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SEROPREVALENCE OF SMALL RUMINANT BRUCELLOSIS AND ITS PUBLIC HEALTH AWARENESS IN SELECTED TWO DISTRICTS OF AFAR REGION, ETHIOPIA

MSc THESIS

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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH (MIVP)

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A thesis submitted to the school of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Science in Veterinary Public Health

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Approval sheet of Thesis

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Anteneh Hailu** entitled seroprevalence of small ruminant brucellosis and its public health awareness in selected two districts of afar region, Ethiopia and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Master of Science in Veterinary Public Health.

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I under sign, declare that the thesis is my original work and has not been presented for a Degree in any University. All the resources and materials used are duly acknowledged.

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LIST OF ABBREVIATIONS

ANRS	Afar National Regional State
ARFEB	Afar Region Finance and Economy Bureau
CBAHWs	Community Based Animal Health Workers
CFT	Complement Fixation Test
CSA	Central Statistical Agency
I-ELISA	Indirect Enzyme Linked Immuno Sorbent Assay
FAO	Food and Agricultural Organization
GDP	Gross Domestic Product
HS	Haemolytic System
ILRI	International Livestock Research Institute
MoARD	Ministry of Agriculture and Rural Development
mRBPT	Modified Rose Bengal Plate Test
NGOs	Non Governmental Organizations
NVI	National Veterinary Institute
OIE	Office International des Epizooties
PA	Peasant Association
S-LPS	Smooth lipopolysaccharide
SPSS	Statistical Package for Social Sciences
SRBC	Sheep Red Blood Cell
SSA	Sub-Saharan Africa
VCM	Veronal Calcium Magnesium
WHO	World Health Organization

ABSTRACT

Between Nov. 2013 and Apr.2014, a serological study was conducted on small ruminants to determine the prevalence of brucellosis, identify risk factors and its public health implication. A total of 1190 blood samples collected from sheep and goat populations in Chifra and Ewa woredas (876 caprine and 314 ovine), 155 (13%) tested positive using mRBPT. Further testing of the positive reactors with CFT confirmed that 147(12.35%) samples were positive for brucella infection. The results revealed that among the risk factors considered in the analysis, species, sex, age, parity number and flock size had statistically significant effect on seropositivity ($p<0.05$). Woreda had no statistically significant effect on seropositivity ($p>0.05$). The individual animal level seroprevalence was significantly higher in goats (14.4%) than in sheep (6.7%) populations. The analysis revealed that goats were more than 2 times (OR=2.34) at risk of getting infected with brucella than sheep. The seroprevalence was also significantly higher in female animals (13.8%) than in males (6.5%) with the likelihood of female animals to get infected with brucellosis is higher than that of males. Similarly, Brucella seroprevalence was also significantly increased with age; the odds of seropositivity in older animals are 2.36 times higher than that of younger animals. Seroprevalence rate of brucellosis was also significantly varied with parity, with the likelihood of infection being 2.038 times higher in animals with 3 to 4 parities than otherwise. Regarding flock size individual animal sero-prevalence was higher in larger flocks than in the smaller ones (OR=0.68). Almost all the respondents in the studied areas were not aware of brucellosis as a disease affecting different species of livestock (91.1%) although all of them recognized the existence of abortion among small ruminant flocks and most of them handle abortion materials with bare hand without protecting themselves (82.2%). The habit of drinking raw milk is practiced by the majority of the respondents 45 (100%) while there is no habit of consuming raw meat. Poor awareness of the zoonotic importance of brucellosis and the practices of consuming raw milk and handling potentially infectious materials using bare hands pose a serious danger to small ruminant owners. Finally, this study demonstrated that small ruminant brucellosis is highly prevalent in the study districts. Hence, it is suggested that there is a need for planning and implementation of joint programs by stakeholders in prevention and control of the disease as well as raising public health awareness.

***Key words**: Afar region, Brucellosis, Small ruminants, CFT, Public health, RBPT, Zoonoses*

1. INTRODUCTION

Brucellosis is a zoonotic bacterial disease caused by *Brucella* species and is primarily a disease of animals whereas humans are accidental hosts (Corbel *et al*, 2006). The disease is one of the most widespread zoonoses and is endemic in many countries including Ethiopia. The disease is an important public health problem in many parts of the world (Pal, 2007; Hadush and Pal, 2013). It is also considered a neglected zoonosis by the WHO (WHO, 2006).

Brucellosis is one of the most important bacterial zoonoses worldwide in particular in developing countries and has important economic and public health consequences (Pappas *et al*., 2006; Franco *et al*., 2007). Brucellosis has been virtually eliminated from the majority of the developed countries, but it is still endemic in Africa, the Middle East, Central and Southeast Asia, Central and South America and in most of the Southern European countries (Donev *et al*., 2010). Despite being endemic in many developing countries, brucellosis remains under diagnosed and under-reported. It is an important disease among livestock and people in sub-Saharan Africa (Smits *et al*., 2007).

Infection in animals is strongly correlated with abortions in the last trimester of pregnancy. The most commonly affected animals are cows (*B. abortus*), ovine and caprines (*B. melitensis*), pigs (*B. suis*), and some other domestic animals including Camels. Some wild animals might also be affected by this zoonosis, but these are rarely implicated as sources of human disease. In animals, the primary sign of infection in females is abortion and in males epididymitis and orchitis and diagnosis can only be confirmed by laboratory tests that may even confirm latent infections (Corbel, 2006; Denov *et al*., 2010). Cross-transmission of brucellosis can occur between cattle, sheep, goats, camels and other species (Dawood, 2008).

Brucellosis is one of the most important bacterial zoonoses transmitted to humans mainly from ruminants. This disease is a major cause of direct economic losses resulting from clinical disease, abortion, neonatal losses, reduced fertility, decreased milk production and emergency slaughtering of the infected animals for animal industry. It also causes a barrier for international trade of live animals by being used as an impediment to free animal movement and export (Coelho *et al.*, 2007).

It has been shown that brucellosis cause heavy economic losses in livestock industry. Economic losses in small ruminants stem from breeding inefficiency, loss of lambs and kids, reduced wool, meat and milk production (Renukaradhya *et al.*, 2002).

More than 500,000 human cases are reported worldwide each year, but the number of undetected cases is believed to be considerably higher. This alarming situation can be attributed to the non-specific clinical picture of human brucellosis, low awareness of the disease in non-endemic countries and shortcomings in laboratory diagnosis. The number of human cases is directly correlated with the number of infected animals within a defined region. Effective counter measures to reduce the incidence of human brucellosis are therefore based on surveillance and control of livestock and pasteurization or cooking of contaminated food products. Once the disease has been transmitted from its animal reservoir to humans, only early diagnosis and adequate antibiotic therapy can prevent serious sequelae in patients (Pappas *et al*., 2006; Donev *et al*., 2010).

In Africa, zoonotic diseases are among the major diseases of veterinary and public health importance accorded a very little attention. To that effect, little is known about its epidemiological status in animals and humans and factors contributing to its cross-species and zoonotic transmission (Megersa *et al*., 2011).

A huge and diverse livestock species of Ethiopia are maintained under different agro-ecological zones, management, migration and animal health care system. The predominately extensive animal husbandry practices of the country provide ample opportunities for intermixing of different animal species, communal grazing areas and water points, and composite holding of livestock maintained by nearly 80% of the rural community (Samui *et al*., 2007).

The detection of specific antibodies in serum or milk remains the most practical means of diagnosis of brucellosis (WHO, 2006). Among many serological tests available, the complement fixation test (CFT) is the only test prescribed for confirmation and international trade. The Rose Bengal plate agglutination, complement fixation and indirect ELISA tests are recommended for screening flocks and individual animals (FAO, 2003).

More importantly, a close human-animal contact and tradition of raw animal product consumption make zoonosis among the major public health hazards, with particular implication to pastoral area. This requires a thorough epidemiological investigations including due consideration to identifying the major risk factors that predominantly influence the disease occurrence, and thus contribute to designing appropriate and feasible national controlling strategies. This serological investigation was therefore, designed with the aim:

- To assess sero-prevalence of brucellosis in small ruminants and livestock owners awareness of the problem and its zoonotic impact in Ewa and Chifra districts.

- To identify risk factors of brucellosis and its sources for livestock and people.

2. LITERATURE REVIEW

2.1. Socio Economic Importance of Ovine and Caprines

The World Development Report (2010) estimated that about 410 million people in sub-Saharan Africa still live in absolute poverty, surviving on less than one dollar per day. According to Olayemi (1995), the poor have no access to the basic necessities of life such as food, clothing and decent shelter, are unable to meet social and economic obligations, lack skills for gainful employment, have few if any economic assets and a general lack of self-esteem. In most cases, the poor lack the capacity to liberate themselves from the shackles of poverty, and this perpetual situation makes the conditions of extreme poverty persist and they are transmitted from generation to generation (Obadan, 1997).

Small ruminants are of economic importance to smallholder farmers and especially women. The total income share of small ruminants tends to be inversely related to size of land-holding, suggesting that small ruminants are of particular importance for landless people especially women. In some cultural settings, women are often not entitled to own land and since agriculture (crop production) provides only seasonal employment, rearing small ruminants would provide employment and income as a subsidiary occupation. Livestock are often regarded as producers of milk and meat, income generators and reservoirs of wealth (Coppock *et al.*, 2006).

In fact, among the pastoralists, flock building is the main source of survival. Pastoralist women traditionally have exclusive rights to sell milk and milk products to obtain a modest amount of income. However, the low marketable milk output in pastoral area poses limitations on the possibilities of exploring distant but rewarding markets due to high transaction costs arising from transportation and high opportunity costs of labor (Sadler *et al.*, 2009). Moreover, the environmental adaptation of indigenous breeds facilitates livestock production in a wide range of agro-ecological conditions and constraints. Compared to exotic breeds, indigenous varieties entail lower workloads, especially for women (Köhler-Rollefson, 2000).

However, rearing of small ruminants like sheep and goats would have lasting effects in bringing about social change by improving the incomes of the rural people. The ruminants provide their owners with a vast range of products and services. Very often, there are no banking facilities in rural areas and an easy way to store cash for future needs is through the purchase of sheep and goats (IBC, 2004). In fact, in some areas, small ruminants have been described as the 'village bank'. From the foregoing, small ruminants play an important role in ensuring rural women's financial security and data supports that women are better managers of household resources than men. Thus, an improvement in the financial security of rural women through rearing small ruminants would inevitably translate to better living conditions for households (Maxwell, 1990).

