the other hand out of the 143 negative samples by RBT, 28 were positive by IFA and 115 negative. This put the sensitivity of RBT at 69.56%, specificity at 72.78% while the positive predictive value was 59.81% and negative predictive value 19.58%

$$\text{Sensitivity of RBT} = \frac{64\,x92}{100} = 69.56\%$$

$$\text{Specificity of RBT} = \frac{115\,X100}{100} = 72.78\%$$

$$\text{Positive predictive value of RBT} = \frac{64\,X107}{100} = 59.81\%$$

$$\text{Negative predicitive value of RBT} = \frac{28\,X100}{143} = 19.58\%$$

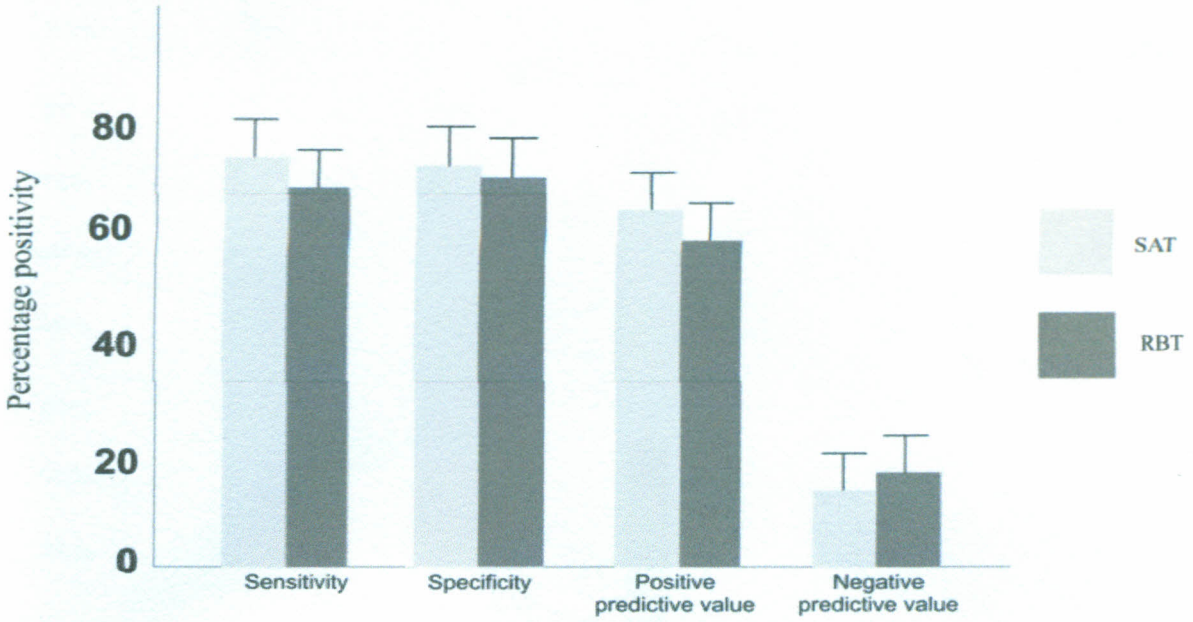


Figure 4.3: Positivity of SAT and RBT using IFA as a gold standard

4.1 Comparison of Rose Bengal Test, Serum agglutination test and Immunoflourescence antibody assay in human samples

Out of 36 samples with fever of unknown origin 2(6%) from Kiambu and 5(14%) from Narok had *Brucella* antibodies by RBT, 3 (8.3%) from Kiambu and 13 (36.1%) from Narok by SAT and 4(11.1%) from Kiambu and 25(69.4%)Narok by IFA(Figure 4.4).

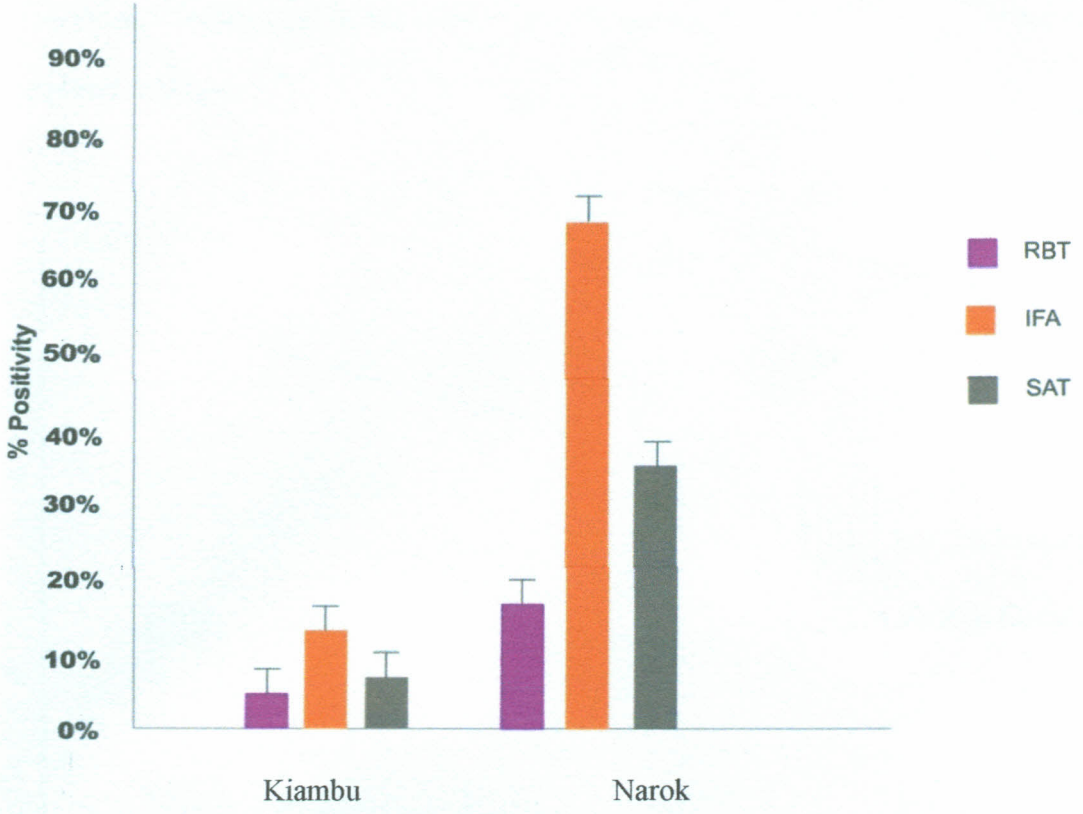


Figure 4.4: Brucellosis positivity by different tests from Kijabe

Positivity of human samples showed a prevalence of 69.4% and 11.1% in Narok and Kiambu respectively by IFA as a gold standard test. This was followed by SAT at 36.1 % and 8.3% andlowest was at 14% and 6% by RBT in Narok and Kiambu respectively.IFA was the gold standard for diagnosis of brucellosis in human.

4.4.2Brucella antibodies in milk samplesfrom Kiambuand Narokby one step bovine brucella rapid antibody test and Immunoflourescence antibody assay.

A total of 125 milk samples each from Kiambu and Narok were screened using one step bovinebrucella rapid antibody agglutination tests and results confirmedusing Immunoflourescence antibody assay. With one step bovine Brucella rapid antibody test 15 samples representing (12%) fromNarokand 2 samples (1.6%) from Kiambu had Brucella antibodies. Using immune

flourescence antibody assay, the results were 68(54.4%)and 18(14.4%) for Narok and Kiambu respectively(Figure 4.5).

