ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICNE

SEROPREVALENCE OF BRUCELLOSIS IN SMALL RUMINANTS AND HUMANS IN SELECTED PEASANT ASSOCIATIONS OF ADAMI-TULU-JIDO-KOMBOLCHA DISTRICT, OROMIYA REGIONAL STATE, ETHIOPIA

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in Partial fulfilment of Requirement for the Degree of Master Of Science in Tropical Veterinary Public Health

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"Trust in the Lord with all thine heart; and lean not unto thine own understanding. In all thy ways acknowledge Him, and He shall direct thy paths".

~Proverbs 3:5& 6

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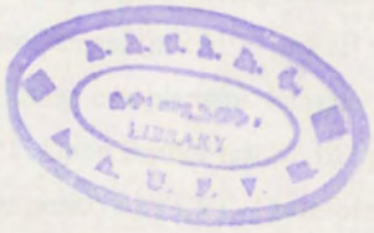
LIST OF ABBREVIATIONS

BPAT	Buffered plate agglutination test
CDC	Centers for Disease Control
CFSPH	Centers for Food Security and Public Health
CFT	Complement Fixation Test
CSA	Central Statistical Agency
cELISA	Compitetive enzyme-linked immunoassay
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FPA	Fluorescence polarization assay
FPSR	False positive sero-reactors
GAIN	Gloval agricultural information center
CH	Haemolytic unit of complement
iELISA	Indirect enzyme- linked immunosorbent assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
masl	meter above sea level
MHD	Minimum hemolytic dose
MLA	Multiple locus analysis
MMWR	Morbidity and Mortality Weekly Report
MRT	Milk Ring Test
MZNT	Modified Ziehl Nielsen Test
OIE	Office International des Epizooties
OPS	O-polysaccharide
PAHO	Pan American Health Organization
PCR	Polymerase Chain Reaction
RB	Rough *Brucella*
RBPT	Rose Bengal plate Test
SAT	Serum Agglutination Test

SDTH	Skin delayed –type hypersensitivity
SLPS	Smooth lipopolysaccharide
SRBC	Sheep red blood cells
TSA	Trypticase soy agar
VNTR	Varible number tandem repeats
WHO	World Health Organization



ABSTRACT

A cross-sectional study was conducted in selected 10 peasant associations found in Rift Valley area of Adami-Tulu-Jido-Kombolcha District, Oromiya Regional State, 160km South of Addis Ababa between October 2008 to March 2009 to determine the seroprevalence of brucellosis in small ruminants and human risk groups (farmers), as well as to investigate the potential risk factors associated with seroprevalence of brucellosis. Proportional sampling method was employed to include 160 households and 2340 small ruminants (1565 goats and 775 sheep). The sampling involved small ruminants above 6 months of age and human risk groups (farmers), who had close contact with small ruminants, visited the District's health center and had the symptoms suggestive of brucellosis.The primary sampling unit was a flock or a household having at least one or more sheep/goat. The 10 peasant associations were selected porpusibely based on accessibility of flocks and other logistics like transport facilities. Convenient samling method was employed to gather 230 sera from human risk groups (especially farmers). About 8ml and 5ml of blood samples were collected from jugular and brachial veins of shoats and humans, respectively using plain vacutainer tubs. Blood samples were allowed to clot at room temperature and the harvested sera were stored at -20^{o}C until analysis. Blood sample collection and public health surveys were conducted in collaboration with the district's health center institutions; ethical issues were observed in the studies and sampling involving human subjects. All the serum samples were screened using Modified Rose Bengal (mRBPT) and all the positive sera and 10% of the negative sera were further tested using complement fixation test. Out of 2340 blood samples, 107 sera were positive by mRBPT. Upon further test of 107 positive and 230 negative sera only 90 of 107 and 16 of 230 sera were positive by CFT. Therefore, only 90 serially positive sera were used for the subsequent data analysis. The overall individual and herd level seroprevalences were 3.8% and 28.1%, respectively. The seroprevalence was 4.8% in goats and 1.9% in sheep, whereas, 4.2% in females and 2.2% in males. Statistically significant difference was observed among different age groups, in which the seroprevalence of brucellosis was 6.5% in young ages, 3.1% in adult ages and 3.8% in old ages. Both univariable and multivariable

logistic regression analyses showed the significant association of species with the seropositivity of small ruminant brucellosis. The serum samples of 230 farmers gathered from the district's health center in collaboration with the public health institutions showed two positive sera by mRBPT, but all the human sera were negative by CFT. In conclusion,the present studies served to demonstrate the widespread occurrence of *Brucella* antibodies seroprevalence in small ruminants.

Key words: mRBPT, CFT, Brucellosis, Small Ruminants, Human Risk Groups, Seroprevalence.

1. INTRODUCTION

Livestock plays a crucial role in the livelihoods of the majority of Africans. Over the last decades, the population growth in sub-Saharan Africa, combined with rising per capita income has resulted in high demand for food of animal orign. The World Bank has estimated that demand for milk and dairy products in sub-Saharan Africa will increase by 5.5 million tons by the year 2025, with an annual growth rate of 4%, questioning the supply side (World bank, 1992).

Livestock contributes about 15 and 33% of the national and agricultural GDPs, respectively. Hides and skin contribute 17% of the country's annual export earning, while other livestock products and live animals contribute about 1-25%. The greatest share of this income is from small ruminants. But, the contribution of livestock is inconsistent and fluctuating due to several factors such as poor livestock management, prevallence of different livestock diseases, inadequate veterinary services, lack of market-oriented livestock production reflected by lack of trade routes to the market,, using animals for meat as secondary to draught power, high rates of smuggling of livestock from the pastoral areas, and reccurent severe drought were among them (CSA, 2004).

Small ruminants are associated with the rural poor. They play a big role in supporting the livelihood system of the poorest men and women livestock keepers, especially in the marginalized areas. Inspite of its livelihood contribution and contribution to the livestock economy, this sub-sector received only very small attention in the poor countries and diseases of small ruminants affect the incomes of smallholder farmers in sub-Saharan Africa by reducing product ivity or through loss of the animal (Ibrahim, 1999; Sebastian, 2004).

Brucellosis is a major zoonotic disease, widely distributed in both humans and animals, especially in the developing world. The occurrence of the disease in humans is largely dependent on the animal reservoir and high rates of brucellosis infection in sheep and goats usually cause the greatest incidence of infection in humans (WHO, 1997; Banai, 2002; Cutler *et al.*, 2005).

In Ethiopia, prevalence of human brucellosis of 12.5% (Yirgu, 1991), 2.4% (Tolosa, 2004), 5.3% (Asmare, 2004) and 3.78% (Hailemelekot, 2005) have been recorded, and fragment studies have revealed the occurrence of small ruminant brucellosis (Tekelye and Kasali, 1990; Yibeltal, 2005; Mengistu, 2007; Teshale *et al*, 2006; Ashenafi *et al*, 2007). Particularly, small ruminants play major economic roles in the low land pastoral areas of the country, where they serve as sources of milk and meat and as milk from sheep and goats is consumed raw, it could be possible to expect human brucellosis caused by *B. melitensis*. Good quality data on the impacts of these different diseases and their control on animals and humans populations in sub-Saharan Africa are usually lacking (Banai, 2002; McDermott and Arimi, 2002).

Therefore,the objectives of this study were:

- To determine the seroprevalence of brucellosis in small ruminants.
- To investigate the potential risk factors and their associations with the disease.
- To investigate the seroprevalence of human brucellosis.

2. LITERATURE REVIEW

2.1. Etiology

Brucella species are comprised of obligate intracellular organisms, requiring an animal host for their survival and maintenance and in nature they are pathogens that do not multiply in open environments. *Brucellae* are small Gram-negative, aerobic, capnophillic, nonsporformig, non capsulated, nonmotile, coccobacilli or short rods measuring from 0.6 to 1.5 µm long and 0.5 to 0.7 µm wide. Apart from *Brucella ovis* and *Brucella neotomae*, all are oxidase-positive. All *Brucella* species are urease-positive except *Brucella ovis*. *Brucella ovis* and some biotypes of *Brucella abortus* require 5 to 10% C_2O for primary isolation (Quinn *et al.*, 2005). They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucellae* is fairly constant, except in old cultures, where pliomorphic forms may occur. Six species are recognised within the genus: *B. abortus*, *B. Melitensis*, *B. Suis*, *B. Canis* and *B. Neotomae*. With respect to the current classification of *Brucella* species according to the preferential host, two new species have been recognized: *B. Pinnipediae* (for pinniped isolates) and *B. Cetaceae* (for cetacean isolates) (Moreno *et al.*, 2002; Bricker, 2002; Bounaadja *et al.*, 2007). *Brucella melitensis* (biovars 1, 2 and 3) is the main causative agent of ovine and caprine brucellosis. Sporadic cases caused by *B. abortus* and *B.suis* have been observed but clinical disease is uncommon (Walker, 1999; Seifert, 1996; Banai, 2002; Cutler *et al.*, 2005; OIE, 2000a).

Some of *Brucella* species are further classified into biotypes (biovars). The numbers of biovars of the different species are shown in Table 1.The biotypes within the species are differentiated on the basis of combinations of characteristics. These include carbon dioxide requirement, hydrogen sulfide production, urease activity, and growth in the presence of dyes, serological specificity, phage typing, and oxidative metabolic profiles (Baron and Petersons, 1994; Quinn *et al.*, 2002).

Brucella organisms are catalase positive and contain cytochrome C. They usually do not ferment carbohydrates or reduce nitrates, do not produce indole, utilize citrate, liquefy gelatin, lyse blood, change litmus milk or produce acethylmethyl carbinol. The guanine plus cytosine (G+ C) content of *Brucella* is 55-58 mole %. Differences between species of *Brucella* depend on biochemical tests, including CO_2 requirement, H_2S production, growth on dye media, agglutination with

monospecific sera, phage lysis, and oxidation of different substrates. *Brucellae* contain characteristic surface antigens. Two specific antigens have been characterized as A (*abortus*) and B (*melitensis*) antigens, respectively. They have been shown to be similar O-polysaccharide chains of a smooth lipopolysaccharide (SLP) attached to the outer cell membrane. Both A and B antigens are present in varying amounts in all smooth *Brucella,* with one type predominant in some species and the other type predominant in other species. Similar antigens found in other bacteria such as *Yersinia enterocolitica* O: 9 can cause cross-reactions with *Brucella*. The rough *Brucellae*: *B. ovis* and *B. canis* and rough strains of smooth biovars do not present the A and M antigens on their cell surface, but, instead present other antigens on the LPS and other outer membrane proteins (Hugh *et al*., 1994).

Table 1: Species, biovars and reservoirs of brucellosis

Species of *Brucella*	No of Biovars	Reservoir hosts
B. melitensis	3	Sheep, goat, cattle
B. abortus	7	Cattle
B. suis	5	Swine
B. canis	1	Dogs
B. ovis	1	Sheep
B. neotomae	1	Rodents
*B. pinnipediae**	Not determined	Otter, seal
*B. cetaceae**	Not determined	Dolphins, porpoise

* Species not yet approved Source: Cutler and Cutler (2006)

Human brucellosis can be caused by *B. abortus, B. melitensis* and *B. suis* biovars 1-4 and, rarely *B. canis* or marine mammalian *Brucella*. Live vaccines for *B. abortus* and *B. melitensis*, as well as the *B. canis* M- strain are also pathogenic for humans. *Brucella ovis*, *B. neotomae* and *B. suis* biovars 5 have not been linked to human disease (Renukaradhya *et al.*, 2002). The species of *Brucella* affecting human beings and the levels of their pathogenicity are summarized in Table 2.

Table 2: Typical host specificity *of Brucella* species

Brucella Species	Animal Host	Human Pathogenicity
B. suis	Swine	High
B. melitensis	Sheep, goats	High
B. abortus	Cattle, bison	Intermediate
Marine species	Marine mammals	Rare
B. ovis	Sheep	None
B. neotomae	Rodents	None

Source: Chan and Hardiman (1993)

2.2. Epidemiology

2.2.1. World distribution

Brucella melitensis is primarily found in intensive sheep and goats raising areas and is a serious problem in almost any area of the world where large numbers of sheep and goats are found (Corbel and *Hendy*, 1985; Quinn *et al.,* 2002). Factors influencing the epidemiology of *Brucella* infection in any geographic location can be classified into factors associated with transmission of the disease among herds and factors influencing the maintenance and spread of the infection within the herd (Crawford *et al.,* 1990). Further factors associated with brucellosis include: host factors (age, sex and breed), agent and extrinsic factors (environment factors) including management and ecology influence its transmission and maintenance among and within hosts (Nicoletti, 1980; Mikolon *et al.,* 1998; Garin-Bastuji *et al.,* 2006).

Brucella melitensis infection in sheep appears to occur endemically in the Mediterranean region, especially along its northern and eastern shores, stretching through Central Asia as far south as the Arabian peninsula and as far east as Mongolia. Parts of Latin America are also seriously affected, especially Mexico, Peru and northern Argentina. The disease also occurs in Africa and India. However, North America (except Mexico) is believed to be free, as are Northern Europe (except for sporadic incursions from the south), Southeast Asia, Australia and New Zealand. Of

the three different biovars of *Brucella melitensis,* biovar 3 predominates almost exclusively in Mediterranean countries and Middle East, while biovar 1 seems to predominate in Latin America. The biovars 1 and 2 have also been reported in some southern European countries. However, the precise recognition of biovar 3, especially its differentiation from biovar 2 appears sometimes equivocal. (FAO/OIE/WHO, 1997; Elzer *et al.*, 2002).