2.2. Etiology

Brucella species are facultative intracellular gram-negative cocco-bacilli, non-spore-forming and non-capsulated. Although *Brucella* species are described as non-motile, they carry all the genes except the chemotactic system, necessary to assemble a functional flagellum. 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* (Sriranganathan *et al*., 2010) 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 *Brucella* have no classic virulence genes encoding capsules, plasmids, pili or exotoxins and compared to other bacterial pathogen 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; Sriranganathan *et al*., 2010).

2.3. Epidemiology

2.3.1 Distribution of the disease

Brucellosis is a widespread disease and of major economic importance in most of the countries in the world, particularly among cattle. In small ruminants the disease is more restricted to the Mediterranean region including southern Europe, West and Central Asia, South America and Africa (Corbel, 1997; Godfroid *et al*., 2005) with considerable variation between flocks and between areas and countries.

B. melitensis is the most virulent species of the *Brucella* genus and has three biovars, with biovars 1 and 3 being the ones isolated most frequently in small ruminants in the Mediterranean, the Middle East and Latin America (Lucero *et al*., 2008; Blasco and Molina-Flores, 2011). Brucellosis is a barrier to trade in animals and animal products and causes significant losses from abortion, as well as being a serious zoonosis (Benkirane, 2006; Banai, 2007; Seleem *et al.*, 2010).

Goats are the classic and natural host of *B. melitensis* and together with sheep are its preferred hosts. In pathological and epidemiological terms, *B. melitensis* infection in small ruminants is similar to *B. abortus* infection in cattle: the main clinical manifestations of brucellosis in ruminants are abortion and stillbirths, which usually occur in the last third of the pregnancy following infection and usually only once in the animal's lifetime (Elzer *et al*., 2002; Blasco and Molina-Flores, 2011).

The geographical distribution of brucellosis is constantly changing, with new foci emerging or re-emerging. The epidemiology of human brucellosis has drastically changed over the past few years because of various sanitary, socioeconomic, and political reasons, together with increased international travel. 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).

Table 1: Prevalence status of small ruminant brucellosis in some countries of the world including Africa

Species	Authors	Country	Prevalence (%)	Year
Ovine	Gupta *et al*.,	India	59	2006
Ovine & Caprine	Coelho *et al*.,	NE Portugal	8.9	2007
Caprine	Bokaie *et al*.	Iran	3.4	2008
Ovine	Celebi and Atabi	Turkey	36.7	2009
Ovine	Bertu *et al*.,	Nigeria	14.5	2010
	Abdel-El *et al.,*	Egypt	3.5	2010
Caprine	Bertu *et al*.,	Nigeria	16.1	2010
Caprine	Ahmed *et al*.,	Libya	31	2010

In Africa, the occurrence of brucellosis in sub-Saharan countries (either prevalence or incidence) is not well documented and reports submitted to the World Organization for Animal Health (Office International des Epizooties) are largely confined to serological surveys, and mainly conducted for cattle and less for sheep and goats. McDermott and Arimi (2002) referred to a great variation in prevalence in sub-Saharan Africa (ranging from 4.8 to 41%) in pastoral systems. In comparison with bovine brucellosis, brucellosis in sheep and goats caused mainly by B.*melitensis* has with only a few exceptions a low or sporadic degree of incidence throughout the African continent (McDermott and Arimi, 2002).

2.3.2. Distribution of Brucellosis in pastoral and agro pastoral areas of Ethiopia

The pastoral and agro-pastoral production system represent approximately 45-55% of the cattle, 75% of the small ruminants, 20% of the equines and 100% of the camels of the total national livestock population. The main mobile pastoralists in Ethiopia are the Somalis (Somali region) in the east, the Afars (Afar region) in the northeast, the Borena Oromos

(Oromiya region) in the south and south-east and the Southern Omo people (SNNPS region) in the south and partly in the Gambela and Benishangul regions and around the Dire Dawa Administration. Despite the large size of the regional livestock population, its economic contribution to the regional and national economy is not significant, mostly due to natural and human limitations (Amaha, 2006).

Most of the studies which conducted on brucellosis were entirely based on estimation of seroprevalence of the disease. There are very few reports on the Seroprevalence of brucellosis, which are conducted on different livestock species and human in pastoral and agro-pastoral areas of the Ethiopia. Additionally, pastoral households often keep a diverse composite of livestock species as part of a coping mechanism for uncertainties and risks. Such conditions certainly increase aggregation and interaction of different animals at villages, grazing fields and water points, thus, facilitate transmission of the disease. The dynamics and frequent migration of pastoral flocks might increase the chance of coming into contact with other potentially infected flocks and exposure to geographically limited or seasonally abundant diseases. Mobility also increases the opportunity of interactions with wild animals. This has already been confirmed by (Samui *et al*., 2007; Megerssa *et al*., 2011) in that flocks coming into contact with wildlife had higher likelihood of acquiring infection than those without contact.

Table 2: Seroprevalence of brucellosis in small ruminants of different pastoral and agro pastoral areas of Ethiopia

Authors	Region	Prevalence (%)	Year
Teshale *et al*.,	Afar	1.7 (Caprine)	2006
Teshale *et al*.,	Somali	3.2 (Ovine)	2007
Teshale *et al*.,	Afar	16.5 (Caprine)	2007
Ashenafi *et al*.,	Afar	5.8 (Caprine)	2007
Wesinew *et al*.,	Afar	7.1 (Ovine)	2012
Wesinew *et al*.,	Afar	13.6 (Caprine)	2012

2.3.3. Transmission

B. *melitensis* primarily affects small ruminants and while the bacteria shows strong host preference B. *melitensis* can also be found in other species, due to the bacteria's ability to cross-infect to other animal species. Transmission occurs mainly after abortion when the bacteria can be found in fluids and tissues connected with pregnancy like the placenta, dead foetuses and the udder (Corbel *et al.*, 2006; OIE, 2009).

B. *melitensis* is highly pathogenic and infection in sheep and goats create a great risk for people living in close vicinity to their animals to contract the disease. Animal owners generally mix small ruminant from different flocks to higher extent than cattle and the density of the flocks and flocks causes a high number of bacteria shed in the environment and consequently generate a main route for animal-to-animal transmissions (Corbel *et al.*, 2006; FAO, 2010).

The disease in goats is usually more severe and prolonged than in sheep due to the fact that the susceptibility to B. *melitensis* is generally higher in goats compared to in sheep (Quinn *et al.*, 2002).

Healthy animals can be exposed to *Brucella* infection in many ways, as a large number of bacteria are shed in the birth fluids or fetus, placenta and abortion secretions of infected females. The bacteria have the ability to survive several months outdoors, especially in cold, wet conditions, where they remain infectious to other animals, mainly through ingestion. Brucellae also colonise the udder and contaminate milk (Banai, 2007; Blasco, 2010). Although females calve apparently normally in pregnancies following the first abortion, they continue to shed large numbers of bacteria into the environment. As with *B. abortus* infection in cows, *B. melitensis* can be transmitted congenitally in utero but only a small proportion of lambs and kids are infected in this way and most latent infections of *B. melitensis* are probably acquired by ingesting colostrum or milk (Grillo *et al.*, 1997). Despite the low transmission rate, the existence of such latent infections makes it even more difficult to eradicate the disease because, as the bacteria persist without inducing detectable immune response, infected animals are silent carriers of the disease. It is therefore recommended that infected females and their offspring be culled as part of an eradication programme in infected flocks (Banai, 2007). The exact mechanism enabling latent *Brucella* infection to develop is unknown (Blasco and Molina-Flores, 2011).

The isolation of *B. melitensis* in dogs has been demonstrated and this has been observed to favour incidence of the disease, as dogs can drag placentas or aborted fetuses to uninfected areas (Mikolon *et al*., 1998; Hinić *et al*., 2012). There is one report of *B. melitensis* biovar 3 having been isolated from a black bullhead catfish (*Ameiurus melas*) but clarification is needed as to whether such animals can act as disease carriers or whether the finding was a result of water pollution (El-Tras *et al*., 2010).

Transmission of infection to humans occurs through breaks in the skin, following direct contact with tissues, blood, urine, vaginal discharges, aborted foetuses or placentas. Food-borne infection occurs following ingestion of raw milk and other dairy products, but rarely from eating raw meat from infected animals. Occupational airborne infection in laboratories and abattoirs has also been documented. Accidental inoculation of live vaccines (such as *B. abortus* Strain 19 and *B. melitensis* Rev.1) can also occur, resulting in human infections. There are also case reports of venereal and congenital infection in humans (Robinson *et al.*, 2003).

2.3.4. Risk factors for transmission

The epidemiological variables which are considered to affect the initiation, spread, maintenance and/ or control of brucellosis can be categorized into those related to the animal population, to management, or to biology of disease (Nicoletti, 1980; Radostitis *et al*., 1994). The factors influencing the transmission of Brucella species in a geographical region can be classified into two categories: those associated with transmission of disease between flocks (purchase of infected animals, proximity of infected flocks to clean flocks, sharing pastures, dip tanks, watering points, and strays of infected animals into clean flocks), and those influencing the maintenance and spread of infection within flocks (unvaccinated animals in infected flocks, flock size, population density, method of housing and use of maternity pens) (Nocoletti, 1980; Radostitis *et al*., 1994).

2.3.5. Host factors

Susceptibility of livestock to Brucella infection is influenced by the age (young animals are less susceptibile to Brucella than older animals), sex and reproductive status of the individual animal (sexually mature, pregnant animals are more susceptible to infection with the organism than sexualyy immature animals) (Nicoletti, 1980). Placental trophoblasts produce erythritol in increasing amounts during the later stages of pregnancy which coincides with the period when pregnant sheep and goats are more susceptible to infection with B.*melitensis.* The preferential utilization of erythritol rather than glucos is characterstic of pathogenic *Brucella* strains. Erythritol promotes the growth of some strains of *Brucella* and the metabolic pathway for degradation of erythritol, the role of this sugar in the virulence of the organisms has been put into question (Sangari *et al*., 2000).