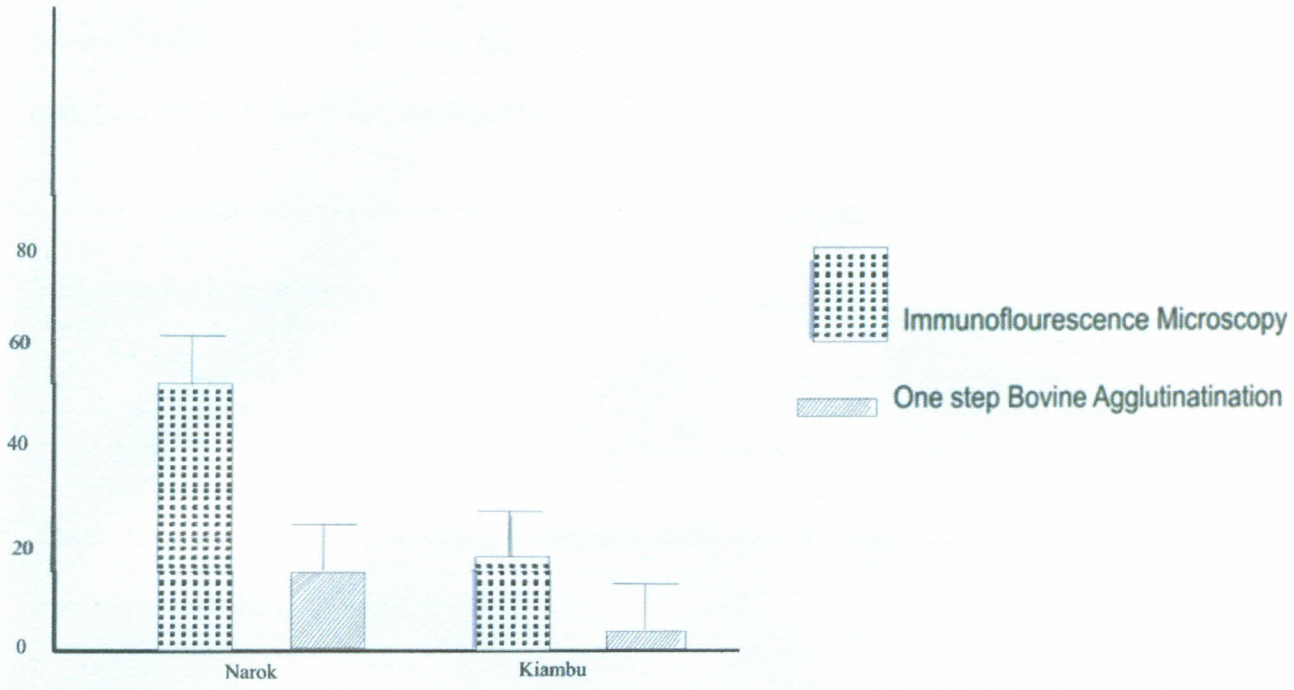


Figure 4.5: *Brucella* antibody positivity of milk using one step

***Brucella* rapid antibody test and IFA tests**

The difference in positivity to *Brucella* antibodies in milk samples in Narok and Kiambu was statistically significant ($P<0.001$), $n=250$, $df=1$, $r = 1$.There was a strong correlation of *Brucella* antibodies in milk samples in Narok and Kiambu counties.

4.4.3 Correlation of *brucella* antibodies in milk and human samples in Kiambu and Narok counties

There was no significant relationship in *brucella*antibodiesin milk from that in human in Kiambu county (r=-0.975, P= 0.143). Also there was no significant difference in *Brucella* antibodies in milk samples from that in human detected in Narok (r =-0.771, P = 0.440) (Table:4.5).

Table:4:5 Prevalence of brucellosis in human samples and milk samples

Milk sampleHuman serum

Narok	Kiambu	Narok (%)	Kiambu (%)
IFA 54.4%	IFA 14.4%	IFA 70.4	3.2
		SAT77.6	10.4
		RBT 80	5.6

There was no significant relationship in *Brucella* antibodies in Kiambu to those in Narok in milk and human samples (r = -0.674, P = 0.097).

4.4.4 Validation ofOne Step Bovine Brucella Ab Rapid Antibody Testusing IFA

The IFA was used as a gold standard to determine the actual disease status in the milk sample. Out of 86 samples considered positive by IFA, 17 were positive by one step bovine rapid agglutination test. On the other hand out of 164 negative by IFA, 78 samples were negative by One step bovine rapid agglutination test. This put the sensitivity of one step bovine *Brucella* Ab rapid antibody test at 16.5%, specificity at 53.06%, positive predictive value at 19.76% and negative predictive value at 52.43% (Table: 4.6).

Table 4.6 Validation of One Step Bovine Rapid Agglutination Test using IFA

True disease status		Positive	Negative	Total
Test result	Positive	17	69	86
	Negative	86	78	164
	Total	103	147	250

Sensitivity of one step bovine ab rapid agglutination test = $\frac{17x100}{103} = 16.5\%$

Specificity of one step bovine ab rapid agglutination test = $\frac{78x100}{147} = 53.06\%$

PPV of one step bovine ab rapid agglutination test = $\frac{17x100}{86} = 19.76\%$

NPV of one step bovine ab rapid agglutination test = $\frac{86x100}{164} = 52.43\%$

4.5Occupational Exposure

Out of the total positive cases house wives constituted 15.91% and 50%, abbatoir workers 32.95% and 25%, veterinarinas 4.55% and 0%, farmers 15.91% and 0%, herdspersons 21.59% and 0% and butchers 9.09% and 25% in Narok and Kiambu counties respectively. In all occupations except the house wives and butchers Narok county had significantly higher positive cases when compared to Kiambu county (Table: 4.7).

Table 4.7: *Brucella* antibodies in occupation groupsin risk population as determined by IFA test(n= 250)

Occupation	Narok(n %)	Kiambu (n %)
Housewife	14(15.91%)	2(50%)
Abbatoir work	29(32.95%)	1(25%)
Veterinarians	4(4.55%)	0(0%)
Farmers	14(15.91%)	0(0%)
Herdsperson	19(21.59%)	0(0%)
Butchers	8(9.09%)	1(25%)
Total	88 (70.4%)	4 (3.2%)

To test for association between patient's occupation and place of sampling that were positive(n=92), df 2, the calculated χ^2 was 1.4904 with an associated p value of 0.4746. There wasno evidence to show that patient's occupation was associated with Narok and Kiambu. Also the correlation coefficient was 0.0867 and the associated p-value was 0.2694 which was not significant.

To test for association between patient's occupation and disease occurrence for total positive and negative samples (n=250), df 2, the calculated χ^2 was 4.6003 with an associated p value of 0.1002. There was no evidence to show that occupation is associated with Narok and Kiambu. Also the correlation coefficient was 0.1356 and the associated p-value was 0.0311 which was significant at 5% level (Table:4.8).

Table 4.8: Seroprevalence of brucellosis among risk groups in Narok and Kiambu counties by IFA (n= 250)

Place of sampling	Positive	Negative	Total
Narok	88	37	125
Kiambu	4	121	125
Total	92	158	250

Estimation of the Relative Risk (Row1/Row2)

Type of Study	Value	95%	Confidence Limits
Case-Control (Odds Ratio)	71.9459	24.7395	209.2292
Cohort (Col1 Risk)	22.0000	8.3327	58.0843
Cohort (Col2 Risk)	0.3058	0.2329	7 0.4015

Sample Size = 250

The calculated χ^2 was 121.4 with an associated p value of <0.0001, (n=250), df 1. In conclusionthere was strong evidence to show that disease occurrence differed across Narok and Kiambu. Relative risk of acquiring brucellosis was higher in Narok (22.000) than Kiambu (0.3058). The prevalence in Narok was 70.4 %while in Kiambu it was 3.2%by IFA. Also the correlation coefficient 0.6967 and the associated p-value was <0.0001 which was highly significant.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The overall prevalence of brucellosis was 70.4% and 3.2% by Immunoflourescence antibody assay in Narok and Kiambu respectively. The results are in agreement with the study conducted in Nigeria where a prevalence rate of 7.6% for *Brucella* RBPT obtained in the study agrees with the findings of other workers who reported high seroprevalence of the range of 6 % and 28% among hospital patients(Asande and Agbede, 2001). The findings also concur with the study conducted among hospital patients in Nigeria where 63 positive samples (RBPT) were retested with SAT and a prevalence of 90.5% were obtained (Ofukwu*et al.*, 1994).

In this study the overall prevalence of brucellosis was higher in Narok than Kiambu county (table 4.3). In Narok also women had higher prevalence than men with the age group 21-30 years recording the highest followed by 31-40 years for both males and females. The lowest prevalence was recorded in age groups above 61 years old and those below 10 years old for both males and females. It is noteworthy that in Narok women were the ones found herding animals while men were left to deal with security matters. The age group 21-30 was the most active group and interacted more with possible sources of infection like livestock and livestock products. The age group above 61 years old were too close to retirement and their movement was minimal hence there was less chances of infection, while those below 10 years were too young to adequately interact with sources of infection. This results differs with those obtained from Egypt where the prevalence was found to increase in the age group 51-60 years followed by 14-20 years and 7-13 years (Asmaa *et al.*, 2005). In Saudi Arabia and Bangladesh brucellosis was found to be 60% in 13-40 years and 57.14% in 21-40 years old respectively (Noor, 2009, Wafa Al- Nassir *et al.*, 2013).