Although *Brucella melitensis* has been eradicated in most industrialized regions, its occurrence is increasing in developing countries, particularly in some Mediterranean and Middle Eastern countries. Available information indicates that *B. melitensis* infection is mostly widespread in Egypt, Sudan, Syria, Morocco, Turkey, Greece, Spain, and Italy, and in some Latin American countries countries. It is also a common infection of animals and humans in countries neighbouri countries (Argentina, Mexico, Peru and Brazil). It is also a common infection of animals and humans in countries neighbouring Turkey. *Brucella melitensis* has been reported to be the primary agent responsible for sheep abortions in Turkey (Sahin *et al.*, 2008). Areas currently listed as high risk are the Meditterean Basin (Portugal, Spain, Southern France, Itally, Greece, North Africa), South and Central America, Eastern Europe, Asia, Africa, the Caribbean and Middle East Region (Taleski *et al.*, 2002).

In humans caprine and ovine brucellosis caused by *B. melitensis* is by far the most important clinically apparent disease with a limited geographical distribution, but remains a major problem in the Mediterranean region, western Asia, and parts of Africa and Latin America. Although sheep and goats and their products remain as the major source of infection, *Brucella melitensis* in cattle has emerged as an important problem in some southern European countries, Israel, Kuwait and Saudi Arabia. Thus, bovine *Brucella melitensis* infection is emerging as an increasingly serious public health problem in some countries. In some Latin American countries, particularly, Brazil and Colombia, *B. suis* is reported in cattle, in which they become more important than pigs as source of human infection. The true incidence of human brucellosis is unknown and reported incidence in some endemic areas varies widely from < 0.01 to > 200 per 100,000 populations. While some areas such as Peru, Kuwait, and parts of Saudi Arabia have a very high incidence of acute infection (Corbel, 1997).

Human brucellosis caused by *B. melitensis* shows wide geographic distribution in the world. The highest prevalence of human brucellosis is seen in the Iberian Peninsula on the Mediterranean littoral especially in France, Portugal, Italy, Greece, Spain and Turkey (Memisoglu *et al.*, 2008). On the basis of the provisions of Directive 91/68/EC, nine member states are recognized free of *B.melitensis*. These include: Belgium, Denmark, Finland, Germany, the Netherlands, Ireland, Luxebourg, Sweden and the United Kingdom. In these countries, an annual monitoring program is carried out in accordance with the rquirements of Directive 91/68/EC to confirm the freedom from B.melitensis (Godfroid and Kasbohrer, 2002).

There are so many factors that can affect the prevalence of brucellosis in various species of livestock. Prevalence of brucellosis can vary according to climatic conditions, geography, species, sex, age and diagnostic tests applied. Though its distribution is worldwide; yet brucellosis is more common in countries with poorly standardized animal and public health programme (Capasso, 2002). New *Brucella* strains or species may emerge and existing *Brucella* species adapt to changing social, cultural, travel and agricultural environment (Godfroid *et al.*, 2005). Bio-varieties of *Brucella* vary with respect to geographic region. *B. melitensis* biovar 1 from Libya, Oman and Israel and *B. melitensis* biovar 2 from Turkey and Saudi Arabia have been isolated. *B. melitensis* biovar 3 is the most commonly isolated species from animals in Egypt, Jordan, Israel, Tunisia and Turkey (Refai, 2002).

In general, brucellosis can be found in any season of a year. The epidemic peak occurs from February to July and is closely related to the months associated with delivery and abortion in animals (Shang *et al.*, 2002). In humans, prevalence of the disease is high (39.5%) in summer season (Salari *et al.*, 2003). Notifications of human brucellosis, which are mandatory in Italy, reach a peak between April and June. However, considering the standard incubation period of 2-4 weeks, and the fact that lamb slaughter is traditionally at a peak during the Easter period, it might be expected that occupational exposure would result in a peak of human cases between March and May. The observed peak between April and June could be related to the production and consumption of fresh cheese, starting just after lamb slaughter (De-Massis *et al.*, 2005).

Worldwide, the true incidence of human brucellosis is unknown. Reported incidence in endemic-disease areas varies widely from < 0.01 to > 200 per 100 000 population. For example, Egypt, the Islamic Republic of Iran, Jordan, Oman, Saudi Arabia and Syria and Arab Republic reported a combined annual total of more than 90 000 cases of human brucellosis in 1990. The low incidence reported in known brucellosis-endemic areas may reflect the absence or the low levels of surveillance and reporting. In 2000, altogether 2 857 human cases (in 14 Member States) were reported in the EU compared to 3 899 cases (in 13 Member States) in 1999 (Rafi, 2002).

Brucella melitensis is the major cause of abortion in sheep and goats in many countries and the infection is widespread in India. Resent serological surveys of small ruminants brucellosis indicated varying levels of infection in different states of India. A number of 4.9% of sheep and 7.6% goats in Karnataka (Desai *et al.*, 1995); 11% of sheep and 18% of goats in northern state of Delhi; 50% of sheep and 16% of goats in Punjab and 33% of sheep and 30% of goats in the western state of Rajasthan (Kumar *et al,* 1997b); 55% of goats in Andhra Pradesh (Mrunalini *et al.*, 2000); and 24% of goats and 4.7% of sheep in Uttar Pradesh (Singh *et al.*, 2000) have been recorded. In a national survey of sheep and goats brucellosis, tested serum samples originating from 10 states, which included 6305 from sheep, and 3849 from goats indicated the widespread prevalence of small ruminant brucellosis in the country (Isloor *et al.*, 1998b; Renukaradhya *et al.*, 2002).

Brucellosis is present in many parts of the world. But, information on its distribution is rather sparse in these parts of the world. This is probably due to the fact that the disease, which belongs to OIE List of diseases remains insidious, in which many countries with limited resources give priority to more other specular diseases like foot and mouth disease, sheep pox, rift valley fever, pests des petitis ruminants, caprine contagious pleuropneumonia (Benkirane, 2006). Ovine and caprine brucellosis is distributed worldwide and is a major problem of Arabian Peninsula, West Asia, and parts of Latin America and Africa (FAO, 2002).

Prevalence varies within and among countries, which could be due to different factors such as herd size, management, ecology and biology of the disease. The seroprevalences carried out in different African and Middle East countries revealed prevalence that ranged from 0.3% to 21% in

goats and 0.6 % to 22% in sheep, in which most prevalence fell less than 10% at individual level. The outbreak that occurred in Algeria in 1989 showed seropositivity of 2.2% in sheep and 12% in goats. Whilst the herd level prevalence were 43.5% and 42%, respectively. While in Tunisia in 1991, in a place called Gafsa, a herd prevalence of 12.1% and 2.4%, respectively in sheep and goats were recorded (Benkirane, 2006). The prevalence distribution of small ruminant brucellosis due to *B. melitensis* and *B. ovis* in some African countries is given in Table 3.

Table 3: Prevalence distribution of *B. melitensis* and *B. ovis* in some African countries

Country	*B. melitensis*	*B. ovis*
Egypt	+	ND
Ethiopia	+	ND
Kenya	+	ND
Sudan	+	ND
Somalia	+	ND
Eritrea	ND	+
Libya	+	ND
Algeria	ND	+
Tunisia	++	ND
Niger	+	+
Nigeria	+	ND
Cote devour	ND	+
Zimbabwe	+	+
Botswana	ND	+
South Africa	+	+

++: High prevalence; +: Sporadic low prevalence; ND: No data.
Sources: FAO (2002) and Corbel (1997)

2.2.2. Occurrence of small ruminants brucellosis in Ethiopia

The status of small ruminant brucellosis in Ethiopia is not well known. This may be due to the little attention given to small ruminants. Absence of research activities in animal diseases, poor veterinary development, and lack of awareness about economical and zoonotic impacts of the disease have contributed to the paucity of information. Llimited serosurveillance carried out so far indicates that brucellosis may be one of the most important diseases in goats and sheep in many communities. A serosurveillance study carried out in small ruminants in Afar and Somali Region in 2005, demonstrated that the disease exists in Ethiopia. The serosurveillance findings

were 14.6% in sheep and 16.2% in goats in Afar; 1.7% in goats and 1.6% in sheep in the Somali Region (Yibeltal, 2005). In another survey in the Central highlands of Ethiopia, a seroprevallence of 1.5% in sheep and 1.3% in goats were recorded (Tekelye and Kasali, 1990). The existence of the disease was also confirmed and reported in Southern Nations Nationalities and People's Regional State, according to the annual report of Sodo Regional Veterinary Laboratory in 2005.The status of small ruminant brucellosis in Ethiopia is shown in table 4.

Table 4: Prevalence of brucellosis in small ruminants in Ethiopia

Country	Sheep	Goats	Source
Ethiopia: Central high lands	1.5%	1.3%	Tekelye and Kasali, 1990
Afar Region	14.6%	16.2%	Yibeltal, 2005
Somali Region	1.6%	1.7%	Yibeltal, 2005
Afar Region	3.2%	5.8%	Ashenafi et al, 2007
Borena	1.09% (RBT)	_	Teshale et al, 2006
Konso and AAC	1.6%	3.2%	Mengistu, 2007

AAC: Awassa agricultural college; RBT: Rose Bengal Test.

2.2.3 Transmission and source of infection

According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Office International des Epizooties (OIE), brucellosis is considered as the most wide-spread zoonosis. The importance of the disease lies on the severe public health hazard it causes and the economic impact on the animal industry resulting in effect on total animal protein supply (Mustofa and Nicoletti, 1993).

Human infection occurs due to direct contact with blood, placenta, fetuses, or uterine secretions of infected animals. Although consumption of raw and infected animal products especially milk and milk products represents an important health hazard to consumers. These organisms localize in the supramammary lymph nodes and lymph glands in 80% of infected animals, which continue

to excrete these organisms in their milk throughout their lives and have significant role in the epidemiology of the diseases (Hamdy and Amin, 2002;Chand *et al.*, 2005; Gupta *et al.*, 2006).

Brucellosis is one of the most important bacterial zoonoses transmitted to humans mainly from mruminants. Animals acquire the infection through oral (ingestion), skin (wound) and sexual routes. The main sources of infection are milk and milk products and aborted materials. Ovine brucellosis is more likely to be introduced through importation of infected animals, semen or embryos (Banai, 2002).

Brucella melitensis is usually transmitted between animals via contacts with the placentas, fetus, fetal fluids and vaginal discharges from infected animals. Animals are infectious after either abortion or full term parturition. Entry into the body occurs by ingestion and through the mucous membranes, broken skin and possibly intact skin. Most or all *Brucella* species are also found in semen. Males can shed these organisms for long periods or lifelong. *Brucella* can be spread by fomites including feed and water (CFSPH, 2007; CDC, 2007).

The source of infection is an aborting animal. As in cattle, the surroundings where lambs are born to infected ewes or where abortion takes place can become greatly contaminated. Animals may contract brucellosis by oral or cutaneous routes, or at birth. Infection by inhalation is also possible when healthy and aborting animals are kept in overcrowded pens with poor sanitary measures. Transmission of *B. melitensis* from flock to flock usually follows the movement of infected pregnant females. However it can also occur via infected males. Wild animals and dogs may transmit parts of aborted fetuses to other areas (Corbel, 1997; Radostits *et al.*, 2000).*Brucella* can be found in milk, urine, feces, placenta, and vaginal secretions that accompany natural birth or abortion. In the case of normal full-term births, kids from infected does are often infected and capable of spreading the disease (Maria, 2006).

Humans are generally infected through one of the three ways: eating or drinking foods contaminated with Brucella, inhalation and through skin wounds. The most common way for infection is by eating or drinking contaminated milk and milk products. When sheep, goats, cows, or camels are infected, their milk is contaminated with the bacteria. If the milk is not pasteurized,

these bacteria can be transmitted to persons who drink the milk or eat cheeses made of it. Inhalation of *Brucella* organisms is not a common route of infection, but it can be a significant hazard for people in certain occupations, such as those working in laboratories where the organism is cultured and in abattoir employees. Contamination of skin wounds may be a problem for persons working in slaughterhouses or meat packing plants and veterinarians. Hunters may be infected through skin wounds or by accidentally ingesting the bacteria after cleaning deer, or wild pigs (Stauffer, 1998; Cutler and Culter, 2006). The cryopreservation of semen increases the national as well as the international distribution of semen and the possibility of spreading different diseases, including brucellosis among animal population. To prevent the transmission of brucellosis by artificial insemination only *Brucella*-free semen collected from *Brucella*-free donors should be used (Amin *et al.*, 2001; CDC, 2007).

2.2.4 Risk factors

Some of the risk factors, which influence the occurrence of ovine and caprine brucellosis in the world, include lack of corral hygiene, breed, uncontrolled animal movements, shared communal pastures, and watering places (Al-Talafhah *et al.*, 2003).

Management practices

Once infected, the time required to become free from brucellosis is increased by large herd sizes, active abortions, and loose housing (Radostits *et al.*, 2000). Herd sizes and animal densities are directly related to prevalence of the disease and difficulty in controlling infection in a population (Nikoletti, 1980; Walker, 1999).

Unless the hygienic conditions or measures to prevent the introduction of the disease and the resistance of the animals are improved, all together the condition favors rapid spread of the disease and subsequently results in high prevalence of the disease in that particular herd (Nicoletti, 1980; Radostits *et al.*, 2000). Management practices directed at eliminating infected animals and minimizing exposure to aborted tissues greatly reduce the incidence of the disease in commercial livestock operations (Walker, 1999). Management risk factors for high occurrence of infection are high stocking densities, poor housing and lambing, lack of sanitary measures

(cleaning and disinfection) and large herd sizes (Mikolon *et al.*, 1998; Crawford *et al.*, 1990).Host factors

Susceptibility to infection depends on age, sex, and breed and pregnancy status of the animals. Younger animals tend to be more resistant to infection and frequently clear infection, although latent infection does occur. Only 2.6% of animals infected at birth remain infective, as adults and sexually mature animals are much more susceptible to infection, regardless of sex. Most animals infected as adult remain infected for life. After reaching sexual maturity, the state of pregnancy has a greater influence on the degree of susceptibility than age. Because of latent infection, kids and lambs borne of recently infected mothers tend to infect the stock later in their life (Nikoletti, 1980). Introduction of infected animals can lead to rapid spread of the disease /infection within the flock (Walker, 1999; PAHO-WHO, 2001).

