ADLDIS ABABA UNIVERSITY
COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

STUDY ON SEROPREVALENCE OF BOVINE BRUCELLOSIS AND ITS ROLE ON SELECTED REPRODUCTIVE PERFORMANCES OF LOCAL AND CROSS BREED CATTLE IN ALAGE AND ITS SURROUNDINGS

MSc Thesis

BY
HAGOS ASGEDOM

JUNE, 2012
DEBRE ZEIT, ETHIOPIA

STUDY ON SEROPREVALENCE OF BOVINE BRUCELLOSIS AND ITS ROLE ON SELECTED REPRODUCTIVE PERFORMANCES OF LOCAL AND CROSS BREED CATTLE IN ALAGE AND ITS SURROUNDINGS

A thesis submitted to School of Graduate Studies of Addis Ababa University, College of Veterinary Medicine and Agriculture, in partial fulfillment of the requirements for the Degree of Master of Science in Tropical Veterinary Epidemiology

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
AI	Artificial Insemination
am	Anti Meridian
ATVET	Agricultural Technical and Vocational Education Training
C-ELISA	Competitive Enzyme Linked Immune Sorbet Assay
CFT	Complement Fixation Test
^{0}C	Degree Celsius
FAO	Food and Agricultural Organization
FVM	Faculty of Veterinary Medicine
HF	Holstein Frisian
i-ELISA	Indirect Enzyme Linked Immune Sorbent Assay
IgG	Immunoglobulin G
Km	Kilo Meter
µl	Micro Litre
Ml	Millilitre
MRT	Milk Ring Test
NAHDIC	National Animal Health Diagnostic Institutions Centre
NSPC	Number of Service per Conception
OR	Odd Ratio
OIE	Office International des Epizooties
PA	Peasant Association
Pm	Post Meridian
PCR	Polymerase Chain Reaction
RBPT	Rose Bengal Plate Test
SAT	Serum Agglutination test
USA	United States of America
WHO	World Health Organization

ABSTRACT

A cross sectional epidemiological study was conducted between October, 2011 and April, 2012 in Alage and its surroundings. The aim was to determine the seroprevalence of bovine brucellosis, to assess the effect of risk factors on bovine brucellosis and to evaluate the role of brucellosis on selected reproductive parameters of cattle. A total of 804 blood samples (421 from the extensive production system and 383 from the intensive production system) were collected. All cattle above six months of age with no history of previous *Brucella* vaccination were included in the study. Multistage cluster sampling method was used during sample collection. The types of tests used to detect the presence of *Brucella* antibodies were the RBPT as initial screening test and the i-ELISA as a confirmatory test. The overall individual level seroprevalence was found to be 2.4%, and herd level seroprevalence about 37.84%. The seroprevalence of the extensive and the intensive management systems were recorded as 3.3 % and 1.3%, respectively. Significant difference was not observed between the intensive and extensive systems. Among the three sites included in the study area, seropositivity of 3.4% in Naka, 3.3% in Negelewudisha and 1.3% in Alage were recorded. There was no significant difference between bovine brucellosis and the three study sites. Factors such as age, sex, parity, number of service per conception, calving interval and reproductive status were identified as risk and significantly associated with *Brucella* infection. Higher seropositivity was recorded in older age (5.3%), females (3.4%), cattle with 1 parity (6.6%), cows with repeated breeding(34.5%), cows with calving interval above 2 years (33.3%) and pregnant cows (7.4%). Breed and herd size were found not significantly associated with seropositivity of *Brucella* antibody. Other management factors such as examination of cattle before purchasing and awareness to brucellosis were found statistically significantly related to *Brucella* infection. Type of breeding (using bull or AI service) was found not significantly associated with brucellosis.

Keywords: Alage, Brucellosis, Intensive and extensive systems, Reproductive performances, Risk factors, Seroprevalence.

1. INTRODUCTION

Brucellosis is a highly contagious, zoonotic and economically important bacterial disease of animals worldwide and it is considered by the Food and Agriculture Organisation (FAO), the World Health Organisation (WHO) and the Office International des Epizooties (OIE) as one of the most widespread zoonoses in the world (Schelling *et al*, 2003). The disease in cattle, is usually caused by *Brucella* abortus and occasionally by *Brucella* melitensis and *Brucella* suis. It is characterised by late term abortion, infertility and reduced milk production as a result of retained placenta and secondary endometritis. *Brucella* organisms are usually excreted through uterine discharges and milk. Full-term calves may die soon after birth. In fully susceptible herds, abortion rates may vary from 30- 80% (Anonymous, 2007).

In Africa, bovine brucellosis was first recorded in Zimbabwe (1906), Kenya (1914) and in Orange Free State of South Africa in the year 1915 (Chukuwu, 1985). However, still the epidemiology of the disease in livestock and humans as well as appropriate preventive measures are not well understood and such information is inadequate particularly in sub-Saharan Africa. The surveillance and control of brucellosis in this region is rarely implemented outside South Africa (McDermot *et al*., 2002). In dairy production, the disease is a major obstacle to the importation of high yielding breeds and represents a significant constraint to the improvement of milk production through cross breeding (Mustefa and Nicoletti, 1993).

In Ethiopia, there is no documented information on how and when brucellosis was introduced and established. Even though, several serological surveys have showed bovine brucellosis is an endemic and widespread disease in urban, peri-urban, highland and lowland, extensive and intensive farming, smallholder farms and ranches of the country (Dinka and Chala, 2009). However, most of the studies on cattle brucellosis have been carried out in central and northern Ethiopia and do not provide an adequate epidemiological picture of the disease in different agro-ecological zones and livestock production systems of the country (Megersa *et al*., 2011).

The evidences of brucellosis in Ethiopian cattle have been serologically demonstrated by different authors. A high seroprevalence of brucellosis (38.7%) was reported by Rashid (1993) in central high lands of Ethiopia, while a low seoprevalence (0.14%) has recorded in Gonder (Yayeh, 2003) and most of the recent studies suggested a low seroprevalence (below 5%) in cattle under crop-livestock mixed farming (Berhe et *al*., 2007; Ibrahim *et al*., 2010). There is a scarcity of published literature on the status of cattle brucellosis in pastoral areas of the country where large population of cattle are reared. So far, a study carried out in east Showa zone of Ethiopia showed a relatively higher sero-prevalence in pastoral than agro pastoral system (Dinka and Chala, 2009). Similar to this, a high seroprevalence (19.5%) was reported by Yirgu (1991) around the present study area. In parallel with the dynamic nature of the disease to vary over space and time, nothing was studied since Yirgu (1991) study. Another deficiency of his work was that it was limited only to the extensive production system in the area. Hence, the seroprevalence of brucellosis should be studied in the extensive production system to compare with Yirgu (1991) study. The brucellosis status should also be studied in the intensive production system.

Therefore, the objectives of this study were

- ✓ To determine the sero-prevalence of bovine brucellosis in the intensive dairy farm production systems in Alage and in extensive production system surrounding Alage.
- ✓ To evaluate the effect of different risk factors on the sero-prevalence of bovine brucellosis.
- ✓ To estimate the role of bovine brucellosis on selected reproductive performances of local and cross breed of cattle.

2. LITERATURE REVIEW

2. 1. Characteristics of *Brucella* organisms

Brucella species are facultative intracellular, gram negative, non-spore-forming and non-capsulated, partially acid-fast coccobacilli that lack capsules, endospores or native plasmids. They survive freezing and thawing but most disinfectants active against gram-negative bacteria kill *Brucella*. Pasturization effectively kills *Brucella* in milk. The bacterium is of 0.5-0.7μ in diameter and 0.6-1.5μ in length. They are oxidase, catalase and urease positive. Although *Brucella* species are described as non-motile, they carry all the genes except the chemotactic system necessary to assemble a functional flagellum (Fretin *et al.*, 2005). They belong to the alpha-2 subdivision of the Proteobacteria, along with *Ochrobactrum, Rhizobium, Rhodobacter, Agrobacterium, Bartonella, and Rickettsia* (Yanagi and Yamasato, 1993).

Ten genome sequences representing five species of *Brucella* (*B. melitensis, B. suis, B. abortus, B. ovis,* and *B. canis*) are available and about 25 additional *Brucella* strains/species are being sequenced. The genomes of the members of *Brucella* are very similar in size and gene make up (Sriranganathan *et al.*, 2009). Each species within the genus has an average genome size of approximately 3.29Mb and consists of two circular chromosomes, Chromosome I, is approximately on average 2.11 Mb and Chromosome II is approximately1.18 Mb. The G + C content of all *Brucella* genome is 57.2% for Chromosome I and 57.3% for Chromosome II (Halling *et al.*, 2005).

Based on a comparison of 10 published *Brucella* genomes, what is striking are the shared anomalous regions found in both chromosomes consistent with horizontal gene transfer in spite of a predominantly intracellular life style. The *Brucella* have no classic virulence genes encoding capsules, plasmids, pili or exotoxins and compared to other bacterial pathogens relatively little is known about the factors contributing to the persistence in the host and multiplication within phagocytic cells. Also, many aspects of interaction between *Brucella* and its host remain unclear (Seleem *et al.*, 2008).

2.2. Epidemiology of bovine Brucellosis

2. 2. 1. Global distribution of the disease

The geographical distribution of brucellosis is constantly changing, with new foci emerging or re-emerging. New foci of human brucellosis have emerged, particularly in central Asia, while the situation in certain countries of the Middle East is rapidly worsening (Pappas *et al.*, 2006).

The disease occurs worldwide, except countries which include Australia, Canada, Cyprus, Denmark, Finland, Netherlands, New Zealand, Norway, Sweden and the United Kingdom which has eradicated. This is defined as the absence of any reported cases for at least five years. However, the Mediterranean Countries of Europe, Africa, Near East countries, India, Central Asia, Mexico, Central and South America are still not brucellosis free. Although in most countries brucellosis is a nationally notifiable disease and reportable to the local health authority, it is under reported and official numbers constitute only a fraction of true incidence of the disease (Robinson, 2003).