2.3.6. Environmental and Climatic Factors

Atmospheric conditions and seasons of the year may have influence on the management and contact of the infected and susceptible host. In dry areas, water resources are sparsely distributed (Helland 1982). As a result, the congregation of a large number of mixed ruminants at water points facilitates disease spread. The coincidence of parturition in wet season (Schwartz and Dioli, 1992) enhances the viability of the organisms in the environment, thus increasing the chance of infecting susceptible animals (Corbel, 1990). Baumann and Zessin (1992) reported higher brucellosis reactor rate in two wet seasons than dry seasons. The incidence of brucellosis in camel population appears to be related to breeding and husbandry practices. Flock sizes, density of animal population, and poor management are directly related to prevalence (Wernery and Kaaden, 2002).

Zoonoses

Five out of the nine known *Brucella* species can infect humans and the most pathogenic and invasive species for human is *Brucella melitensis*, followed in descending order by *B. suis, B. abortus and B. canis* (Acha *et al*., 2003; Sriranganathan *et al*., 2010). The zoonotic nature of the marine *brucellae* (*B. ceti*) has been documented. *B.melitensis, B. suis and B. abortus* are listed as potential bio-weapons by the Centers for Disease Control and Prevention in the USA. This is due to the highly infectious nature of all three species, as they can be readily aerosolized. Moreover, an outbreak of brucellosis would be difficult to detect because the initial symptoms are easily confused with those of influenza (Sriranganathan *et al*., 2010).

2.4. Pathogenesis

The ability of *Brucella* spp. to cause disease requires a few critical steps during infection. *Brucella* spp. can invade epithelial cells of the host, allowing infection through mucosal surfaces: M cells in the intestine have been identified as a portal of entry for *Brucella* spp. (Ackermann *et al*., 1988; Paixão *et al*., 2009). Once *Brucella* species have invaded, usually through the digestive or respiratory tract, they are capable of surviving intracellularly within phagocytic or non-phagocytic host cells (Carvalho, 2010). *Brucella* has the ability to interfere with intracellular trafficking, preventing fusion of the *Brucella*-containing vacuole (BCV) with lysosome markers, and directing the vacuole towards a compartment that has rough endoplasmic reticulum (RER), which is highly permissive to intracellular replication of *Brucella* (Anderson, 1986; Pizarro-Cerdá, 1998; Pizarro, 2000).

The outcome of infection is dependent on the species of *Brucella* and host. The *Brucella* species that infect livestock are host restricted. For instance, *B.melitensis*, *B. abortus*, *B. suis* and *B. ovis* infect preferentially small ruminants, cattle, pigs and sheep, respectively (Xavier *et al*., 2009). With the exception of *B. ovis*, these *Brucella* spp. have zoonotic potential, with *B. melitensis* being the most pathogenic for humans (Young, 1995).

The mechanisms that allow host cell invasion by *Brucella* species are not completely clear, but although specific host receptors that interact with *Brucella* have not yet been identified, internalisation of *Brucella* into host cells requires cytoskeletal changes (Pizarro *et al*., 1999; Guzmán-Verri *et al*., 2001). Interestingly, invasion through the digestive tract does not elicit any inflammatory response from the host (Paixão *et al*., 2009). Therefore, *Brucella* spp. invade silently or unnoticed by the innate immune system of the host. In fact, *Brucella* spp. have mechanisms that prevent activation of the host innate immune system (Barquero-Calvo *et al*., 2007). Indeed, *Brucella* Toll / interleukin-1 receptor (TIR) domain-containing protein prevents Toll-like receptor (TLR) 2 signalling by interfering with MyD88, and also inhibits DC maturation, cytokine secretion and antigen presentation (Cirl *et al*., 2008, Salcedo *et al*., 2008). *Brucella abortus* also induces suppression of the transcription of pro-inflammatory mediators in trophoblastic cells at very early stages of infection (Carvalho *et al*., 2008). Trophoblasts are placental cells that are targeted during infection of pregnant cows. After an initial suppression of pro-inflammatory transcripts, *B. abortus* induces expression of pro-inflammatory chemokines by cultured trophoblastic cells, which correlates with the profile of expression observed *in vivo* in the placenta of infected cows (Carvalho *et al*., 2008).

Brucella spp. lack classical bacterial virulence factors such as exotoxins, cytolysins, a capsule, fimbriae, flagella, plasmids, lysogenic phages, endotoxic lipopolysaccharide (LPS), and inducers of host cell apoptosis (Moreno and Moriyón, 2006). However, LPS plays an important role in *Brucella* virulence because it prevents complement-mediated bacterial killing and provides resistance against antimicrobial peptides such as defensins and lactoferrin (Allen *et al*., 1998; Lapaque *et al*., 2005). Another important virulence mechanism of *Brucella* is the BvrR/BvrS two-component regulatory system, which is required for modulation of the host cell cytoskeleton upon *Brucella* invasion, and for regulation of the expression of outer membrane proteins, some of which are required for full virulence (López-Goñi *et al*., 2002). Cyclic β-1,2-glucans, which are also part of the outer membrane, are also required for intracellular survival of *Brucella* (Briones *et al*., 2001). *Brucella* spp. express a type IV secretion system (T4SS), encoded by the components of the *virB* operon, that is crucial for intracellular survival in host cells and virulence *in vivo* (O'Callaghan *et al*., 1999; Hong *et al*., 2000).

2.5. Clinical signs

Brucellosis affects many animal species but can cause several problems for livestock holders when the disease occurs among food-producing animals. Important clinical signs are; abortions usually during the last third of pregnancy, premature births, retained placenta, reduced fertility and lowered milk production. Epididymitis and orchitis in males are two important clinical signs. Correct diagnosis is reliant on isolation of the bacteria or detection of; genetic material, antigen, antibodies or cell-mediated immune responses since the clinical signs are not pathognomonic (Corbel *et al.*, 2006).

Clinical manifestation among humans is acute febrile illness which may persist and develop into a chronic disease with serious complications, such as joint illness, organ failure and symptoms of mental illness (Corbel *et al*, 2006; Quinn *et al*, 2002). The mortality rate is relatively low, especially when the patient is treated with adequate antibiotics; however this is not the case for everyone in low income countries (Corbel *et al*, 2006). In endemic countries like Ethiopia humans get infected mainly by drinking unpasteurized milk and/or exposure to aborted foetuses, placentas or infected animals (FAO, 2010).

The agent erythritol (polyhydric alcohol) is found in animal placental tissue but worth mentioning not in human placental tissue. Erythritol acts as a growth factor for *Brucella* species and promotes infection in placenta and foetus and often followed by abortion. The same agent can also be found in mammary glands and epididymis (Quinn *et al.*, 2002).

2.6. Diagnosis

2.6.1. Useful specimen for the diagnosis of brucellosis

For the diagnosis of brucellosis, the organism may be recovered from a variety of materials which usually depends on the presenting clinical signs (OIE, 2009). In animals, the placenta is the most infective and contains the greatest concentration of bacteria; this is followed by the lymph nodes and milk; and from blood in humans (Poiester *et al*., 2010). Furthermore, other materials rich in the organism include: stomach contents, spleen and lungs from aborted foetuses, vaginal swabs, semen, and arthritis or hygroma fluids from adult animals. From animal carcasses, the preferred tissues for culture are the mammary gland, supramammary, medial and internal iliac, retropharyngeal, parotid and prescapular lymph nodes and spleen (OIE, 2009; Ahmed *et al*., 2010).

All specimens must be packed separately, cooled and transported immediately to the laboratory in leak proof containers. For humans, blood for culture is the material of choice, but specimens need to be obtained early in the disease. The samples should be frozen until required for culture (OIE, 2009). There is no ideal tissue for the isolation of *Brucella* from marine mammals, unless gross lesions are found in the tissues. However, the recommended tissues for the recovery of *Brucella* in marine mammals are the spleen, the mammary gland, the mandibular, gastric, external and internal iliac and colorectal lymph nodes, the testes and blood (Foster *et al*., 2002).

Diagnosis and control of the disease in animals must be carried out on a flock basis. There may be a very long incubation period in some infected animals and individuals may remain serologically negative for a considerable period following infection. The identification of one or more infected animals is sufficient evidence that infection is present in the flock, and that other serologically negative animals may be incubating the disease and present a risk. Diagnostic tests fall into two categories: those that demonstrate the presence of the organisms and those that detect an immune response to its antigens (Corbel, 2006).

2.6.2. Bacteriological methods

Direct smear microscopic examination

Marin *et al*., (1996) reported that a presumptive bacteriological diagnosis of *Brucella* can be made by means of the microscopic examination of smears from vaginal swabs, placentas or aborted foetuses, stained with the Stamp modification of the Ziehl-Neelsen staining method. However, morphologically-related microorganisms, such as *Chlamydophila abortus, Chlamydia psittaci* and *Coxiella burnetti* can mislead the diagnosis because of their superficial similarity (Marin *et al.*, 1996; Poiester *et al*., 2010). Accordingly, the isolation of *B. melitensis* on appropriate culture media such as Farrell's selective media is recommended for an accurate diagnosis. Vaginal swabs and milk samples are the best samples to use in isolating *B. melitensis* from ovine and caprines (Marin *et al*., 1996).

Cultural isolation of *Brucella* organism

This procedure may be performed by culturing body tissues or secretions like blood, milk and virginal discharge (Poiester *et al*., 2010). Higher sensitivity and faster culture times may be achieved in patients with previous antibiotic intervention, when the bone marrow is cultured (Mantur *et al*., 2006). *Brucella* species can also be cultured from pus, cerebro-spinal fluid, and pleural, joint and ascitic fluids. Growth of the bacteria in culture media is an unequivocal proof of infection. Blood cultures are useful only in animals with bacteraemia, which may not always occur. However, milk has often been found to contain *Brucella* by this test. Samples like lymph nodes, liver, spleen, udder and other organs at post-mortem can present positive culture results with negative serological tests. In this respect, the culture test has been widely used in research (OIE, 2009; Poiester *et al*., 2010).