There are controversial reports regarding the prevalence of brucellosis in relation to sex of animals, as some of the research workers reported significantly higher prevalence in females than in males. The relatively higher incidence reported among human females than males might be due to more involvement of females in handling of livestock. These females may be highly exposed to the risk of infection through direct contact with animals, consumption of raw milk and milk products. Moreover, risky practices in rural areas such as skinning of stillborn lambs and kids, as well as crushing the umbilical cord of newborn lambs and kids with teeth can also be contributing factors (Hussein *et al.*, 2005). However, some reports indicate that *Brucella* antibody titers are not associated with sex (Muma *et al.*, 2006). Higher prevalence of infection in animals more than 4 years of age compared to younger animals has been reported. It appears that the high prevalence of brucellosis among older cows might be related to maturity with the advancing age. Thereby, the organism may have propagated to remain either as latent infection or it may cause clinical manifestation of the disease (Kazi *et al.*, 2005; CDC, 2007).

Environment

Brucella survives freezing and thawing. Under proper environmental conditions, they survive for up to 4 months in the milk, urine, water and damp soil. In liquid manure, the organism survives for months, in humid faeces for 22 weeks, in aborted fetus up to 4 months and in the dust for 44

days, in tap water for 30 days, in sterile water for 51 days, in desert soil up to 2 months, and in frozen soil for two years. Contaminated straw remains infectious for longer than a month (Weidmann, 1991; Seifert, 1996). *Brucella* organisms do not multiply in the environment, but they persist and their survival is influenced by the environmental factors (Radostits *et al.*, 2000).

The resistance of *Brucella* species to the environmental factors depends on whether they are protected against the sunlight and high temperature. Normal soil pH and moist environments, which are rich in organic materials, are favorable for the survival of the organism. *Brucella* organisms can survive for longer periods in the environment, but does not multiply in the environment (Walker, 1999; Quinn *et al.*, 2002; Anonymous *et al.*, 2007b).

2.3. Economic importance and public health significance

2.3.1. Economic importance

Caprine and ovine brucellosis, caused by the zoonotic bacterium *B. melitensis*, is an economically important cause of abortion in small ruminants. This infection causes significant losses from decreased productivity and loss of trade in much of the developing world. *Brucella melitensis* is considered to be a re-emerging pathogen. The cost of surveillance to remain *B. melitensis*-free is significantly large (CFSPH, 2007). *Brucella melitensis* causes heavy economic losses in animal production resulting from clinical disease, abortion, neonatal losses, reduced fertility, decreased milk production, and emergency slaughtering of the infected animals. In addition, the disease is an impediment to free animal movement and global trade (Radostits *et al.*, 2000). Reproductive diseases and the resulting economic losses are a major problem for the livestock industry. These losses are due to clinical disease, abortions, neonatal losses, reduced fertility, decreased milk production and emergency slaughtering of the infected animals for animal industry, repeat breeding, and cost of treatment and transmission of infectious agents among the herd. It has been estimated that up to 90% of these losses are due to infectious agents. It also causes a barrier for international trade of live animals by being used as an impediment to free animal movements and export (Murray, 1990).

Protein supply is reduced due to livestock losses. In protein-deficient developing countries this is an obvious and serious problem, since potential future supplies of milk and meat are lost through abortion or neonatal death of animals. In developed countries animal loss decreases the potential supply of milk and meat available and results in increased costs to the consumers for these products. Studies have shown that 46% -48% of *B. abortus* infected heifers will lose their caves first year after infection and 3% in each following year. Applying these loss rates to a population of cattle with a 10% animal infection rate and a 20% replacement rate of breeding cows per year would result in a loss of approximately 1,200 newborn animals per 100.000 animals breed. Livestock producer and government costs (testing and vaccination) also result in increased cost of these animal products for the consumer (Hugh *et al,* 1994; Coelho et al., 2007; Maria, 2006).

In the last several years, in Bosina and Herzegovina, outbreaks of brucellosis in small ruminants and humans have caused growing concerns and resulted in significant economic losses. The number of slaughtered animalss has increased severely from 8,788 in 2007 to 32,000 in 2008. In 2008, 205,000 small ruminants were tested and 6,120 were found positive. At the same time, 315,000 small ruminants were tested and 13,500 were found positive. In this country the sheep and goats population is about 1.1 million (GAIN, 2008).

2.3.2. Public health significance

Sheep and goats brucellosis (excluding *Brucella ovis*) is a zoonotic infection with important effects on both public health and animal health and animal production and is widespread in many areas of the world, particularly in some Mediterranean and Middle East countries (EC, 2001). Brucellosis is a zoonotic illness with a prevalence of 60 to 80/100,000 in Mediterranean and most Middle Eastern countries. Iran has a disease incidence of 132/100000. Some occupations have been identified to be at an increased risk. In recent decades the risk factors for infection have shifted from occupational hazards to the consumption of contaminated food, especially milk and cheese. Human to human transmission is rare and its infectiousness by the aerosol route makes it a potential for bioterrorism and transmission in laboratories (Ghaffarpour *et al.,* 2007; CDC, 2007).

Brucellosis is not only a major zoonotic problem but is also linked with bioterrorist attack. The severity of this disease, lack of vaccines suitable for use in man and frequent failure of clinical laboratories to correctly identify isolates led to the investigation of *Brucella* as an agent for bioterrorism. Before 1954, when Britain was focusing on anthrax, brucellosis was the first microorganism chosen by the United States to develop as a weapon. This microorganism could be effectively disseminated in four pound bombs (Yagupsky and Baron, 2005). Indeed, the American military weaponized *Br. suis* in 1954, however, changing global politics resulted in abandonment of these efforts following the biological and toxic weapons convention in 1972 (Youn, 1995; Ramırez *et al.*, 2008; Poester *et al.*, 2002).

Persons with brucellosis often suffer from a long, protracted and sometimes painful illness which can result in an inability to work, loss of income and productivity for months or years. Brucellosis is a zoonosis caused by non-motile aerobic gram negative coccobacilli, which is transmitted to the human population mostly as an occupational disease or through the ingestion of raw goat or sheep milk and/or unpasteurized dairy products. Camels are also believed to be a reservoir for brucellosis. The occupational risk is especially important for *B. abortus* and *B. suis* infections, commonly found among abattoirs workers and those engaged in animal husbandry. Studies within abattoirs have documented the risk of aerosols as a mode of transmission. *B. melitensis* infection, on the other hand, is primarily food borne. While rare cases of brucellosis transmitted by blood transfusion or bone marrow transplantation have been reported, the disease is not readily transmitted between human beings (Shehata *et al.*, 2001; Sharma and Adlakha, 1996).

Aerosol or food contamination could be the sources of dispersion. This microorganism has the advantage of being debilitating without being fatal. The infective dose for these organisms is very low, if acquired via the inhalation route. It has been estimated that 10-100 organisms are sufficient to constitute an infectious aerosol dose for humans. The economic impact of a brucellosis bioterrorist attack would cost US$477.7 million per 100,000 persons exposed (Kaufmann *et al.*, 1997). The countries with the highest incidence of human brucellosis include Saudi Arabia, Iran, Palestinian Authority, Syria, Jordan and Oman. Bahrain is reported to have no incidence (Refai, 2002). The Mediterranean and Middle East Region is considered as the area

where zoonoses are most numerous and widespread. The reasons for and the impact of this concentration are a) biodiversity, b) close co-existence between humans and animals, c) variety of environments and climates, d) human and animal movements and migrations (Seimenis *et al.*, 2006). Prevalence distribution of brucellosis in the region is summarized in Table 5.

Table 5: Reported cases of human brucellosis in selected Mediterranean countries

Country	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003
Algeria	1616	1227	1345	1296	3439	2779	2493	3933	3200	3262	2783
Egypt	256	309	490	872	1006	(b)	2355	2902	3510	4030	4335
France	127	178	100	67	91	48	49	44	(b)	37	33
Greece	158	174	242	240	360	419	539	548	405	331	240
Italy	1120	1285	1370	1896	1681	1461	1324	1268	923	813	631
Jordan	750	770	770	994	953	684	432	268	275	219	159
Lebanone	(c)	(c)	188	302	326	285	191	224	274	290	193
Portugal	1166	1189	915	860	866	794	683	507	381	206	139
S/Arabia	6985	4929	4929	4800	5781	7468	5913	5320	4865	4687	4534
Spain	2842	2842	2708	2005	21154	1545	1553	1104	924	893	642
Syria	1391	3457	7701	6868	6991	7402	6958	6487	9971	11764	23297
Tunisia	801	472	182	490	291	206	355	183	321	250	128
Turkey	6795	8383	8506	9480	11812	12330	11482	10742	15510	17553	14572

(b) no available information; (c) high occurence Soure: Seimenis *et al.*, (2006)

In Saudi Arabia, the prevalence of brucellosis has been largely attributed to the social norms of nomadic people, who consume raw milk and cheese and live in close proximity with livestock such as sheep, goats, and camels in rural areas. While humans can be infected by all the *Brucella* species, except *B. ovis* and *B. neotomae,* infection of people in Saudi Arabia is generally due to *B. melitensis*. Other infectious B*rucellae,* such as *B. suis* and *B. canis*, have not been reported due to the lack and scarcity of domesticated pigs and dogs in Saudi Arabia (Elfaki *et al.*, 2005; Radostits *et al.*, 2000; Quinn *et al.*, 2002).

2.4. Pathogenesis

Brucella species are intracellular pathogens that are capable of survival and replication inside host phagocytic and non-phagocytic mammalian cells, which is essential for virulence. Following penetration of the mucosal epithelium, the bacteria are transported to the regional lymph nodes. The spread and multiplication of *Brucellae* in lymph nodes, spleen, liver, bone marrow, mammary glands, and sex organs occurs via macrophages. The increase of *Brucellae* in the host is mainly due to the organisms' ability to avoid the killing mechanisms and proliferate within macrophages. Virulent *Brucella* species not only resist killing by neutrophils following phagocytosis, but they also replicate inside macrophages and nonprofessional phagocytes. They also appear to be capable of withstanding exposure to reactive oxygen intermediates, acidic pH, and nutrient deprivation during their time inside the host macrophage. There is evidence that smooth LPS probably plays a vital role in intracellular survival since smooth organisms tend to survive much more effectively than rough ones. Survival in macrophages is considered to be responsible for the establishment of chronic infections and allows the bacteria to escape the extracellular mechanisms of host defense, like complement and antibodies (Celli 2006; Seifert, 1996; Quinn *et al.*, 2002).

B. melitensis is also considered the most pathogenic species for humans (Ramı´rez-Pfeiffer *et al.*, 2008). *Brucella s*pecies produce chronic infections in their mammalian hosts and the capacity of these bacteria to persist for prolonged periods in their natural hosts macrophages is critical to their virulence. Numereous studies have implicated the oxidative killing pathways of host phagocytes as the primary mechanism by which these cells control the intracellular replication of *Brucellae* and prewvious biochemical and genetic studies have shown that members of the high temperature- requirement A (HtrA) family of bacterial stress reasponse proteases function as important cellular defense mechanisms against oxidative killing by degrading the oxidatively damaged proteins in the bacterial periplasm before thse damaged proteins reach toxic level (Roop *et al.*, 2001; Sharma and Adlakha, 1996).

Innate, nonspecific defenses at the site of invasion are the first host defenses and may protect entry of low exposure doses. After invasion, *Brucellae* are ingested by local phagocytes and

localized temporarily in the regional lymph nodes, draining the invasion site. *Brucellae* multiply in the cytoplasm of phagocytic cells, eventually killing and rupturing them. After development of cell mediated immunity, the macrophages become proficient in killing the ingested *Brucellae* (Hugh *et al.*, 1994; Stauffer, 1998).

Susceptibility of animals depends on their natural resistance, age, and level of immunity and environmental factors. If infection is introduced into a non-infected herd in which all animals are immunologically naive to brucellosis, the so-called abortion storms may occur and almost all pregnant female animals will abort (Seifert, 1996). Following exposure *Brucella* penetrates intact mucosal surface in the alimentary tract. The epithelium covering the ileal peyer's patches are the preferred sites for entry. After entry into the host *Brucellae* either free in the extra cellular environment or phagocytic cells or localize in the regional lymph nodes. Once in the blood stream, the organism is seeded to multiple in organs/systems, especially those rich in reticuloendothelial tissue, such as liver, spleen, skeletal and hematopoietic system (Greenfield *et al.*, 2002; Baroso *et al.*, 2002).

In the phagocytic cells the organism is capable of surviving and multiplying. Intracellular survival in the macrophages and to a lesser extent in the neutrophils is enhanced by suppression of the Myeloxidase-H_2O_2 halide system and production of super oxidase dismutase and catalase against oxidative killing. Stress proteins protect the organism from hydrolytic enzymes and oxygen radicals. From the regional lymph nodes the organisms are distributed to the lymphatic system, passes to the thoracic duct and join the blood stream, where it is disseminated to the parenchymatous organs and other tissues such as joints (Walker, 1999; Walravens, 2007; CDC 2007).

The predilection sites for *Brucella* are the reproductive tract of males and females, especially pregnant uterus and reticuloendithelial system. Allantoic factors, erythritol and steroid hormones which are present in the placenta and male genital tract of cattle, sheep, goats, and pigs, but not humans, stimulate growth of *Brucella.* Although the exact mechanism of abortion is unknown, the likely possibilities are: placentitis interfering fetal circulation, direct effect of the organism's toxin, and fetal stress due to inflammation response in the fetal tissue. Generally visible lesions

after abortion are necrotizing placentitis, intercotyledonary thickening with yellow gelatinous fluid, and thick brown exudates, aborted fetus is edematous and abomasal contents may be turbid and have lemon-yellow color (Seifert, 1996; Walker, 1999; Quinn *et al.*, 2002).

