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SCHOOL OF VETERINARY MEDICINE

SEROPREVALENCE STUDY OF BOVINE BRUCELLOSIS IN TWO DISTRICTS OF EAST GOJJAM ZONE OF AMHARA REGIONAL STATE

By:

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
^{O}C	Degree Centigrade
CACC	Central Agricultural Censes Commission
CDC	Center Disease Control
CFT	Complement Fixation Test
CI	Confidence Interval
CO_2	Carbon Dioxide
ELISA	Enzyme-Linked Immunosorbent Assay
EMOA	Ethiopia Ministry of agriculture
EC	European Commission
FAO	Food and Agriculture Organization
GDP	Gross Domestic Product
H_2S	Hydrogen Sulfide
ILCA	International Livestock Center for Africa
MHD	Minimum Hemolytic Dose
OR	Odds Ratio
OIE	Office International des Epizooties
SAT	Serum Agglutination Test
SDTH	Skin-Delayed-Type-Hypersensitivity
WHO	World Health Organization

ABSTRACT

A cross sectional sero-prevalence study was conducted on 600 cattle in Machakel and Dejen districts of East Gojjam administrative zone, Amhara region, Ethiopia, to investigate the status of bovine brucellosis and identify potential risk factors associated with the disease. Sera samples were analyzed using Rose Bengal Plate Test and Complement Fixation Test to detect specific antibodies against *Brucella abortus*. Overall sero-prevalence of bovine brucellosis in the study districts of Amhara region was found to be 5.3% (32/600). However, no statistically significant difference (P>0.05, P=0.467) in seroprevalence was observed between the two study districts, Machakel (6%) and Dejen (4.7%). The sero-prevalence was closely associated with some of the risk factors considered at individual animal. Both univariable and multivariable logistic regression analyses showed statistically significant association (p=0.008, OR= 5.7) was observed between brucellosis seropositivity and *Kebeles*. In contrast, risk of seropositivity was no statistically associated (P>0.05) with risk factors such as breed, age, and sex. Questionnaire survey results of 120 respondent farmers (households) to gather information about bovine brucellosis in the study districts showed that, 61(50.8%) of cattle owners were not aware of bovine brucellosis, 81(67.5%) had no separate calving pen, and 107(89.2%) did not practice safe disposal of fetal membrane and aborted fetuses. The occurrence of abortion, stillbirth and retained foetal membranes were also high 79(65.8%) in their animals. Most of peasants, 74(61.7 %) used natural breeding mating, 40(33.3%) opted AI and 6(5.0 %) used both artificial insemination and natural breeding methods. In conclusion, this study showed bovine brucellosis was prevalent in the study area, however, the occurrence of the disease, its effect as a major cause of abortion was not recognized including its public health implication.

Key words: Bovine brucellosis, *Brucella abortus*, Seroprevalence, epidemiological risk East Gojjam, Ethiopia

1. INTRODUCTION

Ethiopia is a country with diverse ecology that makes the country home for large populations of different domestic and wild animals with considerable contribution to the national economy. The livestock sector has the largest resource base in Africa. The livestock population in Ethiopia is estimated to be about 41.6 million cattle, 14.6 million sheep, 13.6 million goats, 5.8 million equines 0.44 million camels and 42.9 million poultry (CACC, 2003). The livestock supports rural and urban population with milk, meat, employment, investment opportunities and draft power for crop production. The agriculture sector in Ethiopia engaging 80% of its population, contributes 52% of the Gross Domestic Product (GDP) and 90% of the foreign exchange (MOA, 2000). Livestock share is estimated at 40% of the annual agricultural output and 15% of the GDP (Tegegn and Gebrewold, 1997).

Although the livestock sector has a significant contribution to the national economy, production per animal is extremely low (Shiferaw *et al.*, 2003). The main technical limitations on livestock development that determine the biological efficiency of production in the country are inadequate feeding, poor animal health and livestock management practices, the genotypes used, reproduction and infectious diseases control (ILCA, 1992). Among the infectious diseases, brucellosis has been known to be a specific genital disease that induces abortion and causes heavy economic losses (Belihu, 2002).

Brucellosis is an infectious disease caused by bacteria of the genus *Brucella.* Various *Brucella* species affect sheep, goats, cattle, deer, elk, pigs, dogs, and humans (CDC, 2002). The disease was also reported in camels (Abbas and Agab, 2002; Teshome *et al.*, 2003; Hegazy *et al.*, 2004) and in marine mammals (seals, sea otters, dolphins, propoises) (Forbes *et al.*, 2000).

Brucellosis was first recognized as a disease of human-beings on the island of Malta in the 19th and early 20th centuries. *Brucella* organisms can be found worldwide, but it is more common in

countries having poorly standardized animal farming systems. The disease is infectious and zoonotic disease, with a great economic impact on cattle farming.

In cattle, the infection is caused by *Brucella abortus*, and it is characterized by abortions, metritis, orchitis, and epididymitis with subsequent infertility in both sexes. The disease in cattle mainly affects the reproductive organs of infected animals (England *et al.*, 2004).

Brucellosis represents a cause of health problems in a herd. It is spread within the herd mainly by ingestion of contaminated material. The initial infection in the reservoir species is often followed by abortion and subsequent delayed or permanent infertility. The infected animals shed the organisms in the uterine discharges following abortion and subsequent parturition, and also in the colostrums and milk (FAO, 2003).

The transmission of the infection to human-beings occurs through breaks in the skin, following direct contacts with tissues, blood, urine, vaginal discharges, aborted fetuses or placentas (Bercovich, 1998). Food borne infection occurs following ingestion of raw milk and other dairy products. Occupational airborne infection in laboratories and abattoirs can occur (FAO, 2003).

The disease occurs worldwide, except in those countries where bovine brucellosis has been eradicated. The disease has negative impacts on exports and constrains efforts in improving breeding (OIE, 2001).

In Ethiopia, bovine brucellosis was first reported in 1970 by the veterinary section of US Navy the Medical Research Unit (EMOA, 1970). However, in the last two decades, several serological surveys have showed that bovine brucellosis is endemic and widespread disease in the country (Asfaw *et al.*, 1998; Bekele *et al.*, 1989; Eshetu, 2005; Gebretsadik *et al.*, 2007; Hadgou, 1987; Molla, 1989; Nurradis, 2007; Shiferaw, 1994; Taddesse, 2008; Yayeh, 2003). The East Gojjam Zone is part of the country whereby different livestock move from neighboring zones and regions into it. In this East Gojjam Zone there are clinical signs that have been observed in animals like abortions, retained placenta, metritis in female animals and orchitis in the males.

Thus, the disease is suspected to be present in the Zone. But there was no available reports that shown the status of brucellosis in the zone in general and the two study districts in particular.