These differences could be explained by different cultural practices in the different communities. However, in all the places association with animals was positively correlated with brucellosis infection.

Although human brucellosis affects all age groups, it is said to be rare in childhood(Wafa Al-Nassir *et al.,* 2013). However, in areas, where *B. malitensis* is endemic, pediatric cases are increasingly encountered (Caksen *et al.,* 2002; Mantur *et al.,* 2004). Brucellosis in children comprises 3-10% of reported cases, worldwide, with heavier burden in endemic areas. Persons in their third and fifth decades of life are most commonly affected by brucellosis (Caksen *et al.,* 2002; Mantur *et al.,* 2004). The available literature suggest that brucellosis may be common in children in developing countries because of ingestion of unpasteurised milk and working in an agriculture society(Wafa Al- Nassir *et al.,* 2013). Transmission to infants may occur through breast feeding or ingestion of raw milk (Caksen *et al.,* 2002; Mantur *et al.,* 2004).

The prevalence of brucellosis was significantly higher in females ($P<0.05$) as compared to males, in Narok but not in Kiambu county. These findings concur with a study conducted in Uganda,where more females were found sero-positive (69.1%) than men (30.9%) (Kobei*et al.,* 2011). In all studies, females were infected more often than males, most likely because of their increased association and exposure with livestock and their products. The findings also concur with the study conducted in Bangladesh where the prevalence of brucellosis was significantly affected by sex (chi-square = 3.87, $P < 0.05$ and df = 1), where female infection was higher than male detected by slide agglutination test (Noor, 2009). Similar findings were also reported from Egypt and Turkey (male (3.1%, female 6.3%) (Asmaa, 2005, Zafer *et al.,* 2005).The results are in constrast with other studies conducted in Jordan where higher incidence in males (3.5%) than females (2.6%) was reported with no statistically significance difference (Abou, 2001). Prevalence

of brucellosis was lower in Kiambu county probably due to the fact that in Kiambu most farmers have zero grazed animals and the level of development is also comparatively higher.

Prevalence of brucellosis from Kijabe hospital records was put at 13.2% by SAT supplemented with clinical symptoms. This low prevalence as compared to>70% observed from this study byIFA showed the level of reliability of this test. In other studies, compared to the tube agglutination test, IFA detected 56/60 TAT-positive sera (sensitivity:93%) and in 96 sera tested negative at both 1:16 and 1:50 screening dilutions using Fc-specific anti-IgG conjugate (100%) (Philip *et al.,* 1978).It is possible most cases diagnosed clinically as typhoid, malaria and pneumonia could actually be brucellosis since all these diseases can present with almost similar symptoms. This misdiagnosis of brucellosis as malaria and other diseases has been reported by other workers (Maichomo *et al.,* 2000). Examples of diseases which are difficult to distinguish clinically withbrucellosis include typhoid, rheumatic feverand other conditions causing pyrexia, frequently diagnosed as pyrexia of unknown origin (Muriuki *et* al., 1997; Mutanda, 1998;Maichomo *et al.,* 2000; Baba *et al.*, 2001). Because of its difficult diffential diagnosis and lack of health facilities in areas where brucellosis is common, human brucellosis is rarely diagnosed and consequently inadequately treated (Muriuki *et al.,* 1997).

The prevalence of brucellosis based on analysis of serum samples from Narok county was 77.6% by SAT while by IFA 70.4%. The difference though not statistically significant may explain the short coming of SAT which includes cross-reactivity with immunoglobulin M (IgM) of other organisms such as *Francisella tularensis, salmonella urbana, Yersinia enterocolitica, Vibrio cholera* and otherbacteria(Wafa Al- Nassir *et al.,* 2013). Prozone phenomenon may also occur in SAT which result in a false-negative or false positive results. To overcome the prozone

phenomenon dilution of the serum beyond 1:320 is recommended (WHO, 2006). Serum agglutination test has been branded not suitable for patient follow-up since titers can remain high for a prolonged period (Pappas *et al.,* 2006). Also the agglutination test is sensitive to antibody resulting from vaccination and therefore could give false positive results in vaccinated animals (Alton, 1990). The SAT detects IgG less efficiently, especially IgG1 resulting in low assay specificity. Therefore, SAT is generally not used as a single test but rather in combination with other tests (Nielsen and Yu, 2010). The production of IgM in response to cross- reacting antigens often induces significant levels of agglutinating antibody which causes specificity problems with the SAT (Nielsen and Yu, 2010). The problem of defining a SAT titre indicative of active infection has yet to be solved (WHO, 2006). In general, each patient produces an individual response and it is not possible to predict the behaviour of this in each case, not to explain why some patients develop high agglutinin titres while others have only low values during the disease (WHO,2006).

The prevalence of brucellosis by RBT (80%) was higher than SAT (77.6%) and IFA (70.4%) in Narok because of short coming of RBT. Serum for detecting antibodies for SAT was diluted at 1:20 upto 1:1280 and results were valid while for RBT, neat serum was used in detecting antibodies. RBT detect IgM antibodies which is cross reactive and persisting form of antibody hence not reproductive. The potential usefulness of the RBT for diagnosing human brucellosis was suggested by the Food and Agriculture Organisation and World Health Organisation Committee (Diaz and Moriyon, 1989). This method has replaced the rapid slide test of Huddleson which was prone to false –negative results (Diaz and Moriyon, 1989). However, the RBT is very sensitive to vaccinal antibody, seriously limiting its use in vaccinated animals (Alton, 1990).

The sensitivity of SAT and RBT against IFA in this study was 75% and 69.56%, specificity was 74% and 72.78%, positive predictive value 63% and 59.81%, negative predictive value was 16.4% and 19.58% respectively. Other studies have recorded sensitivity of 98.3% and 93.1% when interpreted at the 30- and 60-IU levels respectively (Dohoo *et al.*, 1986). The relatively lower sensitivity of SAT recorded in this study probably was caused by prozone phenomenon where a positive serum samples test negative. This phenomen occurs due to blocking antibodies which do not agglutinate non-specific factors or excess antibodies (Cheesebrough, 1984). The prozone phenomenon is of little practical importance as long as serum samples are routinely diluted beyond 1:320 (Filiz and Mehmet, 2012). False negatives are encountered in patients that have a factor serum that block agglutination reaction (Filiz and Mehmet, 2012).

The RBT is a simple screening test but may give false positive results in endemic areas (Filiz *et al.*, 2012). In other studies, SAT sensitivity was found to be less than RBT at 37.40% and 48.09% respectively. The RBT can also give false positives reactions with sera from patients infected with *Yersinia enterocolitica* 0:9, or healthy individuals who have been exposed to smooth *Brucella* strain antigens (Diaz and Moriyon, 1989). However, no false positives reactions were found with patients infected with turalemia or those vaccinated against *Vibrio cholera* (Diaz and Moriyon, 1989).The level of sensitivity of a test is inversely related to the number of false negatives that are generated by the same test. This may translate to delayed diagnosis and consequently delayed administration of treatment. Misdiagnosis may result to administration of drug meant to treat another ailment but which does not effectively destroy *Brucella* bacteria leading to development of drug resistant *Brucella* organism. Immunoflourescence microscopy determines the presence and the quantity of antibodies present. This enhances its sensitivity as compared to SAT and RBT. Specificity of 97.9% and sensitivity estimate of 96.1% of IFA has been reported (Lucero *et al.*, 2003).

The specificity of SAT and RBT against IFA in this study was 74% and 72.78%. The level of specificity of a test is inversely related to the number of false positives generated by the same test. A study conducted in Saudi Arabia to monitor the persistence of *Brucella* antibodies after successful treatment of acute brucellosis in an area of endemicity, clinically cured patient were noted to continue to have various levels of *Brucella* antibodies for a long time after acute brucellosis. Such cases would test positive for brucellosis at low antibody titres (Memish, 2002). The level of specificity of SAT in other studies was reported as 94% and 99.5% when interpreted at the 30 – and 60 –IU levels, respectively (Dahoo *et al.,* 1986). On the other hand, the issue of false positive reactions can also be seen in the SAT test and they occasionally result from cross reactions with antibodies to *Francisella tularemia, salmonella spp., Yersinia enterocolitica, Vibrio cholera, Escherichia coli* 0:157 and otherbacteria (Filiz and Mehmet, 2012). Other studies has recorded specificity of SAT and RBT as 99.04% and 96.19% respectively (Filiz and Mehmet, 2012).