2.5. Clinical findings

The predominant symptoms of *B. melitensis* infection are reproductive disorders such as abortion, stillbirth, delivery of weak offspring, and placenta retention in females, and orchitis and epididymitis in males. Abortion occurs during the last two months of gestation (Sahin *et al.*, 2008). Animals that abort may retain the placenta. Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies. Milk yield is significantly reduced in animals that abort, as well as in animals whose udder becomes infected after a normal birth. However, clinical signs of mastitis are uncommon. Acute orchitis and epididymitis can occur in males, and may result in infertility. Arthritis is seen occasionally in both sexes. Many non-pregnant sheep and goats remain asymptomatic. *Brucella ovis* affects sheep and can cause orchitis, epididymitis and impaired fertility in rams (Corbel, 1997; Walker, 1999; FAO, 2002).

Brucellosis in the primary host animal tends to become chronic or latent. Nannies infected with *B. melitensis* or cows infected with *B. abortus* rarely recover from the infection and may continue to shade the organism from uterine or vaginal discharges when they kid or calve in subsequent parturitions. Under infections and dormant infections of the lymph glands usually persist, particularly in the supramammary lymph glands in both nannies and cows; this continues to be a hazard for other animals and to people who consume their milk. Ewes infected with *B. ovis* usually do not remain infected for more than one gestation period; rams with epididymitis usually will not recover (Hugh *et al.*, 1994; WHO, 1986; Baroso *et al.*, 2002).

Asymptomatic infection 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. Particularly at night chills and sweats can occur.

Occasionally occurring complications include arthritis, spondylitis, chronic fatigue, epididymitis and orchitis, neurological signs (including personality changes, meningitis, and uveitis and optic neuritis), anemia, internal abscess, nephritis, endocarditis and dermatitis. Deaths are usually caused by endocarditis or meningitis (Quinn *et al,* 2002; CFSPH, 2007; EC, 1991).

2.6. Immunity

Although *Brucella* are capable of stimulating large quantities of humoral antibodies in animals, the antibodies alone are not enough protective and animals receiving large doses of passive anti-*Brucella* antibodies still can be infected with virulent field strain of *Brucella.* Natural host defenses against *Brucella* play an important role in protecting animals against infection. Such natural defenses probably account for the variance in host susceptibilities to different *Brucella* species. The primary protective response is a cell-mediated immune response, which is enhanced by humoral antibodies. Cell-mediated immune responses appear to be characterized by activation of macrophages by T-cell lymphokines with direct lysis of the target cells by cytotoxic T-cells. Humoral immune responses in animals are in most species affected by *Brucella.* Soon after invasion of the *Brucella* organisms, anti-*Brucella* IgM begins to appear and may be seen in measurable amounts within 3-4 weeks. Appearance of anti-*Brucella* IgG follows within 1-2 weeks and soon exceeds the quantity of IgM in the serum. Animals infected with field strains of *Brucella* and those which develop persistent infections with vaccine strains develop a persistently high antibody level which consists primarily of IgG1 and IgG2. Continual release of new antigen stimulates the reproduction of new plasma cells to secret humoral antibody as long as significant numbers of infected macrophages and reticuloendithelial cells are present (Hugh *et al.,* 1994; Abdul and Murray; 2007).

2.7. Diagnosis

Control programmes of brucellosis depend heavily on presumptive diagnosis of the disease by serological tests. The accuracy of the serological tests used has considerable impact on the success of the programmes. If the tests are prone to give false positive results, animals may be needlessly condemned, while tests that give false negative results will prolong any control

campaign. Other factors such as test cost, ease of performance, test precision, interference by antibody to vaccine or cross-reacting antigens are also important and turn around time for results (Nielsen *et al*, 2004; Yagupsky and Baron 2005).

2.7.1. Bacteriological methods

Microscopic examination

Microscopic examination of stained smears can be useful for presumptive diagnosis, particularly, if the direct examination is supported by other tests. *Brucellae* are coccobacilli or short rods, usually arranged singly, but sometimes in pairs or small groups. They are not truly acid-fast, however, they are resistant to decolozization by weak acids and stain red against blue background with stamp`s modification of the Ziehl-Neelsen method. *Brucella* species have a characteristic Gram`s stain morphology that is extremely helpful in differentiating them from other Gram-negative organisms. In the modified Ziehl Neelson (MZN) method smears are made from fetal membranes, fetal stomach contents, vaginal swabs, and lamb semen (for *B.ovis*). The smear is dried and fixed over flame, stained with carbol fuchsin for 10 minutes, washed in tape water, washed in 0.5% acetic acid for 30 seconds and washed thoroughly with counter stain using 10% methylene blue for 20 seconds. Brucella stains red with a blue background. This test is not definitive. Other organisms like Chlamydophila abortus, Coxiella burnetti and B.ovis can resemble Brucellae (CFSPH, 2007; Quinn et al., 2002; Nielsen *et al*., 2000).

Isolation and identification of *Brucellae*

The most reliable and the only unequivocal method for diagnosis of animal brucellosis is isolation of *Brucella* species. *B. melitensis* can be diagulturing the microorganism on appropriate culture media (i.e. the Farrell and modified Thayer-Martin selective media). Thus, taking vaginal swabs and milk samples is the best way to isolate *B. melitensis* from sheep and goats. The spleen and lymph nodes (iliac, supramammary and prefemoral) are the best sites for obtaining samples for isolation during post-mortem examination (Garin-Bastuji *et al*., 2006; Weidmann, 1991; Corbel, 1997; McDermott and Arimi, 2002; Nielsen, 2002).

Brucellae are coccobacilli or short rods measuring from 0.6 to 1.5 μm long and 0.5 to 0.7 μm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucellae* is fairly constant, except in old cultures, where pleomorphic forms may occur. *Brucellae* are nonmotile, nonsporing, with out flagella, pili or true capsules, and are Gram-negative and usually do not show bipolar staining. Basal media: Direct isolation and culture of Brucellae are usually performed on solid media. This is the most satisfactory method, which enables the developing colonies to be isolated and recognized clearly. A wide range of commercial dehydrated basal media are available such as: Brucella medium base, tryptose or trypticase soy agar (TSA), serum dextrose agar (SDA) or glycerol dextrose agar. A wide variety of media have been used for cultivation of Brucella. Most laboratories today use a tryptose or trypticase soy agar containing added serum for primary isolation of Brucella. Antibiotics, mycostatic agents, and bacterial growth inhibitors such as crystal (ethyl) violet are added to the media to inhibit the growth of rapidly-growing contaminants. Except B.ovis and some biovars of B.abortus, Brucellae will not grow in an anaerobic environment and the growth rate of Brucella organisms is relatively slow, especially on primary isolation. If the specimens were initially inoculated into a broth medium, it may be desirable to retain the culture for 6 weeks before discarding as negative (Hugh *et al.*, 1994; Quinn et al., 2002; Alton *et al.*, 1975).

After 3-5 days of inoculation on selective serum agar, pinpoint, smooth, glistening, bluish, translucent colonies appear and as the colonies get old, they become dull and opaque. Strain of *B.melitensis, B.suis, B.abortus* and *B.neotomae* are usually smooth in forms when isolated in each of these species colonies, or rough morphology occur on subculture. Colonies of *B. ovis* and *B. canis* are always in rough forms and rough colonies are dull, opaque, and yellowish and when touched with inoculation loop are found to be friable. Differential diagnosis should be made with diseases causing abortion in small ruminants such as chlamydiosis, leptospirosis, campylobacteriosis, salmonellosis, toxoplassmosis and Q.fever (Nielsen, 2002). Appropriate specimenss for culture are stomach contents, lungs, spleen, and meconium of aborted fetus, fetal membranes, vaginal swabs, blood, milk, lymph nodes (Alton and Forsh, 1975). For *B.ovis* the valuable samples for culture are tail of epididymis, seminal vesicles, ampulla, bulbo-urethral gland and scrotal lymph nodes (West *et al.*, 1993; Alton *et al.*, 1988).

Brucella melitensis does not require supplementation of serum and CO_2 for growth. The optimal temperature for growth is $37^{O}C$, but growth can occur between $20^{0}C$- $40^{0}C$. *Brucella melitensis* grows optimally at a pH of 6.6-7.4. The colonies are pinpoint, smooth, glistening, translucent, raised and convex. The organism is catalase and oxidase positive, reduces nitrate to nitrite, usually hydrolyses urease (but some strains may not), does not produce H_2S, does not cause hemolysis on blood agar, does not produce acid on agar containing glucose, and does not ferment lactose; usually grow in the presence of carbol fuchsin and thionin at a standard concentration (Corbel and Hendy, 1985; Quinn *et al.*, 2002).

Molecular techniques

Polymerase chain reaction (PCR) techniques and other genetic techniques such as polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or Southern blotting are available in some laboratories. The routine diagnosis of brucellosis by detecting *Brucella* DNA by PCR has been previously investigated and PCR assay has been shown to be a valuable rapid and sensitive technique to detect *Brucella* DNA in reference *Brucella* strains and local *Brucella* isolates (Amin *et al.*, 1995; Baroso *et al.*, 2002).

Several authors reported good sensitivity of PCR, based on different molecular markers for detecting of *Brucella* DNA with pure culture. However, few studies have been performed with clinical or field samples. And fewer have evaluated the PCR as a diagnostic tool. The possibility of using PCR technique to detect the DNA of dead bacteria or in paucibacillary samples and even samples highly contaminated with other micro-organisms could increase the rate of detecting infected animals. However, up to now, no technique appears sensitive enough to replace classical bacteriology on all kinds of biological samples (Garin-Bastuji *et al.*, 2006 ;).

Molecular technology like polymerase chain reaction (PCR) is a new approach and applied in many diagnostic works to overcome limitation and difficulties in bacterial culture and serological assays. Polymerase Chain Reaction shows high sensitivity, specificity and overcame the extraneous intervention of mimicry antibodies from sources other than actual infection (Radostits *et al.*, 2000; Quinn *et al.*, 2002).

2.7.2. Serological methods

The commonly used serological tests are the Rose Bengal test (RBPT), serum agglutination test (SAT), complement fixation test (CFT), Coombs test, enzyme-linked immunosorbent assay (ELISA), Rivanol test, skin delayed-type hypersensitivity (SDTH) test, fluorescence polarization assay (FPA) and the Milk ring test (MRT) (Baron and Peterson, 1994; Nielsen *et al.*, 2002; FAO, 2002). The sensitivity of RBPT is improved by using 75µl of serum and 25µl of antigen instead of equal volume of each. This simple modification increases the sensitivity of RBPT and minimizes the discrepancies between RBPT and CFT results. Both tests can not differentiate antibodies produced by *B. melitensis* from those produced by vaccination or cross-reacting bacteria such as *Yersinia enterocolitica* 0:9 (OIE, 2008a).

Rose Bengal Test (RBT) and complement fixation test (CFT)

The World Organization for Animal Health (OIE) currently approved tests for diagnosis of brucellosis in sheep and goats are the rose Bengal test (RBT), the buffered plate agglutination test (BPAT) and the complement fixation test (CFT) (WHO, 2006). The rose Bengal plate test (RBPT) uses *B. abortus* S19 or S1119-3 whole cells, stained with rose Bengal, while BPAT uses *B. abortus* S1119-3 whole cells, stained with crystal violet and brilliant green dyes. Both antigens are used at pH of 3.65. The low pH prevents some agglutination by IgM and encourages agglutination by IgG_1, thereby, reducing nonspecific interactions. Both tests are standardized, simple to perform, inexpensive, considered suitable for screening individual animals, however, false negative results occur, mostly due to prozoning antibody, resulting from *B. abortus* S19 or Rev.1 vaccinations and some cross-reacting antibodies. Thus, it is necessary to use other tests to confirm reactor animals as infected (Nielsen, 2002).

The two tests (RBPT and CFT) are approved by European Union for serological diagnosis of brucellosis in small ruminants. The differentiation of antibodies from natural infection and vaccination is not feasible by both tests. Therefore, to overcome this problem either the reduction of the vaccinal dose and/or administration of the vaccine conjunctivally has been proposed. Additionally, more specific tests like competitive ELIZA (cELIZA) and Fluorescence

Polarization Assay (FPA) have been developed. The smooth lipopolysaccharide (S-LPS), which is identical for both vaccinal and field strains, is the antigen used in the rose Bengal test and complement fixation test (Stournara *et al.*, 2007; Ertek *et al.*, 2006).

Although both tests when used singly or in combination are very effective as flock screening tests, they detect only 70% of the infected animals when they are tested individually. This makes the implementation of test and slaughter policy for brucellosis in small ruminants not very effective. The RBPT, due to its low sensitivity on sheep and goats sera, is suggested to be used only for identification of infected flocks (flock screening test) and not for individual animals. Since CFT is regarded as more sensitive and specific, it is used as both screening and confirmatory test. It is accepted that for serological diagnosis of brucellosis to be improved, there is a need of other tests to be used more sensitive than RBPT and CFT. Therefore, iELIZA, FPA and cELIZA havè been developed, which ability of identifying infected animals appears superior to RBPT and CFT (Minas *et al.*, 2005; Burriel *et al.*, 2004).

Complement fixation test is widely accepted as a confirmatory test, but it is complex to perform requiring good laboratory facilities and adequately trained staff to accurately titrate and maintain the reagents. There are numerous variations of the CFT in use. Either warm (at 37°C for 30 minutes) or cold (at 4°C for 14-18minutes) may be used for the inactivation of serum, complement and antigen. Different concentrations of fresh or preserved sheep red blood cells (SRBCs), sensitized with an equal volume of rabbit anti-SRBCs serum, diluted several times (2-5 times) to contain the minimum concentration required to produce 100% lysis of SRBCs in the presence of a titrated solution of guinea pig complement have been proposed. The guinea pig complement is titrated to determine the amount of complement required to produce either 50% or 100% lysis of the sensitized SRBCs in a unit volume of a standardized suspension. These are defined as the 50% or 100% hemolytic unit of complement or minimum hemolytic dose (CH or MHD_{50} or CH or $MHD_{100)}$ respectively (OIE, 2008b; Ferreira *et al.*, 2003).