Therefore, this study was designed with the following objectives:

- To determine the seroprevalence of bovine brucellosis in Machakel and Dejen districts of East Gojjam Zone, Amhara region, Ethiopia
- To assess possible management risk factors associated with the presence of bovine brucellosis in the study areas

2. LITERATURE REVIEW

2.1. Definition and historical aspect of brucellosis

Brucellosis is an infectious, contagious, and worldwide spread zoonotic disease caused by bacteria of the genus *Brucella*. In animals, the disease primarily affects cattle, sheep, goats, swine, and dogs, and is characterized by abortion or infertility and also affects man and other animal species (Sauret and villisova, 2002). In human-being, the disease is characterized by intermittent fever, chills, sweating, headache, myalgia, arthralgia, and a diversity of nonspecific symptoms (Young and Corbel, 1989).

In domestic animals, brucellosis has been commonly known by various names like enzootic abortion or bovine contagious infection, epizootic abortion, infectious abortion, contagious abortion, slinking of claves, Bang's disease, and ram epididymitis. In the case of human brucellosis, it has been described by various names including undulant fever, Malta fever, Mediterranean fever, gastric fever, Mediterranean gastric fever, Gibraltar Rock fever, Cyprus fever, Neapolitan fever, intermittent gastric fever or intermittent typhoid fever, pseudotyphus, febris typho-malariae, and fièvre sudorale (Ray and Steel 1979).

2.2. Etiology

2.2.1. Taxonomic Classification of Brucella abortus

The current taxonomic classification of *Brucella abortus is* as followed (Garrity *et al.,* 2004):
Phylum (BXII): *Protobacteria*
Class (I): *Alphaprotobacteria*
Order (VI): *Rhizobiales*
Family (IV): *Brucellaceae*
Genus (I): *Brucella*
Species: *Brucella abortus*

2.2.2. Morphology and characteristics of Brucella species

Brucella microorganisms are small, non-motile, non-spore forming, Gram-negative coccobaccilli short rods. They grow rather slowly on ordinary nutrient media while their growth is improved by serum or blood. They are an aerobic and they do not grow under strictly anaerobic conditions. The *Brucella* species are intracellular parasites and are usually found in the reticuloendothelial and reproductive systems. Typically *Brucella* species occurs as small gram-negative coccobaccilli, but coccal and bacillary forms also occur. The cells are short and slender; the axis is straight; the ends are rounded; the sides may be parallel or convex outwards. In length they vary from about 0.5 - 0.7 μm, in breadth vary from 0.5 - 1.5 μm, occurring singly, in pairs or short chains (Leslie *et al.*, 1998).

Brucella abortus was discovered by the Danish veterinarian Bernard Bang in 1897, who isolated the organism from cows with an infected abortion. Cattle are the natural hosts of the organism but it can also infect other animals. The organism is catalase and oxidase positive and requires carbon dioxide for growth. It produces hydrogen sulphate from sulphur containing amino acids or protein (Stack and MacMillan, 2006) (Table 1).

Table 1: Growth requirement of *Brucella* Species

No.	Species	CO_2 requirement	Time to positive Urease	H_2S production	Inhabitation by Dye	
					Thionen	Fuchsine
1	*B. abortus*	+/-	2 hr (rare 24 hrs)	+ (most)	+	-
2	*B.melitensis*	-	2 hr (rare 24 hrs)	+	-	-
3	*B. suis*	-	15 min	+/-	-	+ (most)
4	*B. ovis*	+	≥ 7 days	-	-	+ (most)
5	*B. neotome*	-	15 min	+	+	+
6	*B. canis*	-	15 min	-	-	+

Source: Coetzer and Tustin, (2004)

2.3. Epidemiology of Bovine Brucellosis

2.3.1. Occurrence

Brucellosis is found worldwide but it is well controlled in most developed countries. Clinical disease is still common in the Middle East, Asia, Africa, South and Central America, the Mediterranean Basin and the Caribbean. *Brucella* species vary in their geographic distribution. *B. abortus* is found worldwide in cattle-raising regions except in Japan, Canada, and some European countries, Australia, New Zealand and Israel, where it has been eradicated (WHO, 2006). The occurrence of brucellosis is increasing in developing countries in an even aggravating epizootological situation. This depends in the policy of many developing countries of importing exotic breeds without having the required veterinary infrastructure and the appropriate level of development of the socio–economic situation of the animal holders. Furthermore the increasing international animal trades, with increasing movement of animals and the trends toward intensification of animal production have facilitated the spread of brucellosis (Seifert, 1996). The reported incidences and the prevalence of the disease vary widely from country to country (Radostits *et al.*, 2000)

2.3.2. Transmission

Source of infection

The load of *Brucella* is highest in pregnant uterus, the fetus and the fetal membrane, and considered as a major source of infection (Radostits *et al.*, 2000). Aborted fetus, placental membranes or fluids and other vaginal discharges are all highly contaminated with *Brucella* organisms. Infected animals also shed organisms in the milk, therefore raw milk or raw milk products of bovine origin are sources of infection in humans other sources of infection within and between herds are contaminated water and feed supplies (Walker, 1999)

Route of transmission

Brucellosis represents a cause of health problems in a herd. Its spread within the herd is mainly by ingestion of contaminated materials. The initial infection in the reservoir species is often followed by abortion and subsequent delayed or permanent infertility. The infected animals shed the organisms in the uterine discharges following abortion and subsequent parturition, and also in the colostrums and milk (FAO, 2003).

Congenital (in utero) or prenatal infections may also occur, but this is mainly seen with *B. suis* infection. Grazing on infected pasture or ingestion of other feed stuffs and water supplies contaminated by discharges and fetal membranes from infected cows and contact with aborted fetuses and infected new born calves are the most common methods of spreads (Radostits *et al.*, 2000).

The most common route of transmission is the oral route following ingestion of contaminated pasture, feed, fodder, and water. Moreover, cows customarily lick after birth fetuses and new born calves all of which may contain a large number of organisms and constitutes a very important source of infection. Bulls do not usually transmit infection from infected cows to non infected cows mechanically. The use of infected bulls for artificial insemination (AI) constitutes an important risk since the infection can be spread to many herds (Acha and Szyfers, 2001).

2.3.3. Risk factors

Host risk factors

Animal risk factors like age, sex, breed and reproductive status of the individual animal influence susceptibility of cattle to *B. abortus* infection. Infection occurs in cattle of all ages, but persists most commonly in sexually mature animals. Natural exposure to field strains occurs primarily at the time of parturition of infected cows. The greater the number of infected cows that abort or calving, the greater the exposure risk to the other cattle in the herd. Young cattle are

less susceptible to *B. abortus* than older sexually mature cattle (Radostits *et al.*, 2000). Susceptibility increases with pregnancy and as stage of gestation increases (Bishop *et al.*, 1994).