High prevalence (24.5%) was reported from Sudan among African countries as indicated in Table 1. In Ethiopia a high seroprevalence of brucellosis (38.7%) has been reported in central high lands of Ethiopia by Rashid (1993) while most of the studies suggested a low seroprevalence (below 5%) in cattle as summarized in Table 2 below.

Table5. Sero-prevalence of bovine brucellosis in different production systems in some African and Asian countries.

S/No.	Country	No. tested	P	Test applied	Authors and years	Type of system
1	Zambia	1245	14.1	RBPT.ELISA	Muma *et al.*, 1 2007	Extensive
2	Kenya	393	1	c-ELISA	Kangethe et *al.*,2007	Extensive & Intensive.
3	Zimbabwe	1291	5.5	RBPT, ELISA	Matope *et al.*, 2010	Intensive
4	Sudan	574	24.5	RBPT, ELISA	Angara *et al.*, 2010	Intensive
5	Pakistan	200	3.0	MRT, ELISA	Shafee *et al.*, 2011	Intensive
6	Bangladish	188	2.66	RBPT, ELISA	Rahman *et al.*, 2011	Intensive
7	Ghana	224	21.9	MRT, i-ELISA	Mensah *et al.*, 2011	Extensive

Table 2. Seroprevalence of bovine brucellosis in Ethiopia in different geographical areas under different production systems

S/No	Study area	No.ested	prevalence	Type test	Authors and years	Type of system
1	Jimma zone	1,813	0.61	RBPT,CFT	Tolosa, 2004	Extensive & intensive
2	Tigray	1,951	1.49	RBPT,CFT	Berhe, 2007	Extensive & intensive
3	Bahr Dar	1,944	4.63	RBPT,CFT	Hailemelekot, 2005	Extensive & intensive
4	Cent. Orom	1,238	2.99	RBPT,CFT	Jegerfa, 2006	Extensive & intensive
5	AA &Suluta	1,501	1.13	RBPT, SAT	Tefera, 2006	Extensive &intensive
6	Tigray	1,968	4.9	RBPT,CFT	Haileselassie, 2008	Semi-intensive & extensive
7	East Shewa	1,106	11.5	RBPT	Dinka & Chala, 2009	Pastoral & agro-pastoral
8	Sidama zone	1,627	1.66	RBPT,CFT	Asmare *et al.*, 2010	Extensive
9	Jijjiga	435	1.38	RBPT,CFT	Degefu *et al*, 2011	Agro-pastorals
10	South &East Eth	1,623	3.5	RBPT, SAT	Megersa *et al.*, 2011	Extensive

AA----------Adis Ababa
Eth----------Ethiopia
Cent.Orom----------Centeral Oromia

2.2.2. Host range and *Brucella* diversity

The principal strain that infects cattle is *B. abortus*. Cattle can also become transiently infected by *B. suis* and more commonly by *B. melitensis* when they share pasture or facilities with infected pigs, goats and sheep. *B. melitensis and B. suis* can be transmitted by cow's milk and cause a serious public health threat (Acha and Szyfres, 2003). The main etiologic agent of brucellosis in goats is *B. melitensis*. However in certain countries like Brazil where there is no *B. melitensis*, goats can get infected with *B. abortus* (Lilenbaum *et al.*, 2007). Camels can be infected by *B. abortus and B. melitensis* when they are pastured together with infected sheep,goats and cattle. Milk from infected camels represents a major source of infection that is underestimated in the Middle East (Musa et *al.*, 2008). The main etiologic agent for dog brucellosis is *B. canis*, but sporadic cases of brucellosis in dogs caused by *B.abortus, B. suis* and *B. melitensis* have been reported (Acha and Szyfres, 2003).

Nine *Brucella* species are currently recognized, seven of them that affect terrestrial animals are: *B. abortus, B. melitensis, B. suis, B. ovis, B. canis, B. neotomae*, and *B. microti* (Scholz *et al.*, 2008) and two that affect marine mammals are: *B. ceti and B. pinnipedialis* (Foster *et al.*, 2007). The first three species are called classical *Brucella* and within these species, seven biovars are recognized for *B. abortus*, three for *B. melitensis* and five for *B. suis*. The remaining species have not been differentiated in to biovars. The strains of *Brucella* were named based on the host animal preferentially infected (Verger *et al.*, 1997).

2. 2. 3. Sources of infection and modes of transmission

In humans, consumption of dairy products (especially raw milk, soft cheese, butter and ice cream) is the most important source of infection. It is also possible for raw vegetables and water contaminated through excreta and sometimes consumption of under cooked animal muscle /tissue though the bacterial load is very low (Radostits *et al.*, 2000). Transmission by contact predominates in enzootic areas. Man become infected by handling infected tissues of animals, by close contact with other infected materials, presumably the *Brucella* enter through skin abrasions

or possibly through the intact skin, trans-conjunctival and air born transmission are also indicated. Therefore, human brucellosis is an occupational disease of stockyard, slaughter house workers, bluchers and veterinarians (Walker, 1999).

In animals, the concentration of the bacteria is highest in pregnant uterus. The aborted fetus, placental membranes or fluids, and other uterine discharges are considered as major source of infection. Infected animals also shade organisms in milk which serve as source of infection for the new born. Contaminated feed can spread the infection from infected pasture over long distance during purchasing and selling activities. The disease is transmitted to susceptible animals by ingestion of contaminated feed and water, contact with aborted fetuses, fetal membrane and uterine discharges; infection by inhalation is also possible. The use of infected bull for artificial insemination also poses an important risk and spreads the infection to many herds (Acha and Szyfers, 2001).

2. 3. Pathogenesis and Clinical Signs

2. 3. 1. Pathogenesis

Invading *Brucella* usually localize in the lymph nodes, draining the invasion site, resulting in hyperplasia of lymphoid and reticulo-endothelial tissue and the infiltration of inflammatory cells. Survival of the first line of defense by the bacteria results in local infection and the escape of *Brucella* from the lymph nodes in to the blood. During bacteraemic phase, bones, joints, eyes and brain can be infected, but the bacteria are most frequently isolated from supra-mammary lymph nodes, milk, iliac lymph nodes, spleen and uterus. In bulls, the predilection sites for infection are also the reproductive organs and the associated lymph nodes. During the acute phase of infection, the semen contains large number of *Brucella* but as the infection becomes chronic, the number of *Brucella* excreted decreases. However, it may also continue to be excreted for years or just become intermittent (Acha and Szyfres, 2001).

After the *Brucella* organisms spread through the hematogenous route in females then also reach the placenta and finally to the fetus. The preferential localization to the reproductive tract of the pregnant animal is due to the presence of the allantoic fluid factors that would stimulate the growth of *Brucella*. Erythritol (four-carbon alcohol) is considered to be one of the factors, which are elevated in the placenta and fetal fluid from about the fifth month of gestation. An initial localization within erythrophagostic trophoblsates of the placentome adjacent to chorio-allntoic membrane results in rupture of the cells and ulceration of the membrane. The damage to placental tissue together with foetal infection and foetal stress inducing maternal hormonal changes may cause abortion (Radostits *et al*., 2000).

2. 3. 2. Clinical signs

The primary clinical manifestation of brucellosis in animals is related to the reproductive tract. The most obvious signs in pregnant animals are abortion (usually late abortion), birth of weak calves, lowering of fertility with poor conception rates, retained fetal membrane, endometritis and reduced milk yield. Orchitis and epididymitis are typical signs in males, and hygroma is usually common during chronic infection (Corbel, 1997).

2. 4. Diagnosis and its Limitations

Clinician must develop a high degree of clinical suspicion based on epidemiological information and history which are critical to making the clinical diagnosis. In all cases a blood sample should be collected from the patient and laboratory testing should be requested as the definite diagnosis of brucellosis is impossible without laboratory confirmation (Young, 1995). A proper and prompt diagnosis is important as the treatment requires specific and prolonged antibiotics. Laboratory tools include isolation and identification of *Brucella* from clinical samples, detection of antigen, demonstration of genome and demonstration of *Brucella* specific antibodies (Solara *et al*., 1997).

2.4.1. Isolation and identification of *Brucella* species

Blood culture provides definite proof of brucellosis but may not provide a positive result for all patients even under ideal conditions (Colmenero *et al.*, 1997). *Brucella* are relatively slow growing and the culture result may not become available for several days or weeks. They also need special media with carboxyphlic environment. In particular for patients with chronic disease, the sensitivity of culture can be low. Even though blood culture is the method of choice for isolation of the causative agent, specimens need to be obtained early prior to antibiotic administration and need prolonged periods of incubation. In addition, failure to detect the pathogen is a frequent occurrence. The modern automated blood culture systems have somewhat improved the speed of detection but are still too slow to make a rapid diagnosis (Bannatyne *et al.*, 1997). Bone marrow cultures are considered the golden standard for the diagnosis of brucellosis, since the relatively high concentration of *Brucella* in the reticulo-endothelial system makes it easier to detect the organism. Furthermore, bacterial elimination from the bone marrow is equivalent to microbial eradication. However, in some studies results have not been universally reproducible, suggesting that the bacteraemia is as unpredictable as clinical manifestations especially in human brucellosis (Shehabi *et al.*, 1990).