The basis for CFT is that dilutions of serum, antigen and a pretitrated amount of complement are added together. If antibody is present in the serum, it will bind to the antigen and providing the antibody is IgG_1 isotype, complement will be activated. An indicator system is then added. It

consists of SRBCs sensitized with rabbit antibody. If the test serum contained antibody, complement is not available and lysis of SRBCs will not take place. Alternatively, if no antibody was present, the available complement activated by interaction with receptors on the Fc portion of the rabbit antibody will lyse the SRBCs, releasing hemoglobin. There are two main variations to the CFT standardized by Hill and MacKinnon. The procedures are essentially identical varying only in the primary incubation temperature and time, either 37oC for 1h or 4oC for 18h. The CFT is obviously challenging in that a large number of different reagents are necessary, large number of various controls are required, a number of reagent titrations are needed, each time the assay is set up and the results are subjective. Other problems are its inabilities to distinguish vaccinal from infection antibodies and the occasional occurrence of samples that activate complement in the absence of antigen. In spite of all these problems, the CFT is used as a valuable asset in control or eradication programmes as a confirmatory test (Nielsen, 2002; Memish *et al*., 2002).

Indirect enzyme linked immunosorbent assay (iELISA)

Numerous variations of the iELISA have been described (reviewed) employing different antigen preparations, antiglobulin-enzyme conjugates, and substrate/chromogens. Several commercial iELISAs are available, but there is no internationally accepted procedure for standardizing those tests for use for international trade purpose in small ruminant brucellosis (OIE, 2008b). The indirect ELISA (iELISA) was first developed by Carlsson and his colleagues in 1976 for the diagnosis of human brucellosis. Since then, large numbers of variations have been reviewed. However, the most common format uses the S-LPS antigen coated passively onto polystyrene matrix. The OIE approved version of this test uses a purified S-LPS as the antigen, serum diluted 1:50 and a mouse monoclonal antibody specific for bovine IgG_1 conjugated with horseradish peroxidase (OIE Manual, 2000a; Nicoletti, 1990a; Marianelli *et al*., 2007).

Although, the iELISA is highly sensitive and can be used for detection of caprine and ovine antibody to *B. melietnsis*, it is some times incapable of differentiating antibodies resulting from *B. abortus* S19 or *B. melitensis* Rev.1 vaccines from those induced by pathogenic *Brucella* strains or other false positive sero-reactors (FPSR). Therefore, the iELISA should be considered more as a screening than a confirmatory test in testing of vaccinated cattle or herds affected by false

positive sero-reactor problems. For the screening iELISA, preparations rich in smooth lypopolysaccharides (SLPS) should be used (OIE, 2008a).

Competitive enzyme linked immunosorbent assay (cELISA)

Since both the conventional serological tests and the iELISA can not differentiate vaccinal antibodies, cELISAs were developed. The main rationale for these assays was that vaccines induced antibody of lower affinity due to the shorter exposure to antigen due to immune elimination compared to field infection in which antigen persisted, resulting in increased antibody affinity. Thus, a competing antibody could be selected to inhibit binding of vaccinal but not field strain induced antibody and the selected monoclonal antibody should be specific for a common epitope of the O-PS molecule, allowing its use for *B. abortus*, *B. melitensis* and *B. suis* serology (Biancifori *et al.*, 2000).

The most commonly used cELISA utilizes S-LPS from *B. abortus* as an antigen, passively attached to a polystyrene matrix, followed by incubation with competing antibody and appropriately diluted serum. After a suitable period of incubation, a reagent for detecting bound monoclonal antibody, labeled with an enzyme, usually horseradish peroxidase or alkaline phosphatase, is added followed by substrate/chromagin. The cELISA is a prescribed test by OIE for international cattle trade and an alternative test for swine brucellosis (OIE, 2000a, d).

The competitive ELISA, using a MAb specific for one of the epitopes of the *Brucella* species OPS has been shown to have higher specificity than the iELISA (Weynants *et al.,* 1997). This test is accomplished by selecting a MAb that has higher affinity than cross-reacting antibody. However, it has been shown that the cELISA eliminates some but not all false positive reactions (FPSR) due to cross-reacting bacteria. The cELISA is also capable of eliminating most reactions due to residual antibody produced in response to vaccination. The choice of MAb and its unique specificity and affinity will have a distinct influence on the diagnostic performance characteristics of the assay (Nielsen, 2002).

The fluorescence polarization assay (FPA)

This method was developed as a test that could be performed outside the diagnostic laboratory, allowing for rapid and accurate diagnosis. The basis of the test is that a molecule in solution rotates randomly at a rate inversely proportional to its size. If the molecule is labeled with a fluorescent marker and is examined by plane polarized light, a small molecule will rotate through a given angle faster than a larger molecule (Nielsen *et al.,* 2000). For the diagnosis of brucellosis, a small molecular weight fragment (average 22kD) of the O- polysaccharide (OPS) of *B. abortus* S1119-3 SLPS is labeled with fluorescein isothyanate (FITC) and used as the antigen. This antigen is added to diluted serum or whole blood and a measure of the antibody content is obtained in about 2 minutes (for serum) or 15 seconds (for blood) after the addition of antigen using a fluorescence polarization analyzer. The test is relatively inexpensive, simple and rapid and has been validated for a large number of species, including cattle, swine, bison and is an alternative OIE test for bovine and porcine brucellosis (Nielsen and Gall, 2001).

The fluorescence polarization assay is a simple technique for measuring antigen-antibody interaction and may be performed in a laboratory or in the field. It is a homogeneous assay in which analyses are not separated and it is very rapid. The mechanism of the assay is based on random rotation of molecules in solution. Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus, a small molecule rotates faster than a large molecule. If a molecule is labeled with a fluorochrome, the time of rotation through an angle of 68.5^0 can be determined by measuring polarized light intensity in vertical or horizontal plane. A large molecule emits more light in a single plane than a small molecule rotating faster and emitting more depolarized light. For most FPAs, an antigen of small molecular weight, less than 50kD, is labeled with a fluorochrome and added to serum or other body fluid to be tested for the presence of antibody. If antibody is present, attachment to the labeled antigen will cause its rotational rate to decrease and this decrease can be measured (Biancifiori *et al.,* 1996; OIE, 2008b; Nielsen *et al.*, 2000).

Brucella skin test

The brucellin skin test is an alternative immunological test, which can be used for screening unvaccinated flocks, provided that a purified (S-LPS free) and standardized antigen preparation is used. One such commercially available preparation is brucellin, which is prepared from a rough strain of *B. melitensis* (OIE, 2008b). The test has a high sensitivity for *B. melitensis* infection in small ruminants and, in the absence of vaccination, is considered as one of the most specific diagnostic. This test is of particular value for the interpretation of false positive sero-reactors (FPSR) due to infection with cross-reacting bacteria (FPSR) affected animals are always negative in the skin test), especially in the brucellosis-free areas. Despite its high sensitivity, not all infected animals show positive skin test responses to brucellin and moreover, Rev.1 vaccinated animals can react in this test for longer years. Therefore, this test cannot be recommended either as the sole diagnostic test or for the purpose of international trade (Garin-Bastuji *et al.*, 1998).

In *Brucella* skin test guinea pigs are the most satisfactory laboratory animals used for detecting the presence of small numbers of *Brucella* organisms in animal tissues, body secretions and excretions. The inoculation is done by intraperitonial or subcutaneous routes. In male guinea pig, orchitis is developed; when guinea pig is autopsied, lesions are observed in the spleen, lungs and lymph nodes, after 7 days and these organs are minced and cultured in solid media containing no inhibitory dyes or antibiotics. Serum samples are subjected to specific test 3 to 6 weeks after inoculation. A positive reaction in the serum agglutination test, even without a positive culture, result is sufficient to warrant a diagnosis of brucellosis (OIE, 2000a).

2.8. Prevention and control

Despite the considerable increase in knowledge of the epidemiology of *B. melitensis* infection in small ruminants in recent years, many aspects of its control remain unclear and controversial. The test-and-slaughter policies applied for the eradication of bovine brucellosis due to *B. abortus* in developed countries have also been considered for the control of *B. melitensis* infection in sheep and goats. However, these policies have failed to control small-ruminant brucellosis in some southern European countries and are largely inapplicable in most developing countries, where vaccination programmes are indispensable (Blasco, 2006). Other aspects of control involve

quarantine of infected flocks, sanitation, disinfection of livestock conveyances, and concentration points of infected animals. Laws and regulations restricting the sale and movement of infected and exposed animals become an important part of any control program (Abela, 1999).

2.8.1. Management

Preventing entry of brucellosis into clean herds or flocks requires obtaining animals from sources that are free of infection. Pretesting of animals by the use of serological tests is the final line of defense against acquiring infected additions, but if it is the only action taken to prevent introduction of disease it will ultimately fail. Any added animals should be kept isolated from the rest of the flock until they have had a second negative test at least 60 days after being acquired. Preventing spread of infection in an already infected flock requires instituting management practices to limit contact between animals during the time of year when they are most likely to be contagious or to spread the infection (Hugh *et al.*, 1994).

Other complementary measures that may need consideration include improved farm hygiene, restriction and control of trade and movement of animals, testing of animals and isolation and removal of infected animals. Any area exposed to infected animals and their discharges should be thoroughly cleaned and disinfected. Infections in other species are generally prevented by controlling *B. melitensis* in sheep and goats. *Brucella* species are readily killed by most commonly available disinfectants including hypochlorite solutions, 70% ethanol, isopropanol, iodophores, phenolic disinfectants, formaldehyde, glutaraldehyde and xylene; however, organic matter and low temperatures decrease the efficacy of disinfectants. Disinfectants reported to destroy Brucella on contaminated surfaces include 2.5% sodium hypochlorite, 2-3% caustic soda, 20% freshly slaked lime suspension, 2% formaldehyde solution (Abela, 1999; Schurig et al., 2002). The prevention of human brucellosis is best achieved by control or eradication of the disease in animals, combined with adequate heat treatment of potentially contaminated food products (WHO, 1997).

Preventive measures essential to minimize the risk of infection of the human population should include improved food hygiene, including the pasteurization of milk and protection from infection of high-risk groups such as milkers and other people working in the dairy industry,

health education of risk groups through community participation and health education programmes could play an important role to increase the acceptance and use of preventive measures (West *et al.*, 1993; Smits and Kadri, 2005).

2.8.2. Immunization

Genrally, there are two types of vaccines used for immunization of small ruminants against brucellosis. These are Rev.1 live vaccine and H-38 attenuated vaccine. Rev.1 live vaccine was obtained by attenuation of B. melitensis smooth strain in 1950s.The strain was originally dependent on streptomycin for its growth, but lost this property upon further culture in mice and guinea pigs. It gives protection to sheep and goats against *B. melitensis* and to rams against *B. ovis*. The vaccine induces abortion when given during the last trimester of gestation and for this reason the vaccine was recommended to be given prior to the first gestation at 3 to 7 months of age. The H-38 is the inactivated vaccine for small ruminants produced from attenuated Brucella melitensis containing aluminum hydroxide as an adjuvant and gives good protection, but the induced immunity is not as solid as live Rev.1 vaccine (Alton and Forsh 1996; Blasco, 2005; Schurig *et a*l., 2002).

In areas with high prevalence of the disease, extensive management systems and low socioeconomic conditions, vaccination is the only practical way to control the disease. It is generally agreed that the living attenuated *B. melitensis* Rev. 1 is the most effective vaccine against brucellosis in small ruminants. However, variability in the protective efficacy and the degree of adverse effects of Rev. 1 has been reported in several countries and considered a problem related to the different origin of the strains used (David and Fleche, 2007; Alton, 1990; Verger *et al.*, 1995).

Development of efficacious vaccines against brucellosis is at the forefront of prevention of the disease. Many experiments have been conducted using killed and live vaccine candidates. Killed vaccine candidates usually confer poor immunity, whereas live-attenuated vaccines of virulent strains typically provide adequate immunity against abortion but can lead to release of the pathogenic organisms and possibly expose susceptible animals to infection. Ideal qualities of a vaccine candidate include: (1) long duration of immunegenesis; (2) minimum interference with

diagnostic tests; (3) easy production and storage of the vaccine with long stability; and (4) minimum adverse effects in vaccinated animals and with no danger to humans in the event of exposure (Nicoletti 1990a). The three primary vaccines used to prevent brucellosis in cattle, goats and sheep include *B. abortus* strain 19 (S19), *B. abortus* strain RB51, and *B. melitensis* strain Rev.1 (Sanmartino 2005).

Rev. 1 vaccine was developed by Elberg and Herzberg in 1957 from strain of B. melitensis 5056 as a live vaccine against B. melitensis in goats and sheep (Sanmartino 2005). Rev. 1 is a smooth bacterium with a complete LPS which induces a similar antibody response as that caused by the field strains and cannot be easily differentiated by conventional serology. The vaccine can become an important health problem if accidents occur involving those persons working with the vaccine. Subcutaneous vaccination with Rev. 1 recommended for goats and sheep between four to six months of age at the standard subcutaneous doses (1 x109 - 2 x109 CFU), yields a 2-3 months widespread and persistent infection, and also induces interference with diagnostic tests (Blasco 2006). Conjunctival vaccination induces sufficient protection in animals three to six months of age and also reduces the possibility of serological interference. Both standard and reduced doses of Rev. 1 induce abortions in sheep and goats vaccinated during pregnancy. Studies have demonstrated that even reduced doses are not entirely safe or protective against B. melitensis in sheep and goats. Nevertheless, conjunctival vaccination of animals with Rev.1 during breeding periods can effectively provide protection and reduce the risk of vaccine induced abortions (Blasco, 2006). Test and slaughter policy is not realistic in many developing countries, where B. melitensis is endemic due to lack of financial resources needed for compensation. Therefore, immunization is the primary control method proposed by international agencies to reduce prevalence of the disease and to ensure test and slaughter programs in eradication of the disease (McDermott *et al*., 2002; Memish *et al*., 2002).