Management risk factors

The spread of disease from one herd to another and from one area to other place is usually due to the movement of infected animal from an infected herd into a non-infected susceptible herd. The unregulated movement of cattle from infected herds or areas to brucellosis free herds or areas is the major cause of breakdowns in brucellosis eradication programs (Radostits *et al.*, 2000). Herd size and animal density are directly related to prevalence of disease and difficulty in controlling infection in a population. Calving practices also play a major role in the spread of brucellosis. Separate calving pens allow for minimizing exposure of uninfected animals. Whether a herd raises its own replacement animals or a purchase replacement animal affects the potential for introduction of the disease into the herd (Nicoletti, 1980; Walker, 1999).

The herd size is not only that affects prevalence, but the intensity of cattle contact within and between herds and with infected pasture and water. This shows that incidence of brucellosis increases with change from a purely extensive system to a more intensive form of cattle management (Thimm and Wundt, 1976).

There is a positive association between population density and disease prevalence which is attributed to increased contact between susceptible and infected animals (Omer *et al.*, 2000). Vaccination level, herd size, population density, method of housing, and use of maternity pens influence the probability of exposure to infection (Crawford *et al.*, 1990). Moreover, poor sanitation condition can favor rapid spread and subsequently high prevalence of the disease in the herd (Nicoletti, 1980).

Environmental risk factors

The survival of the organism in the environment plays a role in the epidemiology of the disease. The resistance of the pathogen depends on whether it is protected against the sunlight and high

temperature or from any harsh environment. Survival is prolonged by low temperatures and the organism remains viable for many years in frozen tissue. Moreover, neutral soil pH and moist protected environment which are rich in organic materials are favorable elements for survival of *Brucella* (Seifert, 1996).

Agent risk factors

Brucella abortus is a facultative intracellular parasite, which is capable of multiplication and survival within host phagocytes. The organisms are phagocytised by polymorphnuclear leukocytes in which some survive and multiply. These are then transported to lymphoid tissue and fetal placenta (Radostits *et al.*, 2000). The inability of the leukocytes to effectively kill the virulent *Brucella abortus* at the primary site of infection is a key factor in the dissemination of regional lymphnodes, other sites such as the reticuloendothelial system and organs such as the uterus and udder. The organism is also able to survive within the macrophages because it has the ability to survive phagolysome. *Brucellae* are able to survive within the host leukocytes and may utilize both neutrophils and macrophages for protection from humoral and cellular bactericidal mechanisms during the periods of hematogenous spread (Enright, 1990).

2.3.4. Pathogenesis

Although infection may occur through the skin, conjunctiva or respiratory mucosa by inhalation (Crawford *et al.*, 1990; Ko and Splitter, 2003), the most common route of infection in cattle is the gastrointestinal tract, from where infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes. Invasion of lymphatic vessels is followed by bacteraemia leading to systemic infection, favoring colonization of the pregnant uterus, male genital organs, and mammary gland (Ko and Splitter, 2003).

B. abortus has a strong tropism to the uterus during the last trimester of gestation, which is thought to be due to high concentrations of erythritol and steroid hormones. Erythritol favors bacterial survival since it can be metabolized by *B. abortus* as source of carbon and energy

(Samartino and Enright, 1996). Erythrophagocytic trophoblastic cells located at the base of chorionic villi of ruminants (Santos *et al.*, 1996) are considered the primary site of invasion of fetal placental tissues, from where *B. abortus* disseminates to intercotyledonary trophoblasts (Anderson *et al.*, 1986). *Brucella* multiplication induces infiltration of inflammatory cells, trophoblastic necrosis, vasculitis, and ulceration of the allantochorion. Consequently, fetal–maternal metabolic exchanges are compromised resulting in abortion (Anderson *et al.*, 1986).

2.3.5. Prevalence

Brucellosis in cattle occurs worldwide, except in countries where it has been eradicated, including Britain, Norway, Sweden, Finland, Denmark, Germany, Belgium, the Netherlands, Switzerland, Austria, Czech Republic, Slovakia, New Zealand, Canada, France, and Italy. In conducting prevalence surveys definite information based on herd size, management method, vaccines uses, and possible access to wild animal reservoirs of infection is highly required (Matyas and Fujikura, 1984).

Brucellosis is the most widespread and economically important zoonotic disease in tropical and subtropical regions. The prevalence rate was 20.2% (McDermott *et al.*, 1987) in crop-livestock production system in South Sudan and 10.8% in Tanzania (Jiwa *et al.*, 1996) pastoral production system. Asfaw *et al.*, (1998) found an overall prevalence of 8.1% of the 1,114 dairy animals tested in and around Addis Ababa. Bekele *et al* (2000) in their study in south eastern Ethiopia recorded a seropositivity of 7.3%. Seroprevalence study of bovine brucellosis in dairy cattle kept under intensive and semi intensive management system in Addis Ababa, Ethiopia 10% prevalence was reported (Eshetu *et al.*, 2005). The surveys have showed that bovine brucellosis is endemic and wide spread disease with high prevalence in organized farms than extensively managed herds (Table 2). Moreover, different studies showed that bovine brucellosis is prevalent in various regions of Ethiopia with significant economic losses to a country (Abay *et al.*, 2000; Tadese, 2003; Asmare, 2004; Berhe, 2005; Nurradis, 2007; Taddesse, 2008).

Table 2: **Prevalence of bovine brucellosis in different areas of Ethiopia**

Location/site	No. examined	Breed	Tests used	Prevalence (%)	Reference
Around Bahir dar	678	Zebu, cross	CFT	9.8	Hadgou (1987)
Arsi region	2178	Exotic, cross, zebu	RBPT	7.62	Molla (1989)
Central Ethiopia	1609	Zebu	SAT	4.2	Bekele *et al.*, (1989)
Around Bahir dar	1855	Cross, zebu	SAT	12.34	Shiferaw (1994)
In and around Addis Ababa	950	Exotic, cross	CFT	8.11	Asfaw *et al.*, (1998)
South east Ethiopia	4243	Cross zebu	CFT	4.9	Bekele *et al.*, (2000)
North Gondar	1447	Zebu, cross	CFT	0.14	Yayeh (2003)
Jima zone	1813	Zebu, cross	CFT	0.61	Tolosa (2004)
Addis Ababa	540	Dairy cattle	CFT	10	Eshetu (2005)
Jimma zone	1595	Zebu, cross	CFT	3.13	Nurradis (2007)
West Amhara	2224	Zebu, cross	CFT	0.49	Taddesse (2008)

2.4. Clinical findings

In natural infection it is difficult to know the duration of the incubation period, since it is not possible to determine when infection has occurred. Experiments have shown that the incubation period varies considerably and is inversely proportional to fetal development. The more advanced the pregnancy the shorter the incubation period. The period of serologic incubation "(from the time of infection to the appearance of antibodies)" lasts several weeks to several

months. The incubation period varied according to factors such as the virulence and dose of bacteria, the routes of infection and the susceptibility of the animal (Acha and Szyfers, 2001).