Identification of *Brucella* strains is done using standard classification tests, including Gram stain, a modified Ziehl-Neelsen (ZN) stain, growth characteristics, oxidase activity, urease activity, H_2S production (four days), dye tolerance such as basic fuchsin (1: 50000 and 1: 100000) and thionin (1:25000, 1:50000 and 1:100000) and seroagglutination. It has been also recommended that Gram stain morphology and modified ZN staining, coupled with the urease test, for rapid identification of *Brucella* to the level of genus where facilities for further identification are not available. Laboratory detection of *Brucella* and species identification is based largely on culture isolation and phenotypic characterization. This process is lengthy and labour-intensive and has been associated with a heightened risk of laboratory-acquired infections. To surmount these problems, nucleic acid amplification has been explored for the rapid detection and confirmation (Mantur *et al.*, 2006).

2. 4. 2. Antigenic and genomic detections

Enzyme linked immune sorbent assay (ELISA): Still now, there is only one report (Al-Shamahy and Wright, 1998) suggesting antigen detection by enzyme-linked immunosorbent assay (ELISA) as an acceptable alternative to blood culture for the diagnosis of brucellosis since sensitivity and specificity are 100% and 99.2% respectively. Antigen detection methods are potentially useful but have not been validated. Though co-agglutination has been reported as a technique for antigen detection, there seems to be paucity of published literature.

Polymerase chain reaction (PCR): is fast and can be performed on any clinical specimen. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, the 16S-23S intergenic spacer region, omp2 and bcsp31 (Navarro *et al.*, 2002) .Recently, Redkar *et al.* (2001) described real-time PCR assays for the detection of *B. abortus*, *B. melitensis* and *B. suis* biovar1. These PCR assays target the specific integration of IS711 elements within the genome of the respective *Brucella* species or biovar. Currently, a real-time multiplex PCR assay has been developed for rapid confirmatory identification of *Brucella* with speciation. The genus, *B. abortus* and *B. melitensis* specific primers confirm the organism from isolates. Although in the last few years, PCR is very promising and PCR-based laboratory tests have been proposed, they cannot be considered a routine diagnostic method yet since standardization of extraction methods, infrastructure, equipment and expertise are lacking and a better understanding of the clinical significance of the results is still needed (Navarro *et al.*, 2004).

Molecular characterization techniques are also very useful tools for differentiating *Brucella* spp. especially follow-up testing of unusual phenotypic results of *Brucella* isolates. The use of molecular methods in *Brucella* endemic areas needs to be explored before they can be applied in these areas to diagnose brucellosis.

2. 4. 3. Serologic tests

The above limitations make serology for antibody detection the most useful tool for the laboratory diagnosis of brucellosis. Antibodies usually begin to appear in the blood at the end of the first week of the disease, IgM appearing first followed by IgG. The serological tests like Rose Bengal Plate Agglutination Test (RBPT), Standard tube agglutination test (SAT), Coombs test, Immune capture agglutination test, Complement fixation test, ELISA, Lateral flow assay-a simplified version of ELISA, Milk ring test (MRG) are commonly used tests in the diagnosis of brucellosis (Lucero *et al.*, 2003).

Rose bengal plate test (RBPT): is often used as a rapid screening test. The sensitivity is very high (>99%) but the specificity is disappointingly as low as 68.8% (Barrsol *et al.*, 2002). However, this is of value as a screening test in high risk rural areas where it is not always possible to perform the tube agglutination titration test. To increase the specificity and the positive predictive value of the RBPT, the test may be applied to a serial dilution (1:2 through 1:64) of the serum sample. The specificity of the test increases when higher dilutions agglutinate and titers of 1:8 or 1:16 and above may be regarded as positive. This approach may result in a lower sensitivity. Whenever possible, a serum that gives a positive result should be confirmed by a more specific test. The RBPT is also of value in the rapid confirmation of neurobrucellosis, arthritis, epididymo-orchitis, hydrocele due to *Brucella* if the neat is positive in CSF, synovial fluid, testicular fluid /semen and hydrocele fluid respectively (Manture *et al.*, 2006).

Complement fixation test (CFT): Detects specific antibodies of the IgM and IgG1 type that fix complement. The CFT is highly specific but it is laborious and requires highly trained personnel as well as suitable laboratory facilities that makes less suitable for use in developing countries. Although its specificity is very important for the control and eradication of brucellosis, it may test false negative when antibodies of the IgG2 type hinder complement fixation. The CFT measures more antibodies of the IgG1 than antibodies of the IgM type, as the latter are partially destroyed during inactivation Since antibodies of the IgG1 type usually appear after antibodies of the IgM type, control and surveillance for brucellosis is best done by CFT (Buchanan and Faber, 1980).

Serum aglutination test (SAT): Developed by Wright and colleagues remains the most popular and yet used worldwide diagnostic tool for the diagnosis of brucellosis because it is easy to perform, does not need expensive equipments and training. SAT measures the total quantity of agglutinating antibodies IgM and IgG (Young, 1991). The quantity of specific IgG is determined by treatment of the serum with 0.05M 2-mercaptoethanol (2ME), which inactivates the agglutinability of IgM. SAT titers above 1:160 are considered diagnostic in conjunction with a compatible clinical presentation. However, in areas of endemic disease, using a titer of 1:320 as cut off may make the test more specific. The differentiation in the type of antibody is also important, as IgG antibodies are considered a better indicator of active infection than IgM and the rapid fall in the level of IgG antibodies is said to be prognostic of successful therapy (Buchanan and Faber, 1980).

Drawbacks of the serum agglutination test include the inability to diagnose *B. canis* infections; the appearance of cross-reactions of class M immunoglobulins with Francisella tularensis, *Escherichia coli* O116 and O157, *Salmonella urbana, Yersinia enterocolitica* O:9, *Vibrio cholerae, Xanthomonas maltophilia,* and *Afipia clevelandensis*; and the percentage of cases in which seroconversion does not occur. Lack of seroconversion can be attributed to the performance of tests early in the course of infection, the presence of blocking antibodies, or the so-called "prozone" phenomenon (i.e., the inhibition of agglutination at low dilutions due to an excess of antibodies or to nonspecific serum factors). Some of these shortcomings can be overcome by modifications such as the addition of EDTA, 2-mercaptoethanol, or antihuman globulin (Young, 1991).

Indirect enzyme-linked immune sorbent assay (i-ELISA): Typically uses cytoplasmic proteins as antigens. ELISA measures IgM, IgG and IgA, which allows for a better interpretation of the clinical situation. A comparison with the SAT, ELISA yields higher sensitivity (97.5%-100%) and specificity (99.7%-99.8%). ELISA is also reported to be the most sensitive test for the diagnosis of central nervous system brucellosis. Among the newer serologic tests, the ELISA appears to be the most sensitive; however, more experience is needed before it replaces the SAT as the test of choice for brucellosis (Almunneef and Memish, 2003).

The rapid and simple assays like *Brucella* IgM and IgG lateral flow and latex agglutination have been developed recently. The sensitivity and specificity of lateral flow assay for culture confirmed brucellosis is >95%. The sensitivity of the latex agglutination assay was determined to be 89.1% for the initial serum samples collected for the patients with culture confirmed brucellosis and the specificity was 98.2%. Both these tests are ideal for use as field tests in remote areas and as point of care tests in hospitals and health care centers that lack the expertise and facilities to perform the more demanding classic serologic tests (Abdoel and Smits, 2007).

Generally, all told, antibody profiles do not have specific clinical correlations, and titers often remain high for a protracted period. The asymptomatic patient with an isolated positive titer of class G and A immunoglobulins, or A immunoglobulin only, has not been adequately studied. Variations of ELISA exist, such as competitive ELISA and sandwich ELISA, which may prove useful as a follow-up tool (Ariza, 1992).

2. 5. Treatment

Due to the intracellular localization of *Brucella* and its ability to adapt to the environmental conditions encountered in its replicative niche e.g. macrophage (Seleem *et al.*, 2008), treatment of domestic animals with antibiotics is not usually successful. Even though, treatment failure and relapse rates are also high in humans, treatment depend on the drug combination of doxycycline with streptomycin which is currently the best therapeutic option with less side effects and less relapses, especially in cases of acute and localized forms of brucellosis (Seleem *et al.*, 2009). Neither streptomycin nor doxycycline alone can prevent multiplication of intracellular *Brucella* (Shasha *et al.*, 1994). Although the doxycycline-streptomycine regimen is considered as the golden standard treatment, it is less practical because the streptomycin must be administered parentrally for 3 weeks. A combination of doxycycline treatment (6 weeks duration) with parentrally administered gentamicin (5 mg/kg) for 7 days is also considered an acceptable alternate regimen (Glynn and Lynn, 2008).

2. 6. Prevention and Control Measures

The initial aim of surveillance and control programs is the reduction of infection in the animal populations to reduce the effect of the disease on animal health and production, thus minimizing its impact on human health. An effective control of animal brucellosis requires the following elements: 1) surveillance to identify infected animal herds, 2) prevention of transmission to non-infected animal herds, and 3) eradication of the reservoir to eliminate the sources of infection in order to protect vulnerable animals or herds coupled with measures to prevent re-introduction of the disease (Gwida *et al.*, 2010).

In areas where a brucellosis free status has been established or where such a status is assumed from epidemiological data, the risk of importing the disease by means of animal movement must be protected. Movement of infected animals must be prohibited and import permissions should be given only to certified brucellosis-free farms or areas. This is also true for national and international transport of animal products, in accordance with the general principles and procedures specified in the International Zoo-Sanitary Code of the OIE. This code also describes the testing procedures for animals and quarantine measures (OIE, 2009).