3. MATERIALS AND METHODS

3.1. Study area

The study was undertaken in selected peasant associations in Adami -Tullu –Jido- Kombolcha District from October 2008 to March 2009. The district is situated in the mid-rift Valley, East Showa zone of Oromiya Regional State, 160kms south of Addis Ababa, Ethiopia. It has an altitude of 1650 meter above sea level with a bimodal unevenly distributed rainfall pattern. The average annual rainfall is 760.9 mm and consists of short rainy season (February to April) and long rainy season (July to September). The District has a maximum and minimum temperature of 35^{o}C and 12.7^{O}C, respectively, and a relative humidity of 760. Vertisol is the predominant soil type with sand-silt-clay in the proportion of 33:48:18 respectively and has a pH of 7.9. The predominant land area is suitable for livestock production, goats being the most selected animals for breeding purpose next to cattle. There are 43 peasant associations, about 176,186 cattle, 105,085 goats, 30,899 sheep, 20254 donkeys, 518 mule, 1,258 horses and 120,991 poultry and the total human population is about 159,159, of which 65,487 males and 69,012 females which are contained in 24660 households (23,242 male and 1,418 female headed households) (Adami-Tulu-Jido-Kombolcha District, 2009).

3.2. Study animals

The study animals were goats and sheep above six months of age. In addition, human risk groups (especially farmers), who had close contact with sheep and goats and visited the District`s health center with symptoms suggestive of brucellosis were included in the study. The predominant goat breed in the study area was Arsi-Bale breed, which was managed under extensive farming system. Traditional feeding and housing systems were practiced in the area.

3.3. Study design

A cross-sectional study was conducted between October 2008 and March 2009 in selected peasant associations of Adami Tulu Jido Kombolcha District to determine the seroprevalence of brucellosis in sheep and goats and purposive sampling was conducted to include occupationally

related human sampling units (farmers) in the study from those patients visiting the district's health center with the symptoms suggestive of human brucellosis. The sampling involved sheep and goats above 6 months of age with no histry of vaccination. Risk factors for the seroprevalence of small ruminant brucellosis such as species, sex, age, herd size and pregnancy status were considered during questionnaire survey and blood sample collection.

3.4. Questionnaire survey

The information on potential risk factors of small ruminant brucellosis was gathered using a pre-tested questionnaire. Risk factors such as species, sex, age, herd size, pregnancy status and source of replacement stock, grazing and breeding systems together with the reproductive disorders such as history of abortion, stillbirths and other potential risk factors were recorded. Relevant risk factors for human brucellosis such as removal of retained fetal membranes, consumption of raw milk and milk products, contact with infected sheep and/ goats were recorded with questionnaire format during blood sample collection.

3.5. Sample size determination and sampling method

Proportional determinatoin was employed to include 160 households/flocks from the selected 10 peasant associations, found in Adami -Tulu –Jido- Kombolcha District to obtain a total sample of 2340 small ruminants (775 sheep and 1565 goats). The primary sampling unit was a flock defined as a household having at least one sheep or goat. Out of 43 peasant associations, 10 PAs were included in the study purposively. This porpusive approach was based on accessibility of flocks and other logistics , fro example availability of transport. Convenient sampling was also used to gather 230 ssera from human risk groups, who visited the District`s health center and with the symptoms suggestive of brucellosis.

3.6. Collection of blood samples

Approximately 8 ml of blood samples were collected from the jugular vein of sheep and goats aged above 6 months and from non-vaccinated ones against *Brucella melitensis* using sterile

plain vaccutainer tubes and needles. Blood samples were properly labeled and left for 24 hours at room temperature to allow clotting and the harvested sera were stored at -20^{O}C until tested for the presence of *Brucella* antibodies. About 5ml of blood samples was collected from the brachial veins of humans in collaboration with the District's health institutions. During blood sample collection from human patients proper ethical procedures were observed.

3.7. Serological tests

3.7. 1. Modified rose bengal plate test (mRBPT)

All serum samples (2340 animal and 230 human sera) were screened using modified Rose Bengal plate test according to the procedures described by OIE (2008a). The antigen used was Rose Bengal antigen, which constitutes a suspension of *Brucella abortus* (obtained from the Institute Pourquier, Montpellier, France), inactivated by heat and 0.5% phenol, adjusted to pH 3.65 and colored with Rose Bengal. Briefly, 75µl of serum was mixed with 25µl of antigen on a glass plate and shacked. After 4 minutes of gentle shacking , any visible agglutination was considered as positive (OIE, 2008a).

3.7.2. Complement fixation test (CFT)

All sera which tested positive by modified Rose Bengal plate test and 10% of the nagative sera were further tested using complement fixation test for confirmation. For there was no *Brucella melitensis* antigen, Standard *Brucella abortus* antigen S99 for CFT (from the Veterinary Laboratory Agency, Addlestone, United Kingdom) and a 2% sheep red blood cells (SRBCs) obtained from National Veterinary Institute, Debre Zeit, Ethiopia, was used to detect the presence of anti-*Brucella* antibodies in the sera. The control sera and complement were obtained from the Federal Institute for Health Protection for Consumers and Veterinary Medicine, Berlin, Germany. All the reagents used in CFT were titrated. A 2% sheep red blood cell (SRBC) suspension was 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 Health protection for Consumers and Veterinary Medicine Service Laboratory, Berlin, Germany (Nielsen and Dunkan, 1990).

Interpretations were as follow: sera were classified as positive: sera with a strong reaction of approximately 100% fixation of the complement (4+) at a dilution of 1:50; about 75% fixation of the complement (3+) at a dilution of 1:10; about 50% fixation of the complement (2+) at a dilution of 1:20 and about 25% fixation of the complement (1+) at a dilution of 1:40 (Alton, 1975; OIE, 2008).

3.8. Public health survey

Blood samples were collected from risk groups (farmers) in collaboration with the District's health officers in Zeway town, Southern Ethiopia. The serological tests employed were mRBPT for screening and sera tested positive to mRBT were further tested with CFT for confirmation.

3.9 Data management and analysis

Data obtained from both serological tests and questionnaire survey were stored in Microsoft excel spreadsheet program. Descriptive statistical analysis for Brucella seroprevalence rate was carried out using SPSS 15.0 for windows. The seroprevalence, 95% confidence interval (CI), univariable and multivariable logistic regression were used to analyse aqssociations of various risk factors such as species, sex, age, herd size, pregnancy status, presence of abortion and still birth with the seroprevalence of the disease. Sheep and goats tested positive to both mRBT and CFT tests serially were said to be positive. Clusters (flocks) having at least one seropositive animal were considered as positive. The individual animal level seroprevalence was calculated on the basis of mRBT and CFT positive results divided by the total number of animals tested. Similarly, household (flock) level seroprevalence was computed as the number of households (flocks) with at least one positive animal divided by the total number of households (flocks) tested (Thrusfield, 2005).

The results obtained from questionnaire survey were compared with those of serological tests. Generally, the kinds of descriptive statistics were frequency tables, and quantification of the disease was accomplished by rate, proportion. Kinds of analytical statistics employed were

univariale and multivariable logistic regression analyses and Pearson's Chi-square test (χ^2). These tests determined the association of risk factors with the seroprevalence of brucellosis. The degree of association between potential risk factors and seroprevalence were computed using Odd's ratio. During analysis of the data seroprevalence was considered as dependent variable and and potential risk factors that would likely predict the outcome variable were considered as independent variables.

4. RESULTS

4.1. Overall herd level seroprevalence of *Brucella* antibodies

The overall herd level seroprevalence of *Brucella* antibodies in the study area was 28.1% (Table 6). There were statistically significant ($\chi 2$=571.55, p=0.000) variations among herds with the highest seroprevalence recorded in Kamo Garbi (75%), and the lowest in Dodicha (11.1%) and Bochesa was negative.

Table 6: Overall herd level seroprevaence of *Brucella* antibody by the serial test

PA	No. tested	No. of + ves and (%)	95% CI	χ^2	P- Value
Bochassa	4	0	0.0-48.99		
Dodicha	18	2 (11.1)	3.1-32.8		
W/Woshgula	5	1 (20.0)	3.6-62.5		
A/Germam	56	8 (14.3)	7.4-25.7		
W/Boremo	14	5 (35.7)	16.3-61.2		
K/Garbi	4	3 (75.0)	30.1-95.4		
W/Bula	23	10 (43.5)	25.6-63.2		
G/Asebo	10	2 (20.0)	5.7-51.0		
H/Gulenta	12	4 (33.3)	13.8-60.9		
A/Shisho	14	10 (71.4)	43.4-88.3		
Total	160	45 (28.1)	21.7-35.6	571.55	0.000

4.2. Individual level seroprevalence of *Brucella* antibodies

A total of 2340 sheep and goats were tested with modified Rose Bengal plate test for the presence of antibodies against *Brucella* infection from which one hundred seven (107) sera were positive. In further confirmation test, 107 positive sera were tested with complement fixation test (CFT),

of these 90 out of 107 sera were positive by CFT. The subsequent data analyses were based on the sera (90) that were positive to both modified rose Bengal (mRBPT) and complement fixation tests. Thus, the overall individual animal level seroprevalence of mRBPT and CFT were 4.6% (107) and 3.8% (Table 7).

Table 7: Individual level Seroprevalence of *Brucella* antibodies using mRBPT and CFT tests

PA	No. tested by mRBPT	mRBPT +ves and (%)	Prev (%)	95% CI	Serial +ves	Prev (%)	95% CI
Bochessa	52	0	0	0.0-6.88	0	0	0.0-6.88
Dodicha	257	2	o.8	0.21-2.80	2	0.8	0.21-2.80
W/Woshgula	76	2	2.6	0.72-9.09	1	1.3	0.23-7.09
A/Germam	816	12	1.5	0.84-2.55	8	1.0	0.50-1.92
W/Boremo	202	11	5.4	3.07-9.49	7	3.5	1.69-6.99
K/Garbi	60	3	5.0	1.71-13.70	2	3.3	0.92-11.36
W/Bula	341	43	12.6	9.50-16.55	39	11.4	8.48-15.26
G/Asebo	152	3	2.0	0.67-5.64	2	1.3	0.36-4.68
H/Gulenta	177	4	2.3	0.88-5.67	4	2.3	0.88-5.67
A/Shisho	207	27	13.0	9.12-18.31	25	12.1	8.32-17.22
Total	2340	107	4.6	3.80-5.49	90	3.8	3.14-4.71

In the present study the overall individual animal level seroprevalence of the serial test was 3.8%. There was statistically significant ($\chi 2$ =123.07, P=0.000) difference, with the highest seroprevalence of 12.1% in Aneno Shisho and the lowest 0.8% in Dodicha whereas, Bochessa had negative results. The distribution of *Brucella* antibody in sheep and goats in selected PAs ranged from 0% to 12.1% (Table 7).

4.3. Potential risk factors of small ruminant brucellosis

In oreder to assess the potential risk factors, associations of seroprevalence of *Brucella* antibody with respect to species, sex, age, herd size and pregnancy status were determined based on risk factor analysis in small ruminants brucellosis. Except herd size, the others species, sex and age showed significant difference ($p< 0.05$), (Table 8).

There was statistically significant ($p< 0.05$) difference between species of small ruminants where the higher seroprevalence of the disease was observed in goats 4.8% (75) than in sheep 1.9% (15) (Table 8). This was also analysed using univariable logistic regrassion in which the odds of the disease in goats was 2.55 times the odds of the disease for the reference category (sheep) (Table 9).

There was significant difference in susceptibility to brucellosis between the two sex groups. The seroprevalence was 4.2% (80) and 2.2% (10) in female and male, respectively. Statistically significant ($p < 0.05$) difference was observed between the different sexes, where the odds of the disease in females was 1.94 times the odds of the disease for the reference category (male) (Table 9).

There had been observed a high variation in seroprevalence of *Brucella* antibody among different age groups. Age was categorized exclusively as young, adult and old shoats. The seroprevalence in age groups was 6.5% (18), 3.1% (29) and 3.8% (43), respectively, in 0.6 - ≤1year , 1 - < 4 years and ≥ 4 years old small ruminants. Statistically significant ($p<0.05$) difference was found among different age groups, in which the odds of the disease in younger group was 1.73 times the odds of the disease in old age group (Table 9).

The seroprevalence at herd level was 2.8% (1), 3.1% (3) and 3.6% (1) with the herd categories of < 10, 10-20 and >20, respectively. There was no statistically significant ($p>0.05$) difference among herd groups (Table 8). The univariable logistic regression analyais model was not

significant (p = 0.98) and thus the seroprevalence of brucellosis was not associated with herd size (Table 9).

Table 8: Ch-square test analysis for possible associations of potentiiial risk factors with the seroprevalence of small ruminant brucellosis

Risk factors		No. of animals tested	No. of positives	Prevalence (%)	95% CI	χ^2	p- value
Species							
	Sheep	775	15	1.9	1.18-3.17		
	Goats	1565	75	4.8	3.84-5.96		
	Total	2340	90	3.8	3.14-4.71	11.4397	0.001
Sex							
	Female	1891	80	4.2	3.41-5.23		
	Male	449	10	2.2	1.22-4.05		
	Total	2340	90	3.8	3.14-4.71	3.938	0.047
Age							
	0.6-≤1year	277	18	6.5	4.01-10.25		
	1- <4years	946	29	3.1	2.15-4.37		
	≥4 years	1117	43	3.8	2.87-5.15		
	Total	2340	90	3.8	3.14-4.71	6.827	0.033
Herd size							
	< 10	36	1	2.8	0.49-16.21		
	10- 20	96	3	3.1	0.81-9.52		
	>20	28	1	3.6	0.63-17.71		
	Total	160	5	3.1	1.16-7.53	0.033	0.984
Pregnancy status							
	Nonpregnant	1502	70	4.7	3.70-5.85		
	Pregnant	389	10	2.6	1.40-4.67	3.330	0.68

Univariable logistic regression on the effect of potential risk factors on the seroprevalence of small ruminant brucellosis was analysed. Statistically significant associations were observed between different risk factors such as species, but there was no statistically significant association with sex, age, herd size and pregnancy status (Table 9). Multivariable logistic regression was used to check for the presence of associations between potential risk factors and seroposetivity. There were statistically significant ($p<0.05$) associations observed between species, but, no significant ($p>0.05$) associations between age groups and seropositivity (Table 10).