The issue of false positive is important because it poses a challenge to diagnosis of brucellosis in patients with signs and symptoms suggestive of brucellosis when their symptoms are caused by other infectious or non-infectious disease. The implication may be misdiagnosed of the disease and exposing patient to unnecessary anti-*Brucella* treatment (Morata *et al.,* 1997). The inability of SAT to predict correctly that a person who tested positive is actually infected can be attributed to the fact that it is a qualitative test that does not address the issue of quantities of antibodies (Cheesebrough, 1984).Other studies conducted in eastern part of Turkey has recorded positive predictive value and negative predictive value of SAT as 100% and 90.9% respectively. Other studies have recorded positive predictive value of SAT and RBT as 0.98 and 0.94 while negative predictive value of SAT and RBT as 0.559 and 0.368 respectively (Filiz and Mehmet).

IFA was the gold standard for detecting *Brucella* antibodies because it the most specific and sensitive test compared to other methods. Robustness of IFA when compared to SAT and RBT in this study was demonstrated by evaluating of *Brucella* antibodies in 36 human samples with fever of unknown origin where prevalence of 69.4%, 36.1% and 14% were recorded from Narok respectively. The IFA test was developed to be performed outside the diagnostic laboratory, allowing for rapid and accurate diagnosis (Lucero *etal.,* 2003). The test is rugged, relatively inexpensive, simple and rapid (Nielsen, 2002). The test is accurate, and its sensitivity and specificity canbe manipulated by altering the cut-off value between positive and negative reactions to provide a highly sensitive screening test as well as a highly specific confirmatory test. The test has also been shown to distinguish vaccinal antibody in most vaccinated animals and eliminates reactivity by some cross-reacting antibodies as well (Nielsen and Yu, 2010). However, IFA requires skilled personnel and more inputs such as flourescence microscopes and flourescence isothiocyanate (FITC).

The seroprevalence of *Brucella* antibodies in milk samples in Narok and Kiambu by one step bovine agglutination and IFA in this study showed statistically significant difference ($P \leq 0.05$). This again shows the robustness of IFA over the former test. More milk samples from Narok a pastoral area had *Brucella* antibodies than those in Kiambu where zero-grazing system is practiced. The findings compare well with a study conducted in Nakasongola, Ugandawhere positivity of 34 % was recorded in pastoral dairy systems while 13.6% and 3.3 % were recorded in the zero-grazing systems (Kobei *et al.,* 2011). There was presence of *Brucell*a antibodies in milk and human serum in Narok and Kiambu counties in varying percentages by IFA. Poor correlation of *Brucella* antibodies in human serum and milk samples was detected in Kiambu and Narok counties ($r = -0.975$, $P = 0.143$) and ($r = -0.771$, $P = 0.440$) respectively. This findings agrees with the study conducted in Kampala, Uganda where significant prevalence of human brucellosis was shown and

poor correlation between the distribution of human cases (urban) and cows (peri-urban and rural) suggesting that brucellosis infections were occurring through dairy market chains (Makita *et al.*, 2008).

In this study, the results indicate that abbatoir workers and herdsperson occupations were significantly more prone to infection compared to other occupations and the difference was statistically significant ($P \leq 0.05$). This could be attributed to the fact that the workers are in close proximity to animals and animal products. On the other hand veterinarians had the lowest prevalence both in Narok and Kiambu counties most probably due to knowledge they possess on disease and its transmission hence are able and can afford to protect themselves. The relative risk of acquiring brucellosis was higher in Narok than in Kiambu. The findings compare well with a study conductedin Pakistan where prevalence was found high in abbatoir workers (Masoumi *et al.*, 1992; Fatima, 2010). High prevalence of brucellosis in people working in close proximity to animals was also reported in India and Saudi (Fatima, 2010).

5.2 Conclusions

1. Brucellosis is more prevalent in Narok than Kiambu county and persons particularly women in the age group 21-30 years are most affected.
2. Prevalence of brucellosis in human samples was 70.4% and 3.2% by Immunoflourescence antibody assay, 77.6% and 10.4% by Serum agglutination test, 80% and 5.3% by Rose Bengal test in Narok and Kiambu respectively. Prevalence of brucellosis in milk samples was 54.4% and 14.4% by IFA and 12% and 1.6% in Narok and Kiambu respectively.
3. Validation of SAT against IFA gave sensitivity (75%), specificity (74%), positivepredictivevalue (63%) and negative predictive value (19.58%). Validation of RBT against IFA gave sensitivity (69.56%), specificity (72.78%), positive predictive value

(59.81%) and negative predictive value (19.81%). Validation of one step bovine *brucella* Ab rapid antibody test and IFA was sensitivity (16.5%),specificity (53.06%), positive predictive value (19.76%) and negative value (52.43%).

4. (a) Immunoflourescence antibody assay (IFA) is the most sensitive and specific diagnostic techniquefor brucellosis from human sera and should replace SAT and RBT in hospitals to give the actual prevalence of brucellosis.

(a) IFA is also more reliable than one step bovine agglutination test for detection of *Brucella* antibodies from milk and should be adopted for screening whenever there is suspicion. Immunoflourescence antibody assay (IFA) is the most sensitive and specific diagnostic techniquefor brucellosis from human sera and should replace SAT and RBT in hospitals to give the actual prevalence of brucellosis. IFA is also more reliable than one step bovine *Brucella* Ab rapid antibody test for detection of *Brucella* antibodies from milk and should be adopted for screening whenever there is suspicion.

(5) Persons in direct contact with animals and animal products such as abbatoir workers and farmers are at a higher risk of contracting brucellosis especially when there is low hygiene.

(6) Individuals who ingest unpasteurized milk especially from areas of endemic infection are at a significant risk of foodborne brucellosis.

5.3 Recommendations

1. Wright's serum agglutination (SAT) and Rose Bengal Test (RBT) tests should be replaced with the more specific and sensitive Immunoflourescence antibody assay (IFA) test for routine diagnosis of brucellosis in humans and animals.
2. Create awareness to the public about the risks of consuming raw milk.

3. Ensure there are safety procedures and strict hygienein slaughter houses to prevent exposure, including the use of personal protective equipment, covering skin scratches and good ventilation.

4. Education to farmers, veterinarians, herds person and butchers on handling of animals and animals products.

5. Further research to check prevalence of *Brucella* in meat sold in butcheries and supermarkets is necessary.

6. More surveillance on disease prevalence, treatment and prognosis is required in Narok county.

REFERENCES

Abou, A. M. (2001). Epidemiological aspects of brucellosis in Jordan.*European Journal of Epidemiology*, **16**:581-584.

Al Dahouk, S., Tomaso, H., Nockler, K., Neubauer, H. and Frangoulidis, D. (2005). Laboratory –based diagnosis of brucellosis –review of the literature. Part II: Serological tests for brucellosis. *Clinical Laboratory*, **49**:487-505.

Alan, J.and Robin, T. (1987). Immunochemistry in Practice. Blackwell Scientific Publisher, USA. Second edition, 131-133.

Alton, G.G. (1990). Animal brucellosis. CRC Press , Inc. Boca Raton. Fla, 383-409.

Alton, G. G., Jones, L. M. andPietx, D. E. (1975). Laboratory Techniques in Brucellosis. Geneva. Second edition; *FOA/WHO Monograph Series*, **50**:34-56.

Alton, G. G., Nielson, K. D. and J. Robert (1990). Animal Brucellosis.CRC Press, Boca Raton, Florida. *Brucella Malitensis*, **382**:409-415.

Asanda, N. N. and Agbede, S. A. (2001). Brucellosis in cattle and Human in Adamawa state.Sokoto.*Journal of Veterinary Science*, **3**:34-38.

Asmaa, A. A.H., Amal, S. M. S.and Elfaky, M. A. (2005). Seroepidemiological study on human brucellosis in Assiut Governorate.*The Egyptian Journal of Immunology*. **12**:49-56.

Baba, M. M., Sarkndered, S. E. and Brisbe, F. (2001). Serological evidence of brucellosis among predisposed patients with pyrexia of unknown origin in northern Nigeria. Cent.*European Journal of Public Health*, **9:**158-161.

Bannatyne, R. M., Jackson, M. C. and Memish, Z. (1997). Rapid diagnosis of Brucella bacteremia using the Bactec 9240 system.*Journal of Clinical Microbiology*, **35**:2673-2674.