Vaccines

In regions with high prevalence of the disease, the only way of controlling and eradicating this zoonosis is by vaccination of all susceptible hosts and elimination of infected animals (Briones *et al.*, 2001). The most commonly used vaccines against bovine brucellosis are *B. abortus* strain 19 and the recently approved strain RB51; the latter unlike strain 19 does not interfere with serological diagnoses (Moriyon *et al.*, 2004). *Brucella melitensis* strain Rev1 although highly infectious to human, is considered as the best vaccine available for the control of ovine and caprine brucellosis, especially when administrated at the standard dose by the conjunctival route. However, the Rev1 vaccine shows a considerable degree of virulence and induces abortions when administered during pregnancy. Also, the antibody response to vaccination cannot be differentiated from the one observed after field infection. The *B. melitensis* Rev1 vaccine for small ruminants has not been fully evaluated for use in cattle. *Brucella abortus* vaccines do not

effectively protect against *B. melitensis* infection, meaning that bovine *B. melitensis* infections may pose a serious problem even for vaccinated cattle. Vaccination alone will not eradicate *Brucella* as the immunity produced by *Brucella* vaccine is not absolute. It is obvious, therefore, that a policy of vaccination is more likely to succeed if combined with good measures of husbandry (Morgan, 1969).

2. 7. Public Health and Economic Importance

2.7.1. Public health Importance

Brucellosis (especially *B. melitensis*), remains one of the most common zoonotic diseases worldwide with more than 50,000 human cases reported annually (Gwida *et al*, 2010). The significance of brucellosis as zonootis has ever increased in recent times, due to the expansion of international commerce in animals and animal products, with increase urbanization, intensive farms and animal products, having nomadic animal husbandry (Bayleyegn, 2007). Despite the advances made in surveillance and control, the prevalence of brucellosis is increasing in many developing countries due to various sanitary, socioeconomic, and political factors (Pappas *et al*., 2006).

Transmission of brucellosis to humans occurs mainly through the consumption of unpasteurized dairy products especially raw milk, soft cheese, butter, and ice cream, through direct contact with infected animal parts (such as the placenta by inoculation through ruptures of skin and mucous membranes), and through the inhalation of infected aerosolized particles. Brucellosis is an occupational disease in shepherds, abattoir workers, veterinarians, dairy-industry professionals, and personnel in microbiologic laboratories. However, consumption of hard cheese, yogurt, and sour milk are less hazardous, since both propionic and lactic fermentation takes place. Bacterial load in animal muscle tissues is low, but consumption of undercooked traditional delicacies such as liver and spleen has been implicated in human infection (Radostits, *et al*., 2000).

Air borne transmission of brucellosis has been studied in the context of using *Brucella* as a biologic weapon. In fact, *B. suis* was the first agent contemplated by the U.S. Army as a potential biologic weapon and is still considered in that category. In a hypothetical attack scenario, it was estimated that release of an aerosolized form of *Brucella* under optimal circumstances for dispersion would cause 82,500 cases of brucellosis and 413 fatalities. Cases of laboratory-acquired brucellosis are the perfect examples of airborne spreading of the disease (Ergonul, 2004).

Most common symptoms of brucellosis include undulant fever in which the temperature can vary from 37^0C in the morning to 40^0C in the afternoon; night sweats with peculiar odor, chills and weakness, insomnia, anorexia, headache, arthralgia, constipation, sexual impotence, nervousness and depression (Acha, 2003). Human brucellosis is also known for complications and involvement of internal organs and its symptoms can be very diverse depending on the site of infection and include encephalitis, meningitis, spondylitis, arthritis, endocarditis, orchitis, and prostatitis. Spontaneous abortions, mostly in the first and second trimesters of pregnancy, are seen in pregnant women infected with *Brucella* (Khan *et al.*, 2001).

Symptoms and signs of brucellosis usually referred as fever of unknown origin can confuse with other diseases including enteric fever, malaria, rheumatic fever, tuberculosis, cholecystitis, thrombophlebitis, fungal infection, autoimmune disease and tumors (Mantur *et al.*, 2007). Because of these rather non-specific signs, brucellosis is constantly mis-diagnosed as malaria, which is very prevalent in sub Saharan Africa (Maichomo *et al.*, 2009). Direct person-to-person spread of brucellosis is extremely rare. Mothers who are breast-feeding may transmit the infection to their infants and sexual transmission has also been reported (Kato *et al.*, 2007).

2.7.2. Economic importance

Brucellosis causes heavy economic losses in livestock producers. The economic losses arises from abortion, reduced milk production, losses of calves due to abortion and still birth, culling of infected cows, hindering animal export trade of a nation, losses of man-hours, medical costs and government costs incurred for research and eradication program (Georgios *et al.*, 2005).

Although estimates of the costs associated with brucellosis infections remain limited to specific countries, all data suggest that worldwide economic losses due to brucellosis are extensive not only in animal production (culling infected animal, reduced milk, abortion and delayed conception), but also in public health (cost of treatment and productivity loss). For example, official estimates put annual losses due to bovine brucellosis in Latin America at approximately $600 million. Although brucellosis eradication programs can be very expensive, they are estimated to save $7 for each $1 spent on eradication. The USA national brucellosis eradication program, while costing $3.5 billion between 1934 and 1997, the cost of reduced milk production and abortion in 1952 alone was $400 million (Sriranganathan *et al.*, 2009).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The present study was conducted in Alage Agricultural Technical and Vocational Education Training (ATVET) College of the Ministry of Agriculture of the Federal Democratic Republic of Ethiopia. The College is situated at 217 km South West of Addis Ababa in the vicinity of Abejata and Shalla lakes, at a distance of 32 kms from Bulbula town to a West direction. Its absolute location is at a longitude of 38° 30' East and a latitude of 7^{0} 30' North. It rests on 105 'gasha'(4200 hectares) of land with an altitude of 1600 meter above sea level (Shimelis, 2009). The area is characterized by sub tropical climate with minimum and maximum temperature ranging from 11 to 29 ^{0}C.The area experiences bimodal rain fall distribution with annual average of 700 and 900mm.The three defined seasons based on rain fail distribution are short rainy season (March to April),long rainy season (June to September), long dry season (October to January). The dominant soil type is black clay soil (verity soil) with a PH of 7.9. The area is suitable for live stock production. The current total cattle population in different farms of the college is about 465.

The study also area includes two peasant associations namely, Negelewudisha and Naka which are found in the surrounding of Alage ATVT College. Negelewudisha is located south west of Alage under the district of Alaba, Southern Nation and Nationalities People Region (SNNPR) where as Naka is situated to north east of Alage which belongs to 'Judo-kombolicha' district, Oromia Regional state. These two peasant associations have high cattle population compared to other peasant associations in the study area. The total cattle population in both peasant associations can reach up to 7550, according to the information obtained from animal health department office of both peasant associations.

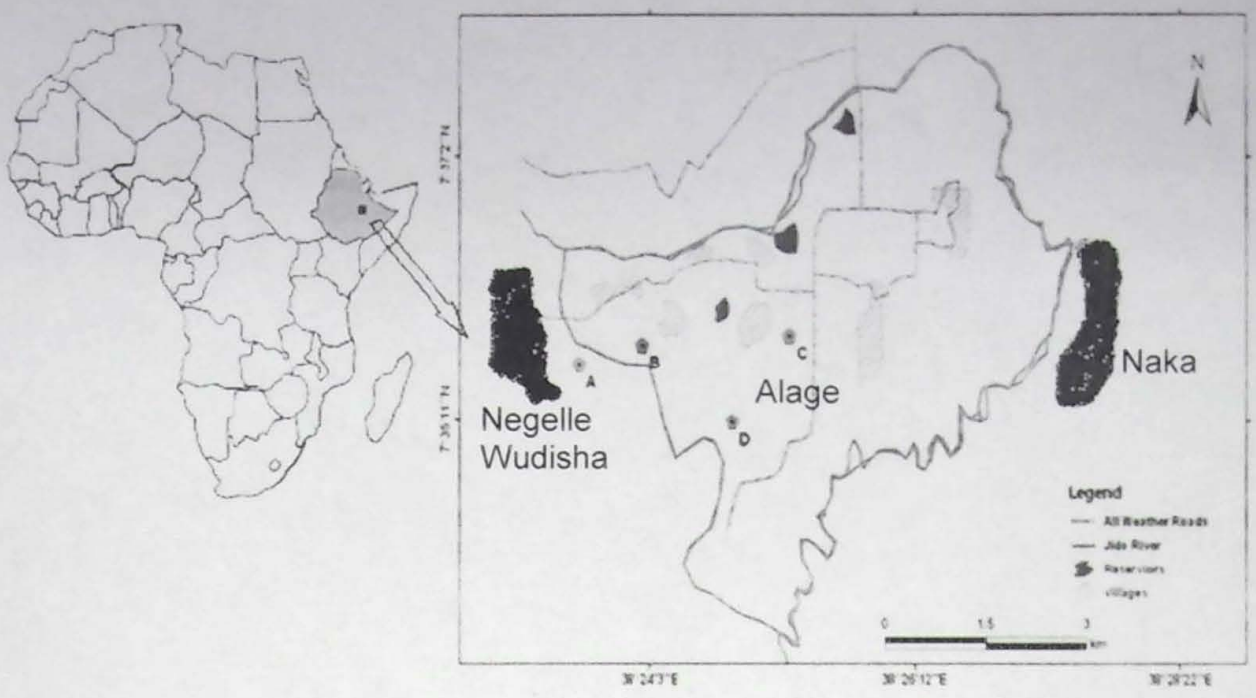

Figure 1: Map of Alage area and location of study sites (A= wheat farm, B= bush land, C= Acacia woodland, D= maize farm) (Addisu, 2007).

3.2. Study Animals

The target population of the present study was cattle including the Borans and Holstein cross breeds which are found in Alage ATVET College intensive farm and the Arsi type of cattle which are found in Negelewudisha and Naka Peasant Association extensive production system. Cattle above six months of age including milking cows, none milking cows, replacement heifers and bulls with no history of *Brucella* vaccination were included in the study.