The identification of *Brucella* species in culture depends on a great deal of phenotypic traits such as: CO2 requirement, phage typing and biochemical tests, which, among other problems, involve time, bio safety, trained personnel and somewhat ambiguous results. Broth or agar can be prepared from powder media for culture of *Brucella* organisms. Due to the low *Brucella* load in the blood and other body fluids, broth or a biphasic medium are preferable for their culture (Bricker, 2002).

Laboratory animal inoculation

Mice have been reported to be the animal model most frequently used in brucellosis research (Mense *et al*., 2001; Silva *et al*., 2011). Nevertheless, it has been reported that guinea pigs are also susceptible and can be used (Avong, 2000; Ocholi, 2005; OIE, 2009). Animal inoculation may be either subcutaneously or through abraded skin in guinea pigs or, preferably, intravenously, intraperitoneally, or through the digestive tract or nasal (aerosol) routes in mice (OIE, 2009; Silva *et al*., 2011). The spleen of mice is cultured 7 days after inoculation, while serum samples of guinea pigs are subjected to specific tests 3 and 6 weeks after inoculation (OIE, 2009). It is noteworthy however, that gastric acid can interfere with the infectivity of *Brucella* in laboratory animals (Silva *et al*., 2011).

2.6.3. Serological methods

Rose Bengal plate test

The RBT is one of a group of tests known as the buffered *Brucella* antigen tests which rely on the principle that the ability of IgM antibodies to bind to antigen is markedly reduced at a low pH. The RBT is a simple spot agglutination test where drops of stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction. The test is an excellent screening test but may be oversensitive for diagnosis in individual animals, particularly vaccinated ones (Corbel, 2006).

Enzyme linked immune sorbent assays test

The ELISA tests offer excellent sensitivity and specificity whilst being robust, fairly simple to perform with a minimum of equipment and readily available from a number of commercial sources in kit form. They are more suitable than the CFT for use in smaller laboratories and ELISA technology is now used for diagnosis of a wide range of animal and human diseases. Although in principle ELISAs can be used for the tests of serum from all species of animal and man, results may vary between laboratories depending on the exact methodology used. Not all standardization issues have yet been fully addressed. For screening, the test is generally carried out at a single dilution. It should be noted, however, that although the ELISAs are more sensitive than the RBT, sometimes they do not detect infected animals which are RBT positive. It is also important to note that ELISAs are only marginally more specific than RBT or CFT (Corbel, 2006).

Complement fixation test

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. It is essential to titrate each serum sample because of the occurrence of the prozone phenomenon whereby low dilutions of some sera from infected animals do not fix complement. This is due to the presence of high levels of non-complement fixing antibody isotypes competing for binding to the antigen. At higher dilutions these are diluted out and complement is fixed. Such positive samples will be missed if they are only screened at a single dilution. In other cases, contaminating bacteria or other factors in serum samples fix or destroy complement causing a positive reaction in the test, even in the absence of antigen. Such “anti-complementary” reactions make the test void and a CFT result cannot be obtained (Corbel, 2006).

2.6.4. Molecular methods

Polymerase chain reaction (PCR) assays can be used to detect *Brucella* DNA in pure cultures and in clinical specimens, i.e. serum, whole-blood and urine samples, various tissues, cerebrospinal, synovial or pleural fluid, and pus (Colmenero *et al*., 2005; Debeaumont *et al*., 2005; Queipo-Ortuño *et al*., 2006; Queipo-Ortuño *et al*., 2009; Colmenero *et al*., 2010). Direct detection of *Brucella* DNA in brucellosis patients is a challenge because of the small number of bacteria present in clinical samples and inhibitory effects arising from matrix components (Queipo-Ortuño *et al*., 2008). Basic sample preparation methods should diminish inhibitory effects and concentrate the bacterial DNA template. Residual PCR inhibition in complex matrices can be unmasked by the use of an internal amplification control (Al Dahouk *et al*., 2007).

The advantages of PCR are numerous. Independent of the disease stage, it is more sensitive than blood cultures and more specific than serological tests. Various PCR assays targeting different gene loci have been successfully used for the diagnosis of human brucellosis (Navarro *et al*., 2004; Al Dahouk *et al*., 2011). By increasing the number of molecular markers, both sensitivity and specificity can be increased accordingly. Molecular assays targeting the *IS711* insertion element, which is found in multiple copies within *Brucella* chromosomes, also improve analytical sensitivity (Hinić *et al*., 2008; Bounaadja *et al*., 2009). The analytical sensitivity can be further increased by using real-time PCR assays, which can detect as few as five bacteria per reaction (Navarro *et al*., 2006; Al Dahouk *et al*., 2007). Moreover, real-time PCR enables high-throughput screening of clinical samples and delivers results within a few hours.

2.7. Economic Impact of Brucellosis

Brucellosis is consistently ranked among the most economically important zoonoses globally. It is a 'multiple burdens' disease with economic impacts attributable to human, livestock and wildlife disease. The epidemiology and economic impact of brucellosis vary by geography and livestock system. In many high-income countries, brucellosis has been successfully controlled or eliminated in livestock populations. Where it persists, wildlife populations have become the main reservoirs (for example, bison and elk in North America). In emerging middle-income countries, the brucellosis picture is much more variable. Middle-income countries tend to report the greatest number of outbreaks and animal losses (Perry *et al*., 2002; Perry and Grace, 2009; WHO, 2009; ILRI, 2012).

Economic impacts vary depending on the main livestock species, management systems, and on the capacity of the country's veterinary and medical systems. In low-income countries, brucellosis is endemic and neglected, with large disease and livelihood burdens in animals and people and almost no effective control (McDermott and Arimi, 2002; Mangen *et al*., 2002; Perry *et al.*, 2002).

The economic impact in terms of human disease has been even harder to gauge; not only must the cost of treatment and diagnosis be considered, but also the cost in terms of disability-adjusted life years. Regardless of the measures used, the economic burden of human brucellosis in endemic areas is high and justifies widespread and sustained control efforts (Roth *et al*., 2003; Smits *et al*., 2007).

2.8. Public Health Importance of Brucellosis

Human brucellosis is a potentially life-threatening multisystem disease with a broad spectrum of nonspecific symptoms (Makis *et al*., 2008). Brucellosis in humans is a major public health hazard which affects social and economic development in various countries due to acute and chronic illness, physical incapacity and loss of manpower (Corbel, 2006). Occupational acquired brucellosis is of special concern for public health because of the high risk of direct transmission from infected animals to persons being employed in animal husbandry. This exposed group includes slaughter men, dairymen, flocksmen and veterinary clinicians. Flocksmen are at the highest risk. The occupational exposure is high especially in countries where flocking of animals is still traditional and unscientific (Straten *et al.,* 1997).

B. melitensis is highly pathogenic for humans; this organism is considered to be the most severe human pathogen in the genus. Occupational exposure is seen in laboratory workers, farmers, veterinarians and others who contact infected animals or tissues. Brucellosis is one of the most easily acquired laboratory infections. People who do not work with animals or tissues usually become infected by ingesting unpasteurized dairy products. The Rev-1 *B. melitensis* vaccine is also pathogenic for humans and must be handled with caution to avoid accidental injection or contamination of mucous membranes or abraded skin. Asymptomatic infections can occur in humans. 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. Some patients recover spontaneously, while others develop persistent symptoms that typically wax / increase and wane / decrease. Occasionally seen complications include arthritis, spondylitis, chronic fatigue, and epididymo-orchitis. Neurologic signs (including personality changes, meningitis, uveitis and optic neuritis), anemia, internal abscesses, nephritis, endocarditis and dermatitis can also occur. Other organs and tissues can also be affected, resulting in a wide variety of syndromes. Treatment is with antibiotics; however, relapses can be seen months after the initial symptoms, even in successfully treated cases. The mortality rate is low; in untreated persons, estimates of the case fatality rate vary from less than 2% to 5%. Deaths are usually caused by endocarditis or meningitis (Straten *et al.*, 1997).

2.9. Control and Prevention

Brucellosis is an infectious disease which has been controlled and eradicated in some countries in the world (Godfroid *et al*., 2005). In sub-Saharan Africa, animal health services delivered by the public sector have greatly decreased over the last 20 years due to various factors such as decreasing government budgets, particularly for operational costs of disease control. Thus, programs that require coordinated surveillance, information exchange and application of control measure are not implemented in many sub-Saharan countries (McDermott and Arimi 2002; Smits and Cutler, 2004).

The B. *melitensis* REV 1 vaccine is an attenuated strain of B. *melitensis* and an effective method to reduce the prevalence of brucellosis among whole flocks or flocks in low income countries and/or endemic countries (Corbel *et al.*, 2006; OIE, 2009).

In unvaccinated animals abortions are more prevalent and the amount of bacteria shed from birth fluids and tissues are much greater than in vaccinated animals. Previous stated enhances the importance to vaccinate small ruminants especially in low income countries (Corbel *et al.*, 2006) where Brucellosis causes substantial economic losses due to abortions, reduced fertility and lowered milk production in livestock (WHO, 2006).

Human brucellosis is usually prevented by controlling the infection in animals. Pasteurization of dairy products is an important safety measure where this disease is endemic. Unpasteurized dairy products and raw or undercooked animal products (including bone marrow) should not be consumed. Good hygiene and protective clothing/equipment are very important in preventing occupational exposure. Precautions should be taken to avoid contamination of the skin, as well as inhalation or accidental ingestion of organisms when assisting at a birth, performing a necropsy, or butchering an animal for consumption. Particular care should be taken when handling an aborted fetus or its membranes and fluids. Risky agricultural practices such as crushing the umbilical cord of newborn livestock with the teeth or skinning aborted fetuses should be avoided (OIE, 2009).

Any strategy for the control or eradication of brucellosis should begin by establishing the different epidemiological contexts within a country or even a region or district, and must have the support and collaboration of farmers. Above all, the effectiveness of any such strategy will rely heavily on the quality of the Veterinary Services and administrative organizations involved, because the requisite diagnostic and prophylactic tools are already fully validated and standardised (Minas, 2006; Blasco and Molina-Flores, 2011).

Disease control in pastoralists and migratory populations has proven especially challenging. Patients may not have access to medical services, and staff at local health-care centers may not be able to make or confirm the diagnosis (Arimi *et al.*, 2005).