In cattle, *B. abortus* causes abortions, stillbirths, and weak calves. Abortions usually occur during the second half of gestation. The placenta may be retained and lactation may be decreased. After the first abortion, subsequent pregnancies are generally normal; however, cows may shed the organism in milk and uterine discharges. Epididymitis, seminal vesiculitis, orchitis and testicular abscesses are sometimes seen in bulls. Infertility occurs occasionally in both sexes, due to metritis or orchitis/epididymitis. Hygromas, particularly on the leg joints, are a common symptom in some tropical countries. Arthritis can develop after long-term infections. Systemic signs do not usually occur in uncomplicated infections, and deaths are rare except in the fetus or newborn. Infections in non pregnant females are usually asymptomatic (OIE, 2009b).

Other signs of brucellosis include an apparent lowering of fertility with poor conception rates, retarded heat, after births with resulting uterine infection (and occasionally) enlarged arthritic joints. In male animals the epididymes are usually affected but also the accessory sexual glands reveal painful, necrotic tissue degeneration and decrease in semen quality (OIE, 2009b).

In humans brucellosis is a multisystemic disease with a broad spectrum of symptoms. Asymptomatic infections are common. In symptomatic cases, the disease is extremely variable and the clinical signs may appear insidiously or abruptly. Typically, brucellosis begins as an acute febrile illness with nonspecific flu-like signs such as fever, headache, malaise, back pain, myalgia and generalized aches. Drenching sweats can occur, particularly at night. Splenomegaly, hepatomegaly, coughing and pleuritic chest pain are sometimes seen. Gastrointestinal signs including anorexia, nausea, vomiting, diarrhea and constipation occur frequently in adults but less often in children (OIE, 2009a).

2.5. Diagnosis

2.5.1. Principles

Even in aborting animals or animals having epididymitis, the clinical diagnosis of brucellosis in infected animals is difficult. The isolation of the organisms is the only way to make a positive diagnosis. The factors determining the diagnosis of bovine brucellosis are: absence of clinical signs other than abortion, the incubation period, and the high proportion of in apparent infections, the degree of resistance, either natural or resulting from vaccination, and the presence of natural or nonspecific agglutinins (Morgan, 1982).

The diagnosis should be based upon the disease history of the herd, epidemiological observations, serum antibody tests, cell mediated immunity, and the demonstration of the causal organism. Because of the costs, difficulty of performance, and lack of sensitivity of culture procedures, there is an indirect method of diagnosis by way of serological tests. There are many serological tests for demonstrating that *Brucella* antibodies exist, which can be found in the serum, the commonly used tests are the milk ring test (MRT), serum agglutination test (SAT), Rose Bengal plate test (RBPT), anti-globulin (Coombs) test, 2-mercaptoethanol, rivanol, and the enzyme-linked Immunosorbent assay (ELISA) (Morgan, 1982).

The reliability of serological tests to detect brucellosis depends on antibodies present at the time of the examination and the fact that infected animals may escape from detection. In order to improve the detection of brucellosis, the skin-delayed-type-hypersensitivity (SDTH) (intradermal test) appears to offer a convenient alternative to the serological tests (Fensterbank, 1984).

2.5.2. Serological tests

Rose Bengal Plate Test (RBPT)

According to the European Union, requirements the RB antigen needs to be standardized. It is a suspension of *B. abortus* smooth cells stained with the Rose Bengal dye, buffered to pH ± 3.65. It is commercially available. The RBP test is the most sensitive and rapid screening method (EC, 2002). The RBPT is a simple test for the diagnosis of bovine brucellosis by using serum samples as screening. The underlying principle is the buffered – acid antigen stained with Rose Bengal used for the early detection of *Brucella* specific agglutinins which is an excellent screen test, but may be over sensitive for diagnosis in individual animals, particularly vaccinated ones (WHO,2006). The RBPT is a rapid agglutination test based on mixing a drop of serum and a drop of antigen together. It is important to note that the antigen has to be shaken before it is used. It must not freeze, and should be stored at 4 °C in a dark place.

Complement Fixation Test (CFT) (A prescribed test for international trade)

The standardization of antigen and sheep erythrocytes is performed as described in the literature. The European Union established a system based on a standard serum that is required to conform to the second international standard anti-*Brucella abortus* serum (ISABS). When a country uses the CFT on a national scale, an agreement on a standard method should be reached among the different laboratories using the test. The CFT was considered the most specific and valuable serological test. It detects mainly immunoglobulin G1 antibodies. However, due to its complexity and delicacy, it is not as widely used as SAT and RBP tests. The CFT is widely used for the diagnosis of brucellosis in cattle, sheep and goat. This test is relatively insensitive to antibodies produced in response to vaccination with the living attenuated vaccine (for *B. abortus, B. melitensis*) whilst being highly sensitive and specific in animals naturally infected with brucellosis. The test is somewhat complex, and in mass tasting a screen test such as the Rose Bengal Plate Test is often used to reduce the number of samples that need to be tested by CFT

(Staak, 2001a). The sensitivity and specificity of the CFT is good, but it is a complex method to perform requiring good laboratory facilities and trained staff. If these are available and the test is carried out regularly with good attention to quality assurance, then it can be very satisfactory (WHO, 2006).

The CFT is widely used not only for the diagnosis of brucellosis in animals, but it is also effective in the diagnosis of human brucellosis. The principle lies in the complement fixation antigen-antibody reaction demonstrated by the binding of the complement. The reaction can be visualized with the aid of an indicator – reacting (hemolytic system). The hemolytic system consists of sheep erythrocytes and an antiserum to sheep erythrocytes (Amboceptor). If no antigen-antibody reaction develops with the consumption of the complement, the erythrocyte will be lysis completely and the hemoglobin will be released. One herd was considered infected at least, and one cattle was positive in the CFT (NHS, 2006).

There are numerous variations of the test, but each may be most conveniently carried out by using microtitration plates. Either a warm or cold fixation may be used for the incubation of the serum, antigen and complement at either 37 °C for 30 minutes or 4 °C for 14 - 18 hours. All dilutions are made in veronal buffer saline prepared from a stock solution of sodium chloride (42.5g), barbituric acid (2.875g), sodium diethyl barbiturate (1.875g), magnesium sulphate (1.018g), and calcium chloride (0.147g) in 1 liter of distilled water and are diluted by addition of 4 volumes of a 0.04% gelatin solution before use. The indicator system is a 3% suspension of fresh sheep red blood cells (SRBC) sensitized with an equal volume of rabbit anti-SRBC serum. This is prepared at a dilution previously determined to contain five times the minimum serum concentration required to produce 100% lysis of the SRBC in the presence of a 1/10 dilution of fresh guinea pig complement. The latter is independently titrated to determine the minimum concentration required to produce 100% lysis of the sensitized SRBC suspension; this is defined as the Minimum Haemolytic Dose (MHD). The complement is used at 1¼ MHD in the test (Forbes *et al.*, 2002).

2.6. Treatment, control, and prevention

Until now, reliable cure for bovine brucellosis in animals and in man has not yet been devised because of its intracellular characteristics. The course of the disease may be modified by tetracycline alone or in combination with streptomycin. However, effective controls must be based on minimizing the potential for infection by sanitary methods, the factors dominant for the spread of the disease, and vaccination (Fensterbank, 1976; Nicoletti and Milward, 1985).

