A COMPARATIVE STUDY OF DIAGNOSTIC ASSAYS AND RISK FACTORS ASSOCIATED WITH HUMAN BRUCELLOSIS TRANSMISSION IN BARINGO COUNTY, KENYA

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DECLARATION

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

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DEDICATION

I dedicate this research to my family.

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TABLE OF CONTENTS

DECLARATION ..... **i**

DEDICATION ..... **ii**

ACKNOWLEDGEMENT ..... **iii**

TABLE OF CONTENTS ..... **iv**

LIST OF FIGURES ..... **viii**

LIST OF TABLES ..... **ix**

LIST OF APPENDICES ..... **x**

LIST OF ABBREVIATIONS ..... **xi**

ABSTRACT ..... **xiii**

CHAPTER ONE: INTRODUCTION **1**

1.0 Introduction 1

1.1 Problem statement 3

1.2 Justification 4

1.3 Research questions 6

1.4 Main objective 6

1.4.1 Specific objectives 6

CHAPTER TWO: LITERATURE REVIEW **7**

2.1 Brucella species and antigenic components 7

2.2 Distribution of brucellosis 7

2.3 Brucellosis in animals 8

2.4 Brucellosis in humans 9
2.5 Prevention and treatment........ 11
2.6 Diagnosis of human brucellosis 12
2.6.1 Serological tests........ 14
2.6.1.1 Serum agglutination test (SAT)........ 14
2.6.1.2 Rapid diagnostic kits (RDTs) 14
2.6.1.3 Rose Bengal Test (RBT)........ 14
2.6.1.4 Enzyme-linked immunoasorbent assay (ELISA). 15
2.6.1.5 Complement fixation test........ 16
2.6.2 Brucella isolation in culture........ 16
2.6.3 Molecular characterization of Brucella species........ 17
2.7 Sensitivity and specificity of diagnostic tests 18
2.7.1 Sensitivity 19
2.7.2 Specificity........ 19
2.8 Predictive value of diagnostic tests 19
CHAPTER THREE: MATERIALS AND METHODS 21
3.1 Study area........ 21
3.2 Study design 25

3.3 Inclusion criteria 25

3.4 Exclusion criteria 25

3.5 Sample size 26

3.6 Patient consenting 26

3.7 Data collection by questionnaires 26

3.8 Specimen collection 27

3.9 Specimen processing and storage 27

3.10 Serological testing 27

3.10.1 Serum agglutination test using rapid diagnostic tests (RDTs) 27

3.10.2 Rose Bengal Test (RBT) 28

3.10.3 Competitive enzyme-linked immunoasorbent assay (c-ELISA) 28

3.11.1 DNA extraction 30

3.11.2 Determination of DNA quality 31

3.11.3 Conventional polymerase chain reaction (PCR) 32

3.12 Data analysis 33

3.13 Ethical considerations 34

4.1 Overview of the collected specimens and tests run 35

4.2 Serological test results 36

4.2.1 Competitive-ELISA results 36

4.2.2 Result of rapid diagnostic kits (RDTs) 37

4.2.3 Rose Bengal Test (RBT) results 39

4.3 Sensitivity and specificity of rapid diagnostic kits (RDTs) compared to c-ELISA .... .40

4.4 Sensitivity and specificity of Rose Bengal Test compared to c-ELISA 41

4.5 Sensitivity and specificity of rapid diagnostic kits compared to Rose Bengal Test 42

4.6 DNA extraction 43

4.7 Polymerase chain reaction results 43

4.8 Clinical information of the patients who participated in the study 46

4.9 Factors associated with brucellosis infection in humans 47

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMEMDATIONS... 50

5.1 DISCUSSION 50

5.2 CONCLUSIONS 56

5.3 RECOMMENDATIONS 56

REFERENCES.. 57

APPENDICES:.. 69

LIST OF FIGURES

Figure 1: Map of Kenya showing Baringo County, 2015…………………………….....23

Figure 2: Map of Baringo showing location of Kabarnet, Marigat, Eldama Ravine district hospitals...24

Figure 3: Reaction of sample 1 and 2 tested with Fortress kit using *B. abortus* (BA) and *B. melitensis* (BM) antigens. ……………………………………….....37

Figure 4: Rose Bengal Test, showing agglutination results for three different serum specimens……………………………………………………………….....39

Figure 5: Agarose gel electrophoresis showing 100bp molecular marker (M), negative specimens 1,2,3,4, positive specimen 5, positive control 6 (*Brucella abortus*) and negative control 7 (double distilled water)……………………………...44

Figure 6: AMOS PCR, gel electrophoresis showing 100bp molecular marker (M) positive human specimens 1, 2, 3, 4, 5, 6, and 7 (amplicon for *B. abortus*, 498bp), negative human specimens 8 (no amplicon), blank well 9 and negative control 10……………...45

Figure 7: Duration of patients' illness by the time reporting to healthcare facility……47

LIST OF TABLES

Table 1: Primers used in the PCR reactions and primer sequences for each primer…...32

Table 2: PCR content per tube (20 ul reaction)……………………………………...33

Table 3: Healthcare facilities sampled and patients' gender in Baringo, 2014………...35

Table 4: Human brucellosis seropositivity in Baringo County as determined by competitive- ELISA in 2014…………………………………………………36

Table 5: Human brucellosis seropositivity in Baringo County as determined by rapid diagnostic kits (RDTs) in 2014 …………………………………………...38

Table 6: Human brucellosis seropositivity in Baringo County as determined by Rose Bengal Test in 2014…………………………………………………………39

Table 7: The agreement on the positive outcomes between c-ELISA, RDTs, RBT serological tests…………………………………………………………….40

Table 8: Comparison of c-ELISA and rapid diagnostic kits used at the healthcare facilities……………………………………………………………........41

Table 9: Comparison of c-ELISA and Rose Bengal Test……………………………...42

Table 10: Comparison of rapid diagnostic kits and Rose Bengal Test…………………43

Table 11: Number of positive specimen on PCR for the healthcare facilities in Baringo County 2014...46

Table 12: Odds ratio estimates with 95% confidence intervals for the factors assessed.49

LIST OF APPENDICES

Appendix 1: Consent Form…………………………………………………………….69

Appendix 2: Fomu ya makubaliano na wagonjwa……………………………………..74

Appendix 3: Questionaire………………………………………………………………79

Appendix 4: Fomu ya mahojiano………………………………………………………84

Appendix 5: Reagent Preparation………………………………………………………89

Appendix 6: Kappa test for evaluation of concordance of the tests……………………90

Appendix 7: Results for RDTs, RBT, C-ELISA and PCR……………………………..92

Appendix 8: Ethical clearance letter…………………………………………………...96

LIST OF ABBREVIATIONS

AFI Acute febrile illness

BA *Brucella abortus*

BM *Brucella melitensis*

C-ELISA Competitive-Enzyme linked immunosorbent assay

CFSPH The centre for food security and public health.

CFT Complement fixation test

CTAB Hexadecyltrimethyl ammonium bromide

DNA Deoxyribonucleic acid

EDH Eldama Ravine District Hospital

EDTA Ethylene diamine tetraacetic acid

ELISA Enzyme linked immunosorbent assay

I-ELISA Indirect-Enzyme linked immunosorbent assay

KDH Kabarnet District Hospital

KEMRI Kenya Medical Research Institute

MDH Marigat District Hospital

OD Optical density

OIE World Organization for Animal Health

OR Odds ratio

PCR Polymerase chain reaction

RBT Rose Bengal Test

RDTs Rapid Diagnostic Test Kits

SAT Serum agglutination test

SDS Sodium dodecyl sulphate

S-LPS Smooth-lipopolysaccharide

TAE Tris-acetate-ethylene diamine tetraacetic acid buffer

TE Tris-ethylene diamine tetraacetic acid buffer

ZN Ziehl Nielsen

ABSTRACT

Brucellosis is a zoonotic disease of public health and economic significance in many developing countries. Clinical diagnosis of human brucellosis is a challenge because of its non specific clinical symptoms. Laboratory diagnosis is based on bacteria isolation from clinical specimens, serological testing for detection of anti-*Brucella* antibodies and molecular methods for detection of *Brucella* DNA. This study was three-fold: first it compared the rapid diagnostic kits (RDTs) used in healthcare facilities in Kenya for brucellosis diagnosis and Rose Bengal Test (RBT) to competitive-enzyme linked immunosorbent assay (c-ELISA), second it identified *Brucella* species infecting humans and third it determined the risk factors associated with transmission of brucellosis in humans in Baringo County, Kenya.

This was a cross-sectional laboratory based study, conducted in three healthcare facilities in Baringo County; Kabarnet district hospital (KDH), Marigat district hospital (MDH) and Eldama Ravine district hospital (EDH). Patients 18 years and older that the attending clinicians suspected to have brucellosis were consented, filled a questionnaire that sought to identify risk factors associated with brucellosis and provided blood specimens. Serum was used to test for brucellosis using the rapid diagnostic kits (RDTs), RBT and c-ELISA. Deoxyribonucleic acid (DNA) extracted from the blood clots was used for identification of infecting *Brucella* species by polymerase chain reaction (PCR) assays.

The percentage positivity for each assays was; c-ELISA, 9.6% (16/166), RDTs, 26.5% (44/166), RBT, 10.2% (17/166). The overall sensitivity of RDTs was 37.5% while the specificity was 74.7% when compared to c-ELISA. The positive predictive value of the RDTs was 13.6% while the negative predictive value was 91.8%. The RDTs used in the healthcare facilities were not able to differentiate between *B. abortus* and *B. melitensis* infection. *Brucella abortus* was the species detected and the risk factors associated with *Brucella* transmission were handling sick animals without gloves and drinking unboiled

milk. Half of the participants were aware that brucellosis could be transmitted through unboiled/unpasteurized milk and 40% of the participants were aware of other ways in which brucellosis could be transmitted.

The study, demonstrated the presence of brucellosis in Baringo County, *B. abortus* was detected and it demonstrated that the RDTs used had low sensitivity and specificity. There is need to use diagnostic tests that are more sensitive and specific for diagnosis of brucellosis for effective treatment. In addition there is need to educate the community on brucellosis and brucellosis preventive measures.

CHAPTER ONE: INTRODUCTION

1.0 Introduction

Brucellosis is a zoonotic disease caused by bacteria of the genus *Brucella.* Brucellosis is mainly a disease of livestock, infecting cattle, sheep, goats and pigs. There are 10 known *Brucella* species; *Brucella abortus* is associated with disease in cattle, *B. melitensis* in sheep and goats, *B. suis* in swine, *B. ovis* in sheep, *B. canis* in dogs and *B. neotamae* in desert wood rats (Al Dahouk *et al.,* 2003). The four species that are of public health importance, causing disease in humans, are *B. melitensis*, *B. abortus*, *B. suis* and *B. ovis* (Foster *et al.,* 2007). In some of the natural hosts like cattle, sheep, goats, pigs and dogs, infection is mainly in the reproductive tract, resulting in placentitis which lead to abortion in pregnant females, epididymitis and orchitis in males (Corbel, 2006). Excretion in genital discharge and milk is common; unpasteurized milk being the major source of human infection. The main clinical signs of brucellosis in animals include abortion, still birth, birth of weak offspring, retained placenta in females and infertility in males.

In humans the disease is acquired directly or indirectly from infected animals and animal products, and manifests as an acute febrile illness (AFI). Clinical signs and symptoms include undulant fever, headache, chills, depression, profound weakness, arthralgia and myalgia. The disease, when not treated persists and progress to a chronically severe complication this include endocarditis, which is the main cause of brucellosis related death. Other complications associated with untreated brucellosis include osteoarthritis which causes pain, stiffness and swelling in joints and epididymo-orchitis (Colmenero *et al.,* 1996).

Infection with *Brucella* species may also affect the central nervous system presenting as meningitis or meningoencephalitis (Turel *et al.,* 2010). Neurobrucellosis is rare and occurs in less than 5% of patients. In endemic region, if uncommon or unexplained

manifestations of neurological disease are seen, *Brucella* psychosis should be considered (Sheybani *et al.,* 2012). Some of the manifestations include depressed mood, amnesia, agitation and nightmares; can occur in acute or chronic phase of the disease (Bidaki *et al.,* 2013).

Diagnosis of brucellosis by bacterial isolation in blood provides an absolute proof of infection. However isolation of *Brucella* has some limitations, it requires Biosafety Level 3 containment, skilled personnel and has long turn-around time (Yu and Nielsen, 2010). Serological tests used in diagnosis of human brucellosis, which detect *Brucella* antibodies in blood are, serum agglutination test (SAT), complement fixation test (CFT) and enzyme linked immunoabsorbent assay (ELISA). Others include antigenic detection using molecular techniques such as polymerase chain reaction (PCR).

Despite being endemic in many tropical and subtropical countries (Pappa *et al.,* 2006), brucellosis remains under-diagnosed and under-reported (Godfroid *et al.,* 2005). In infected livestock the disease causes abortion, reduced fertility and decreased milk yield; which results to substantial economic losses (Corbel, 1988).

In animals, brucellosis diagnosis is well established, but in humans, timely and accurate diagnosis has been a continuous challenge to clinicians. This is because of its non-specific clinical symptoms, which are similar to other causes of febrile illness such as malaria, typhoid. Unavailability of the good *Brucella* diagnostic tests in most healthcare facilities also make *Brucella* diagnosis a challenge. In addition, serological methods are also faced with challenges of cross reactivity with other bacteria such as *E. coli, V. cholera* and *Salmonella spp.* Infection with any of these bacteria results in similar signs and symptoms as brucellosis in humans, thus the challenge in diagnosis. Most healthcare facilities use rapid diagnostic kits which are antigen based and are faced by challenges of cross-reactions leading to false positive results (Samartino *et al.,* 1999).

1.1 Problem statement

Globally an estimated 500,000 human brucellosis cases are reported but these numbers are likely to be an underestimate of the incidence of human brucellosis (Corbel, 2006, Atluri *et al.,* 2011). In brucellosis endemic areas other diseases such as malaria and typhoid are also common. It is therefore common practice for most febrile patients to be initially diagnosed as suffering from malaria. Only a few patients with AFI, usually those who do not respond to malarial treatment, are investigated for brucellosis and other causes of febrile illness (Pappas *et al.,* 2006).

In most African countries including Kenya and in many communities, people live in close proximity to their livestock. The way they inter-mingle with the animals may enhance *Brucella* transmission to the humans. In Kenya, there is limited data on incidence of brucellosis in humans and relationship between the circulating *Brucella* species in humans and livestock. Since over 16% of cattle in Sub-Saharan Africa are estimated to be infected with *Brucella* (Mangen *et al*., 2005), it is likely that a significant number of people in Kenya are exposed to and may be infected with brucellosis. A systemic review of brucellosis in Kenya, estimated a national human seroprevalence of 3.0% (Njeru *et al.,* 2016), and one serological study found a strong statistical association between human and animal seropositivity (Osoro *et al.,* 2015). A study done in Kiambu and Kajiado in Kenya recorded human seroprevalence of 1.2% and 14.1% respectively (Ogola, *et al.,* 2013).

Although mortality rate associated with brucellosis in humans was estimated at 2-5% (Wafa *et al.,* 2009) another estimate of 1-3% has also been recorded (Fact sheet, 2012). Mis-diagnosis or failure to diagnose brucellosis and thus failure to treat the disease may lead to complications such as sterility, endocarditis, osteoarthritis, spondylitis and neurobrucellosis (Colmenero *et al.,* 1996, Sheybani *et al.,* 2012). Brucellosis treatment takes six weeks with a combination of antibiotics; this results in medical expenses and is

quiet traumatizing for patients, especially when a patient has to undergo treatment while in reality they are not suffering from brucellosis.

As indicated earlier, assays that are effective for brucellosis diagnosis (*Brucella* isolation, polymerase chain reaction (PCR), and c-ELISA) are unavailable or unattainable in most healthcare facilities in Kenya. Local clinics are only able to conduct Rose Bengal Test (RBT). Other tests, such as serum agglutination test (SAT), were done in Central Veterinary Laboratories or medical research facilities (Maichomo *et al.,* 2000). Therefore effective test kits are a necessity for accurate diagnosis and treatment of brucellosis.

1.2 Justification

Human brucellosis is a severe disease that requires prolonged treatment with a combination of antibiotics (Smits *et al.,* 2004) therefore, proper rapid diagnostic test are necessary before suspected patient is put on treatment.