3. MATERIALS AND METHODS

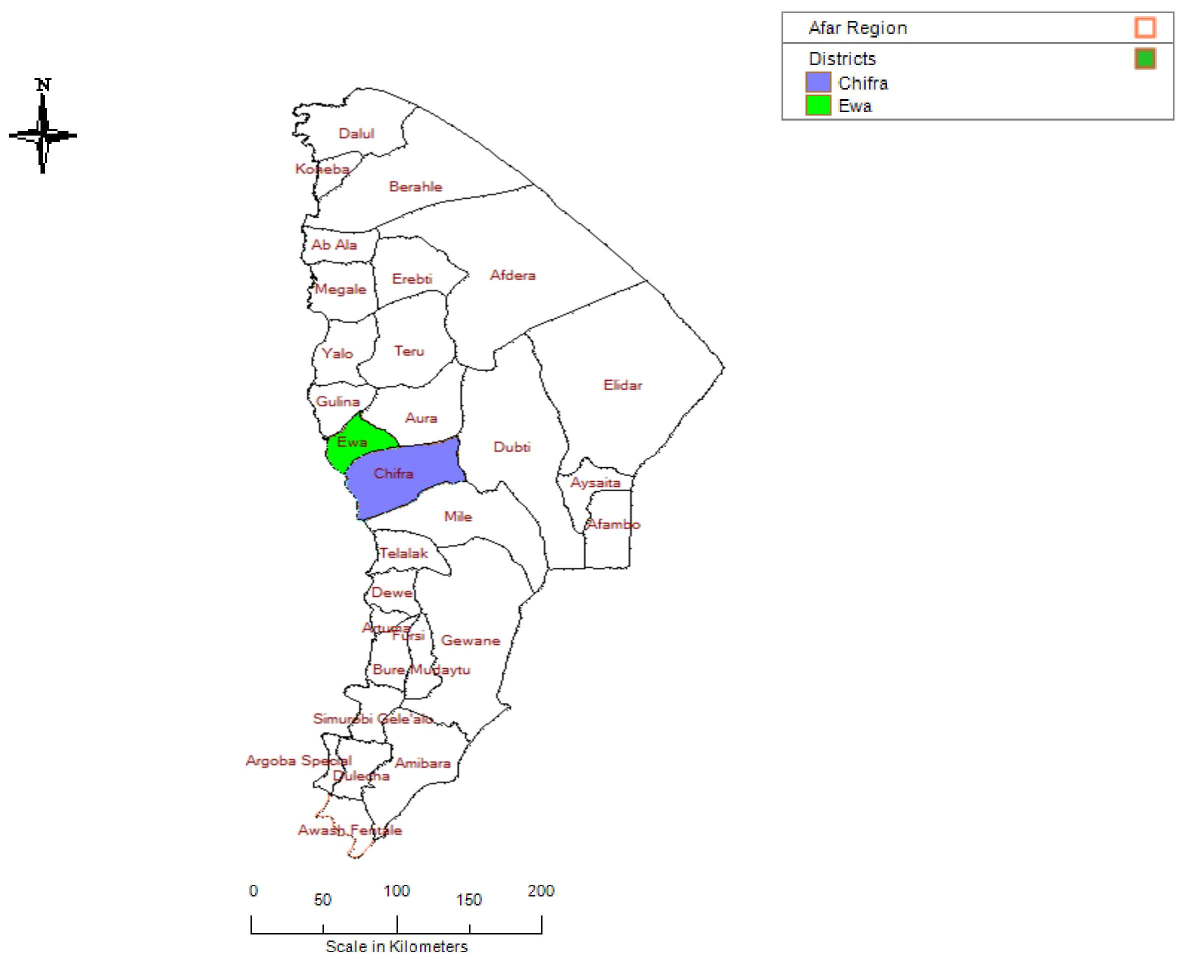
3.1 Description of Study Area

The Afar National Regional State (ARS) is located in the northeast part of the country. The region borders four national Regional States i.e. in the North and Northwest, Tigray Region; in West and South West, Amhara Region; in South, Oromiya Region and in South West, Somalia Region. The ARS also shares international borders with Djibouti and Eritrea to the East and North East, respectively. Administratively, the region is divided into five zones, which are further subdivided into 32 woredas and 358 kebeles. Pastoralism and agro-pastoralism are the two major livelihood ways practiced in the region and according to the official population statistics, the region's population is estimated to be 1.2 million; of which 90% are pastoralists and 10% agro-pastoralists (a mixture of livestock rearing with rain fed and irrigated crop production). In combination with pastoralism and/or agro-pastoralism, small numbers of people benefit from casual employment in agricultural schemes and in urban centers along the main Addis-Djibouti Road. The total surface area of the region is estimated to be 97,970 Km^2 (ARFEB, 2007).

In the region livestock is used as source of income, food and means of transportation. Due to the shortage of rainfall, in the dry season, the pastoralists are forced to move their animals to far distance for water and grazing land. In general, there are 10,179,277 livestocks in the region of which 4,267,969 (41.93%), 2,463,632 (24.20%), 2,336,483 (22.95%), 852,016 (8.37%) are Goat, sheep, cattle and camel, respectively (ARFEB, 2007). The approximate number of sheep and goats in Awesi-Resu (zone one) were 687,551 and 1,083,567, respectively; while in Fanteyna Resu (zone four) approximately 418,206 sheep and 398,127 goats populations exist in the area (CSA, 2011).

The study was carried out in two districts namely Chifra and Ewa, located in Zone one and Zone four respectively of Afar National Regional State (ANRS). Chifra and Ewa districts are found 174 and 219 kilometer far from Semera town respectively. The districts are found adjacently and share boundary with Amhara region. Chifra district has 1 urban and 19 rural peasant associations while Ewa contains 1 urban and 9 rural peasant associations. Ewa is bordered on the North by Gulina, on the East by Aura, on the South East by Chifra and on the west by Amhara region, respectively while Chifra is bordered on the North by Aura, on the East by Dubti, on the South by Mille and on West by Amhara region respectively. The area is characterized by high temperature; it ranges from 25^0C to 40^0C. May/June is the driest season of the year, '*hagay'*. It is said to be unsuitable for browsing since bushes dry up. The main rainy season '*Karma'*, which accounts for above 60% of the annual total rainfall are from July to September. This is followed by the best grazing season of '*Kayra'* that occurs from September to November. Another minor rainy season is *Sugum* and appears during March and April. '*Gilal'* is less severe dry season with relatively cool temperatures (November to March). Occasional rainfalls called *dada* may interrupt *'Gilal'*.

Figure 1: Study Areas of the Region



3.2. Study Population

All indigenous breeds of ovine and caprine kept under extensive management system in the study areas were considered as a study population. They are managed mainly under the pastoral production system, mixed with other species (such as cattle and camels). The animals under study comprised the indigenous Afar goat and Afar sheep from the group known as 'fat tailed hair sheep' (ILRI, 2006).

Ovine and caprine which were above 6 months of age, with no history of vaccination against brucellosis were included in the study. Then individual animal age, species, sex category, flock size and parity were recorded. Moreover, based on their sexual maturity animals were classified into three age groups of 1, 2 and 3 (ARFEB, 2007).

3.3. Study Design

A cross-sectional study design was conducted from November 2013 to April 2014 to determine the seroprevalence of brucellosis in ovine and caprines in the study areas. The study animals consisted of 1190 traditionally managed small ruminants of which 146 ovine and 214 caprine were obtained from Ewa district of Peasant associations namely Huletegna badole, Regden and Duba while the remaining 168 ovine and 662 caprines were obtained from Chifra peasant associations namely Wa'ama, Jarana-Konta, Gurale, Meskid-dora, Semsem and Awgera.

3.4. Sampling and Sample Size Determination

Two districts were selected purposively based on easier accessibility as well as ovine and caprine populations. At the present, there are 20 and 10 PAs in Chifra and Ewa districts, respectively. Peasant associations in the districts were selected simple randomly in the ratio of 2:1 (meaning 6 and 3 kebeles from Chifra and Ewa district, respectively). Peasant association is the lowest administrative unit within a district considered. A total of 1,190 sera samples were collected from 45 flocks of small ruminants.

Two districts from zone one and zone four of the existing five zones were selected purposively; and a multistage random sampling method were used to select the sampling units. Households were the sampling units and the principles of simple random sampling technique to select peasant associations and systematic random sampling to sample the households were followed at each stage of sampling. The numbers of animals included in the study were distributed proportionally over the PAs. The total number of animals to be sampled were calculated using Win Episcope 2.0, an improved epidemiological software for veterinary medicine developed for simple random sampling with the under mentioned assumption and an infinite population and inflated the estimated sample size to 1190 small ruminants (Thrust field, 2005). A 5% absolute precision and 95% confidence interval were used for determining sample size. Since previous study in the region indicated that prevalence rate of 5.7% (Chifra) and 2.4% (Ewa), an expected prevalence of 20 % were used to obtain the maximum sample size. Accordingly, 246 animals were the calculated sample size for each Chifra and Ewa district. In order to get representative sample for both districts inflated the sample size to 1190. Therefore; the appropriate sample size were 830 and 360 ovine and caprine for Chifra and Ewa district respectively. In general 1190 sera samples were collected from 45 flocks of small ruminants.

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

Where n= the total sample size

P= expected prevalence

d^2= absolute precision

3.5. Study Methodology

3.5.1. Questionnaire survey

A structured questionnaire (Annex 1) was administered to livestock flockers after pretesting in the field properly translated to the local language 'Afarigna'. Verbal conset was obtained from the respondents after the objective of the survey is explained to them before starting the interview. This questionnaire was designed for a survey of the potential risk factors associated with the disease in shoat flocks and to gather relevant information on the overall small ruminant flocks management practices, knowledge about zoonotic diseases, habit of consuming raw milk and meat, handling of aborted fetuses and contaminated materials.

3.5.2. Serological survey

Blood Sample Collection

A sample collection format was also prepared to record the history of individual ovine and caprine such as species, sex, age, parity and flock size while taking blood.

Blood sample were collected from the jugular vein of sheep and goats, which were above 6 months of age and with no previous history of vaccination from the selected peasant associations using sterile plain vacutainer tubes. Approximately 8 ml of blood from jugular vein of sheep and goats were collected aseptically using sterile plain vaccutainer tubes and needles. The samples were properly labelled and left for 24 hours at room temperature to allow clotting and the sera were separated within 24 hours through gently transferring to other sterile vials and kept at -20^{O}C (Reviriego *et al.*, 2000) at Samara Regional Veterinary Laboratory until further tested for antibodies in NVI (National Veterinary Institute)**,** Debre Zeit.