The incidence of human brucellosis mainly depends on the extent and density of the infection among livestock and on the closeness of the contacts between humans and animals or their excreta. Nowadays, vaccination plays only a small role in the prevention of the human disease. It includes the live attenuated *B. abortus* strains 19-BA and RB-51 are used in Africa and 104 M (used in Soviet Union and China), the phenol-insoluble peptidoglycan vaccine (used in France), and the polysaccharide-protein vaccine (used in Russia) (Corbel, 1997).

An effective control of animal brucellosis requires the following basic elements: surveillance to find all the infected animals and herds, controlling the transmission of the infection to new animals or herds, and the eradication of the reservoir to eliminate the sources of the infection in order to protect susceptible animals or herds (Metcalf, 1986).

The use of control measures for animals has a beneficial effect on the incidence of the disease in humans. The prevention of disease transmissions within the herd must include: the identification and subsequent slaughtering of infected animals, the isolation of recently calved or aborted animals, the cleaning and disinfection of the quarantine area before other animals are reintroduced, the disposal of all products of abortion by deep burial under a covering of lime, the purchase of herd replacements from disease-free sources, and the isolation of pregnant replacement animals until they have calved and passed a serological test (WHO, 2006).

The prevention of disease transmissions between several herds requires the restriction of the movement of the animals and the quarantine of purchased animals. Prevention and control are conducted by the best country basis, national rules governing; diagnosis; vaccination;

surveillance; the movement of animals, and by the compulsory slaughtering of infected and potentially infected animals providing financial compensation. The restriction of the sale and movement of infected and disease-exposed animals by laws and regulations plays a major role in any control program (Leslie *et al.*, 1998).

The most beneficial control method in bovine brucellosis is vaccination at an early age. The vaccine, which consists of a live suspension of a smooth-intermediate attenuated strain of *Brucella abortus* (strain 19), has found worldwide use in cattle. It fully protects 65 – 75 % of the animals, while the remaining animals are at least partly protected (Alton *et al.*, 1988).

The killing of infected animals is the most effective method of eliminating the reservoir of *Brucella* in individual herds because the organisms do not survive outside of the host. The slaughtering of infected animals while rescuing the meat is a satisfactory method of destroying *Brucella* infected host animals since the organism will not survive in the meat after the slaughtering. The depopulation of infected herds in the final phase of an eradication program is a convenient method of eliminating brucellosis from an area (WHO, 2006).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in Amhara Regional State, East Gojjam administration zone, which constitutes the north-west rangelands of Ethiopia. The zone is bounded on southwest by East Wollega, Oromia region, on East by South Wollo, and in North by South Gonder administration zones and in West, by West Gojjam administration zone. This study was conducted in Enbuli, Girakedamen, and Amanuel peasant associations of Machakel district and Yetnora, Degen, and Subshengo peasant associations in Dejen district. These two districts are known by their immense cattle population and known as the most crop producing areas in the region. The minimum and maximum temperature of East Gojjam ranges from 7.5°C to 25°C and with an annual rainfall ranging from 900-1,800 mm. The altitude of the area ranges from 500 to 3,500 meters above sea level and latitude 10, 5^0 N and 38, 17^0 E. The zone's total cattle population is estimated to be 1,674,891 heads (CSA, 2005).

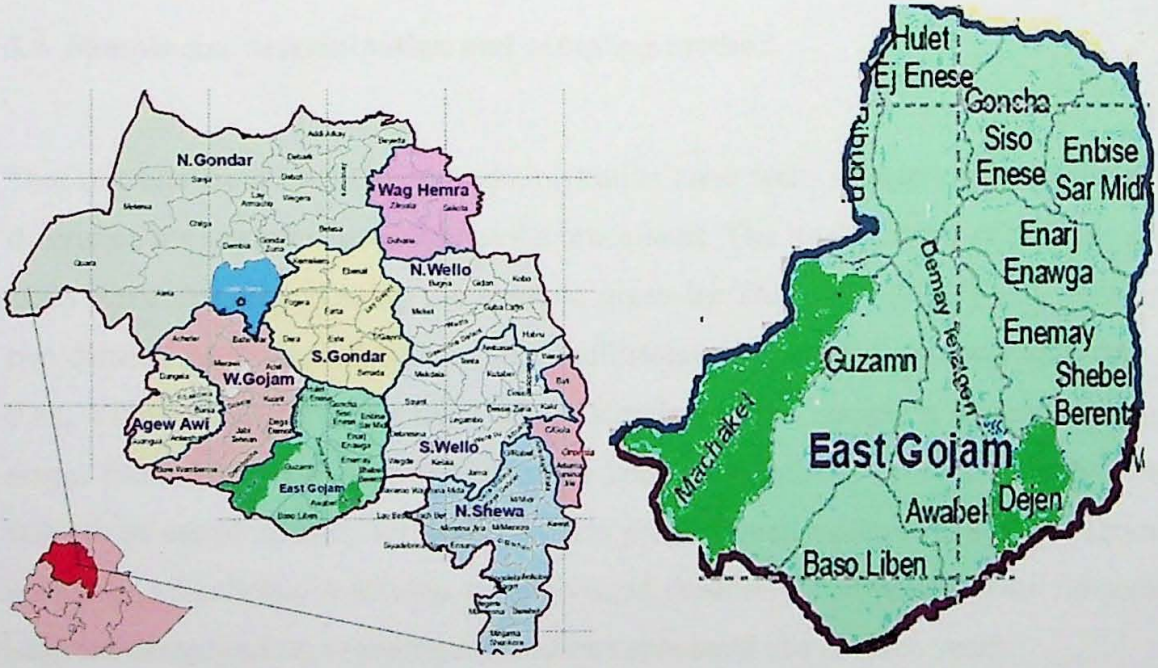

Figure 1: Map of Amhara region, zone and districts of the study area (CSA, 2005)

3.2. Study population

The study animals were cattle population, which are found in the selected peasant associations (PA) and households of the study districts, above three years of age. These animals, chosen for the study, are owned by small holder farmers and which are managed by semi-intensive farming system. The animals under study comprised indigenous local breed zebu and cross breed cattle.

3.3. Study design

A cross-sectional study was conducted from November 2010 to April 2011 to determine the seroprevalence of bovine brucellosis in the study area using serological procedures and questionnaires surveys. The questionnaires have included assessing potential risk factors, host risk factors, such as age, sex, breeds, and management risk factors, densities of animal population, husbandry and mating factors which are thought to be important determinants of the infection dynamics (Omer *et al.*, 2002).