Bikas, C., Jelastopulu, E. and Leotsinidis, M. K. (2003). Epidemiology of human brucellosis in a rural area of North –Western pelponnese in Greece.*European journal of Epidemiology,* **18:**267-274.

BioNote, I. (2005). One Step Bovine *Brucella* Rapid Antibody Ab Test Kit. Htt:/www.binote.co,kr, Korea.

Blood, D.andHenderson, A. (1983). '' Veterinary Medicine''A textbook of disease of cattle, sheep.Pigs, goats and horses.6th Edition.*Bailliere and Tindall,* **154:**464-470.

Caksen, H., Arslam, S., Oner, A. F., Cesur, Y., Ceylan, A, Atlus, B.and Abuhandan, M. (2002). Childhood brucellosis is still a severe problem in the eastern region of Turkey. *Tropical Doctor*, **32**:91-92.

Chantal, J., Bessiere, M. H., Le Guenno B., Magnaval, J. F. and Dorchies, P. (1996). Serological screening of certain Zoonosis in the abattoir personnel in Djibouti.*Bulletin Society Pathology Exotic*, **89**:353-357.

Cheesbrough, M. C. (1984). Medical Laboratory manual for tropical Countries.Toronto: Churchill Living stone, **86**: 353-357.

Cho, D., Naham, H., Kim, J., Hae, E., Cho, Y., Hwang, I., Kim, J.,Kim, J. and Jung,S. (2010). Quantative Rose Bengal Test for diagnosis of bovine brucellosis. *Journal of Immunoassay Immunochemistry*,**31**:120-130.

Cook G.and Zumla, A. (2003). Manson's Tropical Disease. 21st Edition, Saunders:London1085-1089.

Corbel, M. J. (1989). Microbiology of the Genus *Brucella*.CRC Press Boca Raton, Florida, *Clinical and Laboratory Aspects*,53-72.

Corbel, M. J. (1997). Brucellosis: an overview emerging. *Infectious Disease*,**3**:213-221.

Dahoo, I. R., Wright, P. F., Ruckerbawer, G. M., Samagh, B. S.,Robertson, F. J.andForbes, L. B. (1986). Acomparison of five serological tests for brucellosis.*Canadian Journal of Veterinary Research*, **50:**485-495.

Diaz, R., Moriyon, I., Young, E. and Corbel, M. J. (1998). Laboratory Techniques in the diagnosis of human Brucellosis.CRC Press Boca Raton, Florida.*Clinical and Laboratory Aspects*, **60**:73-83.

Diaz and Moriyon (1989). Techniques in the Diagnosis of Human Brucellosis.CRC Press, Inc., Boca Raton, Florida.*Clincial and Laboratory Aspects*, **60**:73 - 83.

El-Ansary E. H., Mohammed B. A., Hamad, A. R. and Korom, A. G. (2001). Brucellosis among animals and human contacts in eastern Sudan. *Saudi Medical Journal*, **22**: 577-579.

Enright, F. M. (1990). The Pathogenesis and Pathobiology of *Brucella*infection Domestic Animals. Florida. *CRC Press Boca Raton*, 301-304.

Fatima, M. (2010). Brucellosis in a high risk occupational group: Seroprevalence and analysis of risk factors. *Journal of Pakistan Medical Association*,**60**:1031.

Fekete, A., Bantle, J. A., Halling, S. M. and Sanborn, M. R. (1990). Preliminary development of a diagnostic test for Brucella using polymerase chain reaction.*Application Bacteriology*, **69**: 216-227.

Filiz, A.and Mehmet, O. (2012). Evaluation of serological diagnostic tests for human Brucellosis in an endemic area,Turkey.*Journal of Microbilogy and infectious Disease, JMID*, **2:**50-56.

Fiori, P. L., Mastrandrea, S., Rappelli, P.andCappuccinelli, P.(2000).*Brucella abortus* infection acquired in microbiology laboratories. *Journal of clinical Microbiol*,**38**:2005-2006.

Foster, G., Osterman, B. S., Godfroid, J. Jacques and Cloeckart, A. (2007). *Brucella Ceti sp. Novel* and *Brucella pinnipidaelis sp.* Novel for *Brucella* strains with cetaceans and seals as their preferred hosts. *Journal of Syst Evolution Microbiology*, **57:** 2688-2693.

Godfroid, J., Cloeckaert, A., Giantard, J. P., Kohler, S., Fredin, D., Walravans, K., Garin Bastriji, B. and Letesson, J. J. (2005). From the discovery of the Malta fever agent to the discovery of a marine mammal. Reservoir, brucellosis has continuously been a re-emerging zoonosis. *Veterinary Research*, **6**:313-326.

Golding, B., Scott, D.E., Scarf, O., Huang, L.Y., Zaitseva, M., Laphan, C., Eller, N. and Golding, H. (2001). Immunity and protection against *Brucella* abortus.Microbes Infectious, **3**:43-48.

Greenfield, R. A., Drevets, D. A., Machado, L. J., Vaskuhl, G. W., Cornea, P.and Bronze, M. S. (2002). Bacteria pathogens as biological weapons and agents of bioterrorism.*American Journal of Medical Sciences,***323**:299-315.

Greenwood, D., Slack, R. and Rutherer, J. (2002). Medical Microbiology. 6th Edition, Toronto: Churchill Livingstone, 322-325.

Gutozzo, E., Carrillo, C., Guerva, J. and Liosa, L. (1986). An evaluation of diagnostic methods for brucellosis –the value of bone marrow culture.*Journal of Infectious Disease,***153**:122-125.

Halling, S. M., Peterson Burch, B. P., Bricker, B. J., Zuerner, R. L., Quig, Z. L., Kapur, V., Alton, D. P. and Olsen, S. (2005). Completion of the genome of Brucella malitensis and Brucella Suis.*Journal of Bacteriology*, **187:** 2715-2726.

Hoover, D. L. Crawford, R. M., Van De Verge, L., Izadjoo, M. J., Bhattachorjee, A. K., Paranavitana, C.M., Warren, R. L., Nikoliach, M. P.andHadfield, T. L. (1999). Protection of mice against brucellosis by vaccination with *Brucella Melitensis. InfectiousImmunology*, **67**:5877-5884.

Karabay, O., Sencan, I., Kayas, D. and Shin, I. (2004). Ofloxacin plus rifampicin versus doxycycline plus rifampicin n the treatment of brucellosis: a randomized clinical trial. *Biomedical Central of Infectious Disease,***4**:18.

Ko, J. and Splitter, G. A. (2003). Molecular host pathogen interaction in brucellosis: Current understanding and future approaches to vaccine development for mice and humans. *Clinical Microbial Revision,***16**:65-78.

Kobei, M., Ferre, E. M., Waiswa. C., Kaboyo, W., Eisler, M. C. and Welburn, S.C.(2011). Evidence based identification of the most important livestock related zoonotic disease in Kampala, Uganda. *Journal of Veterinary Medicine,***73**:991-1000.

Kobei, M., Eric, M. F., Charles, W. M., Winyi, K., Mark, C. E. and Susan, C. W. (2011). Spatial epidemiology of hospital diagnosed brucellosis in Kampala, Uganda. *International Journal of Health Geographics*, **10**:52.

Kochar, D. K., Gupta, B. K., Gupta, A., Kalla, A., Nayak, K. C. and Purohit, S. K. (2007). Hospital-based case series of 175 cases of serological confirmed brucellosis. Bikaner. *Journal of Association Physicians India*, **55**:271-275.

Liaurd, J. P., Gross, A., Dornand, J.andKohler, S. (1996). ''Interactions between professionals phagocytes and Brucella spp.''*Microbiologia,* **12**:197-206.

Lucero, N. E., Flogia, L., Ayala, S. M., Gall, D. and Nielsen, K. (1999). Competitive enzyme Immunoassay for diagnosis of human brucellosis. *Journal of Clinical Microbiology*, **37**:3245-3248.

Lucero, N. E., Escobar, G. I., Ayala, S. M., Siiva, P. and P. Nielson. (2003). Fluorescence polarization assay for diagnosis of Human brucellosis.*Journal of Medical Microbiology*, **52**:883-887.

Maichomo, M. W., McDermott, J. J., Arimi, S. M. and Gathura, P. B. (1998). Assessment of the Rose Bengal plate test for the diagnosis of human brucellosis in health facilities in Narok district, Kenya.*East Africa Medical Journal*, **75**:219-222.