Modified Rose Bengal Plate Test (mRBPT)

The modified Rose Bengal plate test (mRBPT) was done in Semera Regional Veterinary Laboratory in order to screen positive samples by RBPT using RBPT antigen (Institue Pourquier 325, rue de la galèra 34097 Montpellier cedex 5, France). Positive sera were then retested using complement fixation test (CFT) of same origin at the National Veterinary Institute (NVI), Debre Zeit.

All serum samples collected were screened using the RBPT, according to the procedures described by Alton *et al*., (1990) and the World Organisation for Animal Health (OIE, 2004). The antigen used was Rose Bengal antigen, which constitutes a suspension of *B. abortus* (obtained from the Institute Pourquier, Montpellier, France). In brief, 75 µl of serum was mixed with 25 µl of antigen suspension on a glass plate and agitated. After four minutes of rocking, any visible agglutination was considered as positive (OIE, 2004). Agglutinations were recorded as 0, +, ++ and +++, according to the degree of agglutination. A score of 0 indicates the absence of agglutination; + indicates barely visible agglutination; ++ indicates fine agglutination, and +++ indicates coarse clumping. Those samples with no agglutination (0) were recorded as negative while those with +, ++ and +++ were recorded as positive (Nielsen and Duncan, 1990).

Complement Fixation Test (CFT)

The complement fixation test (CFT) was undertaken at the National Veterinary Institute (NVI), Department of Immunology. All the reagents required for CFT were evaluated by titration. A 2% sheep Red Blood Cell (SRBC) suspension were prepared before being used in the test proper. The preparation of reagents and CFT procedures were performed according to the protocols of the Federal Institute for Consumer Protection and Veterinary Medicine Service Laboratory, Berlin, Germany (Nielsen and Dunkan, 1990).

Positive sera with RBPT were further tested with CFT for confirmation using standard *B. abortus* antigen (New Haw, Addleston, Surrey KT15 3NB, UK). The CFT test proper and reagent preparation procedures were following the procedures outlined by (OIE, 2004). Sera with strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of complement (2+) at a dilution of 1:10 and above were classified as positive (OIE, 2004).

3.6. Data Management and Statistical Analysis

Data obtained from both serological tests and questionnaire surveys were stored in Microsoft excel spreadsheet program. Descriptive and analytic statistics were computed using software SPSS® Version 20.0. Logistic regression and Chi-square test (X^2) were employed to identify possible risk factors associated with seropositive ovine and caprines. The degree of association was computed using odds ratio (OR) signified by 95% confidence intervals (Thrusfield, 2005).

4. RESULTS

4.1. Individual and Flock Level Seroprevalence of Brucellosis

The results of serological tests for brucellosis showed that of a total of 1190 blood samples collected from sheep and goat populations in Chifra and Ewa woredas (876 caprine and 314 ovine), 155 (13%) tested positive using mRBPT. Further testing of the positive reactors with CFT confirmed that 147 samples (animals) were positive for *brucella* infection (12.35%). Table 3 depicts the seroprevalence of brucellosis in sheep and goat populations in Chifra and Ewa Woreda. The seroprevalence in Chifra (12.5%) and Ewa (12.0%) woredas was comparable without statistically significant difference ($p>0.05$). The highest individual animal level seroprevalence was recorded in goat from species group in both Chifra and Ewa woredas. The difference in seropositivity between species in both woredas was statistically significant ($p<0.05$). The overall flock level seroprevalence of brucellosis infection was 57.8% while it was 60% and 53.3% in Chifra and Ewa district respectively.

Table 3: Individual animal and flock level seroprevalence of brucellosis in sheep and goat populations in Chifra and Ewa woredas, Afar Region

Zone	District	Species	No. Tested	CFT positive	p-value	Flock level	
						No tested	Positive (%)
	Chifra	Overall	830	104 (12.5%)	0.018	30	18 (60%)
1		Ovine	168	12 (7.14%)			
		Caprine	662	92 (13.8%)			
	Ewa	Overall	360	43(12.0%)	0.005	15	8 (53.3%)
4		Ovine	146	9 (6.16%)			
		Caprine	214	34 (15.89%)			

4.2. Risk Factors Affecting Individual Animal Level Seroprevalence of Brucellosis

The results of univariate logistic regression analysis are presented in Table 4. The results revealed that among the risk factors considered in the analysis, species, sex, age, parity number and flock size had statistically significant effect on seropositivity ($p<0.05$). Woreda had no statistically significant effect on seropositivity ($p>0.05$). The individual animal level seroprevalence was significantly higher in goats (14.4%) than in sheep (6.7%) populations. The analysis revealed that goats were more than 2 times (OR=2.34) at risk of getting infected with *brucella* than sheep. The seroprevalence was also significantly higher in female animals (13.8%) than in males (6.5%) with the likelihood of female animals to get infected with brucellosis is higher than that of males although the odds ratio value is very small probably due to the unbalanced number of female and male animals included in the study. Similarly, *Brucella* seroprevalence was also significantly increased with age; the odds of seropositivity in older animals is 2.35 times higher than that of younger animals (Table 4). Seroprevalence rate of brucellosis was also significantly varied with parity, with the likelihood of infection being 1.2 times higher in animals with 3 to 4 parities than otherwise. Regarding flock size individual animal sero-prevalence was higher in larger flocks than in the smaller ones.

Table 4: Results of univariate logistic regression analysis of risk factors

Risk factors	Category	Prevalence	OR	95% CI	p-value
Woreda	1 (Chifra)	12.5%	0.947	0.648-1.383	0.778
	4 (Ewa)	12%			
Species	*Ovine	6.7%	2.34	1.44-3.79	0.001
	Caprine	14.4%			
Sex	*Male	6.5%	0.43	0.24-0.75	0.003
	Female	13.8%			
Age/Years	*< 2 years	2.8%	2.36	1.84-3.02	0.000
	2-5 years	16.4%			
	> 5 years	20%			
Parity	*0	1.3%	1.298	1.07-1.56	0.006
	1-2	10.2%			
	3-4	22%			
Flock size	*≤25	36.8%	0.68	0.46-0.99	0.011
	> 25	73%			

*Reference category; OR: Odds ratio; CI: Confidence interval.

The risk factors with significant effect after univariate logistic regression test (species, sex, age, parity and flock size) were fitted in a multivariate model and the results showed that all the factors except districts had statistically significant effect on the seroprevalence of brucellosis in sheep and goat populations ($p<0.05$) (Table 5). Age was dropped from the multivariate model due to the likelihood of confounding the effects of parity.

The result of multivariate logistic regression model indicated that caprines were found to be at higher risk of exposure to *Brucella* infection (OR=2.758, CI: 1.673-4.546) than ovine. Similarly female animals were found to be at higher risk of exposure to *Brucella* infection (OR=0.138, CI: 0.059- 0.321) than males (Table 5) and those less than 2 years of age. In statistical terms, animals with multiple parturition were at higher risk of encountering *Brucella* infection (OR=2.038; CI:1.303-3.187) followed by animals having a single or two parity than animals with zero or no parity (Table 5).

Table 5: Multivariate logistic regression analysis of risk factors

CFT	Odds Ratio	(95% CI)	p-value
Species	2.131	1.53-3.44	0.003
Sex	0.138	0.059-0.321	0.000
Parity	2.038	1.303-3.187	0.002
Flock size	0.761	0.684-1.523	0.011

4.3. Risk Factors Affecting The Flock Level Seroprevalence of Brucellosis

Seroprevalence of brucellosis was statistically significant in larger sized flocks than in smaller ones and in Chifra than in Ewa ($p<0.05$).

Table 6: Risk factors associated with seroprevalence occurrence at flock level

Risk factors	Category	Number of flocks	Infected flocks	(95% CI)	p-value	OR
Flock size	≤25	19	7	0.46-0.99	0.011	0.68
	>25	26	19			
Districts	Chifra	30	18	0.648-1.383	0.778	0.947
	Ewa	15	8			

OR: Odds ratio; CI: Confidence interval.

4.4. Public Health Importance of Brucellosis

The results of the questionnaire survey on the perception and practices of livestock owners in the study areas are presented below. Most of the outcomes of the analysis of the questionnaire data showed that livestock owners in the studied areas are at high risk of contracting brucellosis from infected animals. Almost all the respondents in the studied areas were not aware of brucellosis as a disease affecting different species of livestock (91.1%) although all respondents (45 livestock owners) interviewed recognized the existence of abortion (locally called in Afarigna as “Feneg-dalay”) among small ruminant flocks and most of them handle abortion materials with bare hand without protecting themselves (82.2%). The habit of drinking raw milk is practiced by all the 45 interviewed respondents (100%) while there is no habit of consuming raw meat (Table 7).

Table 7: Owners' awareness about small ruminant brucellosis, habit of drinking milk and eating meat and handling of aborted materials of small ruminants.

Variable	Number of respondents	Percentage (%)
Awareness on brucellosis		
Yes	4	8.9%
No	41	91.1%
Removal and disposal of foetal membranes and aborted foetus		
With bare hand	37	82.2%
Glove protected hand	8	17.8%
Habit of drinking milk		
Raw	45	100%
Boiled	0	0%
Habit of eating meat		
Raw meat	0	0%
Cooked meat	45	100%
Both raw and cooked meat	0	0%

5. DISCUSSION

The study demonstrated that the overall individual animal level seroprevalence of brucellosis in small ruminant was 12.35% (95% CI: 1.44-3.79) (14.4% in caprines and 6.7% in ovine). This is fairly comparable to the seroprevalence of 11.6% (13.6% in caprine and 7.1% in ovine) reported in Afar region of Ethiopia (Wesinew *et al.*, 2012), similarly 16% (16.45% in caprine and 14.6% in ovine) in Afar region of Ethiopia (Teshale *et al.*, 2005), 9.11% (9.39% in caprine and 8.77 % in ovine) in Dire Dawa (Negash *et al.*, 2011), 9.6% (11.7% in caprine and 4.9 % in ovine in Yabello (Yohannes *et al.*,2012) and 14.5% in ovine and 16.1% in caprine in Nigeria (Bertu *et al.*, 2010). This could be due to the similarities of animal husbandry in communal grazing range lands and watering areas and possibly similar climatic conditions (Teshale *et al*., 2006). However, the prevalence presently recorded is lower than that recorded by Al-Majali (2005) where 27.7% (305 of 1100) of caprine were seropositive by both RBPT and CFT and Hamidullah *et al*., (2009) in which 34.88% (120 of 344) ovine and caprine were found to be positive for brucellosis using the RBPT and 32.5% using serum agglutination test (SAT) in Kohat, Jordan. The reason for this discrepancy could be variation in management practices and frequent introduction of new animals without proper serological testing and detection and removal of animals with high incidence of abortions (Hamidullah *et al.*, 2009).