Clinical laboratory diagnosis of human brucellosis depends on serological testing (Kunda *et al.,* 2007). Serological diagnoses are faced with challenges of false positives due to cross reactions, inability to distinguish between active and inactive infections caused by antibody persistence after therapy (Lulu, 1988). Therefore false-positive patients (not suffering from brucellosis) end up getting treatment while false-negative ones (suffering from brucellosis) are not treated, hence continue to suffer; this has contributed to disease complication.

Drugs used for treatment of brucellosis are common antibiotics used in treatment of other bacterial infection; considering the prolonged treatment of brucellosis, these may lead to unnecessary medical expense and cases of drug resistance.

Brucellosis is treatable. Proper diagnosis will help in proper patient management and treatment of disease complication(s). The objectives of this study were to compare the

rapid diagnostic kits (RDTs) used in healthcare facilities and Rose Bengal Test (RBT) to c-ELISA in diagnosis of brucellosis in humans, to establish the *Brucella* species that are responsible for the disease in Baringo County and to document the associated risk factors.

Noting that the sensitivity and specificity of the rapid diagnostic kits used in the healthcare facilities were not indicated, this study compared the rapid diagnostic kits (RDTs) to the c-ELISA test and also to the rapid test which is commonly used in veterinary diagnosis-Rose Bengal Test. Finding an effective rapid test, with minimal cross-reactions will reduce false positive results, thus, save patients from the long-term and expensive treatment associated with brucellosis

1.3 Research questions

1. What is the sensitivity and specificity of rapid diagnostic kits used in diagnosis of brucellosis in humans when compared to *Brucella* specific c-ELISA?

2. What *Brucella* species cause human brucellosis in Baringo County?

3. What are the risk factors associated with brucellosis infection in humans in Baringo County?

1.4 Main objective

To carry out a comparative study of diagnostic kits used for diagnosis of human brucellosis and determine risk factors associated with the transmission of the disease in Baringo County, Kenya

1.4.1 Specific objectives

1. To determine the sensitivity and specificity of rapid diagnostic kits (RDTs) used in Kenyan healthcare facilities for human brucellosis when compared to *Brucella* specific competitive-enzyme linked immunosorbent assay (c-ELISA) and Rose Bengal Test (RBT).
2. To identify the *Brucella* species infecting humans in Baringo County.
3. To determine the risk factors associated with transmission of brucellosis infection to humans in Baringo County.

CHAPTER TWO: LITERATURE REVIEW

2.1 Brucella species and antigenic components

Brucella species are intracellular Gram negative and rod shaped bacteria. They are non capsulated, non motile and non spore forming. The outer cell membrane resembles other Gram-negative bacilli with a dominant lipopolysaccharide (LPS), which is the main antigenic component (Araj, 2010).

Other antigenic proteins such as periplasmic, cytoplasmic and outer membrane structural proteins (Omp) are also useful in diagnostic tests (Diaz *et al.,* 1989). Ribosomal proteins and fusion proteins have a protective effect against *Brucella* based on antibody and cell-mediated responses (Oliveira *et al.,* 1996).

2.2 Distribution of brucellosis

Brucellosis is widely spread in the Mediterranean region, Middle East, south and central Asia, Central and South America, North and East Africa, (Corbel, 1997).

In Kenya the prevalence of brucellosis in livestock and human population is not well documented. In a study done in Narok, Kenya 12% of patients with flu-like symptoms were diagnosed with brucellosis using Rose Bengal Test (Maichomo *et al.,* 2000). Reason for the limited reports on the occurrence of brucellosis in pastoralist communities, are thought to be difficulties experienced towards sampling and limited availability of medical facilities and records (Muriuki *et al.,* 1994).

In Kenya a study on brucellosis carried out among pastoralists in Narok district recorded a prevalence of 13.7% (Muriuki et *al.,* 1997), a study done in Kiambu and Kajiado in Kenya recorded human sero-prevalence of 1.2% and 14.1%, respectively (Ogola, *et al.,* 2013) while a systematic review of brucellosis in Kenya, estimated a national human seroprevalence of 3.0% (Njeru *et al.,* 2016). Agglutination for *Brucella* antigen in blood sera among healthy people in Nairobi and Naivasha was 4% and 7%, respectively (Jumba *et al.,* 1996), the reactivity in Naivasha was associated with high contact with

unpasteurized milk compared to that of people in Nairobi. Although brucellosis has been controlled in many developed countries (Smirnova *et al.,* 2013), in Kenya, the infection persists in domestic animals and in the humans.

2.3 Brucellosis in animals

In animals brucellosis predominantly affects the reproductive system, inducing abortion in female and reduced fertility in males (Corbel, 2006) and it is also a common human zoonosis.

The disease in livestock is transmitted mainly due to poor hygienic practice in the farm including: poor disposal of aborted fetus, movement of herds from *Brucella* endemic areas to *Brucella* free areas, use of communal pasture and watering areas, as well as use of manure contaminated with *Brucella* organisms for pasture (Corbel, 2006). Livestock may also become infected with brucellosis through inhalation or ingestion of infected materials, and penetration of the bacteria through the conjunctiva (Corbel, 2006). Intra-uterine infection of fetus and infection through ingestion of milk from infected mother have also been documented (Kusiluka *et al.,* 1996).

Brucellosis is a common disease among pastoralist and nomadic herds in developing countries; however the disease incidence in herds in Africa is largely unknown because regular diagnosis for brucellosis is uncommon (Corbel, 2006). Transmission factors in the pastoralist herds include stock movement and concentration of animals around water points (Waghela, 1976).

Clinical manifestation of *B. melitensis* infection in sheep and goats include: abortion, still birth, and birth of weak offspring (The Centre for Food Security and Public Health, 2009). In cattle, clinical manifestations of *B. abortus* infection are: abortion, reduced milk production; birth of dead calves at term, and high frequency of retained placenta in females. Orchitis and epididymitis may occur in males and can result in infertility.

The bacteria are shed from an infected animal around the time of calving or abortion. Goats usually shed *B. melitensis* in vaginal discharge for at least 2 to 3 months but shedding in milk and semen can be prolonged or life-long; in sheep shedding usually ends 3 weeks after either abortion or full-term parturition (The Centre for Food Security and Public Health, 2009).

Diagnosis of the disease in animals is based on microscopic examination of smears stained with modified Ziehl Nielsen (ZN) technique, serological testing of milk and serum and isolation of bacteria in culture (Ewalt, 1989). The serological tests include serum agglutination test (SAT), complement fixation test Rose Bengal Test (RBT), (CFT), enzyme linked immunosorbent assays (ELISA) and radial immunodiffusion (The Centre for Food Security and Public Health, 2009).

In livestock brucellosis may be controlled through vaccination and improved animal husbandry. Treatment for brucellosis in animals is usually not recommended (The Centre for Food Security and Public Health, 2009). The recommendation is that all infected animals be slaughtered; a practice that is not practical in developing countries due to economical and poor compensation policies by the governments (OIE, 2009).

Prevention and control strategy is the best way of reducing disease burden and limiting transmission to other livestock and humans. Since this is not practical in developing countries, there is need for various stakeholders to be involved in ensuring that effective vaccination is practiced and that farmers are educated on importance of good management and hygiene. These measures are most effective when used in combination (Nicoletti, 2010). People should be informed on how to prevent transmission in livestock and from animals to humans.

2.4 Brucellosis in humans

Human brucellosis has a major impact on public health, the infection normally present as fever with multiple clinical signs and symptoms, such as fatigue and pain in the body

(Al Dahouk *et al.,* 2013). Worldwide 500,000 new cases of human brucellosis are reported yearly but these numbers greatly underestimate the true incidence of human disease (Pappas *et al.,* 2006).

Six major *Brucella* species which cause disease in humans are *B. abortus*, *B. melitensis, B. suis, B. canis, B. ovis* and *B. neotomae* (Glynn and Lynn, 2008). *Brucella melitensis* is the most pathogenic to humans (The Centre for Food Security and Public Health, 2009).

Transmission of brucellosis to humans is mainly by contact with infected placentae, fetal fluids, vaginal discharges and consumption of unpasteurized milk or soft cheese from infected animals (Zerva *et al.,* 2001). Other forms of transmission are through occupational exposure of laboratory workers, veterinarians and slaughter house workers (Corbel, 1997). Accidental injection with some live attenuated vaccines used in livestock, mostly *B. abortus* strain 19, also cause brucellosis in humans. Mother to child transmission through breastfeeding has been recorded and transmission through inhalation has been reported in slaughter houses, ranches and clinical laboratories (Corbel, 1997).

The disease develops gradually from acute, subacute to chronic illness. Acute brucellosis occurs within two to four weeks post exposure, with influenza like symptoms such as intermittent fever, weakness and fatigue. In most acute infections, fever rises and falls for days or weeks then raises again, thus the name “undulant fever”. The patient may be well in the morning but, approaching evening, he/she feels sick with chills, heavy night sweats, headaches and body aches including muscle and joint pains; these may be localized with variable joint swellings. Other signs and symptoms include pain: at the back of the neck, depression, insomnia, irritability and fatigue.

Brucellosis can cause spontaneous abortion in humans especially during the first and second trimesters of gestation. Intrauterine transmission to a fetus can also occur (Young, 1983). There are fewer abortions in humans compared to livestock; this is because of absence of erythritol in human placenta (Poole *et al.,* 1972). Erythritol is a

growth factor and a medium for *Brucella* in the animal placenta. Another reason for fewer abortions in humans is that amniotic fluid has anti-*Brucella* activity (Seoud *et al.,* 1991).

Brucellosis also causes enlargement of lymph nodes, spleen, liver and can lead to neurobrucellosis incase the bacteria invade the central nervous system (Shakir, 1986). Chronic brucellosis may occur a year after infection causing arthritis, weight loss, depression and chronic fatigue. The disease may also persist for months with relapses occurring three to six months after treatment has stopped (Solera *et al.,* 1998). Symptoms vary in severity and depend on the species and the amount of infecting bacteria among other factors.

The duration of illness with brucellosis, which varies from a few weeks to months and its long gradual recovery, makes brucellosis economically important as well as a medical problem for patients in terms of cost of treatment and time lost from normal activity.

2.5 Prevention and treatment

There is limited knowledge and information on brucellosis available to clinicians and other key players in public health in Kenya. This may potentially lead to under-diagnosis, mis-diagnosis and under-reporting on brucellosis (Kunda *et al.,* 2005).

Recommended combination drugs for treatment are oral doxycycline with rifampicin for atleast six to eight weeks (Center for Disease Control and prevention, 2012). Treatment of neurobrucellosis has been successful, using a triple therapy of doxycycline; rifampicin and co- trimoxazole (Mclean *et al.,* 1992).

Brucellosis though can take a chronic form with periods of illness and periods of no symptoms, persistent illness lasting longer than two months may also be as a result of other diseases causing complication of brucellosis. Relapses may occur approximately in 10% of individuals after treatment; in such cases treatment should be repeated. Currently

there is no vaccine against human brucellosis, the animal vaccines *B. abortus* S19 and *B. melitensis* Rev 1 can cause disease in humans and therefore are not candidates for the development of human vaccine (Perkins *et al.,* 2010).

The best measure to prevent brucellosis is to reduce exposure to infected animals and animal products. This may be achieved by adopting preventive measures such as personal hygiene when handling animals and animal products, consuming pasteurized milk and well cooked meat, wearing protective equipment such as gloves when assisting animals during calving, proper disposal of aborted fetuses and afterbirths. Preventing occupational exposure by laboratory, animal health officers and slaughter house workers through use of proper protective equipment, and working in a biosafety cabinet when working with *Brucella* specimens and isolates, among other measures that prevent spread of the disease to humans.

2.6 Diagnosis of human brucellosis

Brucellosis diagnosis is based on isolation and characterization of the bacterium, serological tests and molecular methods (Poester *et al.,* 2010). Several diagnostic tools are available, but implementation is not easy because of unlimited well equiped health facilities especially in developing countries (Ciocchini *et al.,* 2013). Proper diagnosis of human brucellosis is a challenge to clinicians due to its multiple clinical features, slow growth in cultures and complications in serological test (Colmenero *et al.,* 1990).

The definitive diagnosis is by culture and isolation of the *Brucella* organisms however, the sensitivity of this technique strongly varies with the stage of illness (Espinosa *et al.,* 2009), the competence of the laboratory technologists and the time taken to isolate *Brucella* species from blood which is approximately 21 days (Kokoglu *et al.,* 2006).

Brucellosis diagnosis in human although based on clinical observation and laboratory investigation. There are limitations on clinical observation because, brucellosis symptoms are not specific and can be mistaken for other common causes of febrile

disease such as malaria and typhoid (Mutanda *et al.,* 1998). Thus, diagnosis based on clinical presentation should be supported by the use of suitable diagnostic tests to confirm the presence of bacteria, bacterial products or anti-*Brucella* antibodies.

Rapid specific and sensitive assays for brucellosis diagnosis in health facilities are required for better management of brucellosis. Some of the diagnostic tests are effective in diagnosis of brucellosis in animals but have not been validated for diagnosis of brucellosis in humans.

The humoral immune response to *Brucella* is due to antibodies to the O-polysaccharide side chain of the outer membrane. The initial antibody produced is IgM antibodies followed by IgG 1 isotype, IgG 2 and IgA (Beh, 1973). Immunoglobulin M (IgM) antibody would be the right indicator for active brucellosis. But other microorganisms such as *Escherichia coli, Salmonella spp, Vibrio cholerae* and *Yersinia enterocolitica* contain antigen with similar epitopes to those of O-polysaccharide and this has led to cross reactivity (Corbel, 1985). Therefore detection of IgM antibodies sometimes gives false positive reactions in serological tests resulting in low assay specificity. Detection of IgG 2 and IgA antibodies which are produced later in infection would result in low assay sensitivity (Young, 1991).

Some of the tests that have been used for brucellosis diagnosis in humans are serum agglutination tests (SAT), rapid diagnostic kits (RDTs) used for rapid slide screening test and tube agglutination tests, complement fixation test (CFT) and enzyme- linked immunosorbent assay (ELISA) test. These diagnostic tests use antigens as whole bacteria or bacterial protein extracts containing high concentration of the smooth lipopolysaccharides (S-LPS) to detect the presence of anti-*Brucella* antibodies.

2.6.1 Serological tests

2.6.1.1 Serum agglutination test (SAT)

In this test, a mixture of *B. abortus* cells is incubated with the patient's serum in a glass tube, if cell sediment is observed at the bottom of the tube; this is a positive reaction, an indication of infection, while lack of sediment is a negative reaction. The test is done at neutral pH and detects IgM antibody isotype efficiently but detects IgG, especially IgG 1, less efficiently, resulting in low assay sensitivity (Allan *et al.,* 1976). Therefore SAT should not be used as a single test but rather in combination with other tests (Yu and Nielsen, 2010). Serum agglutination test, though good, in chronic or complicated cases can yield false positive result and is time consuming when number of samples are large (Al Dahouk *et al.,* 2003).

2.6.1.2 Rapid diagnostic kits (RDTs)

These kits have attenuated stained smooth specific antigen suspensions of *Brucella,* having specific reactivity towards antibodies to *Brucella abortus* (A) and *Brucella melitensis* (M), for rapid slide agglutination (Eurocell- A/M, West Harrow, UK,). The antigen is mixed with patient's serum for one minute; agglutination indicates presence of *Brucella* antibodies and no agglutination indicates absence of *Brucella* antibodies.

2.6.1.3 Rose Bengal Test (RBT)

This is an agglutination reaction in which patient's serum are mixed with a suspension of whole *Brucella abortus* cell, stained with Rose Bengal dye and buffered at pH of 3.65 to inhibit non specific agglutinins (Corbel, 1997). The test is simple takes 5-10 minutes and the results are good in cases of acute brucellosis, but in chronic and complicated cases false negative results are high (Araj *et al.,* 1988). Long incubation periods may sometimes result in false reactions, due to the formation of fibrin clots. The test is very

sensitive but should be confirmed by other tests such as complement fixation test (CFT) and ELISA.

2.6.1.4 Enzyme-linked immunoasorbent assay (ELISA).

In chronic brucellosis enzyme-linked immunoasorbent assay (ELISA) is one of the tests used, when results are negative and brucellosis is highly clinically suspected (Araj, 2010). It can test for individual specific immunoglobulin (IgG, IgM, and IgA) within four to six hours and has high sensitivity and specificity (Araj *et al.,* 1986).