Table 9: Univariable logistic regession analysis of associated risk factors with serial test seroposetivity

Risk factors	Odds ratio	95% CI for OR	P-value
Species			0.001
Goats	2.55	1.455 -4.470	0.001
*Sheep			
Sex			0.051
Female	1.94	0.997 -3.773	0.051
*male			
Age			0.037
0.6-1yr	1.73	0.248 - 0.832	0.057
1-<4yrs	0.79	0.327 - 1.016	0.330
*≥4yrs			
Herd size			0.984
<10	0.77	0.114 - 11.220	0.857
10-20	0.87	0.078 - 21.681	0.906
*>20			
Pregnancy status			
Nonpregnant	1.85	0.946-3.628	0.072
*Pregnant			

Table 10: Multivariable logistic regression analysis of associations between risk factors and sero prevalence of small ruminant brucellosis

Risk factors	Odds ratio	95% CI for odds raito	P- value
Species			
Goats	3.507	1.668 – 7.375	0.001
*Sheep			
Age			0.198
0.6 - ≤ 1 years	1.50	0.683 – 3.341	0.308
1- <4 years	0.70	0.374 – 1.307	0.262
*≥ 4years			
Pregnancy status			
Absent	2.05	0.900 – 4.675	0.088
*present			

*Reference category

4.4. Reproductive disorders

Statistically significant difference in seroprevalence was observed in abortions 61.1% (33) when compared with nonabortions 2.6% (47) (p=0.000) (table 11). The odds of the disease indicated that the abortions were almost 0.02 times more likely to be seropositive than nonabortions (Table 12). The present study demonstrated that the presence of *Brucella* antibody in the serum of sheep and goats obtained from these peasant associations showed a higher seroprevalence of *Brucella* antibodies in sheep and goats with still births (Table 11).

Table 11: Chi-square test analysis for possible association of reproductive diorders with the seroprevalence of small ruminant brucellosis

Risk factors	No. tested	No. of positives	Prevalence (%)	95% CI	χ^2	p- value
Abortion						
Absent	1837	47	2.6	1.93-3.39	443.893	0.000
Present	54	33	61.1	47.79-72.96		
Still birth						
absent	1853	58	3.1	2.43-4.02	275.641	0.000
presentt	38	22	57.9	42.19-72.14		

Univariable logistic regression analysis demonstrated that the presence of *Brucella* antibodies in the serum of sheep and goats obtained from these peasant associations showed a higher seroprevalence of brucellosis in sheep and goats with still births (Table 11). The seroprevalence was 57.9% (33) in female small ruminants with still births and 3.1% (47) in those without still births. Statistically significant variation was observed (χ^2 =275.641 and p= 0.000) (Table 11).

Table 12: Univariable logistic regression analysis of association between reproductive disorders and seroprevalence brucellosis

Risk factors	Odds ratio	95% CI for odds raito	P- value
bortion			
Absent	0.017	0.009-0.031	0.000
*Present			
Still births			
Absent	0.023	0.012-0.047	0.000
*Present			

*Reference category

Table 13: Multivariable logistic regration analysis of associations between reproductive diorders and seroprevalence of small ruminant brucellosis

Risk factors	Odds ratio	95% CI for odds raito	P- value
Abortion			
absent	0.02	0.008 – 0.35	0.088
*present			
Still birth			
Absent	0.02	0.009 – 0.053	0.000
*present			

*Reference category

Based on multivariable logistic regression analysis, there was no statistically significant (p= 0.088) variation among small ruminants with abortion (Table 13). But, the seroprevalence of small ruminant brucellosis was highly associated with still births (p=0.000) (Table 13).

4.5. Questionnaire survey

In all the study households, 160 individuals were asked about awareness of brucellosis, sources of replacement stock, presence of regular veterinary services, grazing and breeding systems and safe disposal of fetal membrane to find out the presence of association between risk factors and prevalence of small ruminant brucellosis. All households used natural breeding and communal pasture. There were no significant (p>0.05) variations among households in relation to awareness about brucellosis, sources of replacement stock, presence of veterinary service and safe disposal of fetal membrane. Accordingly, 26.7% (40) households with infected flocks had no awareness about brucellosis while only 50.0% (5) were awaare. In addition, 54.55% (6), 26.2% (34) and 26.3% (5) used village, own stock and market, respectively for the replacement of their stock. Out of the total (45) infected households, 26.7% had no regular veterinary services,while the other 50.0% were regularly served. Among the total households with seropositive flocks, 40 owners (27.8%) used unsafe methods to dispose fetal membranes, whereas 5 (31.3%) with infected flocks applied safe disposal of fetal membranes (table 13).

Table 14: Association of management and husbandry risk factors with small ruminant seropositivity

Management and husbandry risk factors	N0. of Respondents and (%)	Herd prevalence (No. and %)	χ^2 -value	P-value
Awareness about brucellosis				
Absent	150 (93.75)	40 (26.7)	2.525	0.112
Present	10 (6.25)	5 (50.0)		
Source of stock replacement				
Village	11 (6.88)	6 (54.5)	4.079	0.130
Own stock	130 (81.25)	34 (26.2)		
Market	19 (11.88)	5 (26.3)		
Presence of regular vet. service				
Absent	150 (93.75)	40 (26.7)	2.525	0.112
Present	10 (6.25)	5 (50.0)		
Grazing system				
coomunal	160 (100.00)	45 (28.1)	-	-
individual	0			
Breding system				
Natural	160 (100.00)	45 (28.1)	-	-
artificial	0			
Disposal of fetal membrane				
Unsafe	144 (90.00)	40 (27.8)	0.086	0.769
Safe	16 (10.00)	5 (31.3)		

4.6. Seropositivity in human risk groups

Out of the two hundred and thirty (230) human sera, only two sera were positive by mRBPT. But, in CFT the two positive and the other 23 negative sera were negative to the disease.

5. DISCUSSION

The herd level seroprevalence of the serial tests was 28.1%. This finding is higher than flock seroprevalence of 22.3% reported in Eastern Amhara Region (Shimeles, 2008) and 12.1% in Moroco (Benkirani, 2006). Flock prevalences of 43% in Uganda (Kabagambe *et al.*, 2001), 37% in West Asia and 49% in North Africa (Benkirane, 2006) and 61% in goats and 30% in sheep in Tunisia (Benkirane, 2006) were higher than the findings in this study. This difference in seroprevalence among flocks might be attributable to poor animal production and management systems, as well as fairly various agro-ecologial conditions, movement of animals in search of pasture and water, trade within and among countries, mixing of animals at market places and watering points.

This study demonstrated that the overall individual animal level seroprevalence of brucellosis in small ruminant was 3.8% (4.8% in goats and 1.9% in sheep). This seroprevalence of small ruminant brucellosis is comparable to the seroprevalence of 3.2% in goats and 1.6% in sheep and overall prevalence of 2.8% in small ruminants reported in Southern Ethiopia (Mengistu, 2007); 3.4% in Afar and 3.9% in Borena (Melese *et al.*, 2007), 1.6% in sheep and 4.1% in goats in Morocco, 3.0% in both species in Iran (Bankirane, 2006) and 3.8% in goats and 1.4% in sheep in Eritrea (Omer *et al.*, 2000).

Other reports comparable to the findings in this study were 3.2% in sheep and 5.8% in goats in Afar Region (Ashenafi *et al.*, 2007), 14.6% in sheep and 16.2% in goats in Afar Region (Yibeltal, 2005), 2.2% in sheep and 12% in goats in Algeria (Benkirane, 2006), 4.0% in sheep and 18.0% in goats in Tunisia (Benkirane, 2006), 8.2% in sheep in Israel (Benklirane4, 2006), 2.4% in sheep and 8.2% in goats in Egypt (Benkirane, 2006), and 14.2% in sheep and 16.7% in goats in Khartoum in Sudan (Benkirane, 2006), 7.2% in sheep and 5.29% in goats (Falade and Hussein, 1979) in Somalia and 6.01% in sheep and goats in Kenya (Waghela,1976).

On the other hand, the overall seroprevalence was higher than that of 1.5% in sheep and 1.3% in goats in central high lands of Ethiopia (Tekelye and Kasali, 1990), 1.6% in sheep and 1.7% in goats in Somali Region (Yibeltal, 2005) and 0.5% in South Omo and 0.11% in Somali Region

(Melese *et al.*, 2007). Furthermore, the overall individual specific seroprevalence was in agreement with prevalences reported in North Africa and Middle East of 0.3% to 21% in goats and 0.6% to 22% in sheep, respectively (Benkirane, 2006). Neverthless, prevalences vary within and among countries, which could be attributed to different risk factors such as herd size, management practices, ecology and biology of the disease.

The present investigation recorded a higher seroprevalence of brucellosis in goats (4.8%) than in sheep (1.9%). There were significant differences between these species. This result is more comparable to 3.2% in goats and 1.6% in sheep reported in Southern Ethiopia (Mengistu, 2007), 3.8% in goats and 1.4% in sheep in Eritrea (Omer,2000) and 4.1% in goats and 1.6% in sheep in East Morocco (Benkirane,2006). Seroprevalences of 16.7% in goats and 14.2% in sheep in Afar (Yibeltal,2005) and 5.8% in goats and 3.2% in sheep in Afar (Ashenafi *et al.*, 2007) were higher than the findings in this study. Most beerds of goats are fully susceptible but the susceptibility of sheep breeds differes widely (EC, 2001).

This difference might be due to the differences in flock sizes and proportions of goats and sheep in the flock (like 1565 goats and 775 sheep in the present study). In addition, sheep are more resistant than goats and they do not shade the bacteria for long time. Flocks with high numbers of sheep would have low prevalence (Radostits *et al.*, 2002). Excretion from the vagina in goats is more copious and prolonged than in sheep and lasts for at least 2-3 months and the goats are considered the principal host of *Brucella melitensis*, notably in Latin America, where, sheep are not significantly infected even when kept in close contact with goats (Alton, 1985). In goats, infection can vary from a short time occurrence to persistent occurrence for years. In sheep, the couurse of infection depends upon the dose of infection and after recovery they are resistant to re-infection (EC, 2001).

The occurrence was higher in females than in males, In this study it was fact tha females were two times more affected than males. This finding is comparable to that of 3.2% and 1.2% in females and males, respectively, reported in Southern Ethiopia (Mengistu, 2007). The highest prevalence of brucellosis in females may be due to high concentration of erythritol, which is scarcely produced in males reproductive organs (EC, 2001; Colmenero *et al.*, 2007).

In this study, age was not found associated with seroprevalence of brucellosis in small ruminants. The highsest occurrence was recorded in sheep and goats below one year. Young animals may be infected, but do not show clinical signs and generally show only a weak and transient serological response. However, susceptibility increases upon sexual maturity and especially with pregnancy. That means sexually mature and pregnant animals are more susceptible to *Brucella* than sexually imature and nonpregnant animals. (Radostitis *et al.*, 2000).

Unexpectedly, pregnancy was not associated with the seroprevalence of the disease. However, sexually mature and pregnant shoats are more susceptible to brucellosis than sexually immature ones (Radostitis *et al.*, 2000). This is due to localization of *Brucella* organisms in the uterus during pregnancy, there is production of erythritol in high concentrations in the placenta of females and also found in other organs such as the mammary gland and epididymis, which are targets for *Brucella* organisms and possibly to steroid hormones (Nicoletti, 1980; Quinn *et al.*, 2002). In female goats, about two thirds of acute infections are acquired naturally during pregnancy, which leads to infection of the udder and excretion of the organisms in the milk during the next lactation. However, excretion may cease during lactation (Alton, 1985).

In this study, *Brucella* seropositivity was stronglly associated with the presence of abortion and still births. Because in both conditions the *Brucella* organisms can be dissiminated in the environment by dogs, carnivorous birds and other contaminated materials, by which the feeding and watering places could be contaminated indirectly for the transmission of the disease.

All the human serum samples (230) were found negative by the confirmatory test. This could be attributable to the collection of blood samples from human risk groups, who had no continuous close contact with infected small ruminants or other livestock, or the blood was collected from farmers with no consumption of raw milk obtained from infected small ruminants.

6. CONCLUSIONS AND RECOMMENDATIONS

The results obtained from the survey are evidences for the presence of small ruminant brucellosis as a problem in the country. The existing traditional husbandry practices of handling multiple species support the spread of brucellosis in the area. This and other facts mentioned herein reveal that brucellosis is becoming one of the impediments to the exploitation of the huge small ruminant population in the country and impairs the export of live sheep and goats, as the importing coutries strictly require *Brucella* free animals. The present study showed moderate number of sero-reactor sheep and goats. These sero-reactor small ruminants were found widely distributed in 10 selected peasant associations of the District. Hwever, there was significant variation in seroprevalence among these PAs. Species and clinical symptoms such as abortion and still births were found to be important risk factors associated with the occurrence of seroreactor small ruminants. However, sex, age and herd size were found less important risk factors associated with seropositivity. The two main clinical symptoms (abortion and still births) were significantly associated with the seroprevalence of *Brucella* antibodies in this extensive production area.

Based on the present study and findings, the following recommendations were put forward:

- Application of well structured and financed veterinary services is one of the primary measures for the control of small ruminant brucellosis in the country.
- Creation of public awareness about the economic and public health importance of the disease by all possble medias (radio, TV, journals, and any published papers).
- Identification of bacterial species for the production of proper vaccines against small ruminant brucellosis
- Independent production of diagnostic kits and vaccines by capacitating research Institetions
- Further well planed study or survey of the disease is warranted.