The individual level prevalence obtained in this study has two and five times increased in Chifra and Ewa district respectively when it is compared with a previous study done by Ashenafi *et al*., (2007) who found 5.7% and 2.4 % in small ruminants in Chifra and Ewa district respectively. Moreover the individual level prevalence obtained in this study using CFT is higher than the findings of 4.8% in ovine and 1.9% in caprine by Aklilu *et al*., (2009) in Adamitulu-Jido-Kombolcha District, Oromia Regional State, Ethiopia, 1.7% in caprine and 1.6% in ovine by Teshale *et al*., (2006) in Somali pastoral area, 5.8% in caprine and 3.2% in ovine by Ashenafi *et al.*, (2007) in Afar region, 1.3% in caprine and 1.5% in ovine by Tekleye and Kasali (1989) in central highlands of Ethiopia, 1.7% in caprine and 1.6% in ovine by Yibeltal (2005) in Somali region and 3.2% in caprine and 1.6% in ovine by Mengistu (2007) in southern Ethiopia and 0.75% in caprine and 0.38% in ovine by Coelho *et al*., (2007) in North east of Portugal. This increase may be due to the seasonal migration of livestock from Zone one and zone five to Zone four (Chifra and Ewa) districts of the region in search of grazing pasture (cotton, maize and sorghum) leftover as an animal feed. Mixing of the different species during migration, at watering or grazing points among different species is a common practice in Afar area. The other contributing factor to the spread of brucellosis may be the movement of animals for grazing and watering as aggregating the animals around watering point will increase the contact between infected and healthy animals thereby facilitating the spread of the disease.

There was no statistical significant difference in the prevalence proportion among the zones and districts. The disease was detected in both districts of investigated. Brucellosis is, therefore, well entrenched across the entire Afar region. This might well be attributable to the use of similar animal production and management systems throughout the zones and districts of the region, as well as fairly similar agro-ecological conditions. Moreover, unrestricted animal movements may have enhanced the spread of infection, such as: movements of animals in search of pasture and water, trade within and between zones and districts, the mixing of animals at market places and watering points, such as the Awash River, especially during the dry season. The flock level prevalence is higher than individual animal level and this characterizes the nature and importance of the disease in the large flock size. This signifies that brucellosis has significant economic implication in its ability to bring about morbidity at flock level.

There was statistical significant difference between ovine and caprine species. The present investigation recorded a higher seroprevalence of brucellosis in caprine 14.4% than in ovine 6.7%. Comparable results were recorded; 13.64% in caprine and 7.1% in ovine in Afar region (Wesinew *et al*., 2012), 11.7% in caprine and 4.9% in ovine in Yabelo district of Borena pastoral area, Oromia National Regional State, Southern Ethiopa (Yohannes *et al*., 2012) and 9.39% in caprine and 8.77% in ovine in Dire Dawa region (Negash *et al*., 2011). Lower seroprevalence of 3.8% in caprine and 1.4% in ovine in Eritrea (Omer *et al*., 2000), 4.1% in caprine and 1.6% in ovine in East Morocco (Benkirane, 2006), 0.75% in caprine and 0.38% in ovine in Northeast of Portugal (Coelho *et al*., 2007). This difference might be due to the differences in flock sizes and proportions of caprine and ovine in the flock (876 caprine and 314 ovine in the present study). In addition, ovine are more resistant than caprine and they do not shade the bacteria for long time. Flocks with high numbers of ovine would have low prevalence (Radostits *et al*., 2007). Excretion from the vagina in caprine is more copious and prolonged than ovine and lasts for at least 2-3 months. In addition, Caprines are considered as the principal host of *B. melitensis*, whereas, ovines are not significantly infected even when kept in close contact with caprine (Alton, 1985). In caprine, infection can vary from a short time occurrence to persistent occurrence for years. In ovines, the course of infection depends upon the dose of infection and after recovery they are resistant to re-infection (European Commission (EC), 2001).

There was statistical significant difference between male and female small ruminants. The present investigation recorded a higher seroprevalence of brucellosis in female ovines or caprines (13.8%) than in male ovines or caprines (6.5%). This finding is in agreement to Mengistu (Mengistu, 2007) who reported brucellosis in 3.2 and 1.2% of females and males, respectively, in Southern Ethiopia and again (Aklilu *et al*., 2009) who reported 4.2% and 2.2 % of females and males, respectively, in Adamitulu-Jido-Kombolcha District, Oromia Regional State, Ethiopia. The high prevalence of brucellosis in females might be due to high concentration of erythritol, which is scarcely produced in males reproductive organs (European Commission (EC), 2001; Colmenero, 2007).

In line with this, ovines and caprines have served as a means of ready cash and a reserve against economic and agricultural production hardship. Most of the time pastoralists supply male small ruminants to both the export and domestic markets than female small ruminants while female small ruminants serve as a source of feed / raw milk for their children. Male small ruminants supply immediately to the livestock trader / public immediately upon maturation. Thus, another factor for the variation may be female small ruminants were found abundantly than male in the study areas.

In statistical terms, parity of animals had shown significant variations in seroprevalence multiple parities (3-4 parities) were three times more affected than in animals with (1-2 parity) and than in animals with zero parity. The present finding is also in consistent with the finding of Yohannes *et al*., (2012), who reported the association of seropositivity with parity. However, the finding of the association of parity numbers with the seropositivity in the present finding is disagreement with that of Yilkal *et al*., (1998). This may be due to host clearing mechanism reduces the colonization bacteria.

A statistically significant variation was also recorded in the prevalence of brucellosis between adults and young animals. A higher prevalence was found in adult ovines and caprines. It has been reported that brucellosis is essentially a disease of sexually mature animals (Quinn *et al*., 1999). Sexually mature and pregnant animals are more prone to *Brucella* infection and brucellosis than sexually immature animals of either sex (Radostits *et al*., 2000). On the other hand, it is also true that younger animals tend to be more resistant to infection and frequently clear an established infection, although latent infections can occur (Walker, 1999). This may result from the fact that sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms, tend to increase in concentration with age and sexual maturity (Radostits., 2000).

Since brucellosis is considered as disease of flock importance, in this study higher flock level seropositivity of 57.8% was found as fairly compared to (Wesinew *et al*., 2012) in Afar (50.51%). This could be due to the presence of large number of small ruminants in the flock and mixing of aborting small ruminants with normally parturient small ruminants. Even though, brucellosis was detected in all the districts with slight variation in prevalence, it was not statistically significant difference ($p>0.05$). This could be attributed to the similarity in agro-ecological conditions and livestock management system in the districts.

Brucellosis is transmissible from animals to humans through contaminated milk, raw milk products, direct contact with infected animals. Almost all small ruminant owners residing in the study area were able to recognize the occurrence of abortion in their flocks but about 91.1% of them lacked knowledge about brucellosis. Furthermore, 86.7% of the respondents had the habit of drinking raw milk and had no habit of eating raw meat of small ruminants while 13.3% had habit of drinking both raw and boiled milk, 82.2% of the respondents used to handle retained fetal membranes and dispose of aborted fetuses using bare hands. Fairly similar findings were reported by (Negash *et al*., 2011). This may be due to lack of awareness about brucellosis together with existing habit of raw milk consumption and close contact with animals can serve as means of infection in human beings.

6. CONCLUSION AND RECOMMENDATIONS

Lack of awareness about brucellosis together with existing habit of raw milk consumption and handling infectious materials can serve as means of infection in human beings. Results of the present study provide the importance of small ruminant brucellosis in Afar pastoral areas and identify the risk factors that contribute to the occurrence of the disease in small ruminants as well as possible zoonotic implications in human beings.

Based on the results of this study the followings are recommended:

- Interdisciplinary collaboration and joint ventures among medical, veterinary and public health professionals is of paramount importance to control this disease that currently perpetuates poverty.

- Awareness creation among pastoralists on modern animal husbandry, disease prevention and risk of zoonotic diseases need to be undertaken.

- Critical assessment of the economic impact of the disease, which emanates from its effect on reproductive and production performance of animals, is worthy. Studies to investigate the link between livestock and human brucellosis and cross infection among species in the region should be conducted to devise appropriate preventive mechanisms.

- Since results indicated that high seropositivity of small ruminant brucellosis in the study areas, further research work that intended to the isolation of causative agent and identification of species and biotypes in Afar region is imperative.