Husbandry and Management Practices of the Intensive and Extensive farms

The intensive production system contains the Borans and the Holstein cross breeds. The purpose of keeping of the Holstein cross breeds are mainly for milk production. This dairy farm was established in 1983 by introducing 300 Holstein heifers from Stella and Holotta private dairy farms. Currently there are about 200 cattle in the dairy farm, out of which 112 are cows but the

rest are heifers and calves. The feeding and the husbandry practices of the dairy farm are intensive. Every morning cows are allowed to exercise around the exercising yard. The farm practices AI service for breeding. Milking activities are done twice a day at 5 am and at 3 pm. Besides the Holstein, the intensive farm contains Boran breeds with a total herd size of 165, out of which 85 are cows and the rest are heifers, calves and bulls. Boran breeds are mainly kept for the demonstration of the practical sections of the College and for fattening practices. In this breed only bull service is used for breeding. Generally, the management and the feeding systems practiced in Boran breeds are intensive but sometimes animals are allowed to graze within the compound of the College.

In the extensive production system, the Arsi types of cattle are the dominant once. Cattle are kept under traditional husbandry practices and the average herd size in those Peasant Associations can reach up to 25 heads of cattle. During dry season usually there is feed and water scarcity in the area. For this reason animals fled to another area including into the College for searching of feed and water. As a result animals from different sites are met in the watering points and seasonal grazing areas. Moreover, farmers in the area don't have the habit of harvesting animal feeds for the dry season that minimizes the problems of feed shortage which is commonly occurred mainly during long dry season. Bull service is the only breeding practice used in this extensive farm system.

3.3. Study Design

A cross sectional epidemiological study was conducted from October 2011 to April 2012 to determine the seroprevalence of bovine brucellosis and the effect of some associated risk factors like age, sex, breed and herd size. In addition a questionnaire Survey was also performed to evaluate the role of bovine brucellosis on selected reproductive performances such as calving interval, parity, number of service per conception and reproductive status of cattle in the study area. The questionnaire survey also contained some management risk factors such as awareness to brucellosis, examination of cattle before purchasing and type of breeding used in the farms.

The hypothesis of this study was, the prevalence of bovine brucellosis would be different between the intensive and the extensive production systems as well as among the different breeds. A higher prevalence was expected from the extensive farm based on the work of Yirgu who reported a prevalence of 19.5% before 21 years ago. However, there was no previous work in the intensive production system.

3.4. Sample Size and Sampling Procedures

The total number of cattle sampled from the two different production systems was 804.

(1) 383 from the intensive production system of Alage farm. All cattle greater than six months of age were conveniently sampled. The sample size for this intensive system was determined according to Thrusfield (2005) using the formula:

$$n = \frac{1.96^2 x pexp(1-pexp)}{d^2}$$

Where, n= sample size estimate for cattle from the intensive system.

α=1.96 (at α=0.05 or 95% confidence interval).

P=expected prevalence (50%)

d=desired absolute precision (5%)

$$n = \frac{(1.96)^2 x 0.5(1-0.5)}{(0.05)^2} = 384$$

Since there was no previous study conducted on this farm, 50% expected prevalence was used in the formula.

(2) From the extensive system (surrounding Alage) two peasant associations that have proximity to the Alage ATVET college were purposively selected. The sample size for these two peasant associations was also determined according to Thrusfield (2005) using the formula:

$$n = \frac{1.96^2 x pexp(1-pexp)}{d^2}$$

Where, n= sample size estimate for cattle from extensive system.

α=1.96 (at α=0.05 or 95% confidence interval).

P=expected prevalence (19.5%)

d=desired absolute precision (5%)

$$n=\frac{(1.96)^2 x 0.1951(1-0.195)}{(0.05)^2}=241$$

However, 19.5% expected prevalence was used in this formula, since there was a study carried out in Abernosa Cattle Breeding Ranch with reported prevalence of 19.5% by Yirgu (1991).

To maximize the precision, about 421 cattle were sampled from this extensive production system. The sampling method used in this system was multi-stage cluster sampling method. The peasant associations and herds were the established clusters.

- Two PA which have proximity to Alage ATVET college were purposively selected
- Each PA was divided in to four sites to cover the whole PA during sampling.
- Name of the farmers with their respective herd size was listed in each site of the PA
- Herd of cattle was categorized in to two groups based on the prevailing herd size in the study area as small (≤20) and large (>20), then herds (from small and large) were selected by simple randomization method from each site.
- Finally, half of the number of herd size with > 6 months of age were sampled from each small and large herds using simple randomization technique.

Based on this, a total of 421cattle from 18 small herds and 19 large herds were sampled in both peasant associations.

3.5. Collection of Blood Samples

Approximately 10ml of blood was taken aseptically from the jugular vein of each animal using plain vaccutainer tubes and needles. The identities of each animal were labeled on each corresponding vaccutainer tube. The tubes were kept tilted over night at room temperature to allow clotting. On the following day, serum was poured from the vaccutainer tube in to cryovial tubes by siphoning and this was labeled similarly as that of the original samples. Finally, serum samples were kept at -20^{0}C in Alage ATVET animal health laboratory until serological tests were conducted in National Animal Health Diagnostic Institution Centre (NAHDIC), Sebeta. Samples were transported to Sebeta National Animal Health Diagnostic Institution Centre using icebox.

3.6. Serology

3.6.1. Rose Bengal Plate Test (RBPT)

The sensitivity of the RBPT is very high which is >99% but the specificity is disappointingly as low as 68.8% (Barrsol *et al.*, 2002). The Rose Bengal Plate Test (RBPT) was conducted in Sebeta National Animal Health Diagnostic Institution Centre (NAHDIC) for screening samples. The RBPT procedure was followed based on the description by Nielsen and Dunkean (1990). The sera and antigen were taken out from the refrigerator and left at room temperature for at least 30 minutes to thaw before the test was done, then 30 µl of test sera was dispensed on each of the 12 cups of the plate. The antigen containing bottle was gently shaken and a drop of RBPT antigen (30 µl) was placed alongside the serum. Negative and positive controls were included in the remaining two cups of the plate. The antigen and the serum were mixed thoroughly using applicator stick and the plate was rocked manually for about four minutes. Finally, results were read in a good light source. Reactions were categorized as 0, +, ++, +++, according to Nielsen and Dunkan (1990), where: 0= no agglutination, + = barely perceptible agglutination (using magnifying glass), ++ = fine agglutination, some clearing and +++ = clumping, definite clearing. Samples identified with no agglutination (0) are regarded as negative, while those with +, ++, and +++, are regarded as positive.

3.6.2. Indirect Enzyme Linked Immuno Sorbant Assay (i-ELISA)

As pointed out by Almunneef and Memish (2003), the i-ELISA appears the most sensitive test (97.5%-100%) with higher specificity (99.7%-99.8%). Confirmation of RBPT positive sera was done by i-ELISA at Sebeta National Animal Health Diagnostic and Investigation Center (NAHDIC). This kit detects anti *Brucella* lipopolisachride antibodies in bovine sera. The i-ELISA test was performed according to the manufacture's manual. Preparation of the sample was done by diluting the sample at 1:10 in sample diluents in a dilution plate. 10 µl of diluted sample was dispensed into 90 µl of sample diluents in the well. After the wells were covered with adhesive film, the plate was incubated at 37 ^{0}c for 1hour. The adhesive film was carefully removed and the plates were washed four times with washing solution. 100 µl of the diluted conjugate (1: 200 in

conjugate diluents) was added to all wells, covered with new piece of adhesive film and then incubated at 37 ^{0}c for 30 minutes. The adhesive film was then removed again and the plates were washed four times. 100 µl of buffered peroxidase substrate was added into each well and mixed by plate agitator to ensure correct homogenization. After incubation at room temperature, shielded from light, 50 µl of stop solution was added and mixed with plate agitator. The sample was then put in to the ELISA reader. The results were read by printing from the computer connected to the ELISA reader. Finally, samples were considered as positive for brucellosis, if they are positive for both RBPT and ELISA tests.

3.7. Data Collection

Questionnaire survey

The questionnaire survey was designed to get information from all herd owners and dairy workers regarding the animal and farm conditions. Data was collected on individual animal bases during blood collection. During blood collection, data related to breed, sex, age, parity number, reproductive statue of the animal, calving intervals and number of service per conception was collected. Furthermore, data regarding the farm management such as type of breeding used (AI or bull service), awareness about brucellosis, examination before purchasing and herd size were collected. Age was stratified into three categories as 0.6 to 3 years, 3 to 6, and > 6years. Herd size was categorized in to two as small having ≤ 20 cattle and large >20 cattle. Similarly, parity number was also categorized in to three: no calved, calved once and with two or more calving. The reproductive status of the cow was also categorized as heifer, pregnant, lactating and dry.

3.8. Data Analysis

Data was entered in to Microsoft (MS) Excel Spread Sheet program. Statistical analysis was computed using cross tabulation for the Chi-square test to compare prevalence differences using SPSS version 16. The total prevalence was calculated by dividing the number of both RBPT and

ELISA positive animals by the total number of animals that were tested in the study period. Herd level seroprevalence was calculated by dividing the number of herds with at least one reactor in both RBPT and ELISA tests by the number of all herds tested (Thrusfield, 1995). Logistic regression by SPSS version 16 was used to determine odds ratio (OR) to measure the degree of association between risk factors and bovine brucellosis. The association between risk factors and seropositivity to *Brucella* infection was considered as significant when $P < 0.05$ at 95% confidence interval.

4. RESULTS

4.1. Seroprevalence of Bovine Brucellosis in the Study Area

In the present study, a total of 804 cattle sera were collected from 37 herds which were not vaccinated against bovine brucellosis before. Out of 804 samples collected, nineteen (19) sera were found to be positive for bovine brucellosis with both RBPT and ELISA tests. An overall individual animal seroprevalence of 2.4% (19 0f 804) and herd level seroprevalence of 45.9% (17 of 37) were obtained.