Briefly, the test is carried out in 96-well microtitre plates that are pre-coated with predetermined *Brucella* antigen. An enzyme-conjugated (horseradish peroxidase) anti-human IgG or IgM is added to the wells after incubation and plates washed. This is then followed by addition of an enzyme substrate and these are incubated. The plates are read at a suitable enzyme wavelength.

Two types of ELISA used in brucellosis diagnosis are competitive-ELISA (c-ELISA) and indirect-ELISA (i-ELISA). Competitive-ELISA method uses a monoclonal antibody (mAb) specific for SLP-*Brucella* molecule which competes with *Brucella* antibody in the test specimen. This makes c-ELISA assay having a higher specificity and eliminates cross-reactions with other antigens, while sensitivity is still retained. The c-ELISA is a suitable test and can be used as a confirmatory test for human brucellosis (Lucero *et al.,* 1999) and can overcome some of the negative challenges of SAT (Pappas *et al.,* 2005).
Indirect-ELISA (i-ELISA) which is based on immobilized antigen, binding specifically to antibodies present in the test specimen. It generally has very high sensitivity even though it can be affected by cross reacting antibodies, thus slightly lowering its specificity (Poester *et al* 2010).

2.6.1.5 Complement fixation test

Complement fixation test detects *Brucella* antibodies that activate complement. The complement system consists of a series of proteins, which if activated by an antigen-antibody complex, reacts leading to cell lysis (Poester *et al.,* 2010). Complement fixation test consists of *B. abortus* antigens (whole cells) this is incubated with heat inactivated serum sample and titrated complement source, usually guinea pig serum.
A sheep erythrocytes coated with respective rabbit antibody (haemolysin) is then added. If a primary immune complex (*B. abortus* cells and anti-*Brucella* antibodies) is formed complement will be activated/fixed and therefore no reaction with the secondary immune complex (sheep erythrocytes and rabbit haemolysin) resulting in no lysis of the erythrocytes. If no primary immune complex is formed, complement will be free to cause lysis of the sensitized sheep erythrocytes. Thus the amount of complement in solution is inversely proportional to anti-*Brucella* antibody activity. Since IgG antibodies fix complement well; the test specificity is high and is used as a confirmatory test although the test is expensive and labour intensive.

2.6.2 Brucella isolation in culture

Brucella isolation in culture is a definitive diagnosis of brucellosis and has been used as gold standard in laboratory diagnosis (Navorra *et al.,* 2004). *Brucella* may be recovered from patient's specimen such as bone marrow, cerebral spinal fluid, wound swab and pus, but blood is the most frequently used specimen. The convectional castaneda method is recommended (Castaneda *et al.,* 1947) though has variable yields of 40% to 90% in acute cases and 5% to 20% in chronic cases (Yagupsky, 1999). Lysis concentration of blood before culture in Castaneda medium has improved the isolation rate (Mangalgi and Sajjan, 2014). Another medium that can be used is Farrell selective medium which inhibits the growth of most contaminants (Vicente *et al.,* 2014); isolates are identified based on colony morphology, Gram stain and modified "ZN" staining.

Culture method sensitivity is low in sub-acute, chronic phase and when antibiotic has been taken before clinical specimens' collection (Yagupsky, 1999). Brucellosis detection in blood culture in patients with persistent or relapsing brucellosis has low yield of microorganisms because the *Brucella* bacteria invade the mononuclear phagocytic cells thus resulting in low bacteremia (Elfaki *et al.,* 2005).

2.6.3 Molecular characterization of Brucella species

Brucellosis diagnosis based on serology and isolation of *Brucella* has limitations which include cross reactivity for serology and the time required for results to be obtained when bacteria isolation is used, thus the need to use other methods such as molecular identification and chararcterization for diagnosis.

Molecular techniques, based on the polymerase chain reaction (PCR), have been used for *Brucella* identification and characterization (Yu and Nielsen, 2010) and monitoring treatment responses (Araj, 2010). The techniques are more sensitive and specific for detecting *Brucella* organisms in blood and serum specimens than blood culture and serological methods (Nimri, 2003).

Brucella DNA detection in clinical specimens is a challenge because of the low bacterimia and inhibitors present in the specimens (Queipo-Ortuño *et al.,* 2008). Polymerase chain reaction (PCR) using single pair of primers specific to the bacterial DNA sequences, such as 16S - 23S rRNA operon, *IS*711 or *BCSP*31 genes is a reliable method for *Brucella* identification (Godfroid *et al.,* 2010). Multiplex PCR which was first developed in 1994 (Bricker *et al.,* 1994), have also effectively been used for diagnosis and identification of *Brucella* (Smirnova *et al.,* 2013). The Multiplex PCR was named AMOS PCR using the first letters of species names that is *B. abortus, B. melitensis, B. ovis and B. suis* which it detects. Improved AMOS PCR can detect and identify *Brucella* S19 and RB51 vaccine strains, therefore used to differentiate *B.*

abortus field strains from vaccine strains (Ewalt, 2000, Ocampo-sosa, 2005). Real-time PCR for detection of *Brucella sp.* are also available. They are fast, can be used for quantitation and PCR products are not analysed in agarose gel electrophoresis (Yu and Nielsen, 2010). When compared to conventional PCR, they have high sensitivity (Alarcón *et al.,* 2006) and reduced sample contamination.

PCR method can be used even when antibiotics have been administered prior to clinical specimen collection (Morata *et al.,* 2001); because PCR does not differentiate between DNA from live and dead organism (Navorra *et al.,* 2004) and the method is also independent of the disease phase (Al Dahouk *et al.,* 2013). Although relatively rapid and accurate, it is expensive and requires expertise (Conchi *et al.,* 1995).

2.7 Sensitivity and specificity of diagnostic tests

Serological tests used in human brucellosis diagnosis have variable specificity and sensitivity. Sensitivity and specificity (as defined in the next page) are terms used in screening tests for diseases; when a test is performed the person may have the infection (positive) or the person may have no infection (negative). Low specificity is the major limitation due to serological cross reactions and low sensitivity due to IgG and IgA which are produced later in infection (Young, 1991). Therefore, distinguishing reactions due to *Brucella* organisms from reaction due to other bacteria that have similar cell surface antigens, for example *Yersinia enterocolitica,* is a challenge. In the early stages of the disease (incubation period), serological tests have low sensitivity because IgM antibody production is low and the tests also have inability to distinguish between active and past infection due to antibody persistence after treatment (Navorra *et al.,* 2004).

2.7.1 Sensitivity

Sensitivity is the probability of a test that a person has the infection/condition that is being tested for when in fact s/he has the infection. It is therefore a measure of how likely it is for a test to pick up the presence of an infection in a person who has the infection (Scheaffer, 1999).

It is calculated using the formula;

Sensitivity = True positive /True positive + False negative

2.7.2 Specificity

Specificity is the probability of a test that a person does not have the infection/condition that is being tested for when in fact s/he is free of the infection (Scheaffer, 1999).

It is calculated using the formula;

Specificity = True negative / True negative + False positive

2.8 Predictive value of diagnostic tests

Predictive values can be used to help predict the likelihood of infection in an individual. It is defined as the likelihood that a positive test result indicates the presence of infection and a negative test results excludes infection (Gaddis and Gaddis, 1990).

A positive predictive value (PPV) is the probability that a subject with a positive test result have the infection.

It is calculated using the formula;

Positive predictive value = True positive / True positive + False positive

Negative predictive value (NPV) is the probability that a subject with a negative test result does not have the infection.

It is calculated using the formula;

Negative predictive value = True negative / True negative + False negative

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study area

This study was carried out in Baringo County, Kenya (Figure 1). Baringo County has an area of 8,655 square km and a human population of 555,561 (KNBS, 2010). About 140.5 square km of the area is covered by water surface. The County borders Turkana, Laikipia, West Pokot, Elgeyo Marakwet, Uasin Gishu, Kericho, Nakuru and Samburu Counties.

Baringo County has some of the most significant geographic features in the country and thus promotes tourism in the country; these include waterfalls, valleys such as Suguta and Kerio valleys, which form part of the Rift Valley and Tugen hills which has the best escarpment in the country (Kenya-information-guide.com/baringo-county, 2015). Temperatures vary from 25°C to 30°C with an altitude of approximately 1000 metres to 2600 metres above sea level. The annual rainfall is between 1000mm and 1500mm in the highlands and 450mm to 900mm in the lowlands. The main economic activities include pastoralism, crop farming, mixed farming and sand harvesting. Other emerging economic activities include bee keeping and Aloe Vera plant cultivation (Kenya-information-guide.com/baringo-county, 2015).

The backbone of the County economy is agriculture. Farming is the main economic activity in the highlands, while livestock keeping is the main economic activity in the lowlands supplemented with crop farming. For the pastoralists in the lowlands, animals kept include cattle, goats, sheep and camels. The livestock occupy a central part in the community's cultural life as food (meat and milk) and as source of livelihood. Baringo County has several healthcare facilities; these include Kabarnet district hospital (KDH), Eldama Ravine district hospital (EDH), Marigat district hospital (MDH) (Figure 2) and Mogotio district hospital among others (Kenya-information-guide.com/baringo-county, 2015).

Baringo County was chosen for this study because no similar study had been carried out in the region and during fieldwork visit in the County hospitals; hospital records shared a significant diagnosis of brucellosis among the patients who visited the hospitals. The County also has pastoralist and agro-pastoralist communities and some of the cultural practices such as consumption of unpasteurized milk among the communities in Baringo were perceived to predispose them to brucellosis. Thus, there was a high likelihood of obtaining *Brucella*-positive specimens in people suspected of having brucellosis, which was valuable in determining the sensitivity and specificity of the rapid diagnostic kits that were used in the healthcare facilities in Kenya for diagnosis of human brucellosis.

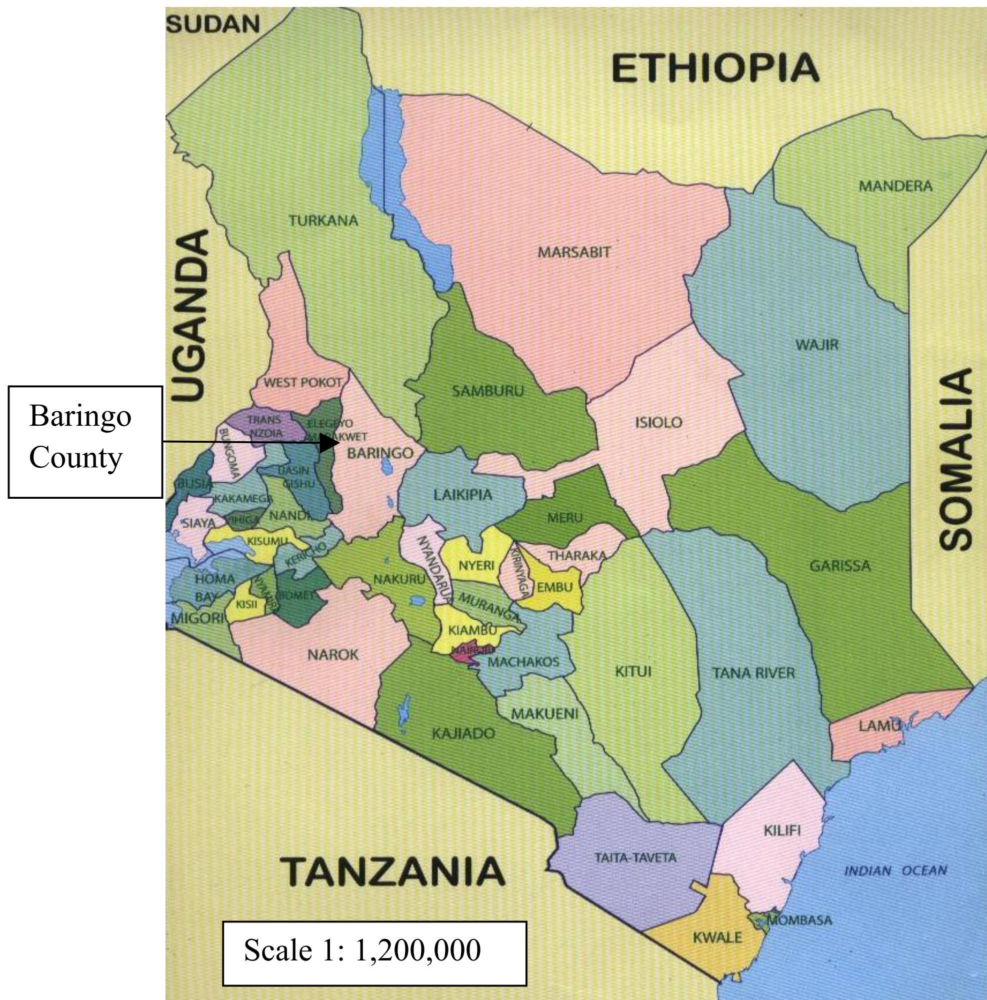

Figure 1: Map of Kenya showing Baringo County, 10/2015
(http:softkenya.com/county/47 counties in Kenya)

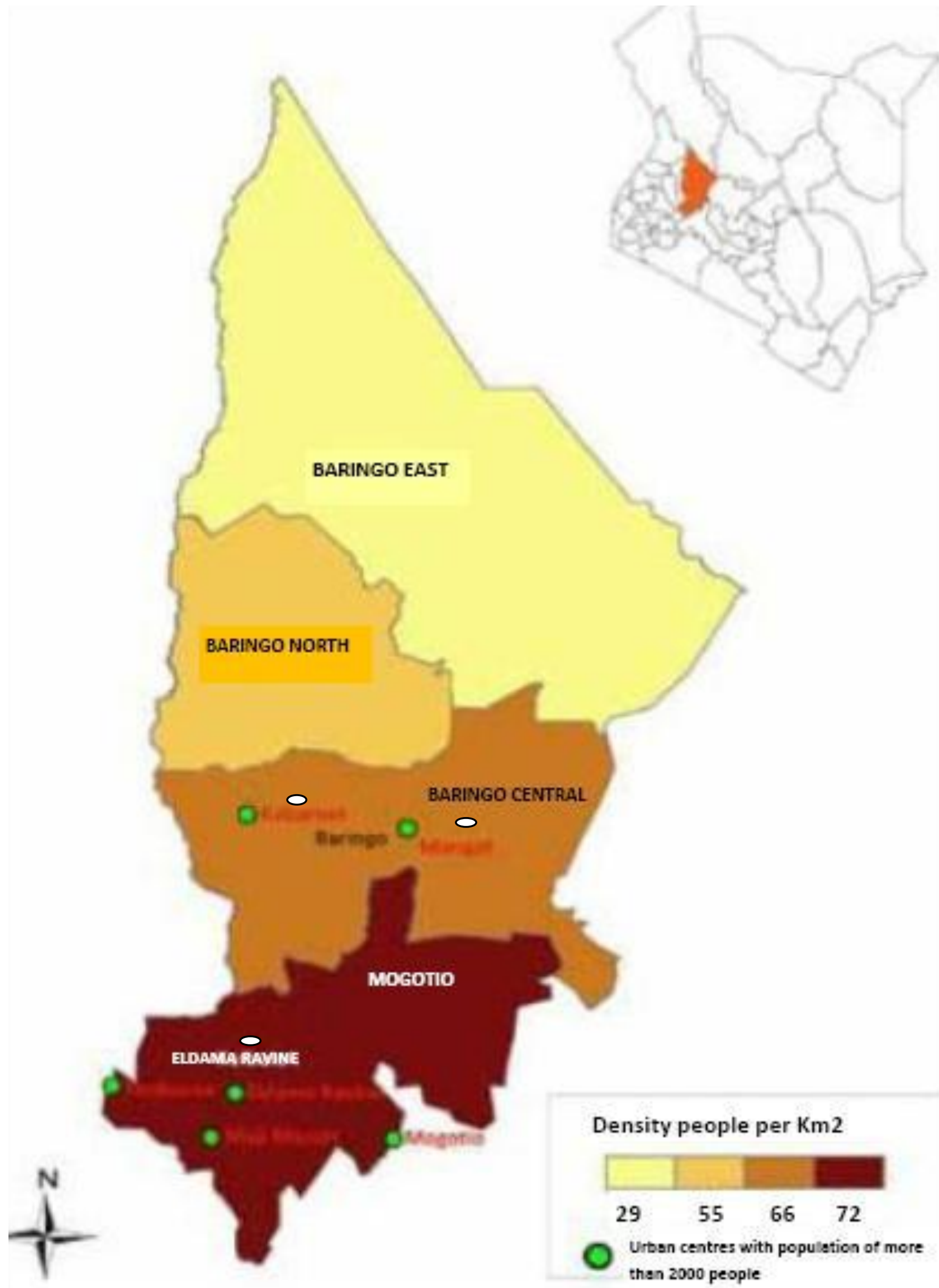

Figure 2: Map of Baringo showing location of Kabarnet, Marigat and Eldama Ravine (White circles) district hospitals (http:softkenya.com/county /baringo-county).