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8. ANNEXES

Annex 1: Modified Rose Bengal Plate Test (m-RBPT)

Materials used:

- mRBT *Brucella* antigen
- Positive control serum
- Negative contol serum
- Enamel plate
- Micropipette
- Micrpipette tips
- Plastic applicator

Test procedure:

All the sera from small ruminants and human beings were screened using mRBT.
Generally, the test sera and the antigen were left at a room temperature for half an hour before the test was conducted.

- 75 µl of the test serum was taken and put on a clean enamel plate.
- 25 µl mRBT antigen was added to the side of each test serum using a micropipette.
- Then the test serum and the antigen were mixed using a plastic applicator.
- Finally, the enamel plate was shacked gently by hands for 4 minutes and the results were read by examining the degree of agglutination in a good source of light with or without a magnifying glass based on the degree of agglutination.

Interpretation: after 4 minutes of gentle shacking, any visible agglutination was considered as positive.

Annex 2: Complement Fixation Test Procedure and Materials required for CFT

Micro titer plates (U-shaped), multi channel and single channel micropipettes, pipette tips
Universal bottles for preparation of solutions, stirrers (magnetic), Cylinders, weighing balance, thermometers, pH indicator, Incubator, water bath, deep freezer, centrifugator, Antigen, complement, hemolysin (amboceptor's), control sera, sheep RBC, veronal buffer, Alsever's solutions and washing buffer.

I. Preparation of sheep red blood cells for the hemolytic system:

10 ml of sheep red blood cells in Alsever's solutions will be centrifuged at 2500 rpm for 5 minutes.
The supernatant will be discarded and replaced by veronal buffer diluents (VBD).
The sheep red blood cells will be resuspended in diluents completely.
This procedure will be repeated 4 timesBefore discarding the supernatant after the last washing, the volume of the packed cell will be measured by placing an identical tube next to the blood containing tube filled up to the level of blood by a measured amount of water.
By the addition of calculated amount of water, a 2% sheep red blood cell suspension will be prepared.

II. Amboceptor's (Hemolysin) titration:
1. Prepare 1:500 dilutions up to 1:8000.
5 test tubes will be prepared
1m of VBD will be added to test tubes 2-5
10µl hemolysin will be mixed with 4990µl VBD in the first tube
1ml will be transferred from the first to the second up to the last tube and 1ml will be discarded.
2. Prepare 1:750 amboceptor's and dilute serially up to 1:12000
5 test tubes will be prepared and 1ml of VBD will be added to test tubes 2 to 5
10µl hemolysin will be mixed with 7490µl VBD in the first tube
1ml will be transferred from first tube to second up to fifth tube and 1ml will be discarded. Put the tubes in order of ascending dilution.

0.5ml will be transferred from each of the above test tubes to a second set of 10 tubes, starting from the 1:12,000 dilutions.

4. 1ml of VBD will be added to each of the test tubes
5. 0.5ml of 2% sheep red blood cell will be added to each of the test tubes and shacked well.
6. The test tubes will be left on the bench for 10minutes.
7. 1ml of complement at working dilution will be added.
8. The tubes will be incubated for 30minutes in a water bath at 37^0 C
9. The last tube showing complete hemolysis, minimum hemolytic dose (MHD) will be read and recorded. The working dilution of amboceptor's is four times the MHD.

III. Evaluation of complement.

Freeze dried complement will be reconstituted according to its instruction.

A 1:100 complement will be prepared

Complement will be added into the 9 tubes increasing by 5 µl every time, starting with 10µl.

Diluent will be added in to the 9 tubes in decreasing amount by 5µl, starting with 40µl.

25µl of diluents will be added into the tubes with the corn well syringe.

The test tubes will be placed in a 37^0C water bath for 1hr.

25µl of 2% sheep red blood cells will be added in all tubes

25µl of amboceptor's at working dilution 1:1000 will be added in all tubes.

The tubes will be properly mixed and put again in the water bath of 37^0C for another 30 minutes

The test will be read by recording the minimum hemolytic dose of complement (MHD), which is represented by the first tube showing complement hemolysis. The next tube contains the full hemolytic dose (FHD).The complement dilution = 2FHD/dilution of complement.

IV. Titration of antigen

Micro titer plate I

25 µl of VBD will be added in all cups (wells).

25µl prediluted antigen will be added to all cups of row A.

By serial doubling (two fold) dilution, 25 µl of antigen will be transferred from row A to B, and again from row B to C, etc. Until row G by multi-channel pipette.

25µl mixture will be discarded from row G (row H will only contain the diluent)

Micro titer plate II

50 µl of VBD will be added to all cups.

50 µl of prediluted (1:2.5) inactivated positive control serum will be added to all wells of column 1.

50 µl will serially be transferred by two fold dilution, from column 1 to 2 and again from column 2, 3 etc. until column 11. 50 µl is to be discarded from column 11.

Mix plate I and II

25 µl will be transferred from plate II to Plate I.

50 µl of complement in 1:40 dilution will be added to all cups of plate I

Plate I will be kept in a refrigerator, covered with second empty plate (cold fixation)

The following day, 50 µl of 2% sheep red blood cells, amboceptor premixture, equal volume, i.e. 25 µl of sheep red blood cells and 25 µl of a 1:100 working dilution of amboceptor, will be added to all cups.

The plate will be covered with sealing tape, shaked well and be kept in water bath at 37^0C for 30 minutes.

The last cup with 50+ sedimentation will be read and recorded. The highest dilution of antigen with 50+ sedimentation is the limiting antigen concentration or the right corner value.

The test proper, multiple sera technique

The sera will be prediluted to 1: 2.5 and incubated at 58^0C in a water bath for 30 minutes in order to inactivate the native complement.

25 µl of diluted test sera will be placed in wells of first and second rows of U- bottom plates and 25µl of VBD will be added to all wells except those of the first.

Serial doubling dilution will be then made by transferring 25µl of serum from the second row onwards continuing for at least four dilutions

25µl of antigen diluted to working dilution will be added to all wells excluding those of the anti complementary controls, which will receive 25µl VBD instead.

25 µl of complement at working dilution (1.25) will be added to all wells except control well. Control wells containing serum+ complement+ diluent, antigen control will have antigen+ complement+ diluent, complement control will have complement+ diluent and hemolytic system will have diluent, set up to 75µl total volume in each case before hemolytic system is added.

The plates will be sealed and incubated at 37^0C for 30minutes with agitation

25µl of sheep red blood cell suspension will be added to each well.

The plates will be sealed and reincubated at 37^0C for 30 minutes with agitation. Before reading the result, the plates will be left in the refrigerator at $+4^0$C for 1-hour in order to allow none lysed cells to settle.

Plates will be taken out from refrigerator and left at room temperature for 10 minutes.

Interpretation: sera were classified as positive: sera with a strong reaction of approximately 100% fixation of the complement (4+) at a dilution of 1:50; about 75% fixation of the complement (3+) at a dilution of 1:10; about 50% fixation of the complement (2+) at a dilution of 1:20 and about 25% fixation of the complement (1+) at a dilution of 1: (Alton et al., 1975; OIE, 2004).

Annex 3: Questionnaire format for serum collection: - format 1

No	Breed	Sex	Age	Source	Pregnancy status			Retention of placenta	Stillbirth	Birth to weak lambs or kids
					Pregnant		Non-pregnant			
					Yes	Month				

Format 2

Region----------.Zone----------District ---------- PA ---------- Village----

Name of respondant ---------- Age ----------Sex-----Date----------.

No	History of abortion					History of fetal membrane retention			
	Y/N	Cause	Rec. date	Frequency	Parity	Y/N	Rec. date	Frequency	Parity/ies

Annex 4: Questionnaire on management and husbandry risk factors

I. General information

Region----------.Zone----------District----------.PA----------

Village----------

Name of respondent----------.Age-------.Sex----------Date----------

Flock size---------- a. Small <5, b. Middle 5-10, c. Large >10

Owner experience------- (qualification) ----------family size----------

What do you do to milk?

A. Home consumption B. Selling for cash income C. both

2. How do you consume milk?

A. Cooked B.Raw C. Both D. Other treatments

3. Do you slaughter animals at home? (Y/N). If yes for what purpose?

Home consumption B. Group share C. Ceremony D.Emergency slaughter

4. How do you consume meat?

Cooked B.Raw C. both D. Other treatments

5. How do you treat your diseased animals?

A. Traditional healers B. Self administered vet drugs C.Vet clinics

6. How do you assist delivery?

A. Hand pulling B. Naturally C. No assistance

7. How do you dispose of fetal membranes?

A. Burring B. offering to dogs C. Leaving in the field

8. Where do you get your replacement stock?

A. Own stock B. Villages` stock C. Both

9. Do you keep dogs at your home? A. Yes B. No

10. List at least five endemic diseases in your area`----------

11. Do you have regular veterinary service?

A. Yes B. No

12. Do you have awareness of small ruminants/humans brucellosis or other diseases? (A). Yes (b)

13. Watering points in the different seasons

I . Watering points in the dry seasons.

A. Rivers B. Ponds C. Artificial/Traditional wells D. Springs.

II. Watering points in the wet seasons

A. Rivers B. ponds C. Artificial/Traditional wells D. Springs

14. What do think about the cause of abortion?

A. Disease B. Mechanical trauma C. Both D. Others

15. What do you do to animals those frequently abort?

A. Sell B. Slaughter C. Keeping D. Others

16. What do you do to animals those do not conceive?

A. Sell B. Slaughter C. Keeping D. Others

17. Do you sell breeding females/males? (Y/N). If yes reasons for selling

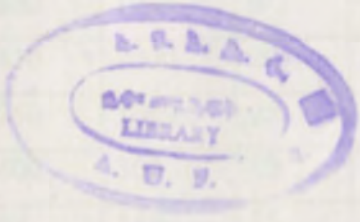
A. Disease B. Infertility C. Shortage of money

18. House types (floor type)?

A. concrete B.Soil D. Others

19. Feed type?

A. Concentrates B. Only grazing C. Grazing and concentrates D. Others



Annex 5: Livestock population of the 43 peasant associations of adami tulu jido kombolcha district

PAs	Cattle	Sheep	Goat	Horse	Mule	Donkey	Poultry
Kamo Garbi	1450	166	695	10	2	250	1500
Gobechecho Asebo	4500	332	1932	40	15	400	2000
Haleku Gulenta	8499	395	2232	13	5	252	3016
Suro Kudusa	6019	460	1984	90	29	680	4854
Wilico Boremo	4550	200	3000	6	1	350	3500
Elka Kalamo	2350	250	1500	8	20	710	1440
Nagaliny	6820	190	2800	2	2	420	4200
Abine Germama	8916	4116	6174	20	2	1029	6700
Gabiba Rassa	4802	1200	2017	32	18	109	4220
Karkafa Waransa	7890	300	2586	70	45	315	4480
Waisco Kankara	3962	120	1091	16	3	295	1197
Adamsho Gogisa	3220	2000	4200	13	7	120	1300
Bochesa	1520	80	720	6	0	75	870
Walin Bula	9970	935	4020	25	10	355	2618
Gallo Hirfe	4930	1230	4230	20	16	1320	4230
Allelan Ababu	3000	800	1000	9	14	500	2200
Liliso Danbe	2210	450	2000	20	10	300	2500
Danbe Adansho	1820	250	1080	25	6	300	2500
Reeji	1000	400	360	10	8	200	620
Naka	2200	89	1490	15	10	230	-------
Adansho Baranota	2413	708	1070	20	10	430	850
Citu Gitu	2061	500	3340	16	10	160	2630
Bara Habicho	3530	560	3420	19	9	220	2530
Edo Gojela	1460	202	670	10	6	107	1970
Jella Aluto	6522	531	2496	20	50	680	4300
Oda Anshura	3068	612	1904	18	1	842	3570

Gali Migira	3479	2025	1302	65	35	250	3545
Habule Gutumuma	3605	404	1968	19	5	672	6157
Desta Abgata	9684	4800	11020	310	30	1703	20217
Arba	5720	644	4765	23	9	1707	------
Ulma Kafara	4626	273	2325	25	14	157	2837
Worja Woshgula	1677	405	538	11	4	218	342
Kormi Bujure	7349	158	6740	6	7	940	2930
Andola Kabi	3999	265	1628	28	6	498	1393
Oitu Basuma	6600	2000	3582	115	10	695	4912
Hurufa Loli	2975	400	1800	15	8	270	1755
G/Wilico Boremo	2412	273	1321	8	3	329	1906
Aneno Shisho	4230	350	2820	2	1	510	3820
Aroresa Kalbo	3200	1200	1490	30	25	400	900
Golba Aluto	1264	42	188	11	4	68	800
Abie Danaba	2762	280	982	22	10	600	1600
Aluto	2400	154	605	15	9	268	257
Dodicha	1522	150	4000	0	29	320	1600
Total	176186	30899	105085	1258	518	20254	120991

9. CURRICULUM VITAE

Name: Aklilu Tesfaye Adane.

Nationality: Ethiopian.

Address: Addis Ababa, Ethiopia.

Marrital status: Married.

Language: Amharic, Geeze, English and Russian languages.

Educational Background:

Elementary school: (6^{th} grade) in Minilik elementary school in 1973, Ethiopian calender

Secondery school: (7-8^{th} grades) in Minilik seconery school from 1974 to 1975 E. C.

High school: (9-12^{th} grades) in Minilik junior high school from 1976 to 1979 E. C.

First degree in veterinary medicine: in Ukrainian agricultural university from 1991 to 1996 European calendar.

- **Work experience:**

- In Afar regional State as field veterinaria from 1990 to 1995 Ethiopian calendar
- In Alage agricultural vocational educational trainig college from 1996 to the present time.

10. SIGNED DECLARATION

I the underlined declare that the thesis is my original work and has not been presented for a degree in any university and that all sources of materials used have been duly acknowledged.

Name ----------

Signature ----------

Date of sumission ----------

Dr. Girma Zewde

This thesis has been submitted for examination with my approval as university adviser.

Name of advisers	Signature
1. Dr. Mosses Kyule	----------
2. Dr. Yilkal Asfaw	----------
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