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8. ANNEXES

Annex 1: Questionnaire Format for individual sampled animals

District __________________PA _______________________ Date__________________

No.	Owner	Species	Sex	Age	Parity	Flock size
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						

Annex 2: Questionnaire format for Sheep and goats owners

Name: ___________Gender: ___ age: ____ address: ____educational level: ________

1. How do you keep your sheep and goats at day time? a. Mixed b. Separated

2. How do you keep your sheep and goats at night? a. Mixed b. Separated

3. Can your flock get contact with other flocks at grazing? a. Yes b. No

4. Who is responsible for milking the sheep and goat? a. Wife b. Husband c. Other house member

5. How do you use the milk? a. Boiled b. As raw c. Other milk forms

6. Do you slaughter sheep and goats at home?a). Yes b). No

7. If yes, for what purpose? a. Home consumption b. Ceremony purpose c. Group share d. Emergency slaughter

8. How do you consume shoat meat? a. Cooked b. Raw c. Both d. With other treatments

9. What is your role in shoat husbandry? a. Flock feeding/ watering b. Milking

c. Delivery assisting d. Mating assisting e. Treating when sick

10. Do you own repeatedly aborting shoat? a. Yes b. No

11. How do you dispose fetal membrane/aborted fetus? a. Throwing on the field b). Offering dogs to eat c. Burning/ burring d. Do not bother about it

12. Do you have any shoat with scrotal or joint swollen? a. Yes b. No

13. What type of breeding method do you use? a. AI b. Natural c. Both

14. Do you have a sick person among your family? a. Yes b. No

15. Does the sick person encountered with the following health problems lasting for 15 days?

a. Head ache------- b. Back pain-------- c. Joint pain-------- d. weakness---------

e. Intermittent fever---------- f. Night sweating--------- f. Pain on testis if male------

16. Does the sick person visit a hospital or a clinic? a. Yes b. No

17. Do you know diseases which can be transmitted from shoat to humans? a. Yes b. No

18. What do you know about brucellosis / feneg dalay?.................................

19. If you know brucellosis, how can be transmitted to human?.....................................

Annex 3: Rose Bengal plate test techniques

Description of the test

An antigen prepared from *B. abortus* (strain 99) stained with rose bengal dye and suspended in acid buffer (PH 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 +20c

Annex 4. Rose Bengal plate test showing agglutination (positivity) and CFT showing sedimentation

	
Fig.1 Serum sample submission for screening	Fig. 2 Sample preservative deep freeze until tested for RBPT
	
Fig.3 Accomplishment of screening test at Semera Regional Vet. Laboratory	Fig.4 Result that show agglutination or positivity
	
Fig.5 Accomplishment of CFT at National Veterinary Institute (NVI)	Fig.6 Result that show absence of haemolysis

1. Purpose /scope/

Activation of classical complement system by antibody bound to antigen results in generation of membrane attack complexes capable of disrupting cell membranes. It is possible to use this reaction to measure serum antibody levels and this test is known as complement fixation test. The principle of CFT is that, if complement is fixed by antigen-antibody immune complex, it is unavailable to lyses the target cells in the indicator system (SRBC bound to Amboceptor). In the absence of antibody, the complement remains unfixed and is available to lyses the **target cells** in the indicator system. In the case of negative sera samples, the **unbound** complement will react with the indicator system results in lyses of SRBCs by activation of complement.

2. Definition and Acronyms

- CFT- Complement Fixation Test
- SRBC- Sheep Red Blood Cell
- VCM- Veronal Buffer with Calcium and Magnesium
- HS-Hemolytic System
- PCV- Packed Cell Volume

3. Safety precautions

Hazards might arise during manipulation of antigens and sera. Hence, appropriate care should always be taken to rescue the operator.

4. Material Required

- Brucella antigens
- Amboceptor (Anti SRBC antibody)
- Complement
- Positive and negative control sera
- Arranged test sera and sheets of plate lay out for record
- Water bath, incubator, and agitator

- Syringe with needle
- Alsever`s solution
- Sheep RBC (SRBC)
- Crystalline Penicillin
- VCM buffer (Veronal buffer with calcium and magnesium)
- Distilled water
- U-shaped micro-plates
- Trough
- Multi-channel and single channel micropipettes, tips
- micropipette tips

5. Procedure

5.1. Preparation of Sheep Red Blood Cells (SRBCs)

- Draw blood from the jugular vein of male sheep freely flowing into a syringe containing Alsever`s solution, take 75 ml sheep blood in 125ml Alsever`s solution and if less blood is required, decrease the volume by the same proportion.
- Add small amount of crystalline penicillin to avoid bacterial contaminants.
- Store at +4^{o}C overnight and the blood can be used for about 2 weeks.
- Sheep blood for CFT should be at least one day old.

5.2. Preparation of Hemolytic System (HS) (indicator)

- Discarded the Alsever's solution very gently or sipped out by using micropipette.
- Wash the sheep blood three times at a dilution of 1:10 by adding VCM at pH 7.2 and centrifugation at 2,500 rpm for 5 minutes.
- Discard the supernatant and re-suspend the SRBCs in VCM, mix gently and centrifuge as above. Repeat this step two times (three total wash).
- Take another tube of identical size and hold it next to the centrifuged tube and measure packed cell volume (PCV) of SRBCs by adding water until you have reached the meniscus of the SRBC (volume of SRBC in ml).
- Dilute the SRBC in VCM to 1% (e.g. 1 ml PCV of SRBC in 99 ml VCM).

- Reconstitute the freeze dried Amboceptor with 1 ml distilled water and keep at +4^{o}C. The working dilution of the Amboceptor is 1:000 (Always draw the Amboceptor sterile from the bottle). Add the reconstituted Amboceptor in a 1:1000 dilution into the 1% SRBC and mix with constant gentle agitation during incubation for 30 minutes (sensitisation) at room temperature.

5.3. Evaluation of Complement

- Evaluate the complement every day when you run the test. Dispense 25 µl VCM into all well of rows A, B, C and D of U-shaped micro-plates
- Add 25 µl of complement at a starting dilution of 1:2 into the first wells of row A, B and C (i.e. A1, B1 and C1). Row "D" is left as HS control where we expect 100% precipitation of SRBC.
- Make two fold dilutions of the complement by transferring 25 µl of the mixture after thorough homogenization to the next wells until A12, B12 and C12 respectively. Discarded the last 25 µl after mixing.
- Dispense 25 µl of the haemolytic system, indicator (Amboceptor + SRBCs) in all wells of rows A, B, C and D and incubate in moist chamber at 37^{o}C with constant agitation for 30 minutes.
- Read and record the last dilution's column showing complete haemolysis and 50% haemolysis of SRBCs by comparing with the HS control. Take the average titre and multiply by the international unit (2.5-5) to get the working dilution of the complement.

5.4. The Test Proper

5.4.1. Before testing pre plate the sera and seal it by a plate sealer, de-complement in a water bath at 58^0C for 30 minutes. Always prepare negative, positive reference sera controls, antigen, complement and hemolytic system (indicator) controls only in a separate plate. We can run CFT for other diseases knowing the cut-off dilutions of sera.

5.4.2. **<u>One-tenth dilution of serum is prepared as follow</u>**

Add 45µl of VCM in rowA1-A12, C1-C12, E1-E12, G1-G12 and transfer 5µl of serum from the U shaper micro pre plate. It gives a total of 50µl. Then homogenize with 12 multichannel micro pipettes and transfer 25µl of the solution into B1-B12, D1-D12, F1-F12, H1-H12 respectively and add 25µl of antigen at working dilution. The remaining 25µl is for (Ac) anti complementary reaction controls without antigen but add 25µl of VCM.

5.4.3. **<u>One-fifth dilution of serum for Brucellosis is prepared</u>**

Add 40µl of VCM in rowA1-A12, C1-C12, E1-E12, G1-G12 and transfer 10µl of serum from the U shaper micro pre plate. It gives a total of 50µl. Then homogenize it and transfer 25µl of the solution with 12 multichannel micro pipettes into B1-B12, D1-D12, F1-F12, H1-H12 respectively. **Ac (Anti-complementary controls):-** The test samples should be always checked for anti-complementary reaction (No antigen is added in these wells as above)

5.4.4. Add 25µl of diluted antigen at working dilution to the wells of rows B, D, F, and H.and add 25µl of VCM to the wells of rows A, C, E, and G and the plates are covered by micro plate sealer to prevent evaporation and incubated at 37^0C in moist chamber for 30 minutes with constant agitation.

5.4.5. Add 25µl of complement at working diluted in all wells and incubate for 30 minutes as above.

5.4.6. Add 25µl HS (indicator) to all wells of the plate and incubated at 37°C for 30 minutes at constant agitation.

5.4.7. The micro plates are centrifuged at 2500 rpm for 4 minutes using sigma centrifuge or put in a refrigerator overnight and read results.

5.4.8. Interpretation:

- Sera samples having SRBCs sedimentation at a dilution ≥ 1:5 are considered to be positive for Brucellosis.

Annex i: CFT plate layout on a U bottomed Micro plate

	1	2	3	4	5	6	7	8	9	10	11	12		
A	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**No Ag**	**Row 1**
B	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**Has Ag**	
C	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**No Ag**	**Row 2**
D	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**Has Ag**	
E	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**No Ag**	**row3**
F	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**Has Ag**	
G	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**Ac**	**No Ag**	**Row4**
H	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**1/10**	**Has Ag**	

6. References/ Associated SOPs and Quality documents

- QMS#: FG001: Internal Sample Submission Form
- QMS #: FG007: External Sample Submission Form

7. Revision History

The Supersede of the current version was existed but not properly identified. Revision of this version shall be envisaged when it's deemed necessary.

8. Appendices

Annex ii: CFT controls layout on a U Micro plate

	1	2	3	4	5	6		↓	7	8	9	10
A	**-S**	**-S**	**Ag**	**Ag**	**C**	**C**	**5U**		**+S**	**+S**	**HS**	**HS**
B	**-S**	**-S**	**Ag**	**Ag**	**C**	**C**	**2.5U**	**d**	**+S**	**+S**	**HS**	**HS**
C	**-S**	**-S**	**Ag**	**Ag**	**C**	**C**	**1.25U**	**i**	**+S**	**+S**	**HS**	**HS**
D	**-S**	**-S**	**Ag**	**Ag**	**C**	**C**	**0.625U**	**l**	**+S**	**+S**	**HS**	**HS**
	-ve serum		**Ag**		**complement**			**u t e**	**+ve serum**		**indicator**	

Contents of the controls (see Annex II)

<u>The negative and positive serum control</u>

- 25µl serum, 25µl Ag, 25µl complement and 25µl haemolytic system.

<u>The Ag control</u>

- 25µl VCM, 25µl Ag, 25µl complement and 25µl haemolytic system

<u>The complement control</u>

- Add 50µl of evaluated complement (5 unite) in the wells A5 and A6, then add 25µl of VCM in to B5, B6, C5-C6, D5 and D6 for dilution.
- Take 5 Unite of 25µl complement to B5, B6, mix it and transfer C5-C6 and transfer to D5 and D6 respectively.
- Discard the last 25µl and add 25µl of haemolytic system in all wells.

<u>The haemolytic system control:-</u>

75µl VCM,25µl haemolytic system. Incubate the controls as the test proper above.

Annex iii: Plate layout

Plate number ______________

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												