3.2 Study design

This was a cross-sectional laboratory based study carried out from June to August 2014. Patients who visited the three healthcare facilities (Kabarnet district hospital, Marigat district hospital and Eldama Ravine district hospital) and the attending clinician requested for brucellosis testing were consented. They filled a pre-tested questionnaire to collect data on knowledge, attitudes and risk factors associated with brucellosis transmission in the community. Patients enrolled in the study provide a blood specimen, which was separated to blood clot and serum and an aliquot of the serum used to test for brucellosis and other tests required for patient management as requested by the attending clinician. The remaining serum and blood clot specimens, in sterile cryovial bottles, were first stored in the healthcare facilities at -20^{0}C and later transported to Biochemistry Department, University of Nairobi where the serum specimens were re-tested for brucellosis using rapid diagnostic kits and also tested using Rose Bengal Test and c-ELISA. In addition, *Brucella* DNA was extracted from the blood clots and PCR carried out to identify the *Brucella* DNA present in the specimens.

3.3 Inclusion criteria

All patients 18 years and older from whom, the attending clinician had requested for a brucellosis laboratory test and who consented to participate in the study.

3.4 Exclusion criteria

All patients less than 18 years old, prisoners, patients from whom brucellosis laboratory test was not requested by the attending clinician and all patients 18 years and older from whom brucellosis test was requested but who did not consent to participating in the study.

3.5 Sample size

The sample size was calculated according to the method of Martin *et al.,* (1987), using the following formula:

- $n = \frac{Z\alpha^2 pq}{L^2}$
- Where;
- n = Sample size
- $Z\alpha$ = confidence level 95% (1.96)
- p = assumed 13.7% prevalence of brucellosis (Muriuki et *al.,* 1997)
- q =1-p
- L = the precision error (5%)
- Sample size =181.67samples

Using this calculation the minimum sample size required for the study was determined to be 182 samples.

3.6 Patient consenting

Patients sent to the laboratory for brucellosis testing were informed about the study and the purpose for the study and requested to consent for participation. The patients who consented signed a consent form (Appendix 1and 2) in either English or Kiswahili; those who could not write provided a thumb print and a person, who was not part of the study, signed the consent form on their behalf.

3.7 Data collection by questionnaires

Patients who consented were interviewed using a semi structured pre-tested questionnaire (Appendix 3 and 4) to collect data based on knowledge, attitudes and possible risk factors associated with brucellosis transmission in humans. The information collected included patient number, gender, residence, contact with animals,

drinking unpasteurized milk, handling aborted fetuses from livestock, assisting animals during abortion and slaughtering of livestock.

3.8 Specimen collection

A blood specimen (5ml) was collected from each patient who consented to be part of the study. Blood specimens were collected asceptically from the cephalic vein into a sterile vacutainer tubes without anticoagulant by the laboratory technologist. The vacutainers were labeled with respective patient's number and hospital initials for ease of identification.

3.9 Specimen processing and storage

The blood specimens were left on the bench for five minutes to clot. They were centrifuged at 7590xg for five minutes and the serum and clot stored in separate sterile cryovials. Part of the serum (about 60µl) was used to test for brucellosis and other tests required for patient management by the laboratory technologist, as requested by the attending clinician. The remaining serum and blood clot specimens were transferred into sterile cryovials and stored at -20^{0}C in the hospital freezer. Specimens were later transported, in a cool box (0^{0}C - 4^{0}C) containing ice packs to Department of Biochemistry, University of Nairobi and further stored at -20^{0}C until additional assays were carried out.

3.10 Serological testing

3.10.1 Serum agglutination test using rapid diagnostic tests (RDTs)

Rapid diagnostic kits (RDTs) were used to test for presence of anti-*Brucella* antibodies in each of the serum specimens according to the manufacturer's instructions. The RDTs used were Fortress *B. abortus* and *B. melitensis* kit (Fortress Diagnostic kit, Antrim, UK), Eurocell *B. abortus* and *B. melitensis* kit (Eurocell Diagnostic Kit, West Harrow,

UK) and Plasmatec *B. abortus* and *B. melitensis* kit (Plasmatec Diagnostic Kit, Bridport Dorset, UK).

Serial dilution of the sera was attempted but aggluitination was only observed when the undiluted specimens were tested, therefore subsequent assays tested only undiluted specimens. Approximately 30µl (a drop) of test serum was mixed with a drop of the antigen on a white tile and mixed using a clean stirring stick for each specimen. The tile was rocked for two minutes while observing for agglutination. Agglutination indicated a positive reaction and lack of agglutination indicated a negative reaction. The results obtained, were then compared to the results obtained in the healthcare facilities using the same RDTs.

3.10.2 Rose Bengal Test (RBT)

Specimens were also tested using Rose Bengal Test; Rose Bengal Test is a stained buffered acidified *Brucella* antigen which permits the serological diagnosis of brucellosis (*B. melitensis, B. abortus* and *B. suis*). *Brucella* antigen and the test serum were first left to attain room temperature. Then 30 µl (one drop) of serum was placed in white tile and 30µl (one drop) of antigen added and mixed using a clean stirring stick. The white tile was rocked for up to four minutes while observing for agglutination. Serum specimens that agglutinated with the antigen were recorded as positive while those for which no agglutination was observed were recorded as negative (Rose Bengal *Brucella* antigen, Morganville, USA).

3.10.3 Competitive enzyme-linked immunoasorbent assay (c-ELISA)

Competitive-ELISA (SVANOVIR® *Brucella*-Ab C-ELISA, Boehringer Uppsala, Sweden) kit for the detection of specific antibodies to *B. abortus, B. melitensis* and *B. suis* was used. All reagents were allowed to attain room temperature before use and the procedure carried out according to manufacturer's instructions. Briefly, a 96 well

microtitre plate coated with *B. abortus* S-LPS antigen was used and 45 µl of sample dilution buffer was added to each well that was used for test serum specimens and control serum specimens. To the wells used for control specimens (positive control serum, weak positive control serum and negative control serum); five microlitres (5 µl) of each control serum specimen were added in duplicate. In the conjugate control well, five microlitres (5 µl) of sample dilution buffer was added. To the remaining wells five microlitres (5 µl) of test serum were added but not in duplicate. Then, 50 µl mouse monoclonal antibody (mAb) solutions were added in all wells. The plate was sealed and the reagents mixed thoroughly for five minutes, then incubated at room temperature for 30 minutes. The plate was then rinsed four times with PBS-Tween buffer and then tapped hard on a paper towel to remove as much fluid as possible from the wells. Conjugate solution (horseradish peroxidase conjugated goat anti-mouse IgG antibodies) 100 µl was added into each well and incubated at room temperature for 30 minutes. The plate was rinsed with PBS-Tween buffer four times. Substrate solution (Tetramethylbenzidine in substrate buffer containing H_2O_2) 100 µl was added in all the wells and incubated for 10 minutes. The reaction was stopped by addition of 50 µl stop solution (2M sulphuric acid) and absorbance read using ELISA reader (MR-96A, Mindray microplate reader, Turkey) at 450 nm. Percentage inhibition for each specimen was calculated as per the formula below and the validity of the test was determined as described. For all tests that met the validity criteria, specimens with percent inhibition (PI) less than 30% were recorded as negative while specimens with PI equal to or more than 30% were recorded as positive.

3.10.3.1 Calculations of percentage inhibition, validity and cutoff values for the specimens

(SVANOVIR® *Brucella*- Ab C- ELISA-Protocol)

$$\text{Percent inhibition (PI)} = 100 - \frac{(\text{OD sample or control} \times 100)}{\text{OD conjugate control (Cc)}}$$

The test was considered to be valid if the value of controls fell within the following limits:

OD (450nm) Conjugate control (Cc) 0.75-2.0

Percent inhibition (PI) Positive control 80-100

Percent inhition (PI) Weak positive control 30-70

Percent inhibition (PI) Negative control (-10) -15

Interpretation

Results for each specimen were recorded as negative or positive using the following criteria:

Percent inhibition (PI) <30% Negative

Percent inhibition (PI) ≥ 30% Positive

3.11 Detection of Brucella DNA in the specimen's using polymerase chain reaction (PCR)

Deoxyribonucleic acid (DNA) was extracted from the blood clot and polymerase chain reaction was carried out, for detection of the *Brucella* species.

3.11.1 DNA extraction

Deoxyribonucleic acids (DNA) were extracted from blood clots from patients suspected to be infected with *Brucella* bacteria using the hexadecyltrimethyl ammonium bromide (CTAB) method described by Chachaty and Saulnier (2000), with a few modifications. Briefly, to 400 µl of blood clot in 1.5 ml eppendorf tube, one ml of erythrocyte lysis solution (Appendix 5) was added and mixed by vortexing, then centrifuged at 5060xg at room temperature for three minutes. The supernatant was discarded and erythrocyte lysis repeated twice or until the leukocyte pellets lost the reddish colour.

The pellet was re-suspended in 567 µl TE buffer (10mM Tris, 1mM EDTA), then 30 µl of SDS (10% in sterile water), 3 µl of proteinase K (20mg/ml in sterile water) and 10 µl lysozyme (10mg/ml in TE buffer) were added, mixed thoroughly and incubated for one hour at 37^0C. Then 100 µl of 5M NaCl and 80 µl of CTAB/NaCl solution (Appendix 5) were added, mixed thoroughly, and incubated for 10 minutes at 65^0C. Phenol/chloroform/isoamyl alcohol (25:24:1) (500 µl) was added, mixed thoroughly and centrifuged at 17700xg for 10 minutes at room temperature. The supernatant was transferred into a fresh micro-centrifuge tube without interfering with the interface. Chloroform/isoamyl alcohol (24:1) (750 µl) was added mixed thoroughly and centrifuged at 17700xg for 10 minutes at room temperature. The supernatant was then transferred into a sterile micro-centrifuge tube. Then 0.6 times volume isopropanol was added to precipitate the DNA; this was kept at -20^0C overnight and then centrifuged at 17700xg for 10 minutes at room temperature, the supernatant discarded and the DNA washed with 500 µl 70% ethanol. It was mixed thoroughly and centrifuged at 17700xg at room temperature. The supernatant was discarded and the DNA left to dry in air for 10 minutes and then re-dissolved in 20 µl double distilled sterile water (Chachaty and Saulnier, 2000).

3.11.2 Determination of DNA quality

To determine the presence and quality of extracted DNA, the DNA was resolved using 0.8% agarose gel in 1 x TAE buffer (Appendix 5) the gel was then stained using ethidium bromide (10mg/ml in sterile water). Ethidium bromide (10mg/ml in sterile water) 5 µl was added in the agarose gel at a temperature of about 50^0C; the gel was poured in the gel casting chamber then allowed to solidify for about 30 minutes. The wells were loaded with 5µl of DNA extracts mixed with 1 µl of 6x loading buffer alongside DNA size markers. Electrophoresis was carried out at 100V for one hour at room temperature and the DNA visualized under UV transilluminator and photographed.

3.11.3 Conventional polymerase chain reaction (PCR)

To determine the presence of *Brucella*-specific nucleic acids in the extracted DNA, improved AMOS PCR assay was carried out (Bricker *et al.,* 2003). Briefly, AMOS PCR is a multiplex PCR assay that uses a five-primer cocktail to test for the presence of DNA of four *Brucella* species: *B. abortus, B. melitensis, B. ovis* and *B. suis*. Each assay included a forward primer for each of these four *Brucella* species and a universal reverse primer (IS7 R). The reverse primer was derived from an insertion element which is found in multiple copies within *Brucella* chromosomes, it improves PCR analytical sensitivity (Bounaadja *et al.,* 2009). The name AMOS is based on the *Brucella* species DNA that may be detected using this assay. The forward primers were specific *B. abortus* (BA F), *B. melitensis* (BM F), *B. ovis* (BO F) and *B. suis* (BS F) (Table 1), which when used in a PCR reaction with the reverse primer (IS7 R) were expected to produce fragments of 489 bp, 731bp, 976 bp and 285 bp, respectively.

Table 1: Primers used in the PCR reactions and primer sequences for each primer

Primers	**Nucleotide sequence 5' to 3'**
IS7 R(Reverse)	TGC CGA TCA CTT AAG GGC CTT CAT
BA F(Forward)	GAC GAA CGG AAT TTT TCC AAT CCC
BM F(forward)	AAA TCG CGT CCT TGC TGG TCT GA
BO F(Forward)	CGG GTT CTG GCA CCA TCG TCG
BS F (Forward)	GCG CGG TTT TCT GAA GGT TCA GG

The PCR reaction was carried out as follows; a 20 µl reaction mixture (Table 2) was prepared having 10 µl two times PCR buffer (50 mM Tris, 1.5 mM $MgCl_2$, 10 mM KCl, 50 mM $(NH_4)_2SO_4$, pH 8.3) (TopTaq™ Master Mix, QIAGEN), 6 µl double distilled sterile water, 1µl cocktail forward primers (BA F, BM F, BO F, BS F) at a final concentration of 0.25 µM each, 1µl IS7 R primer at a final concentration of 1µM, and 2 µl DNA template for each specimen. Two control reaction mixtures were included,

positive control containing 2 µl *Brucella abortus* DNA and a negative control containing 2 µl double distilled water instead of specimen DNA.

Table 2: PCR content per tube (20 µl reaction)

PCR Components	**Volume(µl)**
2x PCR buffer (TopTaq™ master mix)	10µl
Double distilled water	6 µl
Forward primers (1 µM)	1µl
Reverse primer (1 µM)	1µl
DNA template	2µl
Final volume	20µl

The amplification was carried out as follows, initial denaturation at 95ºC for 5 min, 40 cycles of 95ºC for 15 seconds , 52ºC for 30 seconds, 72ºC for 90 seconds and a final extension at 72ºC for 5 minutes, the PCR products were stored at 4^{0}C (Bricker, 2002). The amplified products were separated on 1% agarose gel containing 5µl ethidium bromide (10mg/ml) in 1x TAE buffer at 100 volts for one hour. The DNA bands were observed by UV fluorescence and photographed.

3.12 Data analysis

Sensitivity, specificity**,** positive predictive value and negative predictive value (Gaddis and Gaddis, 1990; Scheaffer, 1999) were calculated using the formulae given below:

Sensitivity = True positive/True positive + False negative (x 100)

Specificity = True negative/True negative + False positive (x 100)

Positive predictive value = True positive/True positive+ False positive

Negative predictive value = True negative/True negative+ false negative

The level of agreement between the serological tests used (RDTs, RBT, c-ELISA) was calculated using Kappa analysis (Appendix 6). Data coding for the qualitative data was done using the Statistical Package for Social Sciences (IBM SPSS Statistics 23, Chicago, USA). Quantative data was entered in Microsoft excel and graphs generated using the same program (Microsoft excel 2007, Microsoft Corporation, Redmond, Washington, USA). Odds ratios (OR) were used to measure level of association for brucellosis seropositivity (Epi info 7, CDC, Atlanta, USA).

3.13 Ethical considerations

Ethical approval to carry out the study was obtained from the Kenyatta National Hospital/ University of Nairobi-Ethics and Research committee (KNH/UON-ERC) P589/11/2013 prior to commencement of the study (Appendix 8). Written consent was obtained from each study participant before enrollment to the study.

CHAPTER FOUR: RESULTS

4.1 Overview of the collected specimens and tests run

Field site visits and key informant interviews were carried out prior to beginning the study to identify and categorize various healthcare facilities in the County and the kits used for brucellosis testing. Three healthcare facilities were selected for the study. These included two healthcare facilities in the highlands, Kabarnet district hospital (KDH) and Eldama Ravine district hospital (EDH) and one healthcare facility in the lowlands, Marigat district hospital (MDH).

A total of 182 patients met the inclusion criteria during the sampling period (from June to August 2014), in the three healthcare facilities but only 166 (116 female and 50 male) consented to participate in the study. Blood specimens were collected from the 166 patients of who included 50.6% (84/166) from Marigat district hospital (MDH), 31.3% (52/166) from Kabarnet district hospital (KDH) and 18.1% (30/166) from Eldama Ravine district hospital (EDH) (Table 3).