3.4. Sample size determination and sampling method

Two districts from East Gojjam administrative zone were selected purposively as study site to determine the seroprevalence of bovine brucellosis. The total number of animals sampled for the study were determined using the formula given by Thrusfiled (2005) and were divided for the two districts proportionally. Generally, multistage cluster sampling technique was used to select PAs, Villages and sampling units where simple random sampling system was applied at each stage. From Machakel district having 24 kebeles, 3 kebeles were randomly selected and the number of sampling units for each PAs was proportionally determined. From Dejen district with a total of 21 kebeles, 3 kebeles were selected randomly. Sampling units from each PAs were selected using random sampling method on cattle aged above three years.

The sample sizes required were estimated using the formula given by Thrusfiled (2005).

$$n = \frac{1.96^2 \times P(1-P)}{d^2}$$

Where:

n = the total sample size

P = expected prevalence,

d^2= absolute precision

Therefore, the estimated minimum sample size was 384. But, to maximize the precision of the estimate to be measured, and minimize errors of the seroprevalence, the sample sizes were increased to 600. These 600 samples were proportionally and equally allocated to the two study districts. For Machakel district 300 and Dejen district 300 samples were collected for this study.

3.5. Study methodology

3.5.1. Serological methods

Collection of blood samples

Blood samples were collected from jagular vein of animals above three years of age and with no previous history of vaccination from the selected PAs. The owners were asked for their consent to collect samples and use it for this study analysis. Approximately 6 ml of blood was collected aseptically using sterile plain vaccutainer tubes and needles. The serum was properly labeled and left for 24 hours at room temperature to allow clotting and the sera was separated within 24 hours through gently transferring to other sterile vials and kept at -20^{O}C until tested for serological test employed such as Rose Bengal plate test and Complement fixation test according to standard procedure (EC, 2002; Staak, 2001b).

Rose Bengal Plate Test (RBPT)

This is a simple spot agglutination test using antigen stained with Rose Bengal and buffered to a low pH, usually 3.65 ± 0.5. In this research, the procedure described by MacMillan (1990) was followed. Accordingly, the sera and antigen were taken out from refrigerator and left at room temperature for 14 – 18 hours and at 37^0 c incubated at 30 minutes before the test commence. Then 25 – 30 microliter test serum was dispensed in each of the plate. The antigen bottle was gently rotated and 25- 30 micro liter of them was placed in front of the serum. The serum and antigen was mixed thoroughly using applicator stick then the plate was rocked manually for about four minutes. Finally, the results were read in a good light source. Reactions are identified as 0, +, ++, +++, according to (Staak, *et al.*, 2000). Where 0 means no agglutination; + = barley precipitation agglutination, + + fine agglutination some clearing and +++ = course clumping; definite clearing. These samples identified with no agglutination were recorded negative, while those with +, ++, and +++, were recorded as positive.

Complement Fixation Test (CFT)

The CFT test was done at National Veterinary Institute Debre Zeit. Sera being positive to RBPT were further tested with CFT to confirm the result according to the previous description (Dohoo, *et al.*, 1996) since such serial testing procedure maximizes the specificity of the test. The CFT test proper was conducted according to the protocols recommended by OIE (2004). Sera with strong reaction, more than 75%, fixation of complement (3+) at a dilution of 1: 5and at least with 50% fixation of complement (2%) at a dilution of 1: 10 and at dilution of 1: 20 are to be classified as positive (OIE, 2004).

3.5.2. Questionnaire survey

Information on potential risk factors of bovine brucellosis was collected using pre-tested questionaire. The information was collected from farmers of the given area with the questionnaire format which enquires like herd size, management and disease; while the blood sample was collected from animals randomly selected.

3.6. Data management and analysis

Data obtained from both serological tests and questionnaire surveys were stored in Microsoft excel spreadsheet program. Categorical variables (district, sex, age, cattle breed, mating) using Intercooled STATA version 10. Descriptive statistics such as proportion were calculated to determine sero-prevalence of Bovine Brucellosis.

To assess association of epidemiological risk factors with the sero-prevalence of the disease, statistical tests such as chi-square test and logistic regression (univariate and multivariate) were done taking 95% confidence interval (95% CI) and 5% level of significance (P-value of 0.05) to determine for the presence of statistical significance difference.

4. RESULTS

This study investigated the seroprevalence of bovine brucellosis and associated risk factors with the seroprevalence of the disease in cattle population of the two study districts of East Gojjam Zone, Amhara regional state, Ethiopia.

Of the total of 600 sera sampled to determine the seroprevalence of the disease, seroprevalence of 5.8% (35/600) was obtained using RBPT. Among 35 RBPT positive sera, 32 of them were confirmed positive for the disease using CFT with overall prevalence of 5.3% (32/600) bovine brucellosis in the study area as shown in Table 3.

Table 3: Seroprevalence of bovine brucellosis using RBPT and CFT in the study districts

Study districts	**No. of sera tested**	**Positive to RBPT (%)**	**Positive to CFT (%)**	**$\chi 2$**	**P-value**
Machakel	300	20(6.66)	18(6)	0.5282	0.467
Dejen	300	15(5)	14(4.7)		
Total	**600**	**35(5.8)**	**32(5.3)**		

However, there was no statistically significant difference ($p > 0.05$, $p = 0.467$) in seropositivity between the two study districts with seroprevalence of 6% in Machakel district and 4.7% in Dejen district using CFT.

Results of multiple logistic regression analysis indicated that seropositivity to bovine brucellosis was significantly associated ($p < 0.05$, $p = 0.008$) with kebeles, ranging from 0 in Amanuel to 15% in Girakedamin kebele as shown below (Table 4).

Table 4: Epidemiological risk factors associated with bovine brucellosis using multiple logistic regressions

PAs	No. of sera tested	RBPT Positive (%)	CFT Positive (%)	$\chi 2$ (LR)	P-value	OR (95% CI)
Enbuli	100	4(4)	3(3)			
Girakedamin	100	16(16)	15(15)	31.9	0.008	5.7 (1.597-20.386)
Amanuel	100	0	0			
Yetnora	100	1(1)	1(1)			0.33 (.0334-3.194)
Dejen	100	4(4)	4(4)			1.35(.294-6.180)
Subshengo	100	10(10)	9(9)			3.2 (.839-12.831)
Total	600	35(5.8)	32(5.3)			

Note: OR = Odds Ratio

OR (95% CI) = 95% Confidence Interval of Odds ratio

$\chi 2$ (LR) = Logistic Regression Chi-square

Among kebeles, Girakedamin kebele is compared to be significantly associated ($p < 0.05$) with odds ratio (OR) = 5.7 at 95% CI of OR (1.597-20.386).

The results of age category showed there is no statistically significant difference ($p > 0.05$) among the three age groups. All age group were affected by the disease and young sexually mature groups showed higher seropositivity than old animals. There was no statistically significant association ($p > 0.05$) between sex and seropositivity to brucellosis. However, higher seropositivity was recorded for males (6.8%, 10/148) than females (4.6%, 22/452) (Table 5).

There was no statistically significant difference ($p > 0.05$) between local and cross breed cattle to seroprevalence of bovine brucellosis. Similar seropositivity was recorded for the two breeds, 5.3% (29/544) and 5.4 % (3/56) for local and cross breed cattle, respectively.