A relatively higher individual seroprevalence was recorded in the extensive production system (3.3%) compared to that of the intensive production system (1.3%). However, it was not statistically significant (Table 3). On the contrary, very high herd level seroprevalence (75%) was observed in the intensive production system whereas the herd level serporevalence in the extensive production system were found to be 42.4% (Table 4). Nevertheless, the intensive farm was observed as a risk factor, it was not statistically significant. Among the three sites in the study area, a relatively low individual seroprevalence (1.3%) was found in Alage farms. However, the individual level seroprevalence of both Naka (3.4%) and Negelewudisha (3.3%) Peasant Associations was almost nearly the same. The odd ratio indicated that animals living in Naka and Neglewudisha were relatively at risk in reference to animals living in Alage. But this risk was not statistically significant ($p>0.05$) (Table 5).

Table 3. Individual level seroprevalence of bovine brucellosis in the intensive and extensive farms of the study area assessed by Chi-square.

Factor	Level	No.exam	Test +ve	P	OR	95% CI	χ^2	P-value
Farm type	Intensive	383	5	1.3	-	-	-	-
	extensive	421	14	3.3	2.60	0.93-7.29	3.54	0.06

*P ----------Prevalence

Table 4. Herd level seroprevalence of bovine brucellosis in the intensive and extensive farms of the study area assessed by Chi-square.

Factor	Level	No.exam	Test+ve	P	OR	95% CI	χ^2	P-value
Farm type	Extensive	33	14	42.4	1	-	-	-
	Intensive	4	3	75	4.07	0.38-43.38	1.52	0.22

P ----------Prevalence

Table 5. Individual seroprevalence of bovine brucellosis in three different sites in the study area assessed by logistic regression.

Factor	Level	No.exam.	Test +ve	P	OR	95% CI	P-value
Sites	Alage*	383	5	1.3	1	-	-
	N/wudisha	246	8	3.3	2.54	0.82-7.86	0.105
	Naka	175	6	3.4	2.68	0.81-8.92	0.107

*: others were computed in reference to this category

P ----------Prevalence

4.1. Risk factors effect on bovine brucellosis in the study area

Age: There was a low serprevalence (0.4%) of bovine brucellosis in animals with less than or equal to three years old. However, 5.3% seroprevalence was observed in animals with greater /equal to seven years old. The logistic regression result also showed that older animals were 11.376 times at risk than younger animals. Hence, age was observed to be statistically significant risk factor for brucellosis by taking cattle with ≤3 years old as a reference (Table 6).

Sex: Based on the result of the ELISA test, 18 (3.4%) female and 1 male (0.4%) positive reactors were detected. The odds ratio assessment for sex indicated that females were 9.36 times at risk

than males, meaning being male was strongly protective from brucellosis. The p-value showed the presence of significant difference between sex and brucellosis (Table 6).

Breed: Even though the seropositivity to *Brucella* antibody was relatively higher (3.2%) in Arsi type of cattle than the Boran (1.3%) and the Holstein (0.7%) breeds, the odds ratio analysis did not express breed as strong correlated factor for brucellosis and the P-value found not to be statistically significant (Table 6).

Herd size: In this study, 13 (2.6%) positive reactors from large herds and 6 (2.3%) from small herds were detected. Nevertheless, the individual level seroprevalence was found a little bit higher in large herd size compared to that of small herd size. Herd size had no statistically significant difference ($p>0.05$) on the effect of brucellosis (Table 6).

Table 6. The effect of some risk factors on bovine brucellosis in the study area assessed by logistic regression.

Factors	Level	No.exam.	Test +ve	P	OR	95% CI	P-value
Age	0.7≤3*	233	1	0.4	1	-	-
	3≤6	308	4	1.3	3.2262	28.32-30.53	0.299
	≥7	263	14	5.3	11.376	90.33-85.47	0.0198
Sex	M*	275	1	0.4	1	-	-
	F	529	18	3.4	9.36	0.014-0.78	0.007
Breed	Arsi *	494	16	3.2	1	-	-
	HF	149	1	0.7	0.1724	0.302-3.75	0.1641
	Boran	161	2	1.2	0.4001	2.02-4.15	0.3914
Herd size	≤20*	232	6	2.3	1	-	-
	>20	572	13	2.6	1.6378	4.30-5.28	0.7199

*: others were computed in reference to this category.

4.2. The role of bovine brucellosis on reproductive performance of cattle in the study area.

Parity: Statistically significant difference ($p<0.05$) in seropositivity between parity and *Brucella* infection was observed by taking animals without parity as a reference. Higher seroreactors (6.6%) were found in cows with one parity than cows with more than/equal to two parity (3.7%) and very low (0.7%) in animals without parity (Table 7).

Number of service per conception (NSPC): Out of 29 animals that needed repeated services (more than or equal to three services), 10 cows (34.5%) were found seropositive for *Brucella* infection, showing the impact of the disease which makes animals not to have conception at first breeding/mating. Odds ratio indicated there was strong association between brucellosis and NSPC with P-value statistically significant difference ($P<0.05$) (Table 7).

Calving interval: The seropositivity of cows that gave birth at an interval of 1year, less/equal to 2 years and above 2 years were 2.1%, 1.8% and 33.3%, respectively. The Odd ratio indicated cows that gave birth above 2 years interval were 27.62 times at risk of getting *Brucella* infection compared to cows calving yearly (Table 7). In addition to the above, strong significant association was observed between the calving interval and brucellosis in reference to cows with calving interval of above 1 and less/equal 2 years. This indicated that brucellosis delayed calving interval and caused wastage of reproduction performance.

Reproductive status: Regarding the reproductive status of the animal, the present study revealed a seropositivity of 1.2%, 1.4%, 3.7% and 7.5% for heifers, lactating, dry and pregnant cows, respectively. According to this, higher seropositivity was recorded in pregnant animals indicating susceptibility to *Brucella* infection was higher in pregnant animals compared to that of dry and lactating cows. Besides, statistical significance ($p<0.05$) was observed between brucellosis and reproductive status by taking heifers as a reference (Table 7).

Table 7. The role of bovine brucellosis on reproductive performance of cattle on the study area assessed by logistic regression.

Factors	Level	N0.exam.	Test +ve	P	OR	95% CI	P-value
Parity	0 parity*	152	1	0.7	1	-	-
	1 parity	106	7	6.6	10.678	85.34-9o.46	0.028
	≥2 parity	271	10	3.7	5.7854	43.81-47.42	0.095
NSPC	1*	262	2	0.8	1	-	-
	2	82	5	6	8.3974	41.96-46.23	0.011
	≥3	29	10	34.5	62.053	303.4-311.55	0.000
Calving interval	1≤2year*	218	4	1.8	1	-	-
	1year	48	1	2.1	8.609	0.005-0.351	0.003
	>2year	33	11	33.3	27.622	0.011-0.127	0.000
Reproductive status	Heifer*	85	1	1.2	1	-	-
	lactating	140	2	1.4	0.821	0.073-9.20	0.87
	dry	107	4	3.7	2.68	0.481-9.91	0.26
	pregnant	148	11	7.4	5.54	1.21-25.46	.028

*: others were computed in reference to this category

4.3. Other factors that affect herd level seroprevalence of bovine brucellosis in the study area

Bull service for breeding showed higher seropositivity (2.8%) than using AI service (0.7%). However, statistically significant difference was not observed between seropositivity to *Brucella* antibody and type of matting/breeding (Table 8). In the case of owner's awareness to brucellosis, higher seroprevalence (4%) was recorded in cattle owned by farmers who had no awareness about brucellosis compared to that of cattle owned by farmers having awareness about brucellosis. Statistically significant difference was also found between awareness to brucellosis and seroposivity to *Brucella* infection (Table 8).

In a similar manner, conducting screening test for animals before purchasing (before introduction in to a herd) was also found statistically significant (Table 8).

Table 8. Other factors that affect herd level seroptrevalence of bovine brucellosis in the study area assessed by logistic regression.

Factors	Level	No.exam	Test+ve	P	OR	95% CI	P-value
Breeding type	AI*	150	1	0.7			
	Bull	654	18	2.8	4.13	0.56-32.25	0.129
Brucella awareness	yes*	531	8	1.5			
	no	273	11	4	2.67	1.07-6.89	0.026
Examn.before purchasing	yes*	310	3	1			
	no	494	16	3.1	3.35	1.1-11.9	0.039

*: others were computed in reference to this category

5. DISSCUSSION

The result of the present study showed that the overall seroprevalence (2.4%) of bovine brucellosis in Alage and its surrounding was at low level. A prevalence of 3.3% was recorded in the extensive management system. In a similar management system, there are studies in agreement with this finding reported by Megersa *et al.* (2011) South and East Ethiopia (3.5%), Wondimu (1989) in centeral Ethiopia (3%), Jegerfa (2006) in three Agro-ecologies of Central Oromia (2.99%), Shiferaw (1987) in Bahir Dar (2.1%), Fekadu (1999) in Amhara National Regional State (1.8%), Asmare *et al.* (2004) in Sidama Zone (1.9%), Berhe (2007) in Tigray Region (1.5%), Kang'ethe *et al.* (2007) in Kenya (1%). However, the present finding was lower than the result of Mensah *et al.* (2011) in Ghana (21.9%), Yirgu (1991) in Abernosa Cattle Breeding Ranch (19.5%), Muma *et al.* (2007) in Zambia (14.1%) as well as Dinka and Chala (2009) in East Showa (11.5%). Most of the recent studies suggested a low seroprevalence (below 5%) in cattle under crop-livestock mixed farming (Berhe *et al.*, 2007; Ibrahim et *al.*, 2010). Such reduction in prevalence could be due to the improvement of management and husbandry practices, specially the raising awareness of the farmers and dairy men about brucellosis.