Table 3: Healthcare facilities sampled and patients' gender in Baringo, 2014

Healthcare Facilities	Number sampled (%)	Gender	
		Female	**Male**
Kabarnet district hospital (KDH)	52 (31.3%)	36 (69.2%)	16 (30.8%)
Marigat district hospital (MDH)	84 (50.6%)	55 (65.5%)	29 (34.5%)
Eldama Ravine hospital (EDH)	30 (18.1%)	25 (83.3%)	5 (16.7%)
Total	166 (100%)	116 (69.9%)	50 (30.1%)

All 166 serum specimens were tested using the rapid diagnostic kits, Rose Bengal Test and competitive-ELISA. Nucleic acid was extracted from the blood clots and polymerase chain reaction (PCR) carried out on the extracted DNA to identify *Brucella* species.

4.2 Serological test results

4.2.1 Competitive-ELISA results

Of the 166 serum specimen tested using c-ELISA (SVANOVIR® *Brucella*-Ab C-ELISA, Boehringer Uppsala, Sweden) kit, 9.6% (16/166) were positive for brucellosis. Table 4, showing number of positive specimen with respect to the three healthcare facilities studied. The c-ELISA was used as the gold standard in the study; therefore the seropositivity of brucellosis in Baringo County was 9.6%.

Table 4: Human brucellosis seropositivity in Baringo County as determined by competitive- ELISA in 2014

Healthcare Facilities	Number of samples tested	Number positive	Percent positive (%)
Kabarnet district hospital (KDH)	52	6	11.5%
Marigat district hospital (MDH)	84	6	7.1 %
Eldama Ravine hospital (EDH)	30	4	13.3 %
Total	166	16	9.6 %

4.2.2 Result of rapid diagnostic kits (RDTs)

Overall 44 of the 166 specimens (26.5%) were seropositive for brucellosis as determined by RDTs (Table 5). An example of a positive and negative result is shown in figure 3. Each specimen that was positive for *B. abortus* (BA), as determined by the RDTs, was also positive for *B. melitensis* (BM) while each specimen that was negative for *B. abortus* was also negative for *B. melitensis*. Therefore, it is possible that the tests did not differentiate between the two antigens (*Brucella abortus* and *Brucella melitensis*). In addition, there were no differences in the test results obtained using each of the three RDTs (Appendix 7) suggesting that the antigens used in the kits may have been similar.

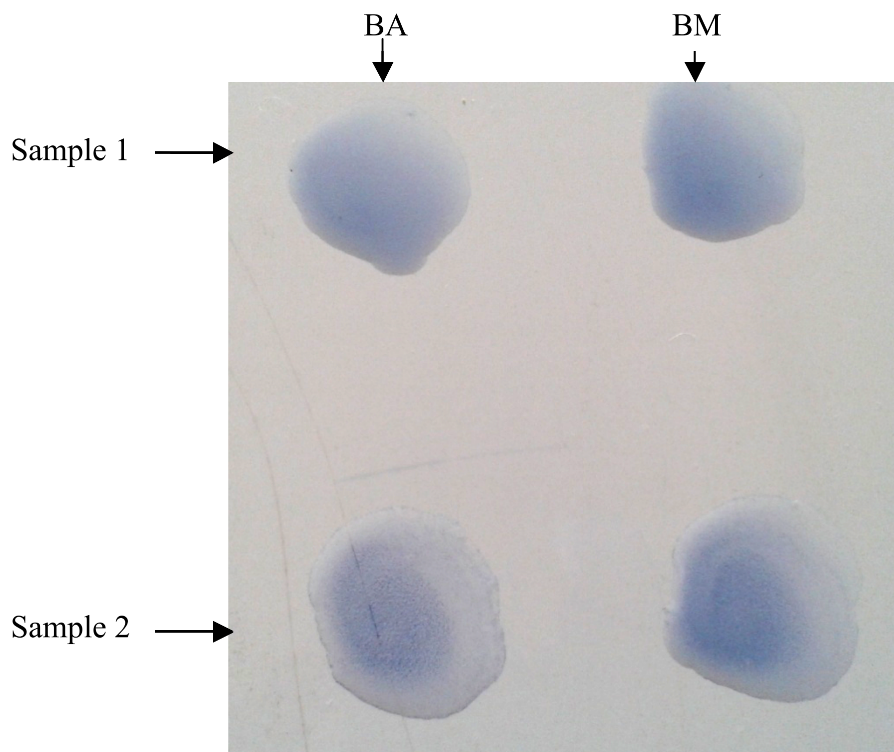

Figure 3: Reactions of samples 1 and 2 tested with Fortress kit using *B. abortus* (BA) and *B. melitensis* (BM) antigens. Sample 1: Negative (no agglutination) for both BA and BM, sample 2: positive (agglutination) for both BA and BM.

Table 5: Human brucellosis seropositivity in Baringo County as determined by rapid diagnostic kit (RDTs) in 2014

Health care facilities	**Number of samples tested**	**Number of positive specimens for each rapid kit**						**Percent positive (%)**
		Fortress kit		**Plasmatec kit**		**Eurocells kit**		
		BA	BM	BA	BM	BA	BM	
Kabarnet district hospital (KDH)	52	9	9	9	9	9	9	17.3%
Marigat district hospital (MDH)	84	30	30	30	30	30	30	35.7%
Eldama Ravine district hospital (EDH)	30	5	5	5	5	5	5	16.7%
Total	166	44	44	44	44	44	44	26.5%

4.2.3 Rose Bengal Test (RBT) results

Overall 10.2% (17/166) were positive for brucellosis using Rose Bengal Test (RBT) (Table 6). The agglutination results for three serum specimens, one positive (agglutination) and two negative (no agglutination) are illustrated in figure 4.

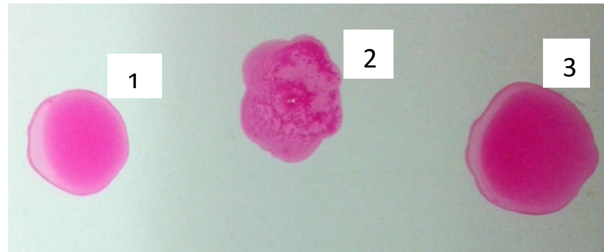

Figure 4: Rose Bengal Test, showing agglutination results for three different serum specimens, 2 agglutination , 1 and 3 no agglutination.

Table 6: Human brucellosis seropositivity in Baringo County as determined by Rose Bengal Test in 2014

Healthcare faclities	**Number of samples tested**	**Number positive**	**Percent positive (%)**
Kabarnet district hospital (KDH)	52	3	5.8%
Marigat district hospital (MDH)	84	13	15.5 %
Eldama Ravine hospital (EDH)	30	1	3.3%
Total	166	17	10.2 %

A comparison of the results obtained using the serological tests are shown in Table 7.

Table 7: The agreement on the positive outcomes between c-ELISA, RBT, Plasmatec, Fortress and Eurocell serological tests

Test	c-ELISA	RBT	Plasmatec	Fortress	Eurocell
C-ELISA	16	6	3	3	3
RBT	6	17	13	13	13
Plasmatec	3	13	44	44	44
Fortress	3	13	44	44	44
Eurocell	3	13	44	44	44

4.3 Sensitivity and specificity of rapid diagnostic kits (RDTs) compared to c-ELISA

The sensitivity and specificity of the rapid diagnostic kits (RDTs) compared to c-ELISA was 37.5% with specificity of 74.7% (Table 8). Rapid kits diagnosed 38 specimens as false positives (specimen positive based on RDTs but negative with c-ELISA) and 10 specimens as false negatives (specimens positive based on c-ELISA but negative with RDTs). False positives may be due to cross reactions; these may be found with *Brucella* antigen in cases of infection with some strains of *Vibrio* (campylobacter), *Pasteulla* and patient vaccinated with *V. cholerae.* The positive predictive value was 13.6% these were patients with positive test results that truly had the infection, the negative predictive value was 91.8% these were the patients with negative test results that truly had no infection). The level of agreement between the two tests (c-ELISA and rapid diagnostic kits) was 7%, which translates to slight agreement according Kappa statistics (Appendix 6).

Table 8: Comparison of c-ELISA and rapid diagnostic kits used at the healthcare facilities

Rapid diagnostic kits	**C- ELISA**		**Total**
	Number positive	Number negative	
Number positive	6	38	44
Number negative	10	112	122
Total	16	150	166
	Sensitivity = 37.5%	Specificity =74.7%	
	Predictive value positive = 13.6%	Predictive value negative = 91.8%	
	Level of agreement = 7%		

4.4 Sensitivity and specificity of Rose Bengal Test compared to c-ELISA

The sensitivity and specificity of the Rose Bengal Test (RBT) compared to c-ELISA was 18.8% with specificity of 90.7% (Table 9). The positive predictive value was 17.6% and negative predictive value was 91.3%, the level of agreement between the two tests (c-ELISA and Rose Bengal Test) was 8%, which translates to slight agreement according to Kappa statistics (Appendix 6).

Table 9: Comparison of c-ELISA and Rose Bengal Test

Rose Bengal Test	C- ELISA		Total
	Number positive	Number negative	
Number Positive	3	14	17
Number negative	13	136	149
Total	16	150	166
	Sensitivity =18.8%	Specificity = 90.7%	
	Predictive value positive = 17.6%	Predictive value negative =91.3%	
	Level of agreement = 8%		

4.5 Sensitivity and specificity of rapid diagnostic kits compared to Rose Bengal Test

The sensitivity and specificity of the rapid diagnostic kits compared to Rose Bengal Test (RBTs) was 76.5% with specificity of 79.2% (Table 10). The positive predictive value was 29.5% and negative predictive value was 96.7%, the level of agreement for the two tests (rapid diagnostic kits and Rose Bengal Test) was 32.7% which translate to fair agreement according to the interpretation (Appendix 6). Thus RDTs and RBT agreement was more than when each of the two tests was compared with c-ELISA.

Table 10: Comparison of rapid diagnostic kits and Rose Bengal Test

Rapid diagnostic kit	**Rose Bengal Test**		**Total**
	Number positive	Number negative	
Number positive	13	31	44
Number negative	4	118	122
Total	17	149	166
	Sensitivity =76.5%	Specificity = 79.2%	
	Predictive value positive = 29.5%	Predictive value negative = 96.7%	
	Level of agreement = 32.7%		

4.6 DNA extraction

Deoxyribonucleic acid (DNA) extraction done on 166 specimens, 89 out of 166 (53.6%) had good quality DNA after running agarose gel electrophoresis.

4.7 Polymerase chain reaction results

Of the 89 DNA positive specimens, PCR was carried out on 79.8% (71/89) specimens in order to identify the *Brucella species* involved. PCR was done on 71 samples because the reagents we had were enough for 71 samples. Figure 5, agarose gel electrophoresis showing negative result, positive result, positive control (*Brucella abortus*) and negative control (double distilled water). Seven out of 71 (9.9%) specimens were positive for *B. abortus* (489 bp) (Figure 6); there were no amplicons observed that corresponded with expected amplicon for *B.melitensis* (731 bp), *B.ovis* (976 bp) or *B.suis* (285bp), results are given on Table 11.

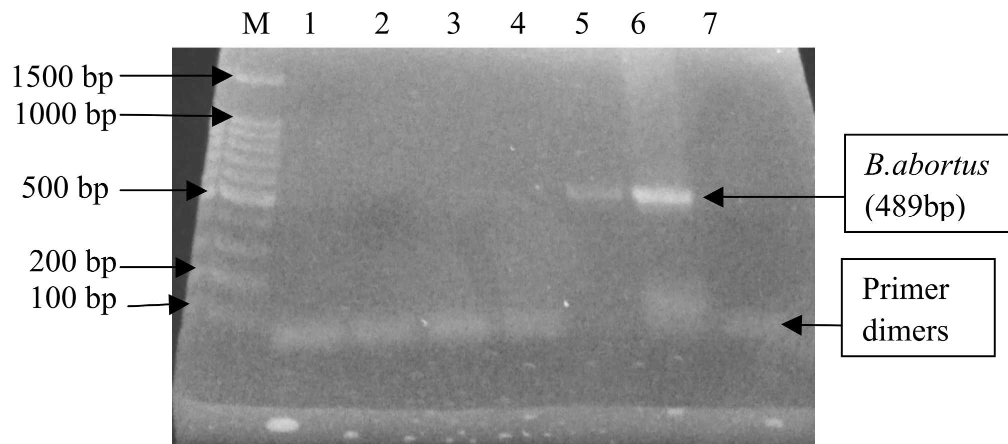

Figure 5: Agarose gel electrophoresis showing 100bp molecular marker (M), negative specimens 1, 2, 3, 4, positive specimen 5, positive control 6 (*Brucella abortus*) and negative control 7 (double distilled water)

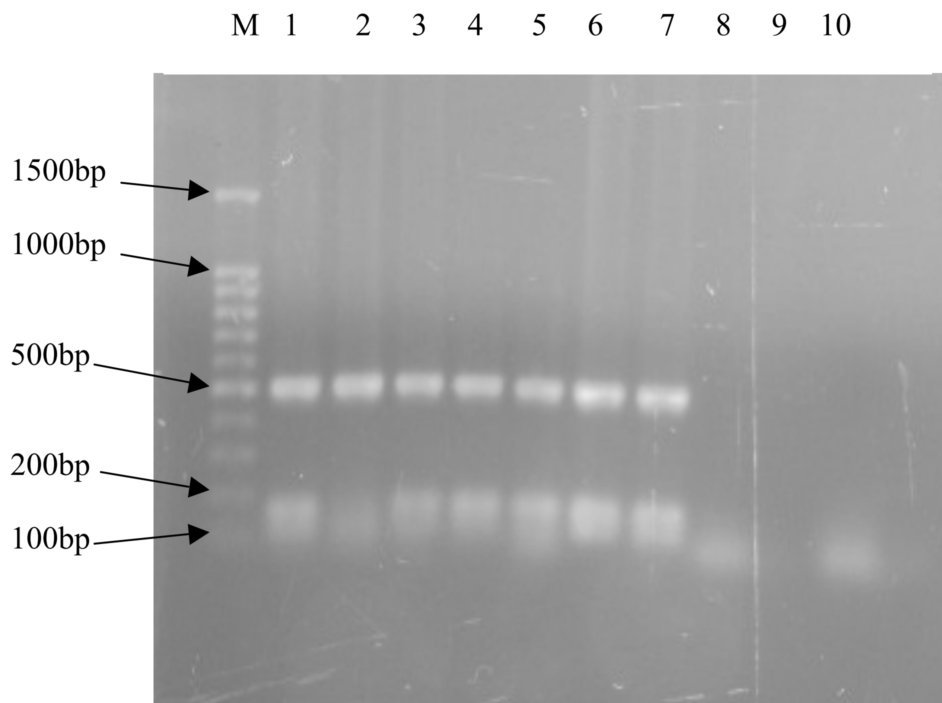

Figure 6: AMOS PCR, gel electrophoresis showing 100bp molecular marker (M), positive human specimens 1,2,3,4,5,6,7 (amplicon for *B. abortus* (489bp), negative human specimens 8 (no amplicon), blank well 9 and negative control 10.

Table 11: Human brucellosis positive specimens in Baringo County as determined by polymerase chain reaction (PCR) in 2014

Healthcare facilities	**Number of samples tested**	**Number positive**	**Percent positive (%)**
Kabarnetdistrict hospital (KDH)	26	1	3.8%
Marigat district Hospital (MDH)	30	6	20.0%
Eldama Ravine Hospital (EDH)	15	0	0.0%
Total	71	7	9.9%

4.8 Clinical information of the patients who participated in the study

The number of patients who consented to participate in the study was 166; these were patients who visited the healthcare facilities and were suspected of suffering from brucellosis. One hundred and fifty seven (157; 94.6%) experienced fever, Figure 8: shows respective durations of the patients' illness.

About 78.9% (131/166) of the patients had used some drugs, including antibiotics 18.1% (30/166), paracetamol 21.7% (36/166) anti-malarial 6.6% (11/166), traditional medicine (herbs) 32.5% (54/166) and others unidentified drugs 21.1% (35/166). The medicine was either self-prescribed or obtained from a healthcare facility.

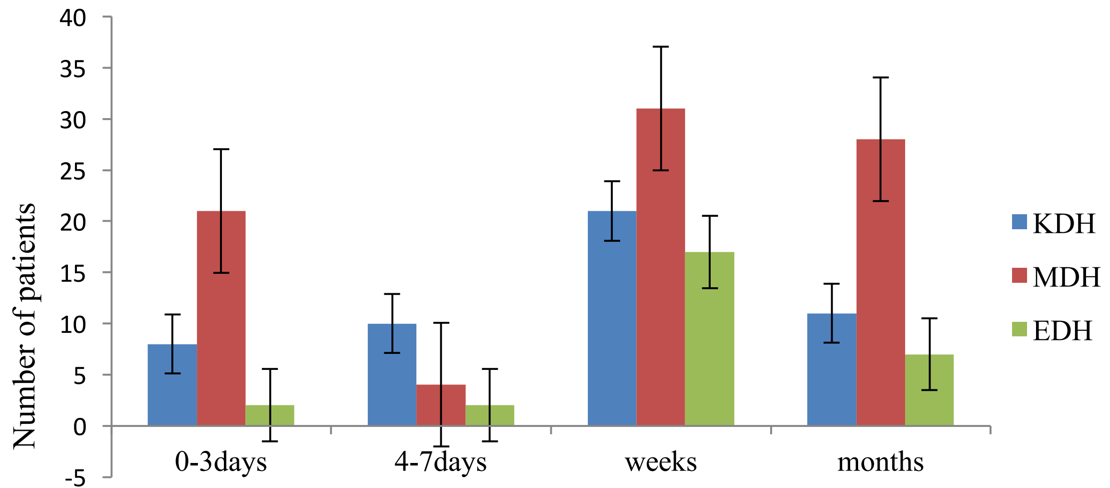

Figure 7: Duration of the patients' illness by the time reporting to heathcare facilit

4.9 Factors associated with brucellosis infection in humans

Approximately 81.3% (135/166) of patients kept animals, 57.8% (96/166) of patients kept cattle, sheep and goats in the same herd, and 45.8% (76/166) of the patients had sick animals and 31.9% (53/166) patients said their animals had experienced abortion. About 58.5% (31/53) patients assisted the animals during abortion without gloves, 18.9% (10/53) called health care workers who assisted the animals, and 11.3% (6/53) used gloves and 11.3% (6/53) did not assist the animals. Retained after birth was not common, but when it happened at home the placenta was given to dogs and when it happened at the field while grazing it was left unburied. Patients involved in slaughtering animals were 25.9% (43/166); while12.0% (20/166) ate meat from sick animals.

About 42.8% (71/166) of the patients used milk from their animals, 26.5% (44/166) bought raw milk from the market, 5.4% (9/166) bought packed milk (pasteurized) and 18.7% (31/166) bought both pasteurized and raw milk, while 6.6% (11/166) did not

drink milk. Those who drank sour milk (mursik) 83.2% (129/155) boiled the milk before preparing "mursik" while 16.8% (26/155) bought already prepared"mursik", from the market and were not sure of how it was prepared. The culture of drinking animal blood and eating raw meat was still practiced in the region with percentage proportion of 42.9% (36/84) of patients from Marigat, 17.3 % (9/52) from Kabarnet and 6.7% (2/30) from Eldama Ravine reporting engaging in the practice.

Overall, 88.6% (147/166) of the participants were aware that there is a disease called brucellosis (ugonjwa ya maziwa), while 11.4% (19/166) were not aware of the disease. In addition, 50.6% (84/166) of the participants knew the disease was caused by drinking unboiled milk, 40.4% (67/166) knew that other causes included consumption of raw meat and raw blood while 9.0% (15/166) did not know the cause of brucellosis.

The odds ratio (OR) estimates for the factors associated with brucellosis (Table 12). Among the factors assessed included keeping animals at home, having sick animals, disposal of placenta, involvement in animal slaughtering, eating meat of sick animals and drinking "mursik" prepared after boiling milk, were all not statistically significant ($P>0.05$) as factors associated with increased risk of brucellosis (OR less than 1.0). Handling sick animals (OR 3.77; CI, 1.17-13.31) and drinking unboiled milk (OR 4.21; CI, 1.02-28.63) were the factors positively associated with brucellosis (in bold, Table 12) and were statistically significant ($P<0.05$). The patients who drank unboiled milk were 4.2 times more likely to have brucellosis than the patients who boiled their milk before consumption. In addition, patients who handled sick animals were 3.8 times more likely to have brucellosis than patients who did not handle sick animals.

Table 12: Odds ratio (OR) estimates with 95% confidence intervals for the factors assessed

Factors	**Odds ratio (OR)**	**95%Confidence interval (CI)**	**P values α = 0.05**
Keeping animals	0.37	0.12-1.50	0.08
Sick animals	0.81	0.21-3.14	0.38
Handling sick animals	**3.77**	**1.17-13.31**	**0.01**
Buried placenta	0.76	0.03-4.88	0.44
Slaughter animals	0.72	0.15-2.56	0.33
Eat meat from sick animal	0.59	0.03-3.94	0.35
Milk from own animals	1.03	0.32-3.18	0.47
Unboiled milk	**4.21**	**1.02 -28.63**	**0.02**
Boiled milk before prepare Mala (Mursik)	0.33	0.08-1.67	0.08

CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMEMDATIONS

5.1 DISCUSSION

Brucellosis diagnosis in humans is based on a patient's medical history, clinical signs and symptoms, and the use of different methods of diagnosis to detect *Brucella* microorganisms (Araj, 2010). Definitive diagnosis of brucellosis is based on isolation and identification of *Brucella* pathogens from clinical specimens (Yagupsky, 1999). None of the three healthcare facilities used *Brucella* isolation method for diagnosis because they lack biosafety level 3 laboratories that would be required for isolation of *Brucella* spp. They all used rapid diagnostic kits.

The minimum sample size calculated for the study was 182, though only 166 patients consented to participate in the study. When calculating sample size the true prevalence for the region should be used but if not known an assumation is made based on studied done in other regions having some similarity to the area of study. In this study brucellosis prevalence was assumed to be 13.7% based on study done in Narok a pastrolist community, which was perceived to be similar to the community in Baringo County. This study revealed a seroprevalence of 9.6% (c-ELISA). When this is considered as the true prevalence of brucellosis in Baringo County, then the minimum sample size required for this study would have been 133. Thus 166 specimens used for this study were sufficient to fulfill the objectives of the study.

The numbers of female participants was greater than that of male participants. This was because sampling was based on the patients who visited the hospital during the sampling period and brucellosis testing was requested by the attending clinician and only those who consented participanted of which it happened that majority were females. This would have affected the study considering that males are the ones who take care of the livestock though few visited the hospital when the study was done.

The RDTs kit indicated that one antigen was specific for *B. abortus* and one for *B. melitensis*; however the RDTs antigens did not differentiate between the two *Brucella* species because the reactions for the two antigens were the same. This may indicate cross reactivity between the two tests possibly because of the use of a similar antigen in the two tests. There is also possibility of mixed infections. These results are in agreement with the statement that *Brucella* specific seropositive response indicates infection, but does not differentiate the *Brucella* species (Smirnova *et al.,* 2013). Since all smooth *Brucella* species share common epitopes in the O-polysaccharide (OPS) and almost all serological tests use *B. abortus* antigen as whole cells, smooth lipopolysaccharides (SLPS) or OPS (OIE, 2008). Only in *B. ovis* and *B. canis* infection, rough lipopolysaccharide (RLPS) antigen is used for detection of *Brucella* because *B. ovis* and *B. canis* lack OPS component (Blasco, 1990). Antigen preparation also has an influence on serological tests and currently there is no standardized antigen for diagnosis of human brucellosis (Al Dahouk *et al.,* 2013).

The seropositivity of brucellosis in the study area was 9.6% based on c-ELISA (gold standard) while rapid diagnostic kits (RDTS) seropositivity was 26.5% and Rose Bengal Test (RBT) was 10.2%. Comparing the three test RDTs had the highest seropositivity this would be as a result of cross reaction found with *Brucella* antigen in cases of infection with micro-organisms such as *Escherichia coli, Salmonella spp, Vibrio cholerae* and *Yersinia enterocolitica.*

The sensitivity and specificity of RDTs compared to c-ELISA were 37.5% and 74.7% respectively, with a positive predictive value (PPV) and negative predictive value (NPP) of 13.6% and 91.8%, respectively. This compared well with results of the study done in Ijara district hospital in Garrisa (Kenya) which used Febrile diagnostic kit; it recorded sensitivity and specificity of 37% and 69%, respectively with positive predictive value of 18% and negative predictive value of 86% (Kiambi, 2014). The low specificity indicates that there are potentially a high proportion of patients who are diagnosed to be

Brucella-positive who may not be suffering from brucellosis and thus are treated for brucellosis when such treatment is not required.

In this study the average Rose Bengal Test seropositivity was 10.2%; however using the same test, other studies done in healthcare facilities in Narok (Kenya) reported higher sero-positivity of 21% (Muriuki *et al.,* 1997) and 13% (Muriuki et *al.,* 1994). Based on individual locations, these high results of Narok compared well with those recorded in Marigat seropositivity of 15.5% based on Rose Bengal Test, an observation that is not surprising as people in Marigat are pastoralists like those living in Narok.

Brucellosis disease occurs in three phases that is acute, subacute and chronic phase, false-negative results in serological testing is due to measuring a single subgroup of immunoglobulins (Gomez *et al.,* 2008). Evaluation of both IgM and IgG antibodies would enable correct staging of the disease and increase the sensitivity of serological test (Fadeel *et al.,* 2011).

Comparing specimens that were positive by RDTs 44 and those positive by c-ELISA 16, only six (6) were positive for both tests. The 16 specimens which were positive for c-ELISA were considered as true cases of brucellosis. For the 10 which were positive with c-ELISA but negative with RDTs, it can be speculated that, may be, RDTs sensitivity cannot detect the antibodies present in the specimens.

Competitive-ELISA detected less positive compared to RBT but more in Kabarnet district hospital (KDH) and Eldama Ravine district hospital (EDH), KDH and EDH being in the highland region of the County people here are mainly farmers therefore earlier exposure to *Brucella* would be low therefore background antibody level would be low also. To distinguish between active and inactive infection, is another challenge due to antibody persistence after treatment. It needs to be noted, that negative agglutination result also does not exclude brucellosis because, early in the disease, (incubation period) IgM levels are low or absent as in subacute phase while in chronic phase IgG antibodies,

are the main ones present (Al Dahuok *et al.,* 2003). This is the reason why during incubation period and following abortion the serum agglutination test (SAT) is negative (Mittal *et al.,* 1983). Therefore, in cases where on testing a patient, the results are negative yet the patient's history, signs and symptoms strongly indicate brucellosis, using a more specific test like c-ELISA should be considered.

Rose Bengal Test (RBT) which is mostly used as a screening test, the sensitivity and specificity is fairly high in unexposed populations thus false positive results are rare (Ruiz-Mesa *et al.,* 2005, Serra *et al.,* 2004). In endemic countries, RBT is used for rapid screening in emergency departments, although its performance is poor in individual who have been earlier exposed to the *Brucella* (Ruiz-Mesa *et al.,* 2005).

The agreement between rapid diagnostic kits (RDTs) and Rose Bengal Test (RBT) was 32.7% which was higher, than when each of the two tests was compared to c-ELISA. This indicates that rapid diagnostic kit test and Rose Bengal Test may be having similar antigens that the antibody in the serum binds.

Comparing the three serological tests' seropositivity c-ELISA was 9.6%; rapid diagnostic kits (RDTs) were 26.5%, and Rose Bengal Test was 10.2%. The seropositivity of brucellosis in the study area is 9.6% compared to 26.5%, this indicate that RDTs over estimated positive specimens. Since studies have shown that c-ELISA has high specificity and RBT is very sensitive (Poester *et al.,* 2010) more work should be done to ascertain the sensitivity and specificity of the RDTs kits. The possibility of false-positive would be as a result of cross-reactivity since *Brucella* S-LPS antigen resembles corresponding epitopes of Gram-negative bacteria such as *Yersinia enterocolitica*, *Vibrio cholerae*, *Escherichia coli*, and *Salmonella urbana* (Nielsen *et al.,* 2004).

Background level of reactive antibodies in a population and cut-off values used also affect sensitivity and specificity of serological tests (Franco *et al*., 2007). In serum

agglutination test (SAT) although cut-off value of 1/160 titres is an indication of infection, lower and higher cut-off values have been detected in active and asymptomatic cases (Lulu *et al.,* 1988). Cut-off values should be established based on local epidemiological conditions of the region being tested, since, in endemic areas high antibody titres could occur and may affect the diagnostic value of the test (Ariza *et al.,* 1992). *Brucella* antibodies tend to persist in patients for long after recovery, and in endemic regions persistent antibody titres are normally detected due to continuous exposure to *Brucella* (Al Dahouk *et al.,* 2013).

When determining cut-off values for serological assays, background prevalence for healthy individuals should be considered. It should also be noted that at higher cut-off values, the specificity is improved but sensitivity drops (Franco *et al.,* 2007). Therefore interpretation of serological result is important when serology test is negative and clinical symptoms of brucellosis are high. This would be the disease is still at an early phases and testing should be repeated one or two weeks later when titre is expected to be higher in active disease phase.

Brucella abortus was the species identified as causing brucellosis as was demonstrated by positive PCR amplification of *B. abortus* (489bp) in seven DNA positive specimens. A study done on prevalence of brucellosis in animals (cattle, sheep and goat) in Kabarnet, Marigat and Eldama Ravine using Rose Bengal Test, *Brucella* positive cases were detected. Polymerase chain reaction detected 15 positive samples; 11 were *B. abortus* from cattle and four *B. melitensis*, three from goats and one from sheep (Kosgei, 2016). The fact that more cattle were infected with *B. abortus* and the same species was also isolated in human specimens, these shows a relationship between human and animal infection.

The risk factors associated with brucellosis included drinking unboiled milk and handling sick animals using bare hands, either by assisting in abortion or removal of

placenta. This observation agrees with those of Kiambi (2014), who documented drinking unboiled milk as a risk factor of brucellosis; however Kiambi's study did not link handling sick animals as a risk factor of brucellosis. Handling sick animals, by assisting in abortion as a risk factor agreed with observation of Kunda *et al.,* (2010); a study done in Tanzania on quantifying risk factors for human brucellosis. This study shows that the residents of these three areas are ignorant on the dangers of these practices and calls for educational programs to enlighten them.

The study limitations were the study was hospital-based therefore the results cannot be generalized to the general population. The method of recruitment of participants was based on clinician's recommendation and differences in clinicians' understanding/view on brucellosis could have lead to differences in patients' recruitment. Most of the patients sampled were females because sampling was done based on patient who came to the laboratory for brucellosis testing and this may have lead to selection bias. The study also did not quantify the amount of DNA in the specimen.

The study has shown that rapid diagnostic kits have low sensitivity. Brucellosis is present in Baringo County with a seropositivity of 9.6%. Drinking unboiled milk and handling sick animals were shown to be factors associated with brucellosis transmission in Baringo County. In addition, the study showed that nearly half of the patients were not aware that brucellosis may be transmitted through drinking of unboiled/unpasteurized milk and nearly 60% were not aware of other practices that may enhance brucellosis transmission to humans. Thus, there is a knowledge gap on brucellosis transmission in Baringo County.

5.2 CONCLUSIONS

The study reveals that brucellosis in humans is present in Baringo County. Rapid diagnostic kits (RDTs) used at the healthcare facilities over estimated the seropositive cases and they had low sensitivity.

Brucella abortus was the main species detected infecting the people who visited the three healthcare facilities studied in Baringo County.

Risk factors associated with brucellosis infection were drinking unboiled milk and handling sick animals without using gloves.

5.3 RECOMMENDATIONS

This study recommends that first the communities in Baringo and the rest of the country need to be sensitized on brucelosis preventive measures including the need to boil/pasteurize milk before consumption, not to consume raw and blood and to use gloves when assist sick animals among other brucellosis prevention strategies.

Second, Healthcare facilities should have positive and negative control serum specimens for validation of each test kit before the kit is used for diagnosis. In addition, more sensitive diagnostic tests such as ELISA and PCR, which are considered expensive and therefore not available in Sub-County hospitals and dispensaries, should be provided in the referral hospital in each County. A proportion of positive samples and negative samples from each health facility in the County should be sent to the referral hospital to validate test results.

Third, there is also need to develop, standardize and validate test kits based on local epidemiological conditions in endemic regions, for correct brucellosis diagnosis.

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APPENDICES:

Appendix 1: CONSENT FORM

Title of the study

Comparative study on diagnostic kits used in Kenya for diagnosis of brucellosis in humans

Introduction

Brucellosis is a zoonotic disease that is of public health importance and occurs worldwide in both animals and humans. It is transmitted from animals to humans through consumption of unpasteurized milk or processed dairy product from infected animals, other mode of transmission would be through contact with fluids from infected animals.

Expectation of study

All the patients 18 years and older who consent to participate in the study, their blood sample will be collected as requested by clinician. We will also ask a few questions using a short questionnaire to establish the risk factor associated with brucellosis infection in this community. This will help in evaluating the best kit in terms of specificity and sensitivity to improve on diagnosis of brucellosis. The result of the study will then be communicated to the health care facilities and the community will also be educated on the risk factors associated with brucellosis.

Objective of the study

Main objective

To compare the diagnostic kits used in Kenya for diagnosis of brucellosis in humans

Specific Objectives

1. To determine the sensitivity, specificity, predictive values (negative and positive) value positive of laboratory tests used for the diagnosis of brucellosis in Kenya.
2. To isolate and identify the *Brucella* species infecting humans in Baringo County.
3. To determine the risk factors associated with transmission of brucellosis infection in humans in Baringo County

Benefits of the study

When you participate in this study you will help us come up with the best kit which will improve on diagnosis of brucellosis. Patient suffering from brucellosis will get treatment promptly and the patients who tested negative though feeling sick, alternative test will be done to establish the cause of their illness. The finding of study will be communicated to the hospital and the hospital will take action if need be on the diagnostic kit they are using. The community will also be educated on the risk factors associated with brucellosis to prevent future infection. Recommendation also will be given to the ministry of health who will be able to recommend the best kit for brucellosis diagnosis to health care facilities.

Risks involved in the study

No additional risk is involved in this study except for minor pain, when the laboratory staff will be collecting the blood sample for normal testing as the clinician has requested.

Compensation mechanism

No form of compensation will be provided to the patient. The hospitals will manage the patients normally but finding from the study will be provided to all participating health facilities and the Kenya Ministry of Health. This will likely provide community benefit from improved diagnosis

Alternative treatment

Not applicable (N/A)

Voluntarism

Only patient who consent will participant in the study, participation in the study is voluntary you are free to participate or not to participate in the study. This will not affect clinical management of the patient whether you participant or not.

Type of specimens and amount to be obtained

The specimen collected will be blood about 10 ml which the hospital laboratory technologist will collect part of which will be used for routine clinical diagnosis of brucellosis. The remaining blood will be used in the study.

Follow up schedule if applicable expected time

Not applicable (N/A)

Information on the researcher and telephone

The researcher conducting this study is Nelly Waringa, Master student at University of Nairobi, Kenya. You may ask any question you have now or later you may contact her through mobile telephone number 0722284239, P. O. Box 26640-00100 Nairobi or email nelly.akinyi@uonbi.ac.ke

Information on the KHN/UON ERC incases to be contacted:

This study has been approved by Kenyatta National Hospital/University of Nairobi ethical research committee.

Telephone number (254-020) 2726300

Email: uonknh-erc@uonbi.ac.ke

Any other necessary information about the study

Not applicable (N/A)

Possible storage of specimen for further analysis with the permission from KNH/UON/ERC

Not applicable (N/A)

Signature for the participant and witness

Please ask any question or clarification before you sign the form to participate in the study

I, Mr. /Miss. /Mrs.
………………………………………………………………………………

have been explained to the purpose of the study titled; **A comparative study of assays used for human brucellosis diagnosis, identification of infecting *Brucella* species and determination of the risk factors associated with human brucellosis transmission in Baringo County, Kenya,** its benefits and risk factors associated with participating in the study. I have had a chance to ask questions and the questions have been answered adequately. I therefore give consent to participate in the proposed study. I consent voluntarily to participate in this study.

I agree to join the study   Left thumb print (for those who cannot sign)

Signature…………………

Date………………………

Witness

Signature…………………….

Date………………………….

Appendix 2: FORMU YA MAKUBALIANO NA WAGONJWA (Consent form Kiswahili)

KICHWA CHA UTAFITI:

Utafiti kulinganisha na vifaa vya uchunguzi kutumika katika Kenya ajili ya utambuzi wa brucellosis kwa binadamu.

MWANZO

Brucellosis ni ugongwa ambao una umuhimu katika afya ya umma na unapatikana ulimwenguni kote kati ya wanyama na binadamu Husambazwa kutoka kwa wanyama hadi kwa binadamu kupitia utumiaji wa maziwa ambayo hayajasafishwa kwa miale ya stima au kwa kutumia bidhaa za maziwa ambazo zinapatikana kwa wanyama walioambukizwa, njia ingine ya kusambazwa kwa huu ugonjwa ni kwa kupitia kwa kushika unyevu wa myama aliyeambukizwa

Matarajio ya utafiti

Wagonjwa wote wenye umri wa miaka 18 na zaidi ambao wamekubali utafiti huu itawabidi wapeleke damu kwa afisa wa maabara wakati damu yao itakapo hitajika. .Tutauliza maswali machache tukitumia fomu ndogo ili tuweze kubainisha hatari ya kuambukizwa ugonjwa huu (brucellosis) katika jamii. Hii itatusaidia kuamua (kutathmini) kifaa kitakacho kuwa maalum na unyeti katika utafiti wa brucellosis. Matokea ya utafiti huu yatapelekwa kwa hopitali, na jamii katika umma watafundishwa hatari zinazohusishwa na ugonjwa huu.

Lengo utafiti

Lengo kuu

Kutathmini maalum na unyeti wa vifaa vya uchunguzi kutumika kwa ajili ya uchunguzi wa Brucellosis kwa binadamu katika Kenya.

Lengo maalum

1. Kutathmini maalum na unyeti wa matarajio ya matokeo ya maabara kwa utafiti wa brucellosis inchini Kenya.
2. Kutenga na kutambua aina ya brucella inayo waambukiza binadamu katika kaunti ya Baringo.
3. Kuamua hatari zinazohusishwa na kusambazwa kwa brucellosis kwa binadamu katika kaunti ya Baringo

Manufa ya utafiti

Utakapo husika katika huu utafiti utatusaidia tuweze kutambua kifaa bora kitakacho boresha uchunguzi wa brucellosis. Wagonjwa wa brucellosis wapewa tiba mara moja. Wale wagonjwa ambao baada ya kufanyiwa utafiti walionekana sio wagonjwa lakini bado wanahisi kuwa wagonjwa, watapewa tiba tofauti ili kiini cha ugonjwa walionao kijulikane. Matokeo ya utafiti huu yatawasilishwa katika hospitali na hospitali itachukua hatua kuhusu kifaa wanachotumia katika uchunguzi wao. Jamii pia wataelimishwa kuhusu hatari zinazohusishwa na brucellosis ilikuzuia uambukizi siku za usoni. Matokeo pia yatapelekwa kwa wizara ya Afya nao watapendekeza kifaa kilicho bora kwa uchunguzi katika hospitali.

Hatari za utafiti

Hakuna hatari yoyote inayoambatana na utafiti huu isipokua uchungu kidogo wakati damu inapotolewa kwa mgonjwa katika maabara kulingana na hitaji la dakari.

Utaratibu wa fidia

Hakuna fidia yoyote atakayopewa mgonjwa. Wagonjwa watachunguzwa na hospitali, lakini matokeo ya uchunguzi yatapelekwa kwa hospitali zote zinazo husika na utafiti huu pamoja na wizara ya afya Kenya. Utafiti huu utaisadia jamii kupata uchunguzi bora.

Matibabu mbadala

Si husika (N/A)

Kujitolea

Ni wale wagonjwa ambao wamejitolea pekee watakao husishwa na huu utafiti, kuhusishwa katika huu utafiti ni kwa kujitolea. Kujihusisha au kutojihusisha katika huu uafiti hakuta athiri usimamizi wa mgonjwa katika hospitali.

Aina ya specimeni na kiasi kitakachohitajika

Specimeni itakayo hitajika ni damu kipimo cha mililita 10 ambazo sehemu itatumika hospitali katika maabara, kwa uchunguzi wa brucellosis. Damu itakayo baki, itatumika kwa utafiti

Kufatilia ratiba na wakati unao tarajiwa

Si husika (N/A)

Habari kuhusu mtafiti na nambari ya simu

Mtafiti afanyae utafiti huu ni Nelly Waringa, mwanafunzi wa shahada ya Masta chuo kikuu cha Nairobi Kenya. Unaweza kumuuliza swali lolote sasa au uwasiliane naye

baadaye kupitia nambari yake ya simu ya mobile 0722284239, au sanduku la posta P. O. Box 26640-00100 Nairobi or email nelly.akinyi@uonbi.ac.ke

Habari kuhusu KHN/UON ERC kama una taka kuwasiliana nao

Utafit huu umepitishwa na Hospitali ya kitaifa ya Kenyatta pamoja na Chuo kikuu cha Nairobi, kamati inayo husika na maadili ya utafiti Nambari ya simuTelephone (254-020) 2726300

Email: uonknh-erc@uonbi.ac.ke

Habari ingine yoyote kuhusu huu utafiti.

Si husika (N/A)

Kuhifadhi specimen, ili iweze kutumika baadaye kwa utafiti baada ya kupewa kibali KNH/UON/ERC

Si husika (N/A)

Makubaliano

Tafadhali uliza swali lolote au ufafanuzi kabla ya kuweka sahihi ili uhusike na huu uafiti

Mimi/ Bwana/ Bi. ………………………………………………………………………………

nimeelezewa lengo la utafiti huu;**Utafiti kulinganisha na vifaa vya uchunguzi kutumika katika Kenya ajili ya utambuzi wa brucellosis kwa binadamu**, faida za utafiti huu na hatari zinazohusika na anaye kubali kuhusishwa na huu utafiti**,** .Nimipata wakati wa kuuliza maswali na nimejibiwa vilivyo. Ninakubali kuhusishwa na huu utafiti. Nimekubali kwa hiari yangu kuhusika na huu utafiti.

Ninakubali kujiunga katika utafiti huu Kidole cha gumba cha kushoto

(Kwa wale ambao hawana sahihi)

Sahihi……………………
Tarehe……………………

Shahidi

Sahihi…………………….
Tarehe…………………….

Appendix 3: QUESTIONAIRE

COMPARATIVE STUDY ON DIAGNOSTIC KITS USED IN KENYA FOR DIAGNOSIS OF BRUCELLOSIS IN HUMANS.

HOSPITAL NAME…………………… **DATE………**

NAME OF INTERVIEWER…………….

SOCIO-DEMOGRAPHIC DATA

PATIENT NUMBER…………………….. GENDER………

RESIDENCE …………………………… COUNTY…….

DISEASE INFORMATION

1). Have you or any of your family members been sick with symptoms that resemble malaria Yes/No.

2) How long have or your family member been sick?

a) 0-3 days………
b) 4-7days……..
c) weeks……..
d) Months…….

3). Have you had any of these signs/ symptoms with the present illness? Yes/No…..

- Fever
- Joint and muscle aches
- Fatigue

Back and neck pain
Sweat chills
Headaches

4). Have you taken any medicine for the disease Yes/No if yes which one

a) Antibiotics
b) Antimalaria
c) Panadol
d) Others (specify)

QUESTION ON POSSIBLE RISK FACTORS

1). Do you keep animals in your home Yes/No

i). If yes which one

a) Cattle
b) Goats
c) Sheep
d) Others (specify)

2). Have your animals been sick recently Yes/No

i). If yes which one

a) Cattle
b) Goats
c) Sheep
d) Others (specify)

3). Have any of your animals had an abortion Yes/No

i). If yes how was the animal assisted during the abortion.

a) With bare hands…………………
b) With protected (gloved) hands…………….
c) Called animal health workers……..
d) Others (specify)…………

4). Have any of your animals had retained after birth (placenta) recently after calving Yes/No

i).If yes how was the retained after birth removed?

a) Removed with bare hands…………
b) Removed with protected hand…….
c) By animal health workers…….
d) Others (specify)…………..

5.) How did you dispose the aborted fetus/placenta?

a) Buried c) Gave it to dogs to eat

b) Throw it in the garbage pits d) Others (specify)

6). Have you been involved in slaughtering an animal recently? Yes /No……

7). Have you eaten meat from animals that died or killed because they were sick? Yes/ No

8).What is the source of your milk?

a) My animals…………... c) Buy from market …….

b) Buy packet milk……… d) Others (specify)………

9).How do you prepare your milk before consuming

a) Boil first b) Make mala

b) Drink raw milk c) Others (specify)

10).Do you boil milk before making sour milk (mala) Yes/No

11). Do you drink boiled/unpasteurized milk Yes/No

i) If yes which animals

a) Cattle……. c) Sheep…………………..

b) Goats……. d) Others (specify)………..

12). In your culture is raw meat eaten or raw blood drank Yes/No

i). If yes which groups eat the raw meat and blood.

a) 10-20 years c) 30-40 years

b) 20-30 years d) 40 years and older.

LEVEL OF DISEASE AWARENESS

1). Have you heard of brucellosis Yes / No ……

2).What causes brucellosis?

a) Drinking raw milk c) Eating raw meat
b) Drinking raw blood d) Others (specify)

3).What do you do when you think you have brucellosis?

a) Go to hospital c) Buy medicine and take
b) Use tradition medicine d) Others (specify)

LABORATORY STAFF

1). Do you test for brucellosis in your Laboratory? Yes/No………….

i) If yes which diagnostic kit do you use for testing?

a). Fortress kit…. c). Plasmatec kit….

b). Rose Bengal kit…. d). Others (specify)….

2). How do you ensure that the result you give are correct

a) Have reference laboratory where we take sample for confirmatory test

b) We do confirmatory test our self

c) Others (specify)………………

3) Which confirmatory test do you or the reference laboratory use?

a) Indirect enzyme linked immunoassay (ELISA)

b) Polymerase chain reaction (PCR)

c) Others (specify)…………..

4). Do you have cases of false positive or false negative results Yes/No…..

5). Do you culture samples for brucellosis Yes/No………

THANK YOU

Appendix 4: FOMU YA MAHOJIANO (QUESTIONNAIRE KISWAHILI)

UTAFITI KULINGANISHA NA VIFAA VYA UCHUNGUZI KUTUMIKA KATIKA KENYA AJILI YA UTAMBUZI WA BRUCELLOSIS KWA BINADAMU

JINA LA HOSPITALI…………………… TAREHE…………

JINA LA ANAYE HOJIWA …………….

NAMBARI YA MGONJWA…………… MUME/MKE……… MAKAO …………………………… KAUNTI…………..

HABARI KUHUSU UGONJWA

1). Ushawahi ugua au familia yako maradhi yoyote yanayofanana na malaria? Ndio/La?

2).Umeugua kwa muda gani?

a) Siku 0-3……… c) wiki……..
b) Siku 4-7…….. d) Miezi…….

3).Umepata maradhi kama haya kwa wakati huu unaougua? Ndio/La?

- Joto Maumivu ya mgongo au shingo
- Maumivu ya viungo vya mwili Baridi mwilini
- Uchovu Kuumwa na kichwa

3). Tangu uanze kuuguwa umemeza dawa zozote? Ndio/La. Kama ndio, gani?

a) Antibiotics c) Panadol
b) Antimalaria d) Mengine (Fafanua)

MASWALI KUHUSU MAAMBUKIZI

1). Wafuga mifugo? Ndio/ La

i). Kama ndio gani?

a) Ng'ombe
b) Mbuzi
c) Kondoo
d) Mengine(Fafanua)

2). Mifugo yako yamekuwa wagonjwa hivi karibuni? Ndio/ La

i). Kama ndio gani?

a) Ng'ombe
b) Mbuzi
c) Kondoo
d) Mengine(Fafanua)

3). Mifugo yako yamewahi avya mimba? Ndio/ La

i). Kama ndio ilisaidiwaje wakati huo?

a) Kwa mikono mitupu…………………
b) Kwa mikono imekingwa (gloves)…………….
c) Uliita wauguzi wa mifugo……..
d) Mengine (Fafanua)…………

4).Mifugo yako hutata shida wakati wa kuza hata kizazi kubaki ndani Ndio/La

i).Kama ndio ilitolewa aje?

a) Kwa mikono mitupu…………
b) kwa mikono imekingwa(gloves)…….
c) Na wauguzi wa mifugo…….
d) Mengine (Fafanua)…………..