Table 5: Summary of seroprevalence, Chi-square test results and p-value of epidemiological risk factors associated with bovine brucellosis

Variables	No. tested	RBPT positive (%)	CFT positive (%)	$\chi 2$	P-value
Sex					
Male	148	11(7.4)	10(6.8)		
Female	452	24(5.3)	22(4.9)		
Total	600	35(5.8)	32(5.3)	0.7884	0.375
Age					
3-5	231	17(7.4)	15(6.5)		
6-7	206	11(5.3)	10(4.9)		
≥8	163	7(4.3)	7(4.3)	1.0579	0.589
Total	600	35(5.8)	32(5.3)		
Breed					
Local	544	32(5.9)	29(5.3)	0.0001	0.993
Cross	56	3(5.3)	3(5.4)		
Total	600	35(5.8)	32(5.3)		

Sensitivity and specificity of RBPT and CFT

A summary of the RBPT and CFT test results for the sera at the individual animal used for the calculation of sensitivity and specificity of the tests is presented in Table 6. Values calculated at the individual animal level for the sensitivity and specificity were, 100% and 99.47% respectively

Table 6: summary of sensitivity and specificity of RBPT and CFT at individual animal

	CFT +ve	CFT –ve	Total
RBPT (+ve)	32	3	35
RBPT (-ve)	0	565	565
Total	32	568	600

Questionnaire survey

About 120 representative cattle owners in PAs of the study area were asked about the method of breeding, way of fetal membrane disposal, and presence of separate calving pen, awareness about the disease and husbandry risk factors. Most of peasant owners 74 (61.7 %) used natural breeding mating, 40 (33.3 %) opted AI and 6 (5.0 %) used both artificial insemination and natural breeding methods.

The questionnaire survey also showed that 79(65.8%) of cattle owners were not aware of bovine brucellosis and 81 (67.5%), had no separate calving pen, and 107 (89.2%) did not practice safe disposal of fetal membrane and aborted fetuses. The occurrence of abortion, stillbirth and retained fetal membranes were also found high 79 (65.8%) in their animals (Table 6).

Table 6: Summary of questionnaire survey results in all peasant associations

Risk factors	No. of respondent	% of respondents
Qualification		
Literacy	97	80.8
Primary	10	8.3
Junior	13	10.8
Livestock keeping		
Cattle	32	26.7
Mixed to others	88	73.7
Grazing system		
Communal	35	29.2
Individual	8	6.7
Both	77	64.2
Breeding method		
Natural	74	61.7
AI	6	5.0
Both	40	33.3

Safe disposal of fetal membrane		
Burry	13	10.8
Throw	107	89.2
Separate calving pen		
No	81	67.5
Yes	39	32.5
Awareness about the disease		
No	79	65.8
Yes	41	34.2
Swelling of testicle and knee		
No	84	70
Yes	36	30

5. DISCUSSION

The present study revealed that the overall seroprevalence of bovine brucellosis in Machakel and Dejen districts was 5.3% (95% CI = 3.7-7.4%). The overall seroprevalence of brucellosis in cattle in this study was lower than the reports of Kebede *et al.* (2008) (11%) and Eshetu *et al.* (2005) (10%) in the central highlands and Addis Ababa, Ethiopia, respectively. This result is very close to the reports in indigenous and cross breed animals in extensive management system, and agrees with the findings of Bekele *et al.* (2000) 4.9% in Arsi, Berhe *et al.*, (2007) 3.17% in Tigray region, by Hailemelekot (2005) 4.63% in Awi, West Gojjam and North Gondar and by Nuraddis (2010) 3.13% in Jimma zone. Nuraddis (2010) indicated that an increase of stocking density and increase of exposure to infection especially following abortion. Extensive management system which allows unrestricted contact between animals might have contributed to the spread of brucellosis in cattle. Hailemelekot *et al.*, (2007) reported a high seroprevalence of 5.97% in semi-intensive farms than extensive production system (3.83%). However, his finding was in contrast with the finding of the present study which was seen high seroprevalence of 5.3% in extensive husbandry or management. This finding might be contributed to high densities of cattle population and free movement to grazing and watering points which contribute to factors of spread of disease in a population of animal.

With regard to association of age with *Brucella* seropositivity, the finding of the present study differs from the reports of Asfaw *et al.*, (1998) who indicated that older cattle were twice more likely to be affected (4%) than younger ones (1.9%) and this variation was statistically significant ($p < 0.05$). Similarly, a serological study conducted by Bekele *et al.*, (2000) indicated prevalence of 1.7% and 11.5% in the age groups of 2-4 years and above 6 years, respectively. The finding of Bekele *et al.* (2000) agreed with the report of Asfaw *et al.*, (1998) with an increase in susceptibility to the disease as age increases. However, the finding of the present study contradicted with the above reports.

The present study classified the study population to 3-5, 6-7 and ≥ 8 years old where the prevalence of the individual age group was 6.5%, 4.9%, and 4.3%, respectively, and this was in agreement with Matope *et al.* (2011) who reported a decrease in seroprevalence with increasing of age. This might be due to the development of host defense against the organism with continuous and subsequent exposure with increasing of age. Sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms, tend to increase in concentration with age and sexual maturity might also contribute (Radostits *et al.*, 2000).

According to the infection rate for females and males in this study, in females the prevalence found to be lower (4.9%, n = 452) when compared to male (6.8%, n = 148). There is no statistically significant difference existed in the prevalence between the two sex (p = 0.375). This finding is different from other reports that indicate higher prevalence rate in females (Asmare *et al.*, 2010; Berhe *et al.*, 2007). This variation might arise from the fact that this study include relatively higher number of male animals than the previous studies such as in Berhe *et al.*, (2007) (n = 8) and in Asmare *et al.*, (2010) (n = 25). In this study area the male and female cattle are kept together for grazing in the pasture and watering points which is an important area for acquiring the infections. The high frequency of the disease in bulls emphasized cautious consideration as far as breeding program and public health issues are concerned.

In the present study, indigenous and cross breed animals which are around, 90.7% and 9.3% of the total sera collected, showed 29 (5.3%) and 3 (5.4%) seropositive results to brucellosis, respectively. In both cases, the serological prevalence is almost similar between the two breeds. This finding is in agreement with the findings of Radostits *et al.,* (2000) who reported no association of breed as epidemiological risk factor with seroprevalence of bovine brucellosis.

The present study revealed that the seroprevalence of brucellosis in cattle in the extensive management system in this study agrees with reports from other areas of the country and countries with similar cattle husbandry practices (Berhe, 2007). In general, in extensive cattle management system in Ethiopia, prevalence figures reported varies with ranges from 0.77% to 8.2% as reported by Tolosa (2004) and Mekonen (2001) and is the range which agrees with the present finding (5.3%) with similar extensive livestock production system in the study area.

