EVALUATION OF SLIDE TEST, 2-MERCAPTOETHANOL TEST AND ELISA IN DIAGNOSIS OF HUMAN BRUCELLOSIS IN NYANDARUA DISTRICT, CENTRAL PROVINCE, KENYA

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156/CE/12254/2004

A thesis submitted in partial fulfillment of the requirements for the award of the Degree of Masters of Science (Infectious Disease Diagnosis) in the School of Pure and Applied Sciences, Kenyatta University

SEPTEMBER 2009
DECLARATION

I declare that the work presented in this thesis is my original work and has not been presented for a degree in any other university or any other award.

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Signature ........................................ Date. 14 SEPTEMBER 2009

We confirm that the candidate carried out the work reported in this thesis under our supervision.

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DEDICATION

This thesis is dedicated to my wife Wahito Ndung'u and my son Gachigua Ndung'u. To my parents Beth Njoki and Gachigua Gatoho. To my aunt Nyambura Mundia who mentored and sowed seed of quest for knowledge.
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<td>GDP</td>
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<td>KDa</td>
<td>Kilodalton</td>
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<td>KEMRI</td>
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<td>OMP</td>
<td>Outer Membrane Protein</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>SAT</td>
<td>Standard Agglutination Test</td>
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<td>SPSS</td>
<td>Statistical Package for Social Sciences</td>
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<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>WHO</td>
<td>World Health Organization</td>
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DEFINITION OF TERMS

False negative result: This is a negative test result while the actual disease status is positive.

False positive result: This is a positive result while the actual disease status is negative.

Negative predictive value: It is a measure of the probability that a person who tests negative by a particular test for a specific disease is truly uninfected.

Positive predictive value: It is a measure of the probability that a person who tests positive by a particular test for a specific disease is truly infected.

Prevalence: It is the ratio of the number of cases of a disease in a population to the total number of persons in the population at a specified time.

Sensitivity: It is the ability of a test to identify correctly persons who have a particular disease.

Specificity: It is the ability of a test to identify correctly persons who do not have a particular disease.
ABSTRACT

Brucellosis is a zoonotic disease caused by organisms in the genus *Brucella*, and has a worldwide distribution. *Brucella abortus*, *B. ovis*, *B. suis* and *B. melitensis* are pathogenic to man, and human infection occurs through contact with infectious blood, urine, placenta and or aborted foetus and consumption of raw contaminated animals products such as milk, cheese and blood. Diagnosis of brucellosis is difficult because clinical manifestations can be misleading, culture lack sufficient sensitivity and require prolonged incubation leading to excessive delay in diagnosis while serological tests are difficult to interpret. This leads to misdiagnosis and the need to evaluate the performance of laboratory diagnostic methods (Slide test, 2-Me and ELISA) in terms of sensitivity, specificity and predictive values. Factors that predisposes humans to *Brucella* infection as well as the level of awareness about the disease particularly symptoms, treatment and prevention in the study population need to be known for public health appraisal. The study population comprised of all who were residents of Nyandarua district and a sample size of 180 studied. The prevalence of human brucellosis was 3.9%. While 2-Me and ELISA had the same sensitivity, there was a statistically significant difference in sensitivity of Slide test compared to both 2-Me and ELISA; $Z_{\text{actual}} (20.05) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$. A statistically significant difference existed between specificity of Slide test compared to 2-Me [$Z_{\text{actual}} (3.81) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$] and ELISA [$Z_{\text{actual}} (3.54) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$]. 2-Me had a statistically significant higher positive predictive value compared to Slide test [$Z_{\text{actual}} (10.7) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$] as was ELISA compared to Slide test [$Z_{\text{actual}} (18.73) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$]. There was no statistically significant difference between negative predictive value of Slide test compared to both 2-Me and ELISA; $Z_{\text{actual}} (0.53) < Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$ and $Z_{\text{actual}} (0.47) < Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$ respectively. Consumption of contaminated animal products was found out to be the major route of transmission in Nyandarua district while awareness pertaining brucellosis symptom, prevention and treatment in the district was found to be low. It is recommended that Slide test be only used for screening while SAT, 2-Me and ELISA may be used as confirmatory tests and 2-Me test be incorporated in routine diagnosis. Further it is recommended from the findings of this study that the government through the Ministry of Public Health creates awareness of brucellosis to the public to strengthen its control and possible eradication.
CHAPTER ONE
INTRODUCTION

1.1 Background information

Brucellosis is a zoonotic disease affecting both man and a wide range of wild and domestic animals such as livestock, rodents, whales, dolphins and seals, and wild ruminants (Talaro, 2005) and has a worldwide distribution. The disease also referred to as Malta fever, undulant fever or Bang disease is caused by organisms in the genus *Brucella* (Kumar and Clark, 2004).

Brucellosis is associated predominantly with occupational contact in slaughterhouses, livestock handling and the veterinary services (Antonio et al., 2005). High concentrations of the disease occur in Europe, Africa, India and Latin America. It has been virtually eliminated from cattle in the UK, but high prevalence still occurs in Mediterranean countries, the Middle East and the tropics, where about 500,000 new cases are reported per year (Kumar and Clark, 2004). Cook and Zumla (2003) pointed out that in the tropics, cases of brucellosis occur predominantly in rural areas among pastoralist people and also occur in urban settings where small numbers of animals are kept in and around the homestead. Human infection occurs through contact with infectious blood, urine, placenta and or aborted foetus and consumption of raw contaminated milk and cheese. *Brucella* organisms enter the body via damaged skin or mucous membrane of the digestive tract, conjunctiva and respiratory tract (Talaro, 2005). Though person-to-person transmission is rare, spread through sexual contact and by tissue transplant has been reported (Greenwood et al., 2002).

The true prevalence of human brucellosis is not accurately known. Reported prevalence in disease endemic areas varies widely from 0.01 to 200 cases per 100,000 persons (Cook and Zumla, 2003). The true prevalence of brucellosis is several times greater than the reported
prevalence (Cook and Zumla, 2003) and Araj (1999) pointed out that the ratio of reported to unreported cases is around 1:26. While some areas, such as Peru, Kuwait and parts of Saudi Arabia have very high incidence of acute infections, low incidence reported in other known brucellosis endemic areas may reflect low levels of surveillance and reporting. The clinical picture in human brucellosis can be misleading and cases in which gastrointestinal, respiratory or neurological manifestation (focal forms) predominates are common, hence clinical diagnosis needs to be supported by laboratory tests (Renner, 1997).

Wright (2004) postulated that the diagnosis of brucellosis is not always easy even when there is a high degree of suspicion because blood cultures lack sufficient sensitivity and require prolonged incubation leading to excessive delay in diagnosis. Serological tests are more sensitive and show a wide range of sensitivity and specificity. However, they may be difficult to interpret in areas where the disease is endemic and in case of cross reaction with other diseases such as salmonellosis. Alternative verification method based on amplification of universal genes in a conventional polymerase chain reaction (PCR) yields promising results. A single tube nested PCR had sensitivity of 96.98% and a specificity of 100% (Wright, 2004). However, due to the high costs of apparatus and reagents used the method cannot be routinely used. Consequently, details of appropriate exposure such as animal contact, animal products or environmental exposure to locations inhabited by potentially infected animals and laboratories coupled with clinical signs and results of laboratory tests form the basis of diagnosis.

1.2 Statement of the problem

Agriculture is the backbone of Kenya’s economy, livestock contributes over 30% of the gross domestic product (GDP) and employs over 50% of agriculture-based labour (Wabwa,
2003) with dairy sub-sector contributing 14% of GDP (Wafula, 2000). Pasteurized milk accounts for 20% while raw milk accounts for 80% of all marketed milk in Kenya (Wafula, 2000).

In Kenya investigations have shown evidence of *Brucella* infection in livestock in areas such as Kiambu, Samburu and Kilifi (Kadohira *et al.*, 1997) as well as *Brucella* contamination in milk marketed in Nakuru, Narok and Nairobi (Kang’ethe *et al.*, 2000) all of which precipitate human infection. Muriuki *et al.* (1997) reported a 21% prevalence of human brucellosis among human flu-like cases reported in health facilities in Narok.

The close contact of humans and livestock and consumption of infective livestock products facilitates the spread of brucellosis. Nyandarua district in Central province is a high potential area for dairy production. The district annual health report (2004) indicated a high number of human brucellosis cases based on presumptive diagnosis and hence need to determine the prevalence.

Correct diagnosis and treatment of brucellosis in humans is an essential component in control of the disease. Diagnosis is based on clinical manifestation, laboratory test results and history of possible exposure (Alton and Forsy, 2002). Wright (2004) postulated that the diagnosis of brucellosis is difficult because clinical manifestations can be misleading, culture lack sufficient sensitivity and require prolonged incubation leading to excessive delay in diagnosis while serological tests are difficult to interpret. This leads to misdiagnosis and the need to evaluate the performance of laboratory diagnostic methods in terms of sensitivity, specificity and predictive values.
Factors that predisposes humans to *Brucella* infection as well as the level of awareness about the disease particularly symptoms, treatment and prevention in the study population need to be known for public health appraisal.

1.3 **Hypotheses**

The study hypothesised that:-

i) The performance of Slide Test, 2-Me and ELISA when used to diagnose human brucellosis is the same.

ii) Personal characteristics (age, sex occupation and diet consumed) do not predispose human beings to *Brucella* infection.

1.4 **Research questions**

i) What is the prevalence of human brucellosis in Nyandarua district?

ii) Are there differences in sensitivity of Slide Test, 2-Me and ELISA when used to detect human brucellosis?

iii) Is the specificity of Slide Test, 2-Me and ELISA when used to detect human brucellosis the same?

iv) Are there differences in negative predictive values and positive predictive values of Slide Test, 2-Me and ELISA when used to detect human brucellosis?

v) Do personal characteristics predispose human beings to *Brucella* infection?

1.5 **Objectives of the study**

1.5.1 **Overall objective**

i) The overall objective of the study was to evaluate the performance of Slide Test, 2-Me and ELISA in diagnosis of human brucellosis in Nyandarua district.
1.5.2 Specific objectives

i) Determine the prevalence of human brucellosis.

ii) Compare the sensitivity and specificity of Slide Test, 2-Me and ELISA in diagnosis of human brucellosis.

iii) Compare the positive and negative predictive values of Slide Test, 2-Me and ELISA in diagnosis of human brucellosis.

iv) Determine the predisposing causes of human brucellosis.

v) Assess the level of awareness of brucellosis among residents of Nyandarua district.

1.6 Justification

When considering research priorities and the tools needed to control neglected diseases in developing countries, there is a tendency to focus on vaccine research and drug development. However, improved and quality assured diagnostics are equally important for disease control. They provide the rational bedrock for the appropriate treatment of patients, for monitoring disease-control efforts and for enhancement of disease surveillance capacity (Ridley, 2006). Generally brucellosis is insidious and its clinical features are non-specific hence the need to consider laboratory test results to avoid misdiagnosis. The knowledge of the performance of laboratory tests in terms of sensitivity, specificity and predictive values is essential for the proper interpretation of their results. This study sought investigate and compare the performance of laboratory tests commonly used as well as those rarely used and the results will facilitate better interpretation of laboratory tests, hence improved diagnosis of human brucellosis in Nyandarua district.

The clinical presentation of brucellosis is variable and polymorphic and can mimic a variety of infectious and non-infectious diseases (Araj, 1999). Availability of data on the prevalence
of brucellosis provides an insight to clinical suspicion which precedes laboratory investigation. The study findings provide information on prevalence of brucellosis which will promote correct diagnosis of the disease and ensure patients get the correct treatment.

Human brucellosis is highly debilitating and has negative economic impact on health delivery system. It is transmitted through occupational exposure and consumption of contaminated animal products and hence preventable. This study investigated and identified the predisposing factor to *Brucella* infections in humans and determined the level of awareness among the study population to be low. These findings provide an avenue to strengthen the control and possible eradication of human brucellosis in Nyandarua district.
CHAPTER TWO
LITERATURE REVIEW

2.1 Aetiology

Brucellosis is a disease caused by organisms of the genus *Brucella* comprising of a group of closely related bacteria that probably represent variants of a single species. For convenience, these have been classified into species that differ from another in their preferred animal host, in genetic characteristics and in oxidation of certain amino acids and carbohydrates (Greenwood *et al*., 2002). *Brucella* are small, non-motile, non-sporulating, non-toxigenic, non-fermenting, and aerobic, gram negative coccobacilli that may, based on DNA homology represent a single species (Romero *et al*., 2001). It belongs to the family *Brucellaceae*.

The species infective to humans are *Brucella suis*, which usually infect swine, *Brucella abortus* predominantly a pathogen for cattle, *Brucella melitensis* found in goat and sheep and *Brucella canis*, a pathogen of dogs. *Brucella neotomae*, which occur in desert wood rats and *Brucella ovis*, which is pathogenic for sheep, are not known to cause human brucellosis (Lennette *et al*., 1980). Wafa and Michelle (2006) has isolated two new strains from marine animals and identified them as *Brucella pinnipediae* and *Brucella cetaceae*. These two are potentially pathogenic to man but they have not been officially integrated into the existing taxons. When *Brucella* organism is cultured the colonies become visible in 2-3 days. The colony morphology is either smooth or rough. *Brucella ovis* and *B. canis* occur normally in the rough form whereas *B. abortus*, *B. melitensis*, *B. suis* and *B. neotomae* have only been encountered in the smooth form (Lennette *et al*., 1980). However the smooth form dissociate, with the loss of the O-chains of the lipopolysaccharide to form rough or mucoid variants (Alton and Forsy, 2002).
2.2 Transmission

The reservoirs of brucellosis are various wild, feral and particularly domestic mammals (Wright, 2004). Ruminants shed enormous numbers of bacteria from infected products of conception whether aborted or born at term (Alton and Forsy, 2002). Transmission of brucellosis is mainly through occupational exposure in slaughterhouses, livestock handling and veterinary trade. Talaro (2005) pointed out that infection can take place through contact with infected blood, urine, placenta and consumption of raw milk and cheese. An outbreak of brucellosis in Andalucia (Spain) in the year 2002 was traced to consumption of unpasteurised raw goat cheese (Mohedano et al., 2002). Products made from unpasteurised milk such as soft cheese and similar dairy products transmit infection because the shorter time for preparation does not allow the pH to fall sufficiently to kill the organism (Cook and Zumla, 2003). Pasteurization before fermentation of milk for preparing cheese is undesirable as it delays ripening (Mohedano et al., 2002).

*Brucella* species have been isolated from milk from serologically negative bovine females (Langoni et al., 2000) because infected animals excrete large numbers of viable *Brucella* in milk for months to years. Apparently normal mammary glands represent important source of infection not only to other lactating cows but also calves and humans who consume raw milk (Harmon et al., 1988). Cases of transmission by sexual contact and tissue transplantation are occasionally reported (Renner, 1997). Mosquitoes and other blood sucking insects may mechanically transfer *Brucella* from animals to humans (Murat and Ersin, 2007) but no biological vector has so far been implicated in the transmission. Congenital infection has been reported, as is infection via human breast milk (Fernandez and Diaz, 2000).

When milk contaminated with *Brucella* species is ingested, the milk neutralizes gastric
acidity and the organisms are able to survive transit through the stomach to the duodenum where they enter the mucosa (Cook and Zumla, 2003). Aerosols occurring in the laboratories or through splashing of amniotic fluid or milk may be infective through inhalation or contact with conjunctiva or nasopharynx (Bogdanovich et al., 2004). Vaccine strains used in animals are not attenuated for humans and may cause human disease from accidental inoculation. Since Brucella is capable of surviving for prolonged periods in the environment, inhalation of contaminated dust in hot dry countries may be a source of infection (Talaro, 2005).

2.3 Distribution and occurrence

Brucellosis occurs in all areas of the world, and more frequently in the rural communities where the infection may go unrecognized. The true prevalence of human brucellosis is several times greater than reported prevalence. Both sexes and all age groups are susceptible to infections, but adults represent the majority of cases mainly due to occupational predisposition (Cook and Zumla, 2003). Bovine brucellosis caused mainly by Brucella abortus is the most widespread form in humans while Brucella melitensis which causes ovine and caprine brucellosis is the most important clinically apparent disease. However, Brucella melitensis in cattle has emerged as an important problem in some Southern Europe countries, Israel, Kuwait and Saudi Arabia. This is because the available Brucella abortus vaccines do not protect effectively against Brucella melitensis infection (Renner, 1997). A related problem has been noted in South American countries particularly Brazil and Colombia where Brucella suis has become established in cattle.

In Europe, all the four types of Brucella spp pathogenic to humans (B. abortus, B. ovis, B. suis and B. melitensis) are present except in Austria, Denmark, Estonia, Finland, Hungary, Iceland, Luxembourg, Moldavia, Netherlands, Sweden, Switzerland and United Kingdom.
All the four types pathogenic to man are present in Asia except in Bahrain, Japan, Malaysia, Philippines and Singapore while in the America all the four types are present except in Barbados, Falkland Islands and Surinam (Renner, 2001). The occurrence of all the four types of brucellosis in Africa remains unclear. In Tanzania, the only form documented is \textit{B. abortus} while in Kenya it is only \textit{B. abortus} and \textit{B. melitensis} while the occurrence of other forms remain unknown (Renner, 2001).

The true prevalence of human brucellosis is not accurately known. Reported prevalence in disease endemic areas varies widely from 0.01 to 200 per 100,000 persons. While some areas, such as Peru, Kuwait and parts of Saudi Arabia have a very high prevalence of acute infections, low prevalence reported in other known brucellosis endemic areas may reflect low levels of surveillance and reporting (Mohedano \textit{et al.}, 2002) although other factors such as methods of food preparation, heat treatment of dairy products and direct contact with animals also influence risk to population. Slaughterhouse workers in Djibouti had a prevalence of 6.5\% (Chantal \textit{et al.}, 1996) while studies of febrile patients in a large hospital in Kampala yielded 13.3\% prevalence (Mutanda, 1998). A seroprevalence of 5.2\% has been recorded in Eastern Ningeria (Baba \textit{et al.}, 2001) 1\% in Eastern Sudan (El-Ansary \textit{et al.}, 2001) a range of between 3.0\% to 7.1\% in Eritrea (Omer \textit{et al.}, 2002) and 3.8\% prevalence in nomadic pastoralists in Chad (Schelling \textit{et al.}, 2003).

Human brucellosis in Kenya has not been thoroughly studied but there is a lot of evidence indicating presence of bovine brucellosis and investigations by Kadohira \textit{et al.} (1997) demonstrated existence of bovine brucellosis in Samburu, Kiambu and Kilifi. Kang’ethe \textit{et al.} (2000) detected \textit{Brucella abortus} in milk marketed in Narok, Nakuru, Nairobi and Kiambu with a prevalence of 3.4\% at the informal market level and 8.2\% in the formal market.
(pasteurised milk). Muriuki et al. (1997) pointed out that 21% of human flu-like cases reported in health facilities in Narok were diagnosed as brucellosis. In Nyandarua district, laboratory records in various hospitals in the district indicate that the proportion of confirmed cases out of the suspected cases ranges from 6% to 34% and the district has an estimated prevalence rate of 9% (Nyadarua District Annual Health Report, 2004).

2.4 Pathogenesis, pathology, clinical signs and symptoms

2.4.1 Pathogenesis

Brucella is a facultative intracellular parasite and upon entry, it is rapidly phagocytosed by polymorphonuclear leukocytes and macrophage. The bacterium has unique abilities of invading both phagocytic and nonphagocytic cells and surviving in the intracellular environment by avoiding the immune system in different ways. As a result, brucellosis is a systemic disease and can involve almost every organ system (Alton and Forsy, 2000). Brucella survives and multiplies in these cells because they inhibit the bactericidal myeloperoxidase-peroxide-halide system by releasing 5-guanosine and adenine, which inhibits phagosomal fusion and oxidative burst activity (Wafa and Michelle, 2006). The lipopolysaccharide of the smooth type probably plays a substantial role in intracellular survival, as smooth organisms survive much more effectively than rough ones (Nidia et al., 1986).

Brucella has relatively low virulence, toxicity and pyrogenicity, making it a poor inducer of some inflammatory cytokines such as tumor necrosis factor (TNF) and interferons. Also it does not activate the alternative complement system and in addition it is thought to inhibit programmed cell death (Wafa and Michelle, 2006).
Alton and Forsy (2000) postulated that, in systematic spread the bacteria are transported within neutrophils and macrophages and also in blood outside cells. The organism often disseminates widely from regional lymphoid tissue appropriate to the portal of entry and may localize in certain organs such as lymph nodes, spleen, liver, bone-marrow and especially in animals' reproductive organs. If unchecked by macrophage microbicidal mechanism, the bacteria destroy their host cells and infect additional cells, and also replicate extracellularly in host tissues. In ruminants, *Brucella* organisms bypass the most effective host defences by targeting embryonic and trophoblastic tissue and grow in phagosome, cytoplasm and rough endoplasmic reticulum, leading to foetal death and abortion (Romero *et al*., 1995).

Susceptibility to intracellular killing differs among species, with *B. abortus* readily killed and *B. melitensis* rarely affected; this might explain the differences in pathogenicity and clinical manifestations in human infections where infection by *B. melitensis* is more severe (Wafa and Michelle, 2006).

### 2.4.2 Pathology

The host cellular response may range from abscess formation with lymphocytic infiltration to granuloma formation with caseous necrosis. There are no characteristic features of granuloma and hence differential diagnosis of febrile illnesses associated with granuloma formation is considerable with tuberculosis being a particularly important alternative cause to consider as the two infections can produce very similar clinical syndromes (Cloeckaert *et al*., 2001). As the organism is widely distributed through the blood stream, these features are found in many organs. Clinically apparent abscess formation is common and occurs in a range of tissues, including the vertebrae and the psoas muscle (Cook and Zumla, 2003).

Continued presence of *Brucella* in the body lead to weight loss. Multiplication of *Brucella* in
internal organs leads to inflammatory responses primarily fever and in extreme cases reduced and eventually total loss of function of infected organs. Hepatomegally, splenomegally and lymphadenopathy are common. Hepatitis and rarely liver abscess also occurs. *Brucella* endocarditis a rare but fatal complication accounts for 80% of death from brucellosis. Central nervous system infection manifests itself as chronic meningoencephalitis (Cassataro *et al*., 2004).

**2.4.3 Clinical signs and symptoms**

Brucellosis may remain latent, be sub-clinical or give rise to symptoms of varying intensity and duration. Brucellosis can present as acute or sub-acute pyrexial illness, which may persist for months or develop into focal infection. The characteristic intermittent waves of elevated temperature arise in long-standing untreated cases (Greenwood *et al*., 2002) and in cycles lasting two to four weeks (Cook and Zumla, 2003). Incubation period is about two to four weeks. The initial illness is non-specific with fever, lethargy, anorexia and abdominal pains followed by typical signs which include malaise, arthragia, lymphadopathy, night sweats, splenomegally and hepatomegally.

In 20 – 40% of all cases the disease presents in focal forms, which have been described almost in all organ systems (Plommet *et al*., 1998). Gastrointestinal symptoms are noted in 40% of patients, with anorexia, constipation and abdominal pain being the most common. Occasionally, *Brucella* hepatitis with abscess formation, splenic abscess, spontaneous rapture of the spleen, cholecystitis, peritonitis, intestinal obstruction or perforation, erosive colitis and pancreatitis may present with localized and intense abdominal pain (Bikas *et al*., 2003). Lung infections have been described which are manifested by respiratory symptoms, mostly cough, dyspnea or pleuritic pain. *Brucella* endocarditis accounts for 80% of deaths from
brucellosis, while central nervous system infection usually manifest itself as a chronic meningoencephalitis, but subarachnoid haemorrhage and myelitis may also occur. Few cases of skin abscess have been reported (Romero et al., 1995). Osteoarticular and genitourinary forms are the most common while those that affect the heart and the central nervous system are most severe (Plommet et al., 1998).

Spontaneous resolution after weeks or months of clinical illness may occur and mortality associated with brucellosis is estimated at up to 7% (Morata et al., 2001). Case fatalities are very low but the syndrome can last for a few weeks to a year even with treatment (Talaro, 2005).

2.5 Antigenic composition and immune response

2.5.1 Antigenic composition

While a substantial number of antigenic components of Brucella have been characterized, the antigen that dominates the antibody response is the lipopolysaccharide. In all the smooth strains the dominant surface antigen is a lipopolysaccharide O-chain, which depending on the three dimensional structure, forms A, M and C epitopes. These are common to all smooth species but the distribution of A and M depends on biovars. Rough strains do not produce the O-chain but have a common R epitope (Corbeil et al., 1988). The lipopolysaccharide has endotoxin activity and elicits antibody mediated protection. More complete immunity is dependent on cell mediated, particularly cytotoxic, responses elicited by ribosomal and other proteins (Greenwood et al., 2002).

Numerous outer and inner membrane, cytoplasmic and periplasmic protein antigens have been characterized. Some are recognized by the immune system during infection and are
potentially useful in diagnostic tests. Hitherto, tests based on such antigens have suffered from low sensitivity as infected person tend to develop a much less consistent response to individual protein antigens than to lipopolysaccharide. Ribosomal proteins have re-emerged as immunological important components as they have been found to stimulate cell mediated responses and as such appear to have potential as candidate vaccine components (Renner, 1997).

2.5.2 Immune response

An infection with *Brucella* organisms induces both cellular and humoural response. Cellular responses are mainly by increased leukocytes and macrophage. However the bacteria can survive in intracellular environment hence hamper the cellular response (Wafa and Michelle, 2006). Two main classes of antibodies involved in the response are IgG and IgM with IgM appearing first. In acute brucellosis very high IgM levels may be found. Following recovery, IgM level decrease slowly and gradually disappear from the blood over a period of several months whereas IgG levels fall rapidly and disappear from the blood within few weeks of infection. Therefore the persistence of IgG antibody in the blood is an indication of continuing *Brucella* activity (Cheesebrough, 1984).

As the disease progresses from acute to chronic phase and the organisms become localized intracellularly in various parts of the body, the IgM antibodies decrease; the agglutination titre falls and may become undetectable even when the patient is still ill and therefore the absence of agglutination for IgM does not rule out the possibility of infection. In addition, after recovery, Low titre agglutinins due to residual IgM may persist for many months or even years after the infection has cleared. ELISA for IgG antibodies shows good correlation with active disease, especially in long standing infections (Greenwood *et al*., 2002).
2.6 Diagnosis
Laboratory tests available include serological tests, culture and molecular methods.

2.6.1 Serological tests
Serological tests are commonly used for they are inexpensive and rapid. The testing of serum for antibodies against Brucella organisms is an important method of diagnosing brucellosis especially the acute form of the disease (Cheesebrough, 1984). In endemic area where the history strongly indicate possible exposure and examination and investigation does not suggest an alternative diagnosis, positive serology may well be satisfactory for management in resource – limited setting (Wright, 2004).

a) Slide Test
Slide Test is an agglutination test in which presence of visible agglutination implies a positive result. It is easy and widely used but has major limitation in that it does not distinguish past from active infection and cross react with other febrile antigens. This test is of value as a screening test in high risk rural area when it is not possible to perform the tube agglutination titration test (Cheesebrough, 1984).

b) SAT
SAT also known as tube test is more sensitive than Slide Test but high titres sera may not cause agglutination in low dilution (the prozone effect) and a range of sera dilution is therefore needed (Greenwood et al., 2002). Whenever possible, a serum that gives a positive result for slide agglutination should be tested by the SAT. In addition SAT should also be performed when patient with a negative Slide Test continue to show symptoms of brucellosis (Cheesebrough, 1984).
This test, which measures the ability of serum to agglutinate killed organisms, reflects the presence of anti O-polysaccharide antibodies. Consequently, a rise in antibody titre is needed to demonstrate an active infection and a four fold rise has been recommended (Kumar and Clark, 2004).

However, Alton and Forsy (2002) pointed out most patients already have high titters at the time of clinical presentation so a four fold rise in titre may not occur or could lead to unnecessary delay in treatment giving room for disease progression and localisation. Serum testing should always include dilutions to at least 1: 320 since inhibition of agglutination at lower dilutions may occur (Romero et al., 1995).

c) 2-Me
This is a modified SAT in which diluent contains 0.05M 2-Mercaptoethanol, which destroy agglutinating ability of IgM and IgA which may persist for months or even years after the infection has cleared. Agglutination of this test is indicative of the continuing presence of IgG and likelihood of active infection.

d) ELISA
*Brucella* ELISA for IgG antibodies shows a good correlation with active disease. Indirect ELISA (IELISA) and competitive ELISA (CELISA) have been designed to test for brucellosis. IELISA cannot identify animals infected with *Yesinia enterocolitica* 0:9 because of epitopes shared within *Brucella spp* resulting in lower specificity value than CELISA which is capable of distinguishing animals infected with cross reacting micro organisms. This was revealed in a study on evaluation of primary binding assay for presumptive serodiagnosis of swine brucellosis in Argentina in 2000 (Paulo et al., 2000). In a different study on use of
an ELISA combining OMP 31 and other *Brucella* proteins showed improved diagnostic sensitivity by the simultaneous measurement of antibodies to different *Brucella* protein (Smits *et al.*, 2003).

### 2.6.2 Culture method

Specimens for culture include blood, bone marrow, solid tissues samples, exudates such as pus (Greenwood *et al.*, 2002). *Brucella* species are slowly growing pathogens and the cultures require prolonged incubation leading to excessive delay in diagnosis (Morata *et al.*, 2001). Success of culture is usually low. In a study carried out in Kuwait, culture was only successful in 89 cases out of 193 cases confirmed by PCR and 199 confirmed by serology (Wright, 2004). Reported frequency of isolation from blood varies widely from less than 10% to 90%, with *Brucella melitensis* being more readily culture than *Brucella abortus*. Culture of bone marrow may increase the yield and this could be because the reticuloendothelial system holds high concentration of the organism (Romero *et al.*, 1995).

*Brucella* organisms are more likely to be isolated from the blood in acute brucellosis during times of fever, but are extremely rare in chronic brucellosis. *Brucella* species are aerobic with *Brucella abortus* requiring a carbon IV oxide enriched atmosphere in which to grow. Blood culture should be kept 4-6 weeks before being reported as ‘no *Brucella* organism isolated’. To reduce the risk of contamination from sub-culturing, the use of a biphasic medium (Castaneda) is recommended. The sensitivity of blood cultures with improved techniques such as Castaneda is further improved by the lysis-centrifugation technique (Wafa and Michelle, 2006). A variety of colonial forms are produced by *Brucella* strains including colourless or grey white, smooth, mucoid, and rough colonies. When sub-cultured on solid agar, colonies usually appear 2-3 days after incubation (Cheesebrough, 1984). Cultural
facilities may not be available in district hospitals making culture difficult.

2.6.3 Molecular methods

This technique which produces the multiplication of predetermined specific nucleotide sequences offer a potential for the microbiological diagnosis of brucellosis because *Brucella* tend to be difficult to isolate by standard culture method and the laboratory risk of handling organisms are considerable. The method has been applied successfully using a range of primers that allow gene specific and species recognition. As yet they are not routinely used because they are expensive and require sophisticated apparatus which are rare in many health laboratories and institutions (Cook and Zumla, 2003).

In a study conducted in Kuwait (Bannatyne *et al.*, 1997) large scale evaluation of single-nested PCR for the laboratory diagnosis of human brucellosis in Kuwait, a single tube nested PCR assay identifying a 52bp fragment from the genus specific *Brucella* 1S711 gene was used prospectively in clinical practice for the diagnosis of human brucellosis. Out of 263 suspected cases of brucellosis, serology was positive in 199 and culture in 89, while the *Brucella* PCR was positive in 193 patients (sensitivity 96.98%). The study recorded two false negative PCR results. Both had been on long-term antibiotics for previously diagnosed brucellosis but their adherence may have been questionable allowing relapse. PCR gave results as good as serological testing with the added advantage of identifying the presence of the organism and was superior to culture. The *Brucella* DNA sequence detected by PCR in culture negative cases may represent intracellular organism incapable of replicating because of factors such as prior antibiotic therapy which can sterilize peripheral blood preventing identification of specimen.
In a study conducted in Spain (Wright, 2004) thirty three out of thirty four non blood samples (97%) from patients with different focal forms of brucellosis were positive by PCR whereas *Brucella spp* were isolated from only 29.4% of conventional cultures. However, two patients (6.2%) from the control group both with tuberculosis vertebral osteomyelitis had a positive PCR which are false positive results. The PCR essay consisted of amplification of a 223 bp fragment from the gene conding for the synthesis of an immunogenic membrane protein of *Brucella abortus* BCSP 31. The protein has a molecular mass of 31 KDa, is specific to the *Brucella* genus and is present in all its biovars.

Although *Brucella* PCR with the B4 and B5 primers have demonstrated a high degree of specificity of the technique, DNA from *Achrobactum spp*, a pathogen very closely related phylogenetically to *Brucella spp* has been amplified with these primers. PCR with primers specific for the OMP2 and OMP25 gene can detect *Brucella* specifically and also give an indication of species and biovars (Greenwood *et al.*, 2002).

### 2.7 Treatment

The treatment recommended by World Health Organization (WHO) for acute brucellosis in adults is rifampicin 600 to 900mg and doxycycline 200mg daily for a minimum of six weeks. Infections with complications such as meningoencephalitis or endocarditis require a combination therapy with rifampicin, tetracycline and aminoglycoside. Rifampicin has been recommended as the treatment of choice for uncomplicated disease in children with cotrimoxazole as an alternative. However, both are associated with a high relapse rate if used singly, but give better results when used in combination (Renner, 1997). Most *Brucella* species are resistant to penicillin and cephalosporin (Greenwood *et al.*, 2002) leading to relapse and prolonged course of treatment and lack of compliance may be a factor in apparent
failure of regimen (Bikas et al., 2003). Organisms used in a biological attack may be resistant to first line antimicrobial agent such as rifampicin, doxycycline, trimethoprim and streptomycin. Effort should be made to obtain tissue and environmental samples for bacteriological culture so that antibiotic susceptibility profile of the infecting Brucella may be determined and therapy adjusted accordingly. Culturing should be done in appropriate biosafety level 2 or 3 laboratory.

2.11 Prevention

In areas with endemic brucellosis, only vaccination will control the disease in animals. Vaccination reduces the number of infected animals and eventually permits disease control. Brucella vaccines in use are the live B. abortus Strain-19 vaccine and to a lesser extent the whole cell killed adjuvant B. abortus 45/20 vaccine. Vaccination with 40-120 x 10^9 Colony Forming Units (CFU) which is a classical dose of living Brucella abortus Strain-19 gives a good protection but it also has some disadvantages (Crawford et al., 1978). It may cause abortion in pregnant cattle and/or induce an antibody response that confuses the serological diagnosis of brucellosis for 12 up to 36 months. The organisms in vaccinated animals are excreted in the milk and may induce brucellosis in humans. To diminish these undesirable effects of vaccination with S-19, two vaccination procedures have been suggested. In one procedure calves are vaccinated once with 3-10 x 10^9 CFU (reduced dose) at an age of 4-8 months and for the second time with 3-10 x 10^9 CFU as adults. The second procedure suggests a conjunctival vaccination of calves with two drops of vaccine containing 4-10 x 10^9 CFU at an age of 4-10 months and a second conjunctival vaccination with the same dose six months later.

In Brazil, the prevalence of brucellosis in cattle decreased from 5.2% in 1965 to 0.33% in
1986 after a period of 20 years of vaccination with *Brucella abortus* S19. The use of *B. abortus* 45/20 vaccine is less common than S-19 because in comparison to S-19 it does not give lasting immunity. The vaccine does not induce detectable agglutinating antibodies and is not harmful but it gives a marked skin reaction on the injection site. Two initial vaccinations at specific intervals and an annual booster are needed for good protection (Crawford *et al*., 1978).

In the last decade a new *B. abortus* vaccine RB51 has been introduced. *B. abortus* strain RB51 used for vaccination was selected by growth of *B. abortus* strain 2308 in the presence of rifampicin. The protective effect of this vaccine in cattle is similar to that of S-19. Compared with S-19, *B. abortus* RB 51 vaccine causes less abortion (Mikolon *et al*., 1998) and does not induce production of agglutinating antibodies of the IgM type, although specific IgG is produced (Nielsen *et al*., 1989). New vaccines have been evaluated for use in animals, including the *B. suis* strain 2 live vaccine given either orally or parenterally. This vaccine has proved inferior to the rev. 1. strain for the prevention of *B. melitensis* infection in sheep and goats and ineffective against *B. ovis* infection in sheep. *B. abortus* strains 19 still appear to be as effective as any for the prevention of *B. abortus* infection in cattle. However, the RB51 strain of *B. abortus*, an R mutant used as a live vaccine has been licensed in the United State. This does not interfere with diagnostic serologic test but in laboratory trials, its efficacy appeared comparable with that of strain 19 (Renner, 1997).

Eradication of brucellosis in animals has been successful in European countries and the possibility of starting a program of eradication of the disease in Africa is envisaged. However, there are challenges such as high cost of surveillance, vaccination and compensation for livestock. The disease also affects wild animals hence complicates the
situations as culling of the infected ones may not be sustainable particularly for endangered species and can upset ecological balance with tragic consequences (Cook and Zumla, 2003).

Prevention of brucellosis in human still depends on the eradication or control of the disease in animal hosts, the exercise of hygienic precautions to limit exposure to infections through occupational activities and the effective heating of dairy products and other potentially contaminated foods. Vaccination now has only a small role in the prevention of human disease, although in the past, various preparations have been used including the live attenuated *B. abortus* strain 19-BA and 104 M (used mainly in the former Soviet Union and China), the phenol – insoluble peptidoglycan vaccine (Formerly available in France), and the polysaccharide – protein vaccine (used in Russia). All had limited efficacy, while live vaccines were associated with potentially serious reactogenicity. Subunit vaccines against brucellosis are still of interest. The live vaccines have provoked unacceptable reactions in individuals sensitized by previous exposure to *Brucella* or if inadvertently administered by subcutaneous rather than percutaneous injection. These will probably require a combination of detoxified lipopolysaccharide- protein conjugate and protein antigens such as the L7/L12 ribosomal protein presented in an adjuvant or delivery system favouring a Th 1 type immune response (Renner, 1997).

Thorough cooking of meat, milk pasteurisation, observing laboratory safety and wearing protective clothing while handling infected material protect humans from contracting brucellosis (Romero et al., 1995).
CHAPTER THREE
MATERIALS AND METHODS

The study involved three major components, namely, laboratory investigations, data collection using questionnaire data analysis.

3.1 Laboratory investigations

3.1.1 Ethical clearance

Ethical clearance was sought from National Ethical Review Committee based at the Kenya Medical Research Institute (KEMRI) (Appendix I: Ethical Clearance; Appendix II: Informed Consent).

3.1.2 Sampling procedure

The study population comprised of all who were residents of Nyandarua district in 2007 (Appendix III: Map of study area). The minimum sample size \( n = 126 \) was determined using Fishers et al., (1998) formula which was appropriate in determining sample size for population greater than 10 000.

\[
n = \frac{Z^2 \times p \times q}{d^2}
\]

Where \( n \) = minimum sample size (for population greater than 10 000), \( Z \) = standard normal deviation=1.96, \( P \) = prevalence of the condition under study, \( q = 1 - p \) and \( d \) = precision required for the study at 95% confidence level = 0.05.

The inclusion criteria for a participant were he/she must have been a resident in the area under study and willing to participate. A total of 180 samples were collected. Cluster random sampling was used because the study population was widely distributed over a large geographical area. For ethical reasons, sampling was done at health centres, hospitals and
nursing homes in Nyandarua district. Each health centre, hospital or nursing home served as a cluster and the cluster to be studied were selected randomly. The names of these health institutions were written on different pieces of paper then folded and put them in a container. They were then mixed thoroughly and one picked from the container. The name on the paper was written down and then the paper was folded and replaced into the container before picking another one. Out of the thirteen clusters (Appendix IV), two government hospitals and two nursing homes were randomly selected and studied. These were Olkalou District Hospital (74 samples), Good Hope Nursing Home (22 samples), Engineer District Hospital (55 samples) and Charity Medical Centre (29 samples) designated as 1, 2, 3 and 4 respectively in the map of study area (Appendix III).

To ensure random sampling of participants, systematic random sampling was used. Sampling interval was determined by dividing the average total annual attendance in each health institution by the required sample size. Sampling was spread over a period of 3 months covering both dry and wet seasons. The months were March; April and May 2007. This was done to cater for temporal variations within the year as dairy products are an important agent of transmission of brucellosis and their availability is influenced by weather.

3.1.3 Specimen collection

Informed consent of the participant was sought and the potential participant was made aware of what to expect. In addition, they were encouraged to seek clarity and their questions were responded to prior to the recruitment. Those who consented were recruited and 3 milliliters of blood was obtained. The blood was centrifuged and the serum put into vacutainer specimen bottle, and stored at 4°C then frozen at -70°C within 2 days.
3.1.4 Specimen laboratory processing

Each sample was subjected to the following tests; Slide Test, SAT, 2-Me and ELISA (Appendix V: laboratory procedures). The Slide Test, SAT and 2-Me used the same antigen. This was Brucella outer membrane bound lipopolysaccharide stained antigen from plasmatic laboratory products. The positive control used contained polyvalent murine antibodies.

Prior to commencing of laboratory processing, all apparatus were cleaned using liquid detergent (biogel) and rinsed with distilled water. They were then sterilized by autoclaving. All reagents and samples to be analysed were brought to room temperature.

a) SAT

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

To all the 8 test tubes 50 μl of Brucella antigen suspension (Brucella abortus preserved with 0.95% sodium azide) was added and the mixture thoroughly shaken to ensure uniformity. The tubes were covered and incubated at 37°C for 24 hours. Presence of agglutination was observed macroscopically in each tube of the dilution series and the antibody titre recorded. Where there was no agglutination the results were recorded as negative.
In this study the dilutions ranged from 1:20 up to 1:1280. However for determining the disease status the cut off was set at 1:160 and was considered together with the appropriate clinical manifestations.

b) Slide Test

The method described by Cheesbrough (1984) was adapted. Twenty micromillilitres (µl) of each serum sample, the positive and negative control were placed onto a glass slide. *Brucella* antigen suspension was shaken gently (to avoid foam formation) and then 20µl of the antigen suspension was added to each circle next to the sample drop. The two were mixed with disposable stirrer and spread over the entire area enclosed by a ring. A new stirrer was used for each sample. Presence of agglutination was recorded as positive result while absence was recorded as negative results.

c) 2-Me

This was a modified SAT in which diluent contained 0.05M 2-Mercaptoethanol, which destroy agglutinating ability of IgM and IGA which may persist for months or even years after the infection has cleared. Agglutination observed in this test is indicative of the continuing presence of IgG and likelihood of active infection. The dilution was done serially for seven times. The end point of the test was determined by the highest dilution that showed agglutination and this final dilution was the antibody titre.

Eight test tubes were placed on test tube rack and labelled 1 – 8. Some freshly prepared 0.05M 2-Mercaptoethanol phosphate buffered saline (1.9 ml) was added to tube number 1 and 0.1 ml of sample serum was added and mixed thoroughly. One millilitre of the diluted
serum from tube number 1 was transferred to tube number 2 and the mixture thoroughly shaken to obtain uniformity. The serial dilution was continued until tube number 7. 1.0ml of dilute serum was discarded from tube number 7. 1.0 ml of 0.05M 2-Mercaptoethanol phosphate buffered saline was put to test tube number 8 to serve as control.

To all the 8 test tubes 50 µl of *Brucella* antigen suspension (*Brucella abortus* preserved with 0.95% sodium azide) was added and shaken to facilitate binding of antigen and antibodies (if present). The tubes were covered and incubated at 37°C for 24 hours. Presence of agglutination was observed macroscopically in each tube of the dilution series and results recorded.

d) ELISA

The method described by Nielsen *et al.* (1996) was adopted. The ready to be used ELISA kit (Diagnostic Automation Inc Ref # 1501-8) incorporated all required materials. Additional apparatus needed were micro and multi-channel pipettes; Microtitre plate reader (450nm); Microtitre plate washer; Reagents tubes for serum dilutions and Bidistilled water.

All the specimens (deep frozen at 70°C) and all reagents were first thawed and together with samples and controls they were brought to room temperature (25°C) before starting the test run. The samples were diluted 1:101 with ready to use sample diluent (Phosphate buffered saline and 0.095% Sodium Azide) by dispensing 10µl of sample and 1 ml sample diluent into tubes to obtain 1:101 dilutions and were thoroughly mixed with a vortex. Positive control (Human serum diluted with phosphate buffered saline containing high concentration of IgG antibodies against *Brucella*) and negative control (protein solution diluted with phosphate buffered saline containing no antibodies against *Brucella*) were ready to use and were not diluted. Prior to commencing the assay, the distribution and identification plan for all
specimens and controls were carefully established on the result sheet.

Sufficient number of microtitre wells (each coated with a *Brucella abortus* antigen) was prepared for the standard, control and the samples. 100μl of each of the diluted (1:101) samples and the ready to use standard and controls were pipetted into their respective wells. One well was left empty for the substrate blank. The plate was covered with a aluminium foil and incubated at room temperature for 60 minutes. The wells were emptied and 300μl of diluted washing buffer solution (Phosphate buffered solution and Tween 20) added and then aspirated. This procedure was repeated three times. The rest of the washing buffer was removed by gently tapping the microtitre plate on a tissue cloth.

100μl of ready to use enzyme conjugate (anti-human IgG Horseradish peroxidase (rabbit) in protein containing buffer solution with addition of 0.01% methylisothiazolene, 0.01% bromonitrodioxane and 5mg/L proclin) were pipetted into the wells. The well for substrate blank was not included. The plate was covered with a foil and incubated for 30 minutes at room temperature. The wells were then emptied, 300μl of diluted buffer washing solution added and then removed by aspiration. This procedure was repeated thrice. To remove the rest of washing buffer, the micrototre plate was gently tapped on a tissue cloth. One hundred micromilitres ready to use substrate (tetramethylbenzidine) was pipetted into the wells including the substrate blank. The plate was covered with a foil and incubated at room temperature for 20 minutes in the dark (drawer).

To terminate the substrate reaction, 100μl of ready to use stop solution (0.5M sulphuric acid) was pipetted into wells including the substrate blank. After thorough mixing and wiping of the bottom of the plate the reading of the absorption was performed at 450 nm within 5
minutes. A print out showing the results was produced and the results were matched to the identification plan.

3.1.5 Laboratory data recording

Data for the 180 serum samples was organised and recorded into prevalence, sensitivity, specificity, positive predictive value and negative predictive value. SAT was used as gold standard for rating other tests.

Table 1 shows the components of formula used to compute prevalence, sensitivity, specificity, positive predictive and negative predictive values.

Table: 1 General plan for determining components of formula

<table>
<thead>
<tr>
<th>True disease status</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>a</td>
<td>b</td>
<td>a + b</td>
</tr>
<tr>
<td>Negative</td>
<td>c</td>
<td>d</td>
<td>c + d</td>
</tr>
<tr>
<td>Total</td>
<td>a + c</td>
<td>b + d</td>
<td>a + b + c + d = 180</td>
</tr>
</tbody>
</table>

True disease status refers to the actual health status of the participant, whether he/she is infected with brucellosis or not. Test results refer to the finding of the test, whether it indicates that the person is suffering from brucellosis or not irrespective of the true disease status. The following formulas were used;
Prevalence

Prevalence = Number of positive samples / total number of samples tested.

Sensitivity

Sensitivity = \( \frac{a}{a+c} \).

Specificity

Specificity = \( \frac{d}{b+d} \).

Positive predictive value

Positive predictive value = \( \frac{a}{a+b} \).

Negative predictive value

Negative predictive value = \( \frac{d}{c+d} \).

3.2 Questionnaire

A questionnaire was used to collect data pertaining participant (Appendix VI). The questionnaire was piloted and relevant adjustments made before it was used to collect data for this study. The questionnaire had both structured and unstructured questions and sought to collect data on sex, occupation, age, preparation of fermented milk and cheese, consumption of dairy products and blood products, type of animals handled, awareness of existence, symptom, prevention and treatment of brucellosis, previous treatment for brucellosis and disease symptoms if any. The respondents were taken through the questionnaire and those unable to write were assisted.
3.3 **Data analysis**

The data recorded during specimen analysis and gathered using the questionnaires were analysed. The Excel and Statistical Package for Social Sciences (SPSS) programmes were used to analyse the data. The raw data was coded and analysed into frequencies, percentages and correlation coefficient and statistical significance tests computed. The results were presented in form of tables and figures. The results were compared with the objectives to assess their relationship.
CHAPTER FOUR
RESULTS

4.1 Prevalence of brucellosis

The prevalence of human brucellosis in Nyandarua district was 3.9%, determined by SAT.

4.1.1 Prevalence of brucellosis infections by sex

A total of 111 female and 69 male participated in the study. Table 2 shows the prevalence of brucellosis infections by sex.

Table 2: Prevalence of brucellosis by sex

<table>
<thead>
<tr>
<th>Sex</th>
<th>SAT(1:160)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>4.4% (n = 3)</td>
</tr>
<tr>
<td>Female</td>
<td>3.6% (n = 4)</td>
</tr>
</tbody>
</table>

There were a higher number of cases among males compared to females but the difference was not statistically significant, $\chi^2$ actual (0.063) < $\chi^2$ expected (3.841 at $P = 0.05$).

4.1.2 Prevalence of brucellosis infections by age

The youngest participant was 5 years old while the oldest was 80 years. The participants were categorised into classes with class intervals of 10 years. The disease had highest prevalence (28.6%) in the age groups 21-30, 31-40 and 41-50. The prevalence in the age group of 61-70 was 14.3%. The disease was not reported in the age groups 0-10, 11-20, 51-60 and 71-80.
Table 3: Prevalence of human brucellosis infections in various age groups

<table>
<thead>
<tr>
<th>Age</th>
<th>SAT 1:160</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below 10</td>
<td>0%</td>
</tr>
<tr>
<td>11-20</td>
<td>0%</td>
</tr>
<tr>
<td>21-30</td>
<td>28.6%</td>
</tr>
<tr>
<td>31-40</td>
<td>28.6%</td>
</tr>
<tr>
<td>41-50</td>
<td>28.6%</td>
</tr>
<tr>
<td>51-60</td>
<td>0%</td>
</tr>
<tr>
<td>61-70</td>
<td>14.2%</td>
</tr>
<tr>
<td>71-80</td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td>100%</td>
</tr>
</tbody>
</table>

At a 99% level of confidence, the confidence interval for the mean age of *Brucella* SAT positive participants is $23.3 \leq \mu \leq 61.2$ years (estimated by t test statistic).

4.2 Sensitivity of Slide Test, 2-Me and ELISA used to diagnose human brucellosis

The sensitivity of various tests was as computed as shown in Appendix VI. Slide Test had the highest sensitivity (85.7%), while 2-Me and ELISA each had 71.4%. There is a statistically significant difference in sensitivity of Slide Test compared to both 2-Me and ELISA; $Z_{actual} (20.05) > Z_{expected} (2.33$ at $P = 0.01)$. Figure 1 shows the sensitivity of various tests.
4.3 Specificity of different methods used to diagnose human brucellosis

The specificity of various tests used to determine the prevalence of brucellosis was computed (Appendix VI). 2-Me test had the highest specificity (99.4%) followed by ELISA (98.9%) and Slide Test (90.8%) and is shown in the Figure 2.
Figure 2: Specificity of Slide Test, 2-Me and ELISA used in diagnosis of human brucellosis

Specificity of Slide Test was 90.8%, 2-Me was 99.4% and ELISA was 98.9%

A statistically significant difference exist between specificity of Slide Test compared to 2-Me; $Z_{\text{actual}} (3.81) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$. Although 2-Me recorded a higher prevalence compared to ELISA, the difference was not statistically significant; $Z_{\text{actual}} (0.5) < Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$. Slide Test has a higher specificity compared to ELISA and the difference is statistically significant; $Z_{\text{actual}} (3.54) > Z_{\text{expected}} (2.33 \text{ at } P = 0.01)$. 
4.4 Positive predictive values of Slide Test, 2-Me and ELISA used to diagnose 
human brucellosis

Figure 3 shows the positive predictive value of various test used to detect human brucellosis. 
These values were computed as shown in Appendix VI. 2-Me test had the highest positive 
predictive value (83.3%) followed by ELISA (71.4%) and Slide Test (27.3%).

Figure 3: Positive predictive value of Slide Test, 2-Me and ELISA used in diagnosis of 
human brucellosis

Positive predictive value of was 27.3%, was 83.3% and ELISA was 71.4%

2-Me test had a higher positive predictive value compared to Slide Test and the difference is 
statistically significant; Z actual (10.7) > Z expected (2.33 at P = 0.01). The positive
predictive value of ELISA is statistically significant higher than that of Slide Test; \( Z \) actual (18.73) > \( Z \) expected (2.33 at \( P = 0.01 \)). When positive predictive value of ELISA is compared to that of 2-Me there is a statistically significant difference; \( Z \) actual (2.7) > \( Z \) expected (2.33 at \( P = 0.01 \)).

4.5 Negative predictive values of Slide Test, 2-Me and ELISA used to detect human brucellosis

Figure 4 shows the negative predictive value of the three tests used in diagnosis of human brucellosis as computed in Appendix VI. The highest predictive value was recorded by Slide Test (99.4%), followed by ELISA and 2-Me (98.9% each).

![Figure 4: Negative predictive value of Slide Test, 2-Me and ELISA used in diagnosis of human brucellosis](image)

Negative predictive value of Slide Test was 99.4% and 98.9% for both 2-Me ELISA.
There is no statistically significant difference between negative predictive value of Slide Test compared to both 2-Me and ELISA; \(Z\) actual \((0.53) < Z\) expected \((2.33\) at \(P = 0.01)\) and \(Z\) actual \((0.47) < Z\) expected \((2.33\) at \(P = 0.01)\) respectively.

4.6 **Factors that precipitate brucellosis infections**

The following factors were considered to predispose an individual to infection by *Brucella* organisms and were investigated.

4.6.1 **Occupation exposure**

Based on the methods of transmission of human brucellosis that have been recorded by various researchers the occupation of the participant was categorised into two namely; risky occupation and occupation with no risk. Participants working in the following areas were considered to be at risk:

i) Slaughterhouse workers and meat inspectors, primarily those in the kill areas, become inoculated through aerolization of fluids, contamination of skin infections and splashing of mucous membranes.

ii) Butcher-men who handle animal carcass during transportation and packaging and at the point of sale.

iii) Veterinarians usually are infected by handling infected animals as they work.

iv) Farmers and shepherds who contact animals via milking, feeding and those who have exposure to aborted animals.

v) Laboratory workers who are exposed by processing specimens (aerosols) without special precautions.
The results indicate that more of the individuals with occupational exposure were infected with brucellosis ($n = 5$) as compared to those with no occupational exposure risk ($n = 2$). This difference is not statistically significant ($P>0.05$).

Close human contact with animals increases the chances of transmission of human brucellosis from animals to man. The main avenues of human animal contact are through feeding and milking. 71.4% of brucellosis positive participants had close contact with animals through feeding while 57.1% had exposure through milking.

4.6.2 Consumption of animal products

All participants who were *Brucella* SAT positive had risk of exposure to contaminated animal products. The implicated animal products are fermented milk and cheese prepared using raw milk and foods prepared using animal’s blood. A correlation coefficient (+ 0.98) exists between consumption of dairy products prepared using raw milk and infection with *Brucella* spp. A positive correlation coefficient (+ 0.65) for relationship between consumption of animal blood products and infection with brucellosis was found out.

4.7 Symptoms of brucellosis

The clinical picture of human brucellosis is usually confusing, non specific and is further complicated by cases of brucellosis differentials. These are ailments that show almost similar symptoms. The study investigated the clinical symptoms among all the participants. It was found out that most common symptoms among the *Brucella* infected were fever, anorexia, malaise, body and joints ache and night sweats. Some of these symptoms were also reported in persons who suffered from other ailments in different combinations.
4.8 Participants knowledge of issues relating to brucellosis

The study sought to know the information participants had as pertained to existence, symptoms, prevention and treatment of brucellosis and the following are the findings.

4.8.1 Participant's knowledge of existence of brucellosis

58.9% of the participants reported that they knew there is a disease known as brucellosis while 40.1% did not know.

4.8.2 Participant's knowledge of symptoms

39.1% of the respondents had the correct information pertaining to symptoms of brucellosis while 60.9% either had no information or the information was erroneous.

4.8.3 Participant's knowledge of treatment

23.3% of the respondents had the correct information pertaining to treatment of brucellosis while 76.7% either had no information or the information was incorrect.

4.8.4 Participant's knowledge of prevention

37.1% of the respondents had the correct information pertaining to prevention of brucellosis while 62.9% either had no information or the information was wrong.

4.8.5 Ways of preventing brucellosis

The study sought to know the ways of prevention that are known to the participants. Figure 5 shows a summary of the responses. Thorough cooking of animal products was the most known, followed by wearing protective clothing when handling materials suspected to be contaminated.
4.9 Previous treatment for human brucellosis

Brucellosis can easily relapse and the study sought to know if the participants had previously been treated for brucellosis. 14.3% of the brucellosis SAT positive participants had previous treatment for Brucellosis while 85.7% had no previous treatment.
5.1 Discussion

The prevalence of human brucellosis was determined by SAT based on serial dilution and hence was able to estimate the quantities of antibodies. There were agglutination in dilutions below the cut off and were considered as negative. This finding concurs with that of a study conducted in Saudi Arabia (Memish, 2002).

Slide Test had sensitivity of 85.7% but in other studies it has recorded it to be up to 100% (Greenwood et al., 2002). The relatively lower sensitivity of Slide Test recorded in this study probably was caused by prozone phenomenon where a positive serum sample tests negative. This phenomenon occurs due to presences of blocking antibodies which bind but do not agglutinate non-specific factors or excess antibodies (Cheesebrough, 1984). As these blocking antibodies are present in low titre their effects are readily diluted out and testing at high dilutions give a positive agglutination (Cook and Zumla, 2003). 2-Me and ELISA had relatively lower sensitivity compared to Slide Test due to the fact that cut off standards had to be set, and hence has a quantitative aspect in estimation of the level of antibodies.

The level of sensitivity of a test is inversely related to the number of false negatives that are generated by the same test. This may translate to delayed diagnosis and delay in administration of treatment. Brucella organisms are intracellular and the success of treatment is higher when therapy is administered early in the course of disease (Cook and Zumla, 2003). Moreover, brucellosis is treated using broad spectrum antibiotics which are also used to treat other gram negative bacterial infections. Consequently misdiagnosis may result to administration of drug meant to treat another ailment but which does not effectively destroy
Brucella bacteria leading to development of drug resistant Brucella organisms. Slide Test is a qualitative test that determines the presence and not the quantity of antibody present. This attribute enhances its sensitivity and could account for its higher sensitivity when compared to 2-Me and ELISA.

The level of specificity of a test is inversely related to the number of false positives generated by the same test. A study in Saudi Arabia to monitor the persistence of Brucella antibodies after successful treatment of acute brucellosis in an area of endemicity, clinically cured patient were noted to continue to have various levels of Brucella antibodies for a long time after acute brucellosis. Such cases would test positive for brucellosis at low antibody titres (Memish, 2002). The specificity of the 2-Me and ELISA tests were within acceptable range of 95% and above (Encarnacion et al., 2003). The Slide Test sensitivity was the lowest. This implies a large number of false positive were generated and this can be attributed to the fact that the positive test results are obtained at any level of antibody titre.

The issue of false positive is important because it poses a challenge to diagnosis of brucellosis in patients with signs and symptoms suggestive of brucellosis when their symptoms are caused by other infectious or non-infectious diseases. The implication may be over-diagnosis and exposing patients to unnecessary anti-Brucella treatment (Morata et al., 1997). Brucellosis treatment has a long therapeutic period of intramuscular administration up to 3 weeks (Cook and Zumla, 2003). Such treatments that spread over a long duration can easily lead to non-compliance and can interfere with the drugs efficiency especially if it is a broad spectrum. As well there will be unnecessary increased cost of service delivery in the health sector.
Slide Test had the lowest while 2-Me had the highest positive predictive value. The inability of the Slide Test to predict correctly that a person who tested positive is actually infected can be attributed to the fact that it is a qualitative test that does not address the issue of quantities of antibodies (Cheesebrough, 1984). The relatively higher positive predictive value of ELISA as compared to the Slide Test can be attributed to the fact that ELISA can quantitatively estimate the level of antibodies and a positive test must show a certain level of antibodies. 2-Me test had the highest positive predictive value owing to the fact that the 2-Mercaptoethanol included in the test degrades the IgA and IgM which last long even after the recovery. A positive 2-Me test result confirms presence of IgG which is a clear sign of active infection (Murat and Ersin, 2007).

Slide Test yielded one false negative result (Appendix VII). This could be due to the prozone effect as was reported by Cheesebrough (1984). A unique case was recorded as false positive by 2-Me but could have been actually positive. The antibody titre for SAT was below the cut-off and the clinical symptoms were not very suggestive of brucellosis and hence was categorised as negative. A similar case was reported in India by Bassapa et al. (2006) where 88.7% of all cases that tested positive for brucellosis had no clinical suspicion and the diagnosis was made only by routine serology. A total of 76% of these cases who declined treatment eventually developed clinical symptoms.

All the three tests had negative predictive value above 95% and all the three tests are reliable in their ability to predict whether a person who tests negative is truly uninfected.

Both males and females in this study were found to be susceptible to Brucella infections similar to the report by Cook and Zumla (2003). This study found no increased susceptibility
due to sex. However, while a study in Saudi Arabia and in Turkey documented a higher occurrence among females (Murat and Ersin, 2007) Cook and Zumla (2003) reported a higher occurrence among males. These results although they seem to be conflicting may be correct depending on how the study population assign responsibilities to various gender. The gender with a higher occupational exposure will record a relatively higher prevalence. The highest infection frequencies in this study were recorded in the age groups 21-30, 31-40 and 41-50. This finding compares well with a study carried out in Turkey which reported the disease mostly in the 20 to 40 years old group (Cetin et al., 1990).

Distinct modes of transmission implicated for brucellosis are direct transmission due to occupational exposure and food-borne transmission (Mohedano et al., 2002). The results of this study indicate that food-borne transmission was the highest route of transmission within the district. These findings concur with a study conducted in Brazil (Langoni et al., 2000), where Brucella organisms were isolated from milk samples collected from dairy animals that tested negative for Brucella antibodies. Brucella organisms cause a chronic mastitis which is often clinically inapparent. Such infected animals excrete large number of viable Brucella in milk for months to years and hence serve as reservoirs that represent important source of infection to other lactating animals, calves and humans who consume raw milk (Harmon et al., 1988).

In a study in Brazil by Langoni et al. (2000) farms with different milking management such as manual and mechanical milking were all found to be deficient in hygienic procedures of milking. Such shortcomings allow shedding of Brucella into the environment. Contamination of milk by Brucella spp from environment is possible because Brucella has the ability to survive for long periods in various environments and still remain infective. A study on the
survival of the organism in the environment indicated that Brucella can survive in tap water for several months at 4-8°C, 2-5 years at 0°C and several years in frozen tissue. The organism can survive up to 60 days in damp soil and up to 144 days at 20°C and 40% relative humidity. The organism can also survive for 30 days in urine and 75 days in uterine exudates (Murat and Ersin, 2007). Inhalation of contaminated dust in hot dry countries may therefore be a source of infection (Cook and Zumla, 2003). This strongly indicates that when infected animals shed Brucella organisms into the environment, this becomes a source of human infection.

Boiling of milk before fermentation increases the duration of time needed for the milk to ferment hence not desirable and is not widely practised. A positive correlation between consumption of dairy products prepared using raw milk and infection indicated in this study is in agreement with a study that revealed significant rise in prevalence of human brucellosis associated with the consumption of raw milk (Mohedano et al., 2002). In addition, Langoni et al. (2000) pointed out that the main source of infection for the general population is not only contaminated raw milk but also non-pasteurized dairy products such as cheese. A case control study conducted in Andalucia, Spain revealed that persons who eat unpasteurized raw goat cheese were at a higher risk of contracting brucellosis (Mohedano et al., 2002). Brucella have been isolated from cheese and the fact that boiling milk delays ripening of cheese may lead to preparation of the cheese without boiling of milk and as such if prepared from Brucella contaminated milk the cheese would be infective.

Brucella can survive food processing depending on maturation and acidification periods to which each product is subjected (Plommet et al., 1998). Soft cheese and similar products made from unpasteurized milk may transmit infection because the shorter time for
preparation does not allow the pH to become sufficiently acidic to kill the organisms (Cook and Zumla, 2003). The findings of this study agree with that of a study (Kang’the et al., 2007) that implicated home made fermented milk as a source of milk-borne infections to humans in Kenya. According to Farrell (1996) fermentation of milk lowers the pH of milk from 6.8 to 4.5 which can only mildly affect *Brucella abortus*. This implies that fermentation of contaminated raw milk does not render it safe for human consumption. *Brucella* are heat sensitive and are killed by temperatures of only 60°C and above for 10 minutes but dense suspension such as cultured colonies or aborted foetal tissues require more drastic heat treatment to ensure their inactivation (Greenwood et al., 2002). Infected milk is rendered safe by efficient pasteurisation. However, boiling of milk should take several minutes to ensure that any infective heat sensitive micro-organism is killed.

Animal blood products considered were any food made of animals’ blood among other ingredients. Consumption of these products can serve as route of transmission especially when they are undercooked and this may underlie the positive correlation that was found out.

The clinical manifestation of human brucellosis is usually not clear and may be characterised by non-specific symptoms. This is due the fact that symptoms of brucellosis are protean in nature, and none is specific enough to support diagnosis without laboratory tests. Most of the symptoms overlap and therefore brucellosis is difficult to diagnose especially where there are no laboratory facilities in rural areas.

Previous exposure to antibiotics of an infected person prior to testing for human brucellosis may interfere with serological result by delaying sero-conversion and culturing. One participant was continually on a prophylaxis regimen and laboratory tests were difficult to
interpret since they did not meet the criteria but were suggestive of *Brucella* infections especially when previous treatment was factored.

Adequate knowledge on symptoms, treatment and prevention among members of a population is an important element in disease management and this was generally poor in the population studied. This contributes indirectly to increased prevalence through delay in diagnosis, poor adherence to regimen and increased transmission.

Brucellosis can easily relapse and the study sought to know if the participant had previously been treated for brucellosis. One SAT positive participant had previously been treated for brucellosis three years before but reported complete cure and hence there was no case of relapse that was detected. Those samples that tested positive for 2-Me are an indication of either chronic infection or one that developed insidiously for along time without showing any clinical manifestation.
5.2 Conclusions

i) The prevalence of human brucellosis in Nyandarua district is low.

ii) All the three (Slide Test, 2-Me and ELISA) tests have varying level of sensitivity and Slide Test is the most sensitive.

iii) There is difference in the specificity of Slide Test, 2-Me and ELISA when used to diagnose human brucellosis.

iv) There are statistically significant differences in positive predictive values of Slide test, 2-Me and ELISA when used to diagnose human brucellosis.

v) The negative predictive values of Slide Test, 2-Me and ELISA when used to diagnose human brucellosis have no statistically significant difference.

vi) Consumption of animal products contaminated with infective *Brucella* organism is the major route of brucellosis transmission in Nyandarua District.

vii) Awareness of the study population on issues pertaining brucellosis particularly symptoms, prevention and treatment is low.

From the findings of this study the null hypotheses are rejected and the following alternative hypotheses accepted:

i) There is difference in performance of Slide Test, 2-Me and ELISA when used to diagnose human brucellosis in Nyandarua district.

ii) Personal characteristics (age and diet consumed) predispose human beings to brucellosis infection.
5.3 Recommendations

The following are recommendations arising from the results of this study and emerging gaps for further research.

**Recommendations from the findings of this study**

i) Slide Test should be used as screening test for it is quicker, cheaper and easy to perform and does not require sophisticated apparatus. However, all positive cases by this method should be subjected to a confirmatory test (SAT, ELISA and 2-Me) to get rid of false positives generated by the Test.

ii) Cases that test negative by Slide Test while persistently showing signs suggestive of brucellosis infection should be subjected to SAT, 2-Me or ELISA as Slide Test can yield false negative due to prozone phenomenon.

iii) The 2-Me test should be incorporated in routine diagnosis of human brucellosis.

iv) The public should be educated on ways to curb brucellosis and other zoonotic infections even when there are no epidemics as some infections of low prevalence may be ignored but have high indirect impacts on the health.

**The following emerging gaps are recommended for further research**

i) Other protein modifying reagents should be tested on their ability to improve the agglutination tests as 2-Mercaptoethanol has a very strong and pungent smell.

ii) A national wide study should be carried out of investigate the problem and determine awareness of brucellosis in human population in Kenya. The findings of such a study may be considered when designing intervention programs.
iii) In areas where livestock share grazing field with wild animals the latter which may serve as reservoirs of *Brucella* species should be investigated as well.
REFERENCES


smooth and rough Brucella. Clinical and Diagnostic Laboratory Immunology, 11:111-114.


February 28, 2007

Mr. Simon N Gachigua,
Kenyatta University,
PO Box 43844,
NAIROBI

Through: The Chairman,
Dept. of Biochemistry and Biotechnology,
Kenyatta University,
PO Box 43844 00100
NAIROBI

Dear Sir,

RE: NON-SSC – Prevalence of human Brucellosis in Nyandarua district, Kenya determined by slide test, 2-mercaptaethanol degradation agglutination test and ELISA PI: Simon N Gachigua (Kenyatta University)

Thank you for your letter dated 27th February 2007.

The Committee notes that you have adequately addressed the issue raised. It is now apparent that you will do the consenting before the study participants see the doctor and that you will inform the doctor of any samples that have high titre so that the patients can be contacted and treated. We also note the addition of the paragraph on prevention measures for Brucellosis in the Consent Document.

Due consideration has been given to ethical issues and you may proceed with the study.

You are responsible for reporting to the Ethical Review Committee any changes to the protocol or in the Informed Consent Document. This includes changes to research design or procedures that could introduce new or more than minimum risk to human subjects.

Yours faithfully,

R. C. Kithinji,
For: Secretary,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE
Appendix II: Informed consent

KENYATTA UNIVERSITY

P.O. Box 43844, Nairobi.

PREVALENCE OF HUMAN BRUCELLOSIS IN NYANDARUA DISTRICT, CENTRAL PROVINCE, KENYA

Investigators

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Purpose of study

Brucellosis a disease affecting both man and livestock is spread mainly through consumption of contaminated meat and milk and by handling infected animals. The purpose of this study is to find out the prevalence of brucellosis among residents of Nyandarua district. The study will also compare the commonly used laboratory tests with those rarely used and those recently developed in terms of their ability to identify correctly those who have and those who do not have the disease.

Participation

Your participation in this study is voluntary and you are free to reject a request to participate without giving the reasons. Make sure you read and understand what is contained in this document before agreeing to participate. You are encouraged to ask question in areas that you do not understand. Parents must accept that their children participate in the study before they are included. A child who does not want to participate in the study will not be included even if the parent has accepted. The consenting will be done before the participant reports to the doctor.

Study procedure

Having read and understood and accepted to participate in this study then the following will happen to you: 3 milliliters of blood will be drawn from one of your arm. You will be asked questions concerning your status of health, food you eat and animals you keep at home. You are free not to answer a particular question without giving reasons. This exercise will be done once and there will be no follow up.
Risks and discomforts
During and immediately after drawing of the blood you will feel pain. The area where blood was drawn may swell and/or you may take several minutes before you stop bleeding. You may faint and at times though rarely you may get infection through the site of puncture. To ensure your safety, blood will be drawn by a trained technician who has been allowed to practice. All equipments used are sterilized and needles and syringes used cannot be re-used. In addition this exercise will be done in a hospital laboratory.

Benefits
There are no material benefits for participating in this study. However the findings of this research will benefit the entire community as it will help improve diagnosis of brucellosis.

Costs
You will not spend any money while participating in this study.

Confidentiality
Your names will not be recorded and the blood sample collected will have no marks or labels that can enable anyone to trace from who it was collected. The blood sample will only be used for this study and no other person will be allowed to handle or use it.

How to prevent contacting brucellosis
To prevent brucellosis animal handlers should wear protective clothing when working with infected animals. Meat should be well cooked and milk pasteurized. Laboratory workers should culture the organism only with appropriate biosafety level 2 or 3 containment. Milk should be boiled before consumption or being stored to ferment. Meat should be well cooked
and cross contamination should be avoided.

**Enquiries**

If you need further explanation or you have questions concerning your participation in this study you can contact me through any of the following ways

Simon Ndung’u Gachigua,
P. O. Box 851-20300 Nyahururu,
Email: sngachigua@yahoo.com.
Telephone: 0721285382 or 0734212999.

If you would like to know more about your rights as a participant in this study you can contact the National Ethical Review Committee based at Kenya Medical Research Institute (KEMRI) Nairobi by sending your enquiries to: The secretary Kenya Medical Research Institute or National Ethical Review Committee, P.O. Box 54580-00200, Telephone 020-2722501.

**Questionnaire**

The attached is a questionnaire that will enable gathering of more information that can help me investigate more on brucellosis. All the answers will be treated as confidential and will not be used for any other purpose except for this study. If you wish to know the results of the test please register your contacts.
Appendix III: Map of Nyandarua district

LEGEND
+ Public Hospital
+ Health Centre
+ Dispensary
+ Private Hospital
--- District Boundary
--- Road

8 0 16 24 Km

Prepared by Physical Planning Department
Source: District Planning Office
Appendix IV: List of hospitals, health centres and nursing homes in Nyandarua district

1. Nyahururu District Hospital (Private)
2. Charity Medical Centre (Private)
3. Nyahururu Private Hospital (Private)
4. Olkalou District Hospital
5. Canan Hospital (Private)
6. Goodhope Hospital (Private)
7. Engineer District Hospital
8. Kinagop Mission Hospital (Private)
9. Milangine Health Centre
10. Wanjohi Health Centre
11. Ngano Health Centre
12. Ndaragwa Health Centre
13. Karangatha Health Centre
Appendix V: Laboratory procedures

Slide Test

Material required: *Brucella* antigen suspension, stopwatch, positive control, negative control, isotonic saline, glass slide, pipettes/micropipettes, mixing sticks.

Procedure

Bring all reagents to room temperature.

Place 1 drop (20 μl) of the sample serum.

And 1 drop of each control into separate circles on the glass slide.

Shake the antigen vial gently before using.

Add 1 drop (20 μl) of antigen suspension to each circle next to the sample drop.

Mix with disposable stirrer and spread over the entire area enclosed by a ring. Use a new stirrer for each sample.

Reading

Examine the presence of agglutination within a minute.

Positive results: presence of visible; agglutination.

Negative results: absence of visible agglutination.

SAT

Materials required: Stopwatch, test tubes, test tube racks, pipettes/micropipettes, isotonic saline, positive and negative control, incubator (50°C).

Procedure

Take 8 test tubes and label them 1 to 8.
Pipette 1.9ml of 0.85% isotonic saline to tube No. 1.

To each of the remaining tubes (2-7) add 1.0ml of isotonic saline.

To the tube No.1 add 0.1ml of serum sample to be tested. Mix well.

Transfer 1.0ml of the diluted serum from tube No.2 to tube No.3 and mix well.

Continue this serial dilution till tube No.7.

Discard 1.0ml of the diluted serum from tube No.7.

Pipette 1.0ml isotonic saline into tube No.8, which serves as a negative control.

To all the test tubes add 1 drop of antigen suspensions and mix well.

Cover the test tubes and incubate at 37° C for 24 hours.

Observe for agglutination macroscopically in each tube of the dilution series.

Interpretation of results

The titre of patient serum is reciprocal of the last dilution of the serum sample that gives granular agglutination. In negative reaction, the appearance remains unchanged.

2-Me agglutination test

Materials required: Stopwatch, test tubes, test tube racks, pipettes/micropipettes, isotonic saline, positive and negative control, incubator (50° C).

Procedure

Take 8 test tubes and label them 1 to 8.

Pipette 1.9ml of 0.05M 2-Mercaptoethanol phosphate buffered saline to tube No. 1.

To each of the remaining tubes (2-7) 0.05M 2-Mercaptoethanol phosphate buffered saline.

To the tube No.1 add 0.1ml of serum sample to be tested. Mix well.

Transfer 1.0ml of the diluted serum from tube No.2 to tube No.3 and mix well.

Continue this serial dilution till tube No.7.
Discard 1.0ml of the diluted serum from tube No.7.

Pipette 1.0ml of 0.05M 2-Mercaptoethanol phosphate buffered saline into tube No.8, which serves as a negative control.

To all the test tubes add 1 drop of antigen suspensions and mix well.

Cover the test tubes and incubate at 37^\circ C for 24 hours.

Observe for agglutination macroscopically in each tube of the dilution series.

Interpretation of results

The titre of patient serum is reciprocal of the last dilution of the serum sample that gives granular agglutination. In negative reaction, the appearance remains unchanged.

**Brucella IgG ELISA procedure**

Materials required: the kit incorporates all required materials.

Specimen collection and storage

If the assay is performed within 24 hours after sample collection, the specimen should be kept at 2-8^\circ C; otherwise they should be stored deep-frozen (-20 to -70^\circ C). If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing.

Assay Procedure

It is very important to bring all reagents, samples and controls to room temperature (20-25^\circ C) before starting the test run.

Sample Dilution

Before assaying, all samples should be diluted 1:101 with Sample Diluent. Dispense 10\mu l
sample and 1 ml Sample Diluent into tubes to obtain a 1:10 dilution and thoroughly mix with a Vortex.

Positive and negative controls are ready to use and must not be diluted.

Test Preparation and Procedure

• Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit.

• Select the required number of microtitre strips or wells and insert them into the holder. Please allocate at least: 1 well (e.g. A1) for the substrate blank, 1 well (e.g. B1) for the negative control, 2 wells (e.g. C1+D1) for the cut-off control and 1 well (e.g. E1) for the positive control.

• Perform all assay steps in the order given and without any appreciable delays between the steps.

• A clean, disposable tip should be used for dispensing each control and sample.

• Adjust the incubator to 37° ± 1°C.

Pipetting and Incubation Steps

i) Dispense 100μl controls and diluted samples into their respective wells. Leave well A1 for substrate blank.

ii) Cover wells with the foil supplied in the kit and incubate for 1 hour ± 5 min at 37±1°C.

iii) When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300μl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be more than 5 sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step.
iv) Dispense 100µl Brucella anti-IgG Conjugate into all wells except for the blank well (e.g. A1). Cover with foil.

v) Incubate for 30 min at room temperature. Do not expose to direct sunlight.

vi) When incubation has been completed, remove the foil, aspirate the content of the wells and wash each well three times with 300µl of Washing Solution. Avoid overflows from the reaction wells. The soak time between each wash cycle should be more than 5 sec. At the end carefully remove remaining fluid by tapping strips on tissue paper prior to the next step.

vii) Dispense 100µl TMB Substrate Solution into all wells.

viii) Incubate for exactly 15 min at room temperature in the dark.

ix) Dispense 100µl Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.

x) Any blue colour developed during the incubation turns into yellow.

xi) Measure the OD of the specimen at 450/620 nm within 30 minutes after addition of the Stop Solution.

Note: Highly positive patient samples can cause dark precipitates of the chromogen. These precipitates have an influence when reading the optical density. Predilution of the sample with physiological sodium chloride solution, for example 1:2, is recommended. Then dilute the sample 1:101 with dilution buffer and multiply the results in AU by 2.

Measurement

Adjust the ELISA Microwell Plate Reader to zero using the substrate blank in well A1.

If due to technical reasons the ELISA reader cannot be adjusted to zero using the substrate blank in well A1, subtract the OD value of well A1 from all other OD values measured in order to obtain reliable results.
Measure the OD of all wells at 450 nm and record the OD values for each control and patient sample in the distribution and identification plan.

Dual wavelength reading using 620 nm as reference wavelength is recommended.

Results

Assay Validation Criteria

In order for an assay to be considered valid the positive control must show at least double absorption compared to the cut-off standard.

Interpretation of results

Samples are considered POSITIVE if the OD value is higher than 10% over the cut-off.

Samples with an OD value of 10% above or below the cut-off should not be considered as clearly positive or negative because they fall in to the grey zone. In such a case it is recommended to repeat the test again 2 – 4 weeks later with a fresh sample. If results in the second test are again in the grey zone the sample has to be considered NEGATIVE. Samples are considered NEGATIVE if the OD value is lower than 10% below the cut-off.
Appendix VI: Questionnaire

Tick the appropriate response in the brackets or write down the answer in space provided.

1. What is the sex of the respondent male ( ) female ( )

2. What is the age of the respondent .................................................................

3. What is the respondent’s occupation ...............................................................

4 (a) Does the respondent boil milk meant to prepare the following

<table>
<thead>
<tr>
<th></th>
<th>Yes ( )</th>
<th>No ( )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermented milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cheese</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Please tick the appropriate response for the following question in the table provided

<table>
<thead>
<tr>
<th></th>
<th>Always</th>
<th>Often</th>
<th>Rarely</th>
<th>Never</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermented milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locally prepared cheese</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animal blood products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5 Does the respondent handle any of the following animals in any of the following ways?

<table>
<thead>
<tr>
<th></th>
<th>Feeding</th>
<th>Milking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pigs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dogs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6. Is the respondent aware of existence of brucellosis? Yes ( ) No ( )

7. If yes what does the patient know about the following?

(a) Symptoms of human brucellosis.................................................................
(b) Prevention of human brucellosis

(c) Treatment of human brucellosis

8. Has the respondent ever been treated for brucellosis?  Yes ( )  No ( )

If yes how recent

<table>
<thead>
<tr>
<th>Year</th>
<th>Yes ( )</th>
<th>No ( )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td></td>
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<tr>
<td>2004</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. Is the respondent experiencing any of the following symptoms?

- Intermittent fever ( )
- Night sweat ( )
- General body ache ( )
- Low back pain ( )
- Lack of appetite ( )
- Headache ( )
- Urinary retention ( )
- Adenopathy ( )
- Body lesions ( )
- Chills ( )

Any other (specify) ..................................................

10. Has the respondent attended hospital within the past one month? Yes ( ) No ( )

If yes state reason ..........................................................

11. Please indicate the current ailment(s) diagnosed if any ..................................................

Thank you for your time.
Appendix VII: Computations of sensitivity, specificity, positive predictive value and negative predictive value for Slide Test, 2-Me and ELISA

Slide Test

Number of sample that tested positive by this test were 22 and those that tested negative were 158. One sample that tested positive by all other methods tested negative for Slide Test.

<table>
<thead>
<tr>
<th>True disease status</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slide Test Results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>a = 6</td>
<td>b = 16</td>
<td>a + b = 22</td>
</tr>
<tr>
<td>Negative</td>
<td>c = 1</td>
<td>d = 157</td>
<td>c + d = 158</td>
</tr>
<tr>
<td>total</td>
<td>a + c = 7</td>
<td>b + d = 173</td>
<td>a + b + c + d = 180</td>
</tr>
</tbody>
</table>

Sensitivity = 6/7 X 100 = 85.714 %

Specificity = 157/173 X 100 = 90.751 %

Positive predictive value = 6/22 X 100 = 27.27%

Negative predictive value = 157/158 X 100 = 99.367%

2-Me

Six sample tested positive by this method at the lowest dilution (1:20) and were considered positive as this was the cut off. One sample that tested positive by SAT gave a negative result by this method.
<table>
<thead>
<tr>
<th>True disease status</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Me Positive</td>
<td>a = 5</td>
<td>b = 1</td>
<td>a + b = 6</td>
</tr>
<tr>
<td>2-Me Negative</td>
<td>c = 2</td>
<td>d = 172</td>
<td>c + d = 174</td>
</tr>
<tr>
<td>total</td>
<td>a + c = 7</td>
<td>b + d = 173</td>
<td>a + b + c + d = 180</td>
</tr>
</tbody>
</table>

Sensitivity = 5/7 X 100 = 71.429%
Specificity = 172/173 X 100 = 99.422%
Positive predictive value = 5/6 X 100 = 83.333%
Negative predictive value = 172/174 X 100 = 98.851%

ELISA
Out of 180 samples that were analysed 174 tested negative, 1 was in the grey zone but had tested negative for SAT (and was considered negative) and 5 SAT positive samples tested positive for ELISA.

<table>
<thead>
<tr>
<th>True disease status</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA Positive</td>
<td>a = 5</td>
<td>b = 2</td>
<td>a + b = 7</td>
</tr>
<tr>
<td>ELISA Negative</td>
<td>c = 2</td>
<td>d = 171</td>
<td>c + d = 173</td>
</tr>
<tr>
<td>total</td>
<td>a + c = 7</td>
<td>b + d = 173</td>
<td>a + b + c + d = 180</td>
</tr>
</tbody>
</table>

Sensitivity = 5/7 X 100 = 71.429%
Specificity = 171/173 X 100 = 98.943%
Positive predictive value = \( \frac{5}{7} \times 100 = 71.429\% \)

Negative predictive value = \( \frac{171}{173} \times 100 = 98.943\% \)
Appendix VIII: Cross tabulations

Cross tabulation of SAT qualitative results versus the Slide Test

<table>
<thead>
<tr>
<th>SAT qualitative results</th>
<th>Slide Test results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>positive</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>negative</td>
<td>157</td>
<td></td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>22</td>
</tr>
</tbody>
</table>

Out of the seven samples that were considered positive one tested negative with the Slide Test while all the other six tested positive.

Cross tabulation of SAT qualitative results versus 2-Me results

<table>
<thead>
<tr>
<th>SAT qualitative results</th>
<th>2-Me results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>172</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>174</td>
<td>2</td>
</tr>
</tbody>
</table>

Out of the seven samples that tested positive by SAT two tested negative by 2-Me and one positive for each of the following dilutions, 1:20; 1:40; 1:80; 1:160 and 1:320. One sample that had tested negative for SAT gave false positive result for 2-Me at 1:20.
Cross tabulation of SAT qualitative results versus ELISA

<table>
<thead>
<tr>
<th>SAT qualitative results</th>
<th>ELISA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Grey zone</td>
</tr>
<tr>
<td>positive</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>negative</td>
<td>172</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>174</td>
<td>1</td>
</tr>
</tbody>
</table>

Two SAT positive samples gave false negative results by ELISA while five gave positive results by both methods.
Appendix IX: Summary of samples that tested positive by any method

<table>
<thead>
<tr>
<th>No</th>
<th>Sample No</th>
<th>Slide</th>
<th>SAT 1:20</th>
<th>SAT 1:40</th>
<th>SAT 1:80</th>
<th>SAT 1:160</th>
<th>SAT 1:320</th>
<th>SAT 1:640</th>
<th>SAT 1:1280</th>
<th>2me 1:20</th>
<th>2me 1:40</th>
<th>2me 1:80</th>
<th>2me 1:160</th>
<th>2me 1:320</th>
<th>2me 1:640</th>
<th>2me 1:1280</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
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</tr>
</tbody>
</table>

Legend + = positive, - = negative, ? = grey zone.