Maichomo, M. W., McDermott, J. J., Gathura, B. P. and Arimi, S. M. (2000). Rose Bengal plate test for the diagnosis of human brucellosis in health facilities in Narok district, Kenya.*East Africa Medical Journal*, **75**: 219-222.

Maichomo, M. W., McDermott, J. J., Arimi, S. M., Gathura, P. B., Mugambi, T. J. and Muriuki, S. M. (2000). Study of brucellosis in pastoral community and evaluation of usefulness of clinical signs and symptoms in differentiating it from other flu-like diseases.*African Journal of Health science*,**7**:114-119.

Makita, K., Fevre, E., Waiswa, C., Kabonyo, W., Bronsvoort, B., Eisler, M. and Welburn, S. (2008). Human Brucellosis in Urban and Peri-urban Areas of Kampala, Uganda.*Annals of the New Yolk Academy of Science*,**1149**:309-311.

Mantur, B. G., Akki, A. S., Mangalgi, S. S., Patil, S. V., Gobbur, R. H. and Peerapur, B. V. (2004). Childhood brucellosis – a microbiological, epidemiological and clinical study.*Journal of Tropical Pediatrics*,**50**:153-157.

Mantur, B. G., Biradar, M. S., Bidri R. C., Mulimani, M. S., Veerappa, Kariholu P., Patil, S. B. and Mangalgi S. S. (2006). Protean clinical manifestations and diagnostic challenges of human brucellosis in adults: 16 years' experience in an endemic area. *Journal of Medical Microbiology*, **55**:897-903.

Masouni, J. P., Sheikh, M. A., Ahmad, R., Naeem, M., Ahmad, M.andHussain, I. (1992). Seroprevalence of brucellosis in a sheep, goat and man in Lahore, *India. Indian Journal of Dairy Science*, **45:**298-29.

McLean, D. R., Russell, N. and Khan, M. Y. (1992). Neurobrucellosis: Clinical and therapeutic features. *Journal of Clinical Infectious Disease*,**15**:582-590.

McDermott, J. J. and Arimi, S. M. (2002). Brucellosis in sub- Saharan Africa: epidemiology, control and impact. *Veterinary Microbiology*,**90:**111-134.

McEvoy Gerald, K. (2006). American Society of Health –System Pharmacists, Inc, Bethesda, MD, United States.*AFHS Drug Information*, **1**:37-76.

Memish, Z. A. and Balkhy, H. H. (2004). Brucellosis and international travel.*Journal of Travel Medicine*,**11**: 49-55.

Minas, A., Minas, M., Stournara, A. and selepidis, S. (2004). The 'Effects' of Rev-1 vaccination of sheep and goats on human brucellosis in Greece.*Prevention Veterinary Medicine*, **64**:41-47.

Morata, P., Quipo-ortuno, M. J. and Comenero, J.D. (1998).Strategy for optimizing DNA amplification in a peripheral blood PCR assay used for diagnosis of human brucellosis.*Journal of Clinical Microbiology*,**41**:144-148.

Muriuki, S. M., McDermott, J. J., Arimi, S. M., Mugambi, J. T. and Wamola, I. A. (1997). Criteria for better detection of brucellosis in the Narok of Kenya. *East Africa Medical Journal*, **74**: 317-320.

Nicolleti, P. (1980). The epidemiology of bovine brucellosis. Advanced Veterinary Science *Comparative Medicine***24**:69-98.

Nicoletti, P. (1984). The control of brucellosis in tropical Africa and subtropical regions.*Prevention Veterinary Medicine*,**2**:193-196.

Nicoletti, P. (1989). Relationship between animals and human brucellosis. In Brucellosis: CRC Press, Boca Raton, F.L. *Clinical and Laboratory Aspects*, **97**:120.

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

Nielsen, K. and Yu, W. L. (2010). Serological Diagnosis of Brucellosis. Ottawa Laboratories, Canada. *Canadian Food Inspection Agency*;**1:** 65-89.

Noor, M. (2009). Seroepidemiological study of human brucellosis among the population at risk.Department of Microbiology, Mymensingh Medical Collage Mymensingh, Bangladesh.

Ofukwu, A. R., Yohanna, C. A. and Abuh, H. A. (1994). Brucella Infection Among Hospital Patients In Markudi, North Central Nigeria. Htt://www.priory com/med,brucella,htm.

Omer, M. K., Assefaw, T., Skjerve E., Tekleghiorghis, T.andWoldehiwet, Z. (2002). Prevalence of antibodies to Brucella spp. and risk factors related to high-risk occupational groups in Eritrea. *Epidemiology Infection*,**129**: 85-91.

Omore, A.O., Muriuki, H., Kenyanjui, M., Owango, M. and Staal, S.(1999). The Kenyan Dairy Sub-sector: A rapid Appraisal. Research Report of the MoA/KARI/ILRI Smallholder Dairy (R&D) Project . ILRI (International Livestock Research Institute), Nairobi, Kenya, **51.**

Oomen, L. J. A. (1976). Human brucellosis in Kenya.*Tropical Geographical Medicine*,**28:** 45-53.

Otlu, S., Sahin, M., Atabay, H. I. and Unver, A. (2007). Serological Investigations of Brucellosis in cattle, farmers and veterinarians in the Kars Districts of Turkey. *Journal ofActa Veterinaria Brno*,**77**:117-121.

Ozkurt, Z., Erol, S., Tasyaran, M. A.and Kaya, A. (2002`). Detection of *Brucella melitensis* by the BacT/Alert automated system and *Brucella* broth culture. *Clinical Microbiology Infectious.***8:**749-752.

Palanduz, A., Palanduz, S., Guler, K.and Guler, N. (2000). Brucellosis in a mother and her young infant : Probable transmission by breast milk. *Int. Journal Infectious Disease,* **4:**55-56.

Pappas, G., Acridities, N., Bosilkovski, M. and Tsianos, E. (2005). Brucellosis.*The New England Journal of Medicine*, **2352**: 2325-2336.

Pappas, G., Paragopoulu, P., Christou, L. and Acriditis, N. (2006). *Brucella* as a microbiological weapon.*Cell Molecular Life Science*, **63**: 2229-2236.

Park. M. Y., Lee, C. S., Choi, Y. S., Park, S. J. and Lee, H. B. (2005).A sporadic outbreak of human brucellosis in Korea.*Journal of Korean Medical Science.***20**:941-946.

Philip, R.N., Casper, R. A., Ormbee, M. G. and Burgdorfer, W.(1978). Microimmunofluorescence test for the serological study of Rocky Mountainspotted fever and typus.*Journal of Clinical Microbiolog,***3:**51-61.

Pierce (2005/2006). Application Handbook and Catalog.USA,Pierce Biotechnology,Inc.457.

Reviriego, F. J., Moreno, M. A. and Dominguez, L. (2000). Risk factors for brucellosis seroprevalence of sheep and goats flocks in Spain.*Prevention Veterinary Medicine,***44**:167-173.

Sanchez, S. L. P., Ordonez, B., Diaz, P., Garcio, M. O. and Terres, F. A. (2005). Incidence of brucellosis declining in Spain.*Euro Surveillance,* **10**:50421-50424.

Schelling, E., Diguimbaye, C., Daoud, S., Nicolet, J., Boerlin, P., Tanner, M.andZinsstag, J. (2003). Brucellosis and Q-fever seroprevalence of nomadic pastoralist and their livestock in Chad.*Prevention Veterinary Medicine*, **61**: 279-293.

Scholz, H. C., Hubalek, Z., Sedlacek, I., Vernaud, G., Tomato, H., ALDahoulk, S., Melzer, F., Kampfer, P., Neubaner, H., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore, A. M., Falsen, E., Bahn, P., Gollner, C., Pfeffer, M., Huber, B., Hussel, H. J. and Noucler, K. (2008). Brucella microti sp. novel isolated from the common vole microbus arvalis. *Infectious Journal of Syst Enrolment Microbiology,***58:** 375-382.

Scuring, G. C., Srivanganathan, N. and Corbel, M. J. (2002). Brucellosis vaccine: past, present and future. *Veterinary Microbiology,***90**:479-496.

Sunkon, K. and Kerlikowske(2008). Evaluation of Diagnostic and Screening Tests: Validity and Reliability. Johns Hopkins Bloomberg, School of Public Health.*Journal of American Medical Association,***270**:2444-2450.

SmartTest Diagnostics (2003). Immmunoflourescence Antibody Assay *Brucella* IgG.Orgencs Ltd, part of the Inverness, Medical Innovation Group. Israel.

Thermo Scientific (2008). Pirce FITC Antibody Labeling Kit. Pierce Biotechnology, USA. *Thermo Fisher Scientific Inc.*

Thermo Scientific (2008). Zeba TM Desalt Spin Columns, 0.5. Pierce Biotechnology, USA. *Thermo Fisher Scientific Inc.*

Thrustfield, M. (2005). Veterinary pidemiology. 3rd Edition, Black Well Science Limited, Cambridge, USA, 225-228.

Wafa Al Nassir (2013).''Brucellosis'' eMedicine infectious disease. Internet: eMedicine. Medscape.Com/ artice/213/430-Overview.*Mediscape*.

Whatmore, A. M., Perrete, L. L. and Macmillan, A. P. (2007). Characterisation of the genetic diversity of Brucella by multilocus sequencing.*BMC Microbiol*,7:34.

WHO ''World Health Organization''(1997). Brucellosis. Fact sheet, July , No.173, 3P. WHO, 1211, Geneva.

Wiley, J., Hatakeyama, T. and Zhenhai, L.(1998). Handbook of thermal analysis.Chichester.

Young Edward, J. and Corbel Michael, J. (1989). Treatment of Brucellosis in Humans.CRC press, Boca Raton, Florida.*Clinical and Laboratory Aspects*, **97**:126.

Young, E. J. (1995). An overview of human brucellosis.*Clinical Infectious Disease*, **21**:283-290.

Zafer, C., Ortan, C., Ihsan, H. and Reha, D. (2005). Seroprevalence of Brucellosis in the Rural of Western. Anatolia, Turkey. *Journal of Health Population Nutrition*, **23**: 137-141.

Zuniga, E. A., Mota de-la Garza, L., Sanchez, M. M., Santos Lopez, E. M., Filardo, K. S. and Lopez, M. A. (2005). Survival of Brucella abortus in milk fermented with a yoghurt starter culture. *Journal ofRevision Latinoam. Microbial*,**47**:88-91.

APPENDICES

Appendix: 1Permission Form to use Central Veterinary Labortory

MINISTRY OF LIVESTOCK DEVELOPMENT

Telegrams "VETLAB", Kabete
Telephone Kabete 631390/4/5/7, 631287
and 631291
Fax 631275
When replying please quote

Ref. No. TC/VET4/

and date
All correspondences should be addressed to
The Director of Veterinary Services
Parcels by rail: Nairobi Station

DEPARTMENT OF VETERINARY SERVICES
VETERINARY RESEARCH LABORATORIES
P.O. PRIVATE BAG
POST CODE 00625 KABETE
NAIROBI

Date 18th April 2011

Susan Nyawira Nyagah
P.O. Box 138
Kikuyu.

RE: REQUEST TO USE CENTRAL VETERINARY LABORATORIES KABETE (CVL) TO CONFIRM BRUCELLOSIS UISNG IFA MICROSCOPY

I acknowledge receipt of your letters on the above-mentioned subject dated 18th March 2011 and 6th April 2011. My Department has no objection to your request subject to the following conditions.

1. That a proposal for the planned work for MSc is submitted to the Head of CVL as soon as possible.
2. You should ensure that biosecurity requirements are strictly adhered to during the course of your research at CVL.
3. That the head of Bacteriology Laboratory of the CVL, Dr. Peter M. Mbatha, will be your supervisor to ensure that your work is carried out safely.
4. That any publications emanating from the work done at the Central Veterinary Laboratories (CVL) Kabete using these samples should acknowledge the role played by the Director of Veterinary Services.

By a copy of this letter, Dr. Mbatha is requested to liaise with other heads of laboratories at CVL whose facilities and equipment Susan may require to use to enable the successful completion of her project.

Dr. P.M. Ithondeka, PhD, MBS
Director of Veterinary Services.

cc: Head, Central Veterinary Laboratories, Kabete

Officer in charge, CVL Kabete, Bacteriology Laboratory, CVL Kabete

Appendix ii:Screening Consent Form

I, (name of subject) .....

Address ----------

Agree to take part in screening for the research project:

I confirm that the nature and importance of the screening exercise have been explained to me in the informed consent form, and I accept them. I have had the chance to ask questions and discuss this study and I have received satisfactory answers and I understand that my consent is entirely voluntary and that it does not mean that I have to join the study.

Participant:

Name----------Signature----------

Date---------- Time ----------

Appendix: iii **CONSENT FORM FOR THE PROPOSED STUDY**

KENYATTA UNIVERSITY

P.O.Box 43844, Nairobi.

AN INVESTIGATION ON HUMAN BRUCELLOSIS IN KIAMBU AND NAROK DISTRICTS, KENYA USING IMMUNOFLUORESCENCE MICROSCOPY

Investigators

Susan Nyawira Nyagah (principal investigator)

P.O.Box 64167Nairobi,

Email: snnyagah@ yahoo.com

Telephone: 0727257610

Dr. John N.Mbithi

Department of medical laboratory Science,

School of Pure &Applied Science,

KenyattaUniversity

P.O.Box 43844

Telephone: 0770223045

Dr. Joseph J. N. Ngeranwa,

Department of Biochemistry and Biotechnology,

School of Pure &Applied Science,

KenyattaUniversity

P.O.Box 43844

Telephone: 0722268093.

The principal investigator hereby request for your participation. Therefore your are requested to grant permission for the use of your blood specimens for the

Purposes of the proposed study .The benefit of the proposed study are:-

(1) Through your voluntary participation, the proposed study will provide background Information which will be useful for targeting public health measures, focused on geographic origin and source of infection, modes of transmission, risk factors and regional distribution of the disease.

(2) Various serological diagnoses will be carried out on different groups of sera based on Asymptomatic population of blood donors, suspected *Brucellae* patients and *Brucellae*. Patients to determine the acute and chronic form of diseases and create awareness on human Morbidity due to brucellosis cases.

(3)All cases identified of disease will be informed and accorded medical attention

The only risk involved is experiencing a slight pain during bleeding of your blood specimen. Please sign the consent statement below as an indication that you have granted permission for your specimens to be used for the proposed study.

I.....

Study number.....) Have given consent for my blood to be used ONLY For the proposed study.

Signature.....Date.....

Appendix: ivClinical attributes of 250 participants in the study

Symptoms	No. with symptoms	% n = 250
Fever	36	14.4
Headache	122	48.8
Joint pain	50	20
Flu	125	50
Muscle pain	37	14.8
Body weaknesses	37	14.8
Night sweats	36	14.4
Chills	21	8.4
Heartburn	30	12
Running nose	9	3.6
Diarrhea	60	24
Fatigue	44	17.6
Backache	17	6.8
Neck pains	6	2.4
Sneezing	5	2
General Malaise	13	5.2
Fungal infections	71	28.4
Dermatitis	19	7.6
Constipation	33	13.2
Confusions	19	7.6
Still birth	6	2.4
Abortion	13	5.2

Miscarriages	7	2.8
Chest pain	61	24.4
Testis disorder	17	6.8
Difficult in breathing	44	17.6
Loss of Weight	16	6.4
Rashes	2	0.8
Gastroenteritis	44	17.6
Tumour	1	0.4
Totals	**1001**	**292.4**

Appendix: v Statistic Values

1. Statistics on demographic characteristics of subject by gender in Narok and Kiambu

	Male	females	Total
	32	68	100
	41.00	59.00	
	50	50	100
	41.00	9.00	
Total	82	118	200

$\chi^2 = 1.976 + 1.373 + 1.976 + 1.373 = 6.697$

df = 1, P value = 0.010

2) Demographic characteristic by gender- females in Narok

Paired T- test and cl: male and female (Narok)

Paired T for male and female

	N	Mean	StDev	SE Mean
Male	8	12.49	7.90	2.79
Female	8	12.50	11.14	3.94
Diffe	8	-0.01	4.16	1.47

95% Cl for mean difference :(- 3.50, 3.47)

T-Test of mean difference = 0 (vs not =0: T- value = 0.993

3). Statistical analysis of occupation in Narok and Kiambu

Statistic	DF	Value	Prob
Chi-Square	2	1.4904	0.4746
Likelihood Ratio Chi-Square	2	2.4344	0.2961
Mantel-Haenszel Chi-Square	1	0.6833	0.4084
Phi Coefficient		0.1273	
Contingency Coefficient		0.1263	
Cramer's V		0.1273	

Statistic	Value	ASE
Pearson Correlation	0.0867	0.0695
Spearman Correlation	0.0795	0.0765

Pearson Correlation Coefficient

Correlation	0.0867
ASE	0.0695
95% Lower Conf Limit	-0.0496
95% Upper Conf Limit	0.2229
Test of H0: Correlation =	0
ASE under H0	0.0785

Z	1.1044
One-sided Pr > Z	0.1347
Two-sided Pr > |Z|	0.2694
Sample Size = 92	

(4)Statistics analysis for positive samples

Statistic	DF	Value	Probability
Chi-Square	2	4.6003	0.1002
Likelihood Ratio Chi-Square	2	4.6103	0.0997
Mantel-Haenszel Chi-Square	1	4.5813	0.0323
Phi Coefficient		0.1357	
Contingency Coefficient		0.1344	
Cramer's V		0.1357	

Statistic	Value	ASE
Pearson Correlation	0.1356	0.0627
Spearman Correlation	0.1356	0.0626

Pearson Correlation Coefficient	
Correlation	0.1356
ASE	0.0627
95% Lower Conf Limit	0.0128
95% Upper Conf Limit	0.2584
Test of H0: Correlation	0
ASE under H0	0.0629

Z	2.1554
One-sided Pr > Z	0.0156
Two-sided Pr > |Z|	0.0311

Sample Size = 250

Statistic nalysis in Narok (extensive) and Kiambu (intensive) counties

Statistic	DF	Value	Probability
Chi-Square	1	121.3539	<.0001
Likelihood Ratio Chi-Square	1	141.6753	<.0001
Continuity Adj. Chi-Square	1	118.4817	<.0001
Mantel-Haenszel Chi-Square	1	120.8685	<.0001
Phi Coefficient			0.6967
Contingency Coefficient			0.5717
Cramer's V			0.6967

Fisher's Exact Test

Cell (1,1) Frequency (F)	88
Left-sided Pr <= F	1.0000
Right-sided Pr >= F	5.166E-32
Table Probability (P)	2.856E-30
Two-sided Pr <= P	1.033E-31

Statistic	Value	ASE
Pearson Correlation	0.6967	0.0403
Spearman Correlation	0.6967	0.0403

Pearson Correlation Coefficient

Correlation	0.6967
ASE	0.0403
95% Lower Conf Limit	0.6178
95% Upper Conf Limit	0.7757
Test of H0: Correlation =	0
ASE under H0	0.0454
Z	15.3567
One-sided Pr > Z	<.0001
Two-sided Pr > |Z|	<.0

Appendix: vi Methodology

Immunoflourescence Antibody Assay

The IFA utilized fixed *Brucellae* within a matrix of egg yolk sac sonicate. Canine patient and control sera were diluted to screening dilution in phosphate –buffered saline (PBS) and incubated in the individual slide wells to allow reaction of patient antibody with the solid –phase antigens. The slides were washed with distilled water to remove unreacted serum proteins, and an FITC-labeled anti canine IgG (conjugate) was added to label the antigen-antibody complexes. After further incubation for 30 minutes at 37 $^{0}C\pm$ 0.5 ^{0}C, the slides were washed again to remove unreacted conjugate. The resulting reactions were visualized using standard fluorescence microscopy, where a positive reaction was seen as sharply defined apple- green fluorescent *Brucella* (coccobacilli) against a constrasting red background of the yolk sac material. A negative reaction was seen as either red-counterstained yolk sac alone or fluorescence

Canine brucellosis was characterized by a prompt rise in IFA titer in both IgG and IgM fractions. The IgGtiter was most often followed since the IgM antibody was quite crossreactive and also the persisting form of antibody. The IgG antibody titer, in contrast, was specific and declined to undetectable levels with successful treatment of the infection.

Buffer Exchange Procedure for Dialysis

Additional materials required were variable-speed bench-top, microcentrifuge 1.5-2.0 ml and microcentrifuge collection tubes. The column's closure was removed and cap loosened (the cap was not removed). The column was placed in a 1.5-2.0ml microcentrifuge collection tube. Colunm was centrifuged at 1,500 ×g for 1 minute to remove storage solution. A mark was placed on the side of the column where the compacted resin slanted upward. The column was placed in the microcentrifuge with the mark facing outward in all subsequent centrifuge steps. A total of 300 µl

of buffer on top of the resin was added. Centrifugation was done 2 to 3 times at 1,500 ×g for 1 minutes to remove buffer and discarded.

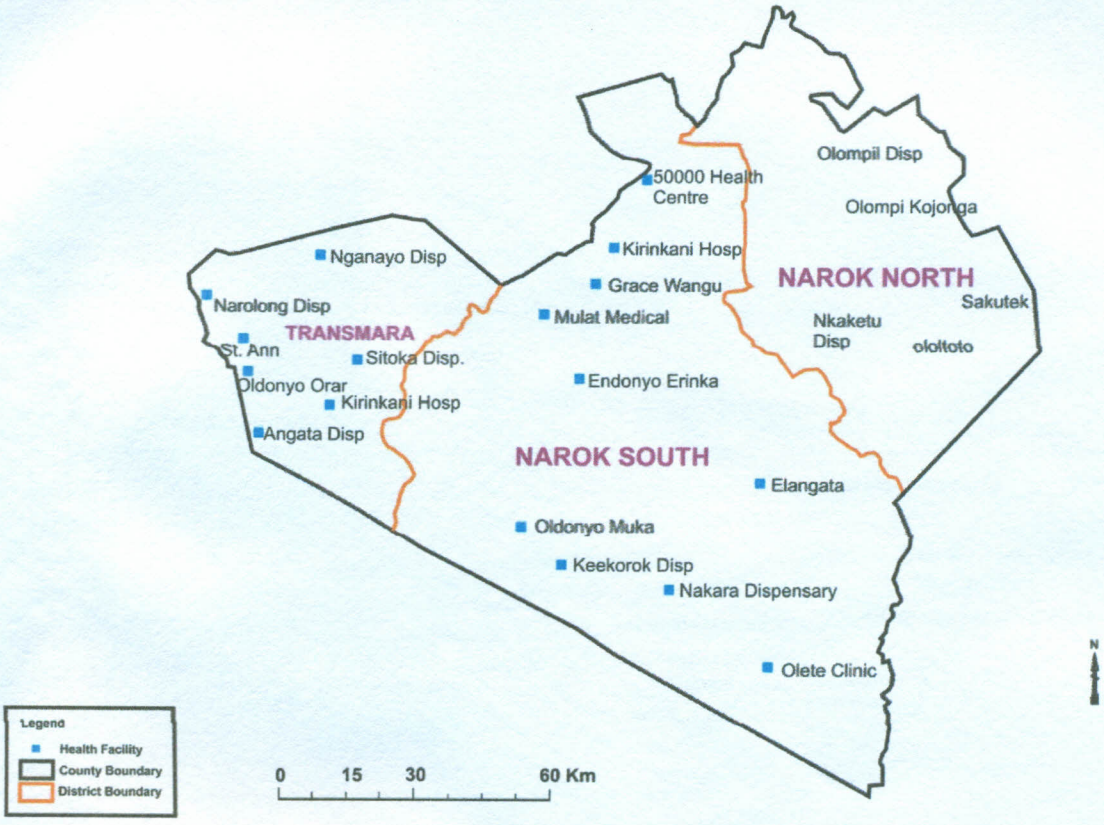
Sample loading was done by placing the column in a new collection tube and the cap removed. A total of 30-130 µl of sample was applied to the top of the compact resin bed. (Optional) For sample volumes less than 70µl a 15 µl stacker of ultrapure water or buffer was applied to the top of the gel bed after the sample had fully absorbed to ensure maximal protein recovery. The column was centrifuged at 1500 ×g for 2 minutes to collect the sample. Desalting column was discarded after use.

Protein Purification for FITC

Two spin columns were placed in separate microcentrifuge collection tubes. Purification resin was mixed to ensure uniform suspension and 400 µl of the suspension added into each spin columns. Tubes were centrifuged for 30-45 seconds at 1000×g to remove the storage solution. Used collection tubes were discardedand the columns placed in new collection tubes. Of the total (250-270 µl) of the labeling reaction was added to each spin column and the sample mixed with the resin by pipetting up and down or briefly vortexing. Columns were centrifuged for 30-45 seconds at 1000 ×g to collect the purified proteins. Samples from both columns was combined (0.5 ml total). Used columns were discarded.

Appendix: vii

NAROK COUNTY MAP



Appendix viii: Map of Kiambu County