Statistical significance difference (p = 0.008) showed in this study among kebeles were influenced by the husbandry and management systems which were found to be important risk factors associated with *Brucella* sero-reactors. Animals aged above four years, large herd size, and herds kept mixed with more livestock species are at increased risk of acquiring *Brucella* infection as indicated by Megersa *et al.,* (2011). In our study area where animals are kept in extensive management practices, these poor feeding and having shared watering points, which might have favored the disease transmission to unaffected animals. Educational status and lack of awareness of farmers about the disease, zoonotic aspect, and its mode of transmission might also contribute to this difference.

The result of the questionnaire survey showed that among one hundred twenty heads of the household farmers (respondents) interviewed, 97 (80.8%) illiterate, 10 (8.3%) primary school and 13 (10.8%) are junior secondary school completed. They were interviewed to assess risk factors for the possible transmission of brucellosis from cattle to cattle and possibility of humans acquiring brucellosis in the study area. The results of the questionnaire showed that there is a lack of awareness about bovine brucellosis and absence of hygienic practices, such as the use of unseparated parturition pen, improper disposal of aborted materials and unprotected handling of parturient animals during and after births. Similar findings have been documented by others researches conducted in the country (Asfaw, 1998; Gebretsadik, 2005; Kebede *et al.,* 2008; Megersa *et al*., 2011; Tadelle, 2004) suggesting that little attention has been given to preventing brucellosis. This might be contributed to the spread and transmission of the infection in the area where lack of awareness of respondents about the disease, its transmission and zoonotic importance.

6. CONCLUSION AND RECOMMENDATIONS

The finding of the present study revealed that bovine brucellosis is widely prevalent in cattle population of the study districts of Amhara regional state, Ethiopia. The disease has been detected in all *Kebeles* except Amanuel. Statistical association was observed between management risk factors and *Kebeles* with brucellosis seroprevalence. Positive serological reaction does not only suggest the occurrence of the disease in cattle population of the study area, but also indicates the presence of foundation (foci) of infection that could serve as source of infection for the spread of the disease into unaffected animals around and elsewhere. It was concluded that lack of awareness of farmers in the area about bovine brucellosis as a major cause of abortion even though high abortion rate, stillbirth, and retained fetal membrane informed by many of the respondents including its zoonotic importance and mode of transmission of the disease favored to the epidemiology of the disease.

Based on the above conclusion, the following recommendations are forwarded:

- Public awareness should be created as to the importance of brucellosis as a major cause of abortion, mode of transmission, proper disposal of aborted materials, and its public health significance of brucellosis at all levels of livestock owners.
- Strong epidemiological reporting system to overtake health measurers to minimize the infection rate of animals and humans as well.
- Seroprevalence and associated risk factors should be conducted in adjacent woredas to undertake prevention and control mechanisms very practical for the area.

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8. ANNEXES

Annexe 1: Questionnaire survey (individual owners)

General information

Date_____region_____Zone_____District_____PA_____

Name of respondant _____ age_____ sex _____

qualification_____ family size_____ herd size_____

Herd size

Breeding females

Dry

Lactating

Breeding males

Un weaned males

Heifers

Un weaned females

Castrated males

Management Information

1. What type of livestock species are you keeping in the area (cattle, sheep, goat and equine)
2. What is your grazing system? (communal, individual, both)
3. Which breed of cattle do you own? (local, cross, exotic)
4. What type of mating do you use? (Natural, Artificial Insemination, Both)

Disease information

Do you know any disease that causes abortion in cattle, sheep and goat? 1. Yes 2. No

If yes what are there? Name symptoms scientific name

What do you do with them after abortion?

Do you separate cows sheep and goat during parturition? 1. Yes 2. No

How you dispose the abortus? 1. Throw 2. Burry

Have you ever observed swelling of testicle or knee in your animals? 1. yes 2. No

Annex 2: Test method to diagnosis of brucellosis

i) Rose Bengal Plate Test procedure

The Rose Bengal or Stained Buffered Acidified Antigen permits the serological diagnosis of brucellosis (Brucella melitensis, abortus and suis) by rapid slide agglutination. It is one of the easiest methods to implement and the most widely used for identifying Brucellosis antibodies in sera.

Instructions for use

- Keep the antigen and sera 30 to 60 minutes at room temperature +21 °C (± 5 °C) before the beginning of the tests. Shake it gently.
- On a plate, place 30µl of each serum to be tested
- Gently shake the antigen vial and put 30 µl besides each serum
- Carefully mix the antigen and the serum
- Shake the plate for exactly 4 min and read immediately

Test Interpretation

No agglutination: absence of antibodies

Agglutination (even slight): presence of antibodies

ii) Complement Fixation Test method

(1) Dilute the samples 1:5 with Veronal Buffered Saline or Diluent (VBS or VBD) and inactivate the complement in a waterbath at 57 °C for 30 minutes.

(2) Using standard 96-well U-bottom microtitre plates, add volumes of 50 µl of the diluted sample to a well in column 1 and column 11.

(3) Add 25 µl of VBD to all wells of column 2 to 10 and column 12.

(4) Transfer 25 µl of the sample from column 1 to 2 until column 10 and then remove 25 µl from column 10.

(5) Transfer 25 µl of the sample from column 11 to 12 and then remove 25 µl from column 12.

(6) Dilute the antigen 1:32 in VBD.

(7) Add 25 µl of diluted antigen to all wells of column 1 to 10.

(8) Add 25 µl of VBD to all wells of column 11 and 12.

(9) Dilute the complement from 1:40 to the appropriate dilution found in the complement titration.

10) Add 50 µl of diluted complement to each well of 96-well U-bottom microtitre plate.

(11) Incubate the plate for 60 minutes at 37 °C in a humid chamber.

(12) Mix equal amounts of amboceptor diluted (1:1000) and 2% SRBC (i.e. hemolytic system).

(13) Add 50 µl of the hemolytic system to each well of the microtitre plate.

(14) Close the plate with adhesive tape and the results are read after the plates have been left incubated in a waterbath at 37 °C for 30 minutes or at 4 °C for 14-18 hours.

Test results

The results should always be expressed in international CFT units (icftu), calculated in relation to those obtained in a parallel titration with a working or national standard serum calibrated against the OIE International Standard Serum (OIEISS, previously the WHO Second International anti-*Brucella abortus* Serum) which contains 1000 icftu. In general, sera giving positive fixation at a titre equivalent to 20 iu or greater are considered to be positive. The test results of CFT read as negative for 100 % hemolysis, 1+ for 75%, 2+ for 50 %, 3+ for 25 %, and 4+ for no hemolysis.

The test is examined by the eye:

Sedimentation of the sensitized RBC = Positive result.

Complete lysis of the sensitized RBC = Negative results

9. SIGNED DECLARATION SHEET

The undersigned, declaration that the thesis is my original work and has not been presented for degree in any university and all source of materials used for the thesis have been dully acknowledged.

Name_____

Signature_____

Date of submission_____

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

Name_____

Signature_____

Name_____

Signature_____

Date_____