5.) Jee fetus iliyo haribika unaifanyaje?

a) Ulizika c) Uliwapa mbwa wale

b) Ulitupa kwa takataka d) Mengine (Fafanua)

6). Umechinja mifugo hivi karibuni? Ndio/ La……

7). Umewahi kula nyama ya mfugo aliyekufa au kuuwawa juu ya ugonjwa? Ndio/ La

8).Maziwa yako yanatoka wapi?

a) Mifugo yangu…………... c) Unanunua sokoni …….
b) Unanunua maziwa ya pakiti……… d) Mengine (Fafanua)………

9).Watengezaje maziwa kabla kunywa

a) Wachemsha kwanza c) Unatengeneza lala

b) Unakunywa ikiwa mbichi d) Mengine (Fafanua)

10).Unachemsha maziwa kabla ya kutengeneza maziwa lala?Ndio/ la

11). Unakunywa maziwa ya kuchemshwa au pakiti? Ndio/ La

i) Kama ndio wanyama wagani

a) Ng'ombe……. c) Kondoo…………………..
b) Mbuzi……. d) Mengine (Fafanua)………..

12). Katika mila zenu mnakunywa damu au kula nyama mbichi? Ndio/ La

i). Kama ndio ni mila gani inakula myama mbichi au kunywa damu

a) Miaka 10-20 c) Miaka 30-40

b) Miaka 20-30 d) Miaka 40 kwenda juu

KIWANGO CHA UFAHAMU WA UGONJWA

1).Ushawahi sikia ugonjwa wa maziwa? Ndio/ La (Brucellosis)

2).Inasababishwa na nini?

a) Unywaji wa maziwa mbichi c)Kuila nyama mbichi

b) Unywaji wa damu mbichi d) Mengine(Fafanua)

3).Uafanya nini ukifikiria una ugonjwa wa maziwa?

a) Unaenda hospitali c) Unatumia madawa ya dukani

b) Unatumia madawa ya kienyeji d) Mengine (Fafanua)

WAFANYIKAZI WA MAABARA

1). Je mnachunguza (pima) brucellosis katika maabara yenu? Ndio/La………….

i) Kama ndio, jee unatumia kifaa kipi kwa uchunguzi?

a). Fortress kit…. c) Plasmatec kit….

b). Rose Bengal kit…. d) Mengine(Fafanua)….

2). Mnadhibitishaje kuwa matukio mnayopeana ni sahihi?

a) Tuko na maabara nyingine ambayo tunapeleka specimeni ili tupate thibitisho la uchunguzi wetu

b)Tunafanya thibitisho la uchunguzi sisi wenyewe

c)Mengine (Fafanua)………………

3) Jee ni thibitisho gani nyie au maabara ingine huwafanyia?

a) Indirect enzyme linked immunoassay (ELISA)

b) Polymerase chain reaction (PCR)

c) Mengine (Fafanua)…………..

4). Jee nini hupata matokeo ya siyo ya kwili? Ndios/La…..

5). Jee mko na culture ya specimen ya brucellosis? Ndio/La………

ASANTE

Appendix 5: Reagent Preparation

1. Erythrocyte lysis solution in 1000mL

-155mM NH_4Cl

-10mM $NaHCo_3$,

-100mM EDTA (pH 7.4)

2. CTAB (hexadecyltrimethyl ammonium bromide)/NaCl

-10% CTAB in 0.7 M NaCl

3. **Tris Acetate EDTA buffer (TAE) 50x (100mL) stock solution**

-Tris base	24.2g
-Glacial acetic acid	5.7mL
-0.5M EDTA	10mL (pH 8.0)

To make 500mL 1x TAE buffer (working solution) dissolve the 10mL 50x TAE stock solution 50 times that is make volume to 500mL using autoclaved distilled water.

Appendix 6: Kappa test for evaluation of concordance of the tests (Antony J *et al.*, 2005)

Test Result (2)	Test Result (1)		
	Yes	**No**	**Total**
Yes	a	b	m_1
No	c	d	m_0
Total	n_1	n_0	n

$P_e = [(n_1/n) \times (m_1/n)] + [(n_o/n) \times (m_o/n)]$, Where;

P_e= Expected agreement (how much agreement would be expected to be present by chance alone)

P_o = Observed agreement (a+d)/n

n_1= No. of times Rapid Diagnostic Rapid Kit indicate positive results

n_o= No. of times Rapid Diagnostic Rapid Kit indicate negative results

m_1= No. of times C- ELISA (Gold standard test) results are positive

m_o= No. of times C- ELISA (Gold standard test) results are negative

n= Total number of samples

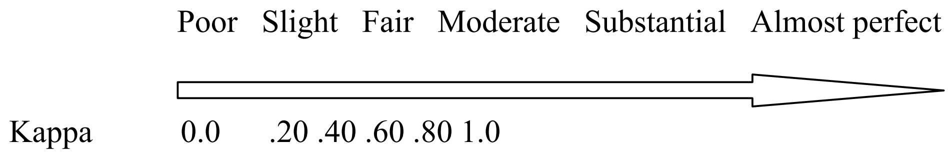
To calculate for the level of agreement (K) between the two tests:

Kappa, $K = (p_o – p_e) / (1 – pe)$,

Where; P_o= observed agreement

P_e= expected agreement

Interpretation of Kappa



Kappa Agreement

< 0 Less than chance agreement

0.01–0.20 Slight agreement

0.21– 0.40 Fair agreement

0.41–0.60 Moderate agreement

0.61–0.80 Substantial agreement

0.81–0.99 Almost perfect agreement

Appendix 7: RESULTS for the RDTS, RBTS, C-ELISA and PCR

Table 1: Kabarnet District Hospital

Sample No.	Fortress Diagnostic Kit	Plasmatec Diagnostic Kit	Eurocell Diagnostic Kit	Rose Bengal test	C-ELISA Kit	PCR Assay
K001	negative	negative	negative	negative	negative	negative
K003	**positive**	**positive**	**positive**	negative	negative	negative
K004	negative	negative	negative	negative	negative	negative
K005	negative	negative	negative	negative	negative	negative
K007	negative	negative	negative	negative	negative	negative
K008	**positive**	**positive**	**positive**	negative	negative	negative
K009	negative	negative	negative	**positive**	**positive**	negative
K010	**positive**	**positive**	**positive**	negative	**positive**	negative
K011	negative	negative	negative	negative	negative	negative
K012	**positive**	**positive**	**positive**	negative	negative	negative
K013	negative	negative	negative	negative	negative	negative
K014	negative	negative	negative	negative	negative	ND
K015	negative	negative	negative	negative	negative	ND
K016	negative	negative	negative	negative	negative	negative
K017	negative	negative	negative	negative	**positive**	ND
K018	**positive**	**positive**	**positive**	negative	negative	ND
K019	negative	negative	negative	negative	**positive**	ND
K020	negative	negative	negative	negative	negative	negative
K021	negative	negative	negative	negative	negative	ND
K022	negative	negative	negative	negative	negative	ND
K023	negative	negative	negative	negative	negative	negative
K024	negative	negative	negative	negative	negative	negative
K025	negative	negative	negative	negative	negative	ND
K026	negative	negative	negative	negative	negative	ND
K027	negative	negative	negative	negative	negative	ND
K028	negative	negative	negative	negative	negative	ND
K029	negative	negative	negative	negative	negative	ND
K026	negative	negative	negative	negative	negative	negative
K027	negative	negative	negative	negative	negative	ND
K028	negative	negative	negative	negative	negative	ND
K029	negative	negative	negative	negative	negative	ND
K030	negative	negative	negative	negative	negative	negative
K031	negative	negative	negative	negative	negative	ND
K032	negative	negative	negative	negative	negative	ND
K033	negative	negative	negative	negative	negative	ND
K034	negative	negative	negative	negative	negative	ND
K036	negative	negative	negative	negative	negative	ND
K037	negative	negative	negative	negative	negative	ND
K040	negative	negative	negative	negative	negative	negative
K041	negative	negative	negative	negative	negative	ND
K042	negative	negative	negative	negative	negative	ND
K043	negative	negative	negative	negative	negative	ND
K044	**positive**	**positive**	**positive**	**positive**	negative	negative
K045	negative	negative	negative	negative	negative	ND
K046	negative	negative	negative	negative	negative	ND
K047	negative	negative	negative	negative	negative	ND
K048	negative	negative	negative	negative	negative	ND
K051	negative	negative	negative	negative	negative	negative
K052	negative	negative	negative	negative	negative	negative
K053	negative	negative	negative	negative	negative	ND
K054	negative	negative	negative	negative	**positive**	ND

Sample No.	Fortress Diagnostic Kit	Plasmatec Diagnostic Kit	Eurocell Diagnostic Kit	Rose Bengal test	C-ELISA Kit	PCR Assay
K055	negative	negative	negative	negative	negative	negative
K056	negative	negative	negative	negative	**positive**	ND
K057	**positive**	**positive**	**positive**	negative	negative	negative
K060	**positive**	**positive**	**positive**	negative	negative	negative

Table 2: Eldama Ravine District Hospital

Sample No.	Fortress Diagnostic Kit	Plasmatec Diagnostic Kit	Eurocell Diagnostic Kit	Rose Bengal test	C-ELISA Kit	PCR Assay
E1	negative	negative	negative	negative	negative	ND
E2	negative	negative	negative	negative	negative	ND
E3	negative	negative	negative	negative	negative	ND
E4	negative	negative	negative	negative	negative	negative
E5	**positive**	**positive**	**positive**	negative	negative	ND
E6	**positive**	**positive**	**positive**	negative	negative	negative
E7	negative	negative	negative	negative	negative	ND
E8	negative	negative	negative	negative	negative	negative
E9	**positive**	**positive**	**positive**	negative	negative	ND
E11	negative	negative	negative	negative	negative	ND
E12	negative	negative	negative	negative	negative	ND
E13	negative	negative	negative	negative	negative	ND
E14	**positive**	**positive**	**positive**	**positive**	negative	ND
E15	negative	negative	negative	negative	negative	ND
E16	negative	negative	negative	negative	negative	ND
E17	negative	negative	negative	negative	negative	ND
E18	negative	negative	negative	negative	negative	ND
E19	negative	negative	negative	negative	negative	ND
E20	negative	negative	negative	negative	negative	ND
E21	negative	negative	negative	negative	negative	negative
E22	negative	negative	negative	negative	negative	ND
E23	negative	negative	negative	negative	negative	ND
E24	negative	negative	negative	negative	negative	ND
E25	negative	negative	negative	negative	negative	ND
E26	negative	negative	negative	negative	negative	ND
E27	negative	negative	negative	negative	negative	negative
E28	negative	negative	negative	negative	negative	negative
E29	negative	negative	negative	negative	negative	ND
E30	negative	negative	negative	negative	negative	negative
E31	negative	negative	negative	negative	negative	negative
E32	**positive**	**positive**	**positive**	negative	**positive**	negative
E33	negative	negative	negative	negative	**positive**	negative
E34	negative	negative	negative	negative	negative	negative
E36	negative	negative	negative	negative	negative	ND
E37	negative	negative	negative	negative	**positive**	ND
E38	negative	negative	negative	negative	**positive**	ND

Table 3: Marigat District Hospital

Sample No.	Fortress Diagnostic Kit	Plasmatec Diagnostic Kit	Eurocell Diagnostic Kit	Rose Bengal test	C-ELISA Kit	PCR Assay
M1	**positive**	**positive**	**positive**	**positive**	negative	negative
M4	negative	negative	negative	negative	negative	ND
M5	negative	negative	negative	negative	negative	ND
M6	negative	negative	negative	negative	negative	ND
M7	negative	negative	negative	negative	negative	ND
M8	**positive**	**positive**	**positive**	negative	negative	ND
M9	**positive**	**positive**	**positive**	negative	negative	negative
M10	negative	negative	negative	negative	negative	ND
M11	**positive**	**positive**	**positive**	negative	negative	negative
M12	**positive**	**positive**	**positive**	negative	negative	negative
M13	negative	negative	negative	negative	negative	ND
M14	negative	negative	negative	negative	negative	ND
M15	**positive**	**positive**	**positive**	negative	negative	negative
M16	negative	negative	negative	negative	negative	ND
M17	negative	negative	negative	negative	negative	ND
M18	negative	negative	negative	negative	negative	ND
M19	**positive**	**positive**	**positive**	negative	negative	ND
M20	negative	negative	negative	negative	negative	ND
M21	**positive**	**positive**	**positive**	negative	negative	ND
M22	negative	negative	negative	negative	**positive**	ND
M23	**positive**	**positive**	**positive**	negative	negative	negative
M24	negative	negative	negative	negative	negative	ND
M25	negative	negative	negative	negative	negative	ND
M26	**positive**	**positive**	**positive**	**positive**	negative	negative
M27	negative	negative	negative	negative	negative	ND
M28	negative	negative	negative	negative	negative	ND
M29	negative	negative	negative	negative	negative	ND
M30	negative	negative	negative	negative	negative	ND
M31	**positive**	**positive**	**positive**	**positive**	negative	ND
M32	negative	negative	negative	negative	negative	ND
M33	negative	negative	negative	negative	negative	ND
M34	**positive**	**positive**	**positive**	negative	negative	ND
M35	**positive**	**positive**	**positive**	negative	**positive**	**positive**
M36	**positive**	**positive**	**positive**	negative	**positive**	negative
M37	negative	negative	negative	negative	negative	negative
M38	**positive**	**positive**	**positive**	negative	negative	negative
M39	negative	negative	negative	negative	negative	negative
M40	negative	negative	negative	negative	negative	ND
M41	negative	negative	negative	negative	negative	ND
M42	negative	negative	negative	negative	**positive**	ND
M43	negative	negative	negative	negative	negative	ND
M44	**positive**	**positive**	**positive**	negative	negative	negative
M45	negative	negative	negative	negative	negative	ND
M46	negative	negative	negative	negative	negative	ND
M47	negative	negative	negative	negative	negative	ND
M48	negative	negative	negative	negative	negative	ND
M49	**positive**	**positive**	**positive**	**positive**	**positive**	negative
M50	negative	negative	negative	negative	negative	ND
M51	negative	negative	negative	negative	negative	negative
M52	negative	negative	negative	negative	negative	ND
M53	negative	negative	negative	negative	negative	ND
M54	negative	negative	negative	negative	negative	ND
M55	**positive**	**positive**	**positive**	negative	negative	negative
M56	negative	negative	negative	negative	negative	negative

Sample No.	Fortress Diagnostic Kit	Plasmatec Diagnostic Kit	Eurocell Diagnostic Kit	Rose Bengal test	C-ELISA Kit	PCR Assay
M56	negative	negative	negative	negative	negative	ND
M57	negative	negative	negative	negative	negative	ND
M58	negative	negative	negative	negative	negative	negative
M59	negative	negative	negative	negative	negative	ND
M60	**positive**	**positive**	**positive**	negative	negative	negative
M61	**positive**	**positive**	**positive**	**positive**	negative	negative
M62	negative	negative	negative	negative	negative	negative
M66	negative	negative	negative	negative	negative	ND
M67	**positive**	**positive**	**positive**	negative	negative	negative
M68	negative	negative	negative	negative	negative	negative
M69	**positive**	**positive**	**positive**	**positive**	**positive**	**positive**
M70	negative	negative	negative	negative	negative	ND
M71	**positive**	**positive**	**positive**	negative	negative	**positive**
M72	**positive**	**positive**	**positive**	negative	negative	ND
M73	**positive**	**positive**	**positive**	negative	**positive**	negative
M74	negative	negative	negative	negative	negative	ND
M75	negative	negative	negative	negative	negative	negative
M76	negative	negative	negative	negative	negative	negative
M77	**positive**	**positive**	**positive**	negative	negative	negative
M78	**positive**	**positive**	**positive**	negative	negative	ND
M79	**positive**	**positive**	**positive**	negative	negative	negative
M80	negative	negative	negative	negative	negative	ND
M81	negative	negative	negative	negative	negative	ND
M82	**positive**	**positive**	**positive**	**positive**	negative	**positive**
M83	negative	negative	negative	negative	negative	ND
M84	negative	negative	negative	negative	negative	ND
M85	**positive**	**positive**	**positive**	**positive**	negative	**positive**
M86	**positive**	**positive**	**positive**	**positive**	negative	negative

Abbreviation: ND…………..Not determine

Appendix 8: Ethical clearance letter.