The present study also showed very low seroprvalence (1.3%) in the intensive production system. In a similar management system, this finding was in consistent with the result of Shafee *et al.* (2011) in Pakistan (3%), Rahman *et al.* (2011) in Bangladish (2.66%), Tefera (2006) in Addis Ababa and Sululta (1.13%) and Anteneh (2010) in Addis Ababa and its surrounding (0.9%). However this low level seroprevalence was at variance with works reported by Rashid (1993) in central highlands of Ethiopia (38.7%), Sintaro (1994) in Cheffa Dairy Farm (22%), Gebremariam (1985) in Addis Ababa (19.1%). On the other hand, moderate level of seroprevalence of bovine brucellosis was reported by Matope et *al.* (2010) in Zimbabwe (5.5%), Gebreyesus (2001) North West Amhara (8.2%), Asfaw *et al.* (1998) in Addis Ababa (8.11%), Hadgu (1987) around Bahr Dar (9.8%).The study also showed that antibodies to *Brucella* infection were prevalent throughout the study areas. This is in consistency with seroprevalence reported by megersa *et al.* (2011) where the positive reactors were distributed across his study areas of Eastern and Western Ethiopia. The reason for the relatively low prevalence in the intensive production system

compared the extensive system might be due to the difference in management and husbandry conditions practiced. In the intensive system, cattle are examined before introduced in to the farm, awareness about brucellosis is relatively higher, AI service is usually used, management of abortion and other hygienic conditions are practiced in better conditions.

The result of the present study revealed the presence of significant difference between sex and *Brucella* infection. Almost all (94.7%) of the seropositive animals were females. This result was in slight difference with Anteneh (2010), Berhe (2007), Tolosa (2004) who reported the absence of male seroreactors in their study. Tefera (2006) also showed the susceptibility to *Brucella* infection was higher in females (3.05%) than males (0.84%). In addition Walker (1999) pointed out that bulls are more resistant to *Brucella* infection than females. The reason for the present study might be explained due to the fact that sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms, tend to increase in high concentration in females and also with age and sexual maturity (Walker, 1999).

The present study also showed the presence of statistically significant difference between age and brucellosis. Out of 19 *Brucella* positive cattle, 18 (94.7%) were detected in cattle with more than 3 years old. Furthermore, from the three age categories the seroprevalence of brucellosis was found higher (5.3%) in cattle with greater than 6 years old indicating that the susceptibility to brucellosis increases as the age of the animal increases. This result was in consistency with the report of Asmare *et al.* (2010) that showed 97.87% of the seroreactors were in age groups above 2 years. Another report by Anteneh (2010) also indicated that all serorectors were found greater than two years old. In addition to the above, Chukwu (1987) and Radostitis *et al.* (1994) observed that sexually matured cows and bulls are at high risk of acquiring brucellosis. The above result might strengthen for the idea which says that the growth stimulating factors for *Brucella* organisms become abundant when the animal becomes sexually matured and older.

A statistically significant difference was not observed among the three different breeds (Arsi cattle, Boran and Holstein), although the seropositivity to brucellosis looked like a relatively higher in Arsi cattle (3.2%) than the Boran (1.2%) and Holstein (0.7%) breeds. In agreement with this result, a work by Anteneh (2010) showed the presence of significant difference in

seropositivity between Holstein and Jersy breeds indicating that the Holstein was less susceptible (0.79%) to brucellosis than the Jersy cross breeds (98.33%). In contrary with the present finding, Lundervold *et al.* (2004) in kazakhstan agreed that local breeds are more resistant compared to exotic breeds. The variance in the present study (from the fact exotic breeds are more susceptible to disease) might be explained because of the low seroprevalence recorded in the intensive production system where the Borans and the Holstein are found. This is due to better management practices, applied in the intensive production system compared to the extensive production system where the Arsi (local) types of cattle are found.

Concerning the herd size, Almost the same seroprevalence was recorded in both the small (2.6%) and large (2.3%) herds. Nevertheless, cattle with large herd size were found 1.6 times at risk (as indicated by the odd ratio) than animals with small herd size, the level of risk were not statistically significant. This finding was greatly varied with the report of Anteneh (2010) that showed large herd size was more at risk (1.59%) of getting *Brucella* infection than small herd size (0%). In a similar way, the work by Asmare *et al.* (2010) showed 4.81%, 50% and 70% were the infection rates for small, medium and large herds, respectively indicating the presence of higher seroprevalence (70%) in large herd size. The reason for the deviation occurred in the present study with the above works might be explained because of the presence of larger herd size in the intensive production system (the herd size of the Borans and the Holstein breeds is very large) where a relatively low seroprevalence was recorded.

Regarding the role of the brucellosis on reproductive performance, Parity was found statistically significant with seropositivity to *Brucella* antibodies. The seropositvity was strongly observed in cattle having one parity compared with animals having more than/equal to two parity and without parity. This might be due to the negative effect of the disease on reproductive organs of the animal that subsequently leads to poor conception and infertility which decreases the parity number. On the top of this, the higher seropositivity observed in cattle with one parity could be due to the immunity developed by the older cows. Meaning, as the parity number increase the immunity against brucellosis also increases. In addition serorector cows are usually culled from the herd (in the intensive farm). This result was greatly varied with that of Kohei *et al.* (2011) who reported the absence of significance difference between parity and Brucellosis positive herds.

However, Haileslassie (2008) and Anteneh (2010) reported the presence of significant difference between parity and *Brucella* infection.

In a similar manner, very high seropositivity (33.3%) to *Brucella* antibody was observed in cows which gave birth above two years interval. Meaning, calving interval is longer in cows affected by brucellosis. The possible reason for this could be due to the negative effect of the disease on reproductive tract of the female animal which cause retained fetal membrane that usually leads in to uterine infection and sometimes to pyometra (in chronic cases). This causes longer calving to first service interval with poor conception rate. In line with the present study, Musa *et al.* (1990) observed that 62% of hygromatous cattle tested for brucellosis were suffering from long calving intervals. Similarly, Haileslassie (2008) reported a high seroprevalence of brucellosis (36.1%) in cows calved above 15 months of interval and concluded that the tendency of increasing calving interval with the presence of brucellosis.

The present study also revealed that higher seropositivity to brucellosis (34.5%) was observed in cows that needed above three (3) number of service per conception. In addition, strong statistically significant difference was observed between number of services preconception and brucellosis. In agreement with this study, there are reports by Anteneh (2010), and Bekele *et al.* (1999) that indicated the presence of significant difference between NSPC and brucellosis. The increase number of service per conception (need for repeated services) might be reason out because of the negative effect of brucellosis on reproductive performance of the animal. The number of services per conception increase whenever cows are repeatedly exposed to abortion, retained fetal membrane, dystocia and other reproductive health problems. The fertility of these seropositive cows is often reduced in terms of a delayed calving to first service interval, calving to conception interval and pregnancy rate to first insemination.

Concerning the reproductive status of the animal, the present study showed that higher seropositivity (7.4%) was recorded in pregnant animals compared with dry, lactating and heifer. Accordingly, statistically significant association was found between pregnant cows and *Brucella* infection. The present result was in agreement with Haileslassie (2008) who reported a relatively higher proportion of seoreactivity (8.6%) in pregnant animals. The reason for the finding of

higher *Brucella* seropositive pregnant cows could be due to the preferential localization of *Brucella* organisms in the uterus in which the concentration of allantioc fluid factors such as erythritol, a four carbon alcohol are elevated in the placenta and fetal fluid during gestation. This could stimulate the growth and multiplication of the *Brucella* organisms (Walker, 1999). However there is reduction in the number of the organisms in the months following calving and abortion until the next pregnancy when there is again rapid increase of *Brucella* organisms in the reproductive tract (Coetzer and Tustin, 2004). On the top of that, Radostits *et al.* (2000) indicated that sexually matured and pregnant cattle are more susceptible to *Brucella* infection. However this result was far distant from the findings of Anteneh (2010) and Berhe (2007) who reported significantly higher seopositivity (11.18%) in non pregnant cows. In addition, Tolosa (2004) and Taddesse (2008) found the absence of significant difference in seropositivity between pregnancy status and *Brucella* antibody.

Concerning the managemental risk factors, having awareness about brucellosis and examination of animals before purchasing (before introducing in to brucellosis free herd) were found significantly associated with *Brucella* infection. This point (awareness to brucellosis) might partially answer why a low seroprevalence was recorded in the present study inspite of high prevalence (19.5%) reported by Yirgu (1991) on the same study area. By contrast, Anteneh (2010), Taddesse (2008) and Hailemelekot (2005) didn't observe any significant association of management and husbandry practices with seropositivity of *Brucella* antibody. On the other side, almost all (exept one seoreactor) the positive reactors were found from herds that use bull service and using bull service for breeding was indicated as a risk by odd ratio, However, the association between brucellosis and type of breeding (whether bull or AI) was not found statistically significant. This finding was in contrast with the works of Heileslassie (2008), Berhe (2007) and Anteneh (2010) who reported the presence of statistical significant difference between type of breeding and the occurrence of brucellosis. The variance in the present study could be because of the sample size (small herd size) that use IA service was relatively small so that the prevalence also become small(2.8).

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed a relatively low an overall seroprevalence of bovine brucellosis in Alage and its surroundings. The number of *Brucella* seroreactor cattle was also found relatively low in the intensive management system compared to that of the extensive management system. A comparable result was recorded among the three sites of the study area. Factors such as age, sex, parity, calving interval, number of service preconception as well as reproductive status were found to be associated with brucellosis. Seroprevalence of bovine brucellosis was found higher in older cattle, in females, in pregnant cows, cows with longer calving interval, cows with parity as well as cows that need repeated breeding. Herd size and breed did not show any significant association with *Brucella* infection. Other management factors like awareness to brucellosis and examination of animals before introduction into a herd showed statistically significant difference. Using either bull or AI service for breeding was not significantly associated with *Brucella* infection.

Based on the above conclusion, the following recommendations were forwarded;

- Raising awareness of the farmers and the dairy men about brucellosis is important point for controlling the disease until economically acceptable level.
- Screening test should be carried out before the introduction of new animal/s to any herd particularly to Alage intensive farm.
- The intermixing of cattle between extensive and intensive farms should be strictly avoided to hindered further spread of the disease.
- Farmers and dairy men should regularly practice test and culling control measures which play a great role to decrease the prevalence of the disease in the country.
- Further studies are needed to isolate and characterize the brucellosis agent in different ruminant species of different locations and to assess the role of the disease on other reproductive performances of the different breeds of cattle.

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8. ANNEXES

Annex 1. Rose Bengal Plate Test Diagnosis of Brucellosis

Description of the test

An antigen prepared from B.abortus (strain 99) stained with rose Bengal dye and suspended in acid buffer 9PH 3.65).Used to detect *Brucella* antibodies in serum-using a plate agglutination test. It detects antibodies against B. abortus ,B.melitensis, and B.suis in serum samples.

Test Procedure

- Bring the RBPT antigen and test (sera) to room temperature before beginning the test.
- Place 30µl of each serum sample on the agglutination plate.
- Shake antigen bottle gently before use and place 30µl of RBPT antigen next to the serum sample on agglutination plate. Mix the antigen and the serum.
- Shake the plate for five minutes and read.

Result Interpretation

No agglutination indicates negative sample.

Agglutination indicates positive sample

NB: Slight agglutination was also considered as a positive result

Validation of the Test

VLA positive control (cat≠RAB1003 was used for each batch of samples and for newly opened antigen bodies

Antigen Presentation

10ml of RBPT antigen per bottle

Storage: Store in the dark at $+2^{0c}$

Annex 2. Indirect Enzyme Linked Immuno Sorbent Assay

SERELISA® Brucela OCB Ab Mono Indirect

Kit for detection of Anti *Brucella* lipopolysaccharide antibodies in bovine sera(individual and pools,ovine and caprine sera(individual

.Principle of the test

The "SEELISA® *Brucella* OCB Ab mono indirect kit uses an indirect immuno enzymatic technique allowing the detection of brucellla lipopolysaccharides(LPS) antibodies in individual bovine in and caprine serum samples or pools of 10 bovine sera(in accordance with the applied regulations .The reaction was composed of three steps

1. Each individual or pooled serum sample was placed in a well sensitized with *Brucella* LPS. Antibodies present in the sample bind to bacterial antigen coated to the well.
2. After a wash step, a peroxidase conjugate was added. It fixes to the immune globulin (antibodies) previously captured, forming a complex (AgLPS) –(Ab anti-LPS)-(peroxidase conjugate).
3. Excess conjugate was eliminated by a wash step. The enzyme linked to the complex was revealed by addition of a substrate.Which was transformed in to a coloured product. After stopping the reaction,the optical densities were measured. The presence or absence of antibodies was determined using threshold values obtained from the positive control

Materials and Reagents required

- Distilled or demineralized water
- Adjustable or set pipepettes to measure and deliver between 0 to 100µl.Measurement deviation must be≤10% for volumes ≤10µl and ≤5% for all other volumes
- Graduated cylinder (100ml and 1000ml).
- Manual, automatic or semi automatic washing devices for microtitration plates.
- Microplate reader, fitted with filters for bichromatic reading at 450 and 630 nm. It is also possible to use a monochromatic reader fitted with 450 nm filter.

- **Samples**
- The reaction was performed on individual sera diluted at 1:100 or10 pooled samples diluted 1:20.

Sample storage:

Samples	Cold(+5^{0c})	Freeze(-20^{0C})	Lab.Temperature -20^{0c}
Individual or pooled serum	Maximum 7 days	Yes	No

.Procedure

Strcitly comply with the procedure below .Use negative and positive control in duplicate for each test run.for each plate.

A. PRELIMINARY STEPS

1. Carefully set up the distribution and identification of controls and sample.
2. Prepare the samples to be tested.

Individual samples protocol: dilution 1:10

Dilute the sample at 1:10 in samples diluents(SD) in a test tube or in to a dilution plate. Dilute again at 1:10 in a test tube or directly.in to the wells: dispense 10µl of already diluted samples in 90µl of sample diluents (SD).

Pooled bovine samples protocol: dilution 1:20.

Add 10 µl of each individual serum to obtain 10µl of 10 pooled samples.

Dilute pooled samples at 1; 20 in a test tube or directly in to the wells: dispense 5µl of pooled samples in 95µl of sample diluents (SD).

B. TTEST PROCEDURE

I Control and sample distribution

1. Control distribution

For the two following protocols, 100µl of diluted negative control was dispensed in wells A1 and A2,100µl of positive control was dispensed in wells B1 and B2.

Individual samples protocol: **dilution 1:100.**

After shaking the vials dilute, the controls was diluted at 1:10 in a sample diuluent.(SD). In a test tube or in to a dilution plate,then dilute again at ,1:10 in a test tube: dispense 10µl of already diluted controlin 90µ of sample diluents(SD).

2. Sample Distribution

Strictly comply with the procedure indicated in VI.A2. for the preparation of samples and the distribution directly in the plate.

- The samples can be tested individually or in dubplicates.Distribute 100µl in each well.
- Strips shoul always be placed on the frame so that both washer and reader can be used.
- Cover the wells with adhesive film, cut to the necessary length by the number of strips used.
- Mix by gently shaking the plates manually or by using plate agitator.

3. Incubation of the Plate

1 hour ($\pm 5^{0c}$) at 37^{0c} ($\pm 3^{0c}$)

Washing

Wash buffer: dilute the concentrated washing solution (W) 1:10 in distilled or demineralized water.

Carefully remove the adhesive film and wash 4 times

II. Addition of conjugates

1. preparation of conjugate:

Individual samples protocol: dilution 1:200

Dilute the concentrate (CJ) 1:200 in the conjugate diluents(CD).2ml were needed for one strip, meaning, 20µl of the conjugate in 1.98 ml of CD.

2. Distribution of the conjugate:

Add 100µl of diluted conjugate to all the wells and cover with a new piece of adhesive film.

3. Incubation of conjugates

Incubate for 30 minutes (±5 min) at 37^{0c} (±3 min).

Carefully remove the adhesive film and wash 4 times.

III. Revelation

1. Addition of the substrate

Add 100µl of buffered peroxides substrate (PS) per well. Do not cover with adhesive films at this stage. Mix by gently shaking the plate manually or use a plate agitator to ensure correct homogenization.

2. Incubation of substrate:

30 inutes ±5 minutes at laboratory temperature($+20^{0}c\pm5^{0c}$). shielded from light.

3. Addition of stop solution

   Add 3µl of stop solution (S) per well.

   Mix by gentle shaking the plate manually or by using a plate agitator.

   Make sure that no bubbles occur in the wells.

4. Measure of the optical density: Measure the optical density (OD) bichromatically at 450and 630 nm or monochromatically at 450 nm (in the yellow band).

Reading bichromatically was strongly recommended. Should the monochromatic reader be used, ensure the cleanliness of the bottom of the wells prior to reading

V. Test Validation

The result of each test run was valid if:

ODP ≥0.5 and OD N<0.3*ODP

OD: Average of the ODs for the samples tested in duplicate

VI. Expression and Interpretation of the Result

The presence or the absence of antibodies against LPS of Brcella was determined by comparing the ODs.to the threshold values obtained from the positive control.

Two methods for calculating and interpreting the resultS were possible

Method 1": Index calculation

Positive threshold value in index=0

For individuals and pooled samples:

Sample index: =0.50*(sample OD-0.6*ODP)

Any samples or pool of samples presenting an index ≥0 was considered as positive

Any sample or pool of sample presenting and index ≤ 0 was considered as positive.

Method 2: Analysis of the Optical Densities

For individual and pooled samples:

Positive threshold value: $\alpha = 0.6 * (ODP)$

Compare each sample OD to this threshold value.

Any individual or pooled sample presenting an $OD \geq \alpha$ was consiederd as positive.

Any individual or pooled sample presenting an $OD < \alpha$ was considered as negative.

Annex 3. Data Collection Format on Reproduction of Cattle in Alage and its Surroundings

Animal No./code									
Date of sample collection									
Owner's name									
Region									
Zone									
District									
Peasant association									
Site									
Number of cattle owned									
Sex of the anim. sampled									
Age of the animal sampled									
Coat colour									
Body condition									
Weaning age of new born									
Role of the animal sampled									
Pregnancy stage									
Age at first calving									
Parity number									
Lactation stage									
Lactation length									
Calving interval									
NSPC									
Milk yield									
Method of breeding									
Awareness to brucellosis									
Examination before purchas									
Source of replacement herd									
Common disease in the area									

10. SIGNED DECLARATION SHEET

I declare that this thesis is my original work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted to Addis Ababa University in partial fulfillment of the requirements of an M.Sc. degree and is deposited at the university's library to be made available to borrowers under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the awards of any academic degree, diploma, or certificate.

Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgment of source is made. Request for this manuscript in whole or in part may be granted by the Head of the Department of Microbiology, Immunology, Epidemiology and Public Health or the Dean of the School of Graduate studies when in his or her judgment the proposed use of the material is in the interest of scholarship. In all other instances, however, permission must be obtained from the author.

Name: Hagos Asgedom Signature: _____

Place: Addis Ababa University, Debre Zeit, Ethiopia

Date of submission: June 18, 2012

This has been submitted for examination with my approval as University advisor.

Dr Reta Duguma (DVM, MSc, Assistant Professor) Signature: