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SEROEPIDEMIOGICAL INVESTIGATION OF BOVINE BRUCELLOSIS AND ITS ZOONOTIC IMPLICATION IN SELECTED TOWNS OF OROMIA REGION, CENTRAL ETHIOPIA

MVSc Thesis

BY

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JUNE, 2018

BISHOFTU, ETHIOPIA

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MSc Thesis

A THESIS SUBMITTED TO THE COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, ADDIS ABABA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF VETERINARY SCIENCE IN VETERINARY MICROBIOLOGY

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by **Abeje Abera** entitled: **Seroepidemiological investigation of bovine brucellosis and its zoonotic implication in selected towns of Oromia Region, Central Ethiopia.** And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

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STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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DEDICATION

This paper is dedicated: To my father, Abera Dejenu and my mother Yewubnesh Hailu, who never went to school themselves for raising me up and sending me to school and supporting me financially and morally throughout my academic career.

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LIST OF ABBREVIATIONS

AGID	Agar Gel Immunodiffusion
BSL	Biosafety Level
BZFED	Bale Zone Finance and Economy Development
CELISA	Competitive Enzyme Linked Immunosorbent Assay
CFT	Complement Fixation Test
ELISA	Enzyme Linked Immunosorbent Assay
FPA	Florescent Polarization Assay
I-ELISA	Indirect ELISA
LPS	Lipopolysaccharide
MET	Mercaptoethanol
MR	Methyl Red
MRT	Milk Ring Test
NAHDIC	National Animal Health Diagnostic and Investigation Center
OIE	Office International des Epizooties
OPS	O-Polysaccharide
PCR	Polymerase Chain Reaction
PPE	Personal Protective Equipment
RBPT	Rose Bengal Plate Test
RFM	Retained Fetal Membrane
RSAT	Rapid Slide Agglutination Test
SAT	Standard Slow Agglutination Tube Test
SDFED	Sinana District Finance and Economy Development Office
SNNPR	Southern Nations Nationality People Region of Ethiopia
UI	Unit International
VP	Vogues Proskauer
ZNS	Ziehl-Neelsen Stain
WAO	Woreda Agriculture Office

ABSTRACT

Brucellosis is a zoonotic disease with economic and public health impact, particularly for human and animal populations within developing countries that relay on livestock production. A cross-sectional study was carried out on bovine brucellosis in three zones of Oromia Region, of central Ethiopia from October 2017 to May 2018. Simple random sampling method was employed to sample unvaccinated cattle above 6 months of age. A total of 803 blood samples were collected from non-vaccinated, crossbred and local breeds of 110 farms of dairy cattle. Individual sera samples were tested for the presence of anti-*Brucella* antibody using Rose Bengal plate test and RBPT positive samples were further tested using Competitive Enzyme Linked Immunosorbent Assay (C-ELISA) as confirmatory test. Thirteen out of 803 (1.6%) of the samples were positive by RBPT while 11 out of the 13 RBPT positive sera were confirmed to be positive using C-ELISA. Herd and individual animal level seroprevalence were found to be 5.5% and 1.4%, respectively. Risk factors such as herd size, breed and abortion history were significantly associated with seropositivity of *Brucella* antibodies. Herd level analysis of the risk factors indicated that the odds of having brucellosis was 21 times higher in farms with large herd size (>20 animals), (OR = 20.99, CI=3.45–127.53) than medium (11-20 animals) and smaller herd sizes (5-10 animals). The odds of *Brucella* infection in local breed cows were 10.5 times at higher risk than in cross breeds. Similarly, risk of having brucellosis in those herds experiencing abortion was 29.2 times at higher risk (OR =29.2, CI = 6.61–128.94) than those without. Dairy farmers addressed in this study were used to throw retained fetal membrane, aborted fetus and different discharges to the environment. They also consume both raw milk and meat that may be associated with high risk of public health problem. Results of this study showed that the prevalence of bovine brucellosis in the study areas was low and pre-purchase test, isolation of sick and new entry animals, movement control between different herds and creation of public awareness by educating people are forwarded to reduce disease transmission within animals and from animals to humans.

***Key words*:** Brucellosis; CELISA; Diagnosis; Epidemiology; Ethiopia; Oromia; RBPT

1. INTRODUCTION

Brucellosis is a highly contagious, zoonotic and economically important bacterial disease of animals worldwide and it is considered as one of the most widespread zoonoses in the world (Schelling *et al*. 2003). Bovine brucellosis is known for its impact on reproductive performance of cattle in Africa (McDermott and Arimi, 2002). The disease often persists in the poorest and most vulnerable populations. Human infection is always associated with brucellosis in domestic or wild animal reservoirs (Godfroid *et al.*, 2005). The disease in cattle is primarily caused by *Brucella abortus* and occasionally by *Brucella melitensis* where cattle are kept together with infected sheep or goats (OIE, 2009). Chronic infection of the mammary glands due to *Brucella suis* has also been reported (Lopes *et al*., 2008). Clinically bovine brucellosis is characterized by impaired fertility specifically with abortion, metritis, orchitis and epididymitis (Radostits *et al*., 2007; Seleem *et al*., 2010).

Brucellosis remains the second most common zoonotic disease after rabies in the world, with more than 500,000 new cases reported annually (Godfroid *et al*., 2013); the actual number of cases, including undetected and unreported cases, is believed to be considerably higher (Dahouk *et al*., 2013). Brucellosis is often a neglected disease despite being endemic with high zoonotic potential in many countries (Poester *et al*., 2013). From public health view point, brucellosis is considered to be an occupational disease that mainly affects farm laborers, slaughter-house workers, butchers, veterinarians (Yagupsky and Baron, 2005). Management, animal movement, wide ranges of host, herd size, commingling of different animal species is risk factors for animal brucellosis. Another worrying aspect of the disease epidemiology includes the presence of brucellosis in wildlife, with a potential for continuous transfer to domestic animals. Control of wildlife brucellosis has been the subject of debate between authorities and environmental activists, the latter opposing the 'test and slaughter' policy as it was applied in elks in the US (Pappas and Papadimitriou, 2007). Additional epidemiological aspect that brought back the interest in brucellosis is its implication in travel-related disease, with numerous imported cases reported annually, often resulting in further laboratory-acquired cases (Memish and Balkhy, 2004). The increased interest in the threat of biological weapons in the early 21st century has also

brought *Brucella* back to the epicenter of scientific interest, as it is considered a Class B potential warfare agent (Memish and Balkhy, 2004). The zoonotic pathogens *B. abortus*, *B. melitensis*, and *B. suis* have been identified as category B bioterrorism agents (Rotz *et al.*, 2002).

Clinical manifestations of bovine brucellosis depend on age, reproductive and immunological status, route of infection, and virulence and dose of the *Brucella* strain. Infection occurs most commonly via the digestive tract, the usual source of the organism being an infected placenta or aborted fetus. A precise diagnosis of *Brucella* species infection is important for the control of the disease in animals and consequently in man. Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, requires biosafety level-3 (Robinson, 2010) protocols for the high risk of laboratory acquired infections, molecular methods have been explored in order to overcome these difficulties. Furthermore, the polymerase chain reaction (PCR) based assays have shown a higher sensitivity with respect to the standard microbiological assay for the diagnosis of brucellosis (Boschiroli *et al.*, 2001).

In Sub-Saharan Africa, the introduction of exotic animals with better productivity merit for food of animal origin is partly constrained by infectious diseases of which bovine brucellosis is one of them (Mangen *et al*. 2002; McDermott and Arimi 2002) and since the first report of brucellosis in the 1970 (Domenech, 1977; Meyer, 1980) in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Kebede *et al*., 2008; Ibrahim *et al*., 2010). In Ethiopia, the growing tendency of intensification, in dairy, especially in urban and peri -urban areas has instituted the practice of improving the exotic blood level in local cows for artificial insemination. It has the vision of increasing local animals' productivity, but the limited number of surveys done by Asfaw *et al.* (1998) showed high prevalence of bovine brucellosis in exotic and crossbred animals compared to local zebu cattle in dairy production; as a result the disease is a major obstacle to the importation of high yielding breeds and represents a significant constraint to the improvement of milk production through cross breeding. The prevalence of bovine brucellosis has been intensively investigated in state owned dairy farms, in smallholder farms in

some parts of Ethiopia (Kassahun, 2004; Berhe *et al*. 2007) and in the central highlands of Ethiopia (Kebede *et al.* 2008).

Market- oriented systems are contributing immensely towards filling the gap between demand and supply for milk and dairy products in urban centers, where consumption of milk and milk products is remarkably high (Azage *et al*., 2000). Reproductive problems occur frequently in lactating dairy cows and dramatically affect reproductive efficiency in dairy herd. Some of the most common problems include: abortion, stillbirth, retained placenta and repeat breeder. Deciding whether to breed, treat, or cull dairy cows exhibiting one or more of these reproductive problems is a challenge for both veterinarians and dairy producers. Dairy farming should focus on prevention and control of risk factors associated with each problem rather than on prescriptive therapeutic interventions (Hafez, 2000). Poor reproductive performance is a major cause for voluntary culling and has a negative effect on the productivity of a dairy herd. The animal health services provided in the Oromia region is inadequate; especially the diagnostic services are not readily available to the dairy farmers, furthermore Bovine brucellosis study was not conducted before in Adama, Bale Robe and Goba towns; whereas in Fiche and Asela towns there were very limited and dissimilarity in reporting research efforts so far conducted to explore and overcome the impact of the disease.

Thus the current study was, targeted to generate further required risk factors information gaps on brucellosis which causes dairy cattle underproductive and to address its zoonotic significance which is hampering human health.

Objectives of this study were:

- To determine seroepidemiology of Brucellosis in dairy cattle in Fiche, Adama, Asella, Bale Robe and Goba towns of Oromia Region, Central Ethiopia;
- To identify putative risk factors in selected areas of urban and peri-urban dairy production systems and
- To reflect the awareness of farmers on zoonotic implication of bovine brucellosis in the study areas.

2. LITRATURE REVIEW

2.1. Historical overview of brucellosis

Brucellosis is an ancient disease that can possibly be traced back to the 5 plague of Egypt around 1600 BC. Recent examination of the ancient Egyptian bones, dating to around 750 BC, showed evidence of sacroiliitis and other osteoarticular lesions, common complications of brucellosis (Pappas and Papadimitriou, 2007) and examination of the skeletal remains of the Roman residents of Herculaneum (Naples, Italy) killed by the catastrophic volcanic eruption of Mt.Vesuvius in the late August, 79 AD revealed vertebral bone lesions typical of brucellosis in more than 17% of the residents. Scanning electron microscopy of recovered cheese provided a likely explanation for the high incidence of the disease (Sriranganathan *et al*., 2009). The buried carbonized cheese, made from sheep's milk and found with the bones, revealed the presence of cocco-bacillary forms that were morphologically similar to *Brucella* spp. (Capasso, 2002). Eighteen centuries later, Sir David Bruce isolated *Micrococcus melitensis* (now *B.melitensis*) from the spleen of a British soldier who died from a febrile illness (Malta fever) common among military personnel stationed on Malta, an island not far away from Herculaneum (Godfroid *et al.*, 2005). For almost 20 years, brucellosis was thought to be a vector-borne disease. The zoonotic nature of the brucellosis was accidentally demonstrated in 1905 by isolating *B. melitensis* from goat's milk used for the production of soft cheese in Malta (Nicoletti, 2002a; Godfroid *et al*., 2005; Rust, 2006). After ten years of "*Micrococcus melitensis*" discovery, the Danish scientist Bernhard Bang identified "*Bacillus abortus*" (i.e. *Brucella abortus*) in bovine aborted fetuses. Another organism similar to "*Micrococcus melitensis"* was isolated from aborted pigs in United States, which was finally designated as *Brucella suis* (Traum, 1914). At that time, this disease was further studied by Alice Catherine Evans (an American microbiologist) and based on her findings pasteurization of milk was proposed as a preventive measure (Evans, 1918). Finally in 1920, Louis Meyer and Wilbur Shaw honored David Bruce by proposing a single new genus named *Brucella* to group these pathogenic bacteria (Meyer, 1920).

2.2. Etiology

Brucella species are gram negative cocco-bacilli, which are classified into species by various techniques such as growth patterns on media and phage susceptibility. There are six "classically" recognized species; *B. abortus*, *B.melitensis*, *B.suis*, *B. ovis*, *B. canis*, and *B. neotomae* (Godfroid *et al.*, 2013). Recently, four new *Brucella* species have been recognized and classified, namely, *B. ceti* and *B. pinnipedialis* (dolphins/porpoises and seals respectively), *B. microti* (voles) and *B. inopinata* (reservoir undetermined). Non-bovine animals, and humans, can also contract the disease, which in turn play a significant role in its persistence and transmission. *Brucella abortus* infecting cattle has seven recognized biovars, the most reported of which are biovars 1, 2, 3, 4, and 9, with biovar 1 being the most prevalent in Latin America. The distribution of biovars could be important in ascertaining the source of some infections (Lucero *et al*., 2008).

2.3. Occurrence

2.3.1. Global distribution and economic impact of bovine brucellosis

Brucellosis is considered by the Food and Agriculture Organisation (FAO) (Robinson, 2010), the World Health Organisation (WHO) (Robinson, 2010) and the Office International des Epizooties (OIE) as one of the most widespread zoonoses in the world. According to OIE, it is the second most important zoonotic disease in the world after rabies (Schelling *et al.*, 2003). Brucellosis remains endemic among Mediterranean countries of Europe, Africa, Near East countries, India, Central Asia, Mexico and Central and South America (Robinson, 2010).

Also it is considered as a reemerging problem in many countries such as Israel, Kuwait, Saudi Arabia, Brazil and Colombia, where there is an increasing incidence of *Brucella melitensis* or *Brucella suis* biovar infection in cattle (Cutler *et al.*, 2005). Brucellosis occurs worldwide, except in those countries where bovine brucellosis (Robinson, 2010) has been eradicated. It has been eradicated from Australia, Canada, Cyprus, Denmark, Finland, the Netherlands, New Zealand, Norway, Sweden and the United Kingdom (Robinson, 2010). Bovine brucellosis has also been

eradicated in most developed countries that have implemented eradication programme (Makita *et al.*, 2008). While *B. melitensis* has never been detected in some countries, there are no reliable reports that it has ever been eradicated from small ruminants in any country (Robinson, 2010).

There are many reasons why brucellosis remains endemic. These include expansion of livestock herds and flocks, with associated uncontrolled movements; lack of veterinary support services and vaccines; and husbandry practices (McDermott and Arimi, 2002).The epidemiology of human brucellosis has drastically changed over the past few years because of various sanitary, socioeconomic, and political reasons, together with increased international travel. New foci of human brucellosis have emerged, particularly in central Asia, while the situation in certain countries of the Middle East is rapidly worsening (Pappas *et al*., 2006)

The most significant economic losses are usually incurred following bovine brucellosis. For instance, the serological prevalence of bovine brucellosis in Central America during the last 10 years has been estimated between 4 and 8%, with higher prevalence in dairy herds and with losses calculated at US$ 25 million per year. There is an estimated 25% reduction in milk production in infected cows (Moreno, 2004).

2.3.2. Bovine brucellosis in Sub Saharan Africa (SSA)

Good quality data on the impacts of different diseases and their control on animal and human populations in sub-Saharan Africa are usually lacking. However, a recent study has attempted to assemble expert advice on the potential impacts of animal diseases in the developing world (Perry *et al*., 2002). One of the highest priority diseases, both in sub Saharan Africa and other regions of the developing world was brucellosis. The importance of brucellosis reflects its wide spread distribution and its impacts on multiple animal species, including cattle, sheep, goats, pigs and humans, while the importance of brucellosis is widely assumed, the benefits of programs to control it, relative to their costs, need to be assessed. Since brucellosis is an important cause of abortion especially in first calf heifers, the disease can also cause important economic losses in developing countries. In Africa particularly in Sub-Saharan Africa, the seroprevalence of

Brucellosis is estimated to be ranging from 10.2% to 25.7% (Mangen *et al*., 2002). Some countries in Africa where seroprevalence of Brucellosis had been reported to be less than 10% were Ethiopia 1.9%, Benin 4.3% and Ghana 6.6% (Kubuafor *et al*., 2000; Asmare *et al*., 2013).

2.3.3. Status of brucellosis in Ethiopia

Since the first report of brucellosis in the 1970 (Domenech, 1977; Meyer, 1980) in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Kebede *et al*., 2008, Ibrahim *et al*., 2010). A large number of articles have been published reporting individual seroprevalence ranging from 1.1% to 22.6% in intensive management systems (Asmare *et al*., 2007; Hailemelekot *et al*., 2007; Tolosa *et al.*, 2010; Tesfaye *et al*., 2011) and 0.1–15.2% in extensive management system (Berehe *et al*., 2007; Hunduma and Regassa, 2009; Asmare *et al*., 2010; Haileselassie *et al*., 2011; Megersa *et al*., 2011).

Furthermore sero-epidemiological study of bovine brucellosis was conducted in three separate agroecological areas of central Oromia and the seroprevalence was 4.2% in the lowlands, 1.0% in the midlands and 3.4% in the highlands (Jergefa, *et al*., 2008). Relatively low individual animal sero-prevalence in intensive farms were recorded in different parts of the country (Taddesse, 2003), observed a prevalence of 0.14% in north Gondar zone, (Tadele, 2004) reported 0.77% in southwestern Ethiopia, However, most reports made thus far have either limited geographic coverage or are relatively confined to a single agro-ecology. This warrants the need for a comprehensive understanding of disease occurrence in dairy production systems and spatial distribution across the country, as to shape possible future intervention programmes (Asmare *et al*., 2013). Furthermore Geresu *et al.* 2016 isolated *Brucella* species from dairy cows in and around Bishoftu and Asela towns and (Tekle, 2016) isolated *B. melitensis* from an aborted goat's milk and vaginal swabs in Afar region which was not largely reported in Ethiopia. Hence the studies have been mainly conducted based on seroprevalence, it is clear that there is information gap on the causative agent identification and specific transmission of the disease dynamics within species and agroecological zones of the country.

Table 1: Sero-prevalence of bovine brucellosis from different locations of Ethiopia

Locations	Test type		Reference
	RBPT (%)	CFT (%)	
Jimma zone, S/W Ethiopia	3.3	3.1	(Tolosa, 2004)
Bahr Dar	4.6		(Hailemelekot, 2005)
Tigray (Tabias)	3.3	3.2	(Berhe *et al.*, 2007)
Tigray	4.9		(Haileselassie, 2008)
Wuchale-Jida district	12.5	11.0	(Kebede *et al*., 2008)
East Showa Zone	11.2		(Dinka and Chala ,2009)
Central Oromia		2.9	(Jegerfa ,2009)
Hwassa	3.9		(Abebe *et al.*, 2010)
Arsi-Negele		2.6	(Amenu,*et al*., 2010)
Sidamo zone		1.7	(Asmare *et al.*, 2010)
Addis Ababa dairy farms	2.5	1.5	(G/Yohannes, *et al.*,2011)
Selected Districts of Arsi Zone	0.5	0.5	(Degefa *et.al*., 2011)
Jigjiga zone	1.8	1.4	(Hailu *et al*.,2011)
Southern & Eastern Ethiopia		3.5	(Megersa *et al.*, 2011)
Western Tigray		6.1	(Haileselassie *et al*., 2011)
Guto-Gida district ,East Wollega Zone	3.0	2.0	(Yohannes *et al.*,2012)
Ethiopia		1.9	(Asmare *et al*., 2013)
South East Somali and Oromia		0.9	(Gumi *et al*.,2013)
Benishangul Gumuz	1.2	1	(Adugna *et al*.,2013)
Debre-Zeit, Central Ethiopia	3.3	2	(Alemu *et al*.,2014)
Adami Tulu	4.5	4.3	(Tibesso *et al.*, 2014)
Debrebirhan and Ambo Towns	0.7	0.2	Bashitu, *et al*., 2015
Bishoftu and Asela	2.3		(Geresu *et al*.,2016)
In and around Alage district	2.4	2.4	(Asgedom *et al.*,2016)
North West Gondar	5.4	4.9	(Alehegn *et al*.,2016)
North Shewa	0.78		(Pal *et al., 2016*)
Arsi zone		2.6	(Yohanes *et al., 2016)*
Chencha district, Gomogofa zone	1.04		(Meles and Kibeb., 2018)

2.3.4. Host range and Brucella diversity

The principal strain that infects cattle is *B. abortus*; cattle can also become transiently infected by *B.suis* and more commonly by *B.melitensis* when they share pasture or facilities with infected

pigs, goats and sheep. *B. melitensis* and *B.suis* can be transmitted by cow's milk and cause a serious public health threat (Ewalt *et al*., 1997; kahler, 2000; Acha and Szyfres, 2003). The main etiologic agent of brucellosis in goats is *B.melitensis*.

2.3.5. Transmission

As a herd problem, it is primarily spread by contact and ingestion of contaminated material (Radostits *et al.*, 2000; Boschiroli *et al.*, 2001), while spread between herds is facilitated by introduction of asymptomatic animals (Nicoletti, 2013). The primary routes of infection are through the mucous membranes of the conjunctiva, oral and nasal surfaces (Abernethy *et al.*, 2006) and supposedly through vertical transmission or colostrum (Aparicio, 2013). Humans get infected by direct or indirect contact with infected animals or by ingestion of their products or by-products (Corbel., 2006; Dean *et al.*, 2012; Karadzinska-Bislimovska *et al.*, 2010) and therefore, human brucellosis is an occupational disease of stockyard, slaughter house workers, butchers' and veterinarians (Radostits *et al*., 2000).

2.4. Pathology and immune response to *Brucella* infection

The ability of the pathogen to survive and replicate within different host cells explains its pathogenicity (Muflihanah *et al*., 2013; Ray *et al*., 2009; Alavi and Motlagh, 2012). Pathogenesis depends upon various factors such as the species, size of the inoculum, modes of transmission and the immune status of the host (Djønne, 2007). Extensive replication in placental trophoblasts is associated with abortion (WHO, 2006; Djønne, 2007; Rodriguez *et al*., 2012), and persistence in macrophages and other cell types leads to chronic infections (Muflihanah *et al., 2013*). Protective immunity to the host is conferred by T-cell mediated macrophage activation by the antigenic protein of *Brucella* and the production of corresponding antibody along with other elements of immune response such as tumor necrosis factor (TNF), interferons and complement. Following infection, the immunoglobulin M (IgM) titer increases initially followed by the

immunoglobulin G (IgG) titer. Thus, the appearance of IgM indicates an early immune response against brucellosis and IgG correspondingly indicates chronic infection or relapse (Young, 2005; McDonald, *et al*., 2006).

2.5. Diagnosis

2.5.1. Diagnostic procedures

Control of brucellosis is dependent on reliable methods for the identification of *Brucella* in livestock, wildlife and humans. The diagnosis of brucellosis is based on serological, bacteriological, allergic skin reaction, and molecular methods (Simsek *et al*., 2004). Most serological tests are based on the agglutination of antigens by specific antibodies present only in the blood of infected animals. Misdiagnosis due to infections from cross reacting bacteria is rare but significant. Therefore the second component of brucellosis diagnosis is the isolation and characterization of the disease causing agent. The most important confirmatory method of *Brucella* infection is bacteriological diagnosis since its specificity is much higher than that of other diagnostic methods and it is used as a gold standard diagnostic method. The existence of different *Brucella* biotypes among the *Brucella* spp. and their identification is important to confirm the infection and trace the source of the infection (Guler *et al*., 2003).

Because of the complications involved in the diagnosis of the disease, including the difficulties in distinguishing between infected and vaccinated animals by conventional serological tests, bacteriological isolation and identification of biotypes of the etiological agent are necessary steps in the design of epidemiological and eradication programs (Refai, 2002; Zinstag *et al*., 2005). The indigenous difficulties in culturing *Brucella* are widely recognised: apart from the low bacterial load needed to induce infection, *brucellae* also exhibit slow doubling time (on average 2–3 hour), and tend to grow in specialised media, of which Castaneda's remained the most efficient and popular for decades (Al Dahouk *et al*., 2003), although it is not solving the problem of sub-culturing. There have been numerous reports in the literature (Bourantas *et al*., 1997) of positive results emerging after four or more weeks of culture and subculture. The emergence of

automated systems in the last decade has improved the rapid detection of the pathogen, with identification time falling to as little as 2–4 days in most reports (Bannatyne *et al*., 1997; Yagupsky, 1999; Baysallar *et al*., 2006). Samples for cultural growth under BSL-3 conditions should be taken prior to the onset of antibiotic therapy. Serum should be shipped cooled or frozen (Schwarz *et al*., 2017). While this approach is definitive, it has several draw backs. It is time consuming to culture *Brucella* from biological samples. The specific tests used to characterize the bacteria are complicated and must be performed by highly skilled personnel. Finally, because *Brucella* is a zoonotic disease, handling the live agent is hazardous to laboratory personnel. For this reason genetic characterization using molecular DNA techniques has been pursued. Molecular diagnostic methods are also currently being used for the detection of *Brucella* spp. in various samples (Sahin *et al*., 2008). Sample transport has to be rapid and cultural growth should start within 1-2 hours after sample taking. For longer transport times, clinical samples should be cooled to 2-8 °C, tissue samples have to stay moist during transport (Schwarz *et al*., 2017).

2.5.2. Direct methods for diagnosis of brucellosis

Isolation of the organism is considered the gold standard diagnostic method for brucellosis since it is specific and allows biotyping of the isolate, which is relevant under an epidemiological point of view (Bricker, 2002; Al Dahouk *et al*., 2003). However, in spite of its high specificity, culture of *Brucella* spp. is challenging. *Brucella* spp. is a fastidious bacterium and requires rich media for primary cultures. Furthermore; its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory (Adams, 2002; Refai, 2003; Seleem *et al*., 2010).

Smooth *Brucella* inhibit host cell apoptosis, favoring bacterial intracellular survival by escaping host immune surveillance, while rough *Brucella* mutants (*Brucella canis* and *Brucella ovis* are two exceptions) induce necrosis in macrophage (Pei *et al*., 2006). *Brucella* uses a number of mechanisms for avoiding or suppressing bactericidal responses inside macrophages. The smooth lipopolysaccharides (LPS) that cover the bacterium and proteins involved in signaling, gene

regulation, and transmembrane transportation are among the factors suspected to be involved in the virulence of *Brucella* (Lapaque *et al*., 2005). LPS is vital to the structural and functional integrity of the Gram-negative bacteria outer membrane (Cardoso *et al*., 2006). The smooth phenotype of *Brucella* is due to the presence in the outer cell membrane of a complete LPS, which is composed of lipid A, a core oligosaccharide, and an O side- chain polysaccharide. Rough (vaccine) strains (i.e., strains with lipopolysaccharide lacking the O-side chain) are less virulent because of their inability to overcome the host defense system. The LPS of *Brucella* exhibits properties distinct from other LPSs. In contrast to classical entero bacterial LPS, those of *Brucella* are several hundred-times less active and less toxic than Escherichia coli LPSs (Lapaque *et al*., 2005).

Contamination of clinical samples is a complicating factor for *Brucella* spp. isolation. Therefore, the use of nutrient-rich media supplemented with antibiotics (polymixin B 5,000 UI/L; bacitracin 25,000 UI/L; cyclohexamide 100 mg/L; nalidixic acid 5 mg/L; nystatin 100,000 UI/L and vancomycin 20 mg/L) is used to inhibit growth of contaminants that may prevent isolation of *Brucella* spp. (De Miguel *et al*., 2011).

Another limiting factor for culturing *Brucella* spp. is the requirement for appropriate laboratory conditions and personnel training so the procedure can be performed safely. *Brucella* spp. is classified as a Biosafety level 3 organism, whose manipulation should be performed in biosafety level-3 laboratories (Lage *et al*., 2008; da Silva Mol *et al*., 2012). Importantly, brucellosis is one of the most common accidental laboratory infections; particularly in research laboratories (*Seleem et al*., 2010; da Silva Mol *et al.*, 2012*;* Sam *et al.*, 2012). Samples for *Brucella* spp. isolation from cattle include fetal membranes, particularly the placental cotyledons where the number of organisms tends to be very high. In addition, fetal organs such as the lungs, bronchial lymph nodes, spleen and liver, as well as fetal gastric contents, milk, vaginal secretions and semen are samples of choice for isolation (Poester *et al*., 2006; Lage *et al*., 2008; Padilla Poester *et al*., 2010). Vaginal secretions should be sampled after abortion or parturition, preferably using a swab with transporter medium, allowing isolation of the organism up to six weeks post parturition or abortion (Poester *et al*., 2006; Padilla Poester *et al*., 2010). Milk samples should be

a pool from all four mammary glands. Non-pasteurized dairy products can also be sampled for isolation (Lage *et al*., 2008; Padilla Poester *et al*., 2010).

Brucella spp. colonies are elevated, transparent, and convex, with intact borders, smooth, and a brilliant surface. The colonies have a honey color under transmitted light. Optimal temperature for culture is 37°C, but the organism can grow under temperatures ranging from 20°C to 40°C, whereas optimal pH ranges from 6.6 to 7.4. Some *Brucella* spp. requires CO2 for growth. Typical colonies appears after 2 to 30 days of incubation, but a culture can only be considered negative when there are no colonies after 2 to 3 weeks of incubation. False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low (Padilla Poester *et al*., 2010).

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

Molecular Technique: Molecular biology as a diagnostic tool is advancing and will soon be at the point of replacing actual bacterial isolation. The use of the Polymerase Chain Reaction(PCR) to identify *Brucella* DNA at genus, species and even biovar levels has becoming extended to improve diagnostic tests and a diversity of methods have been developed. Applications for PCR methods range from the diagnosis of the disease to characterization of field isolates for epidemiological purposes including taxonomic studies. PCR-based assays are also useful in chronically infected animals and humans where the yield of bacteria from blood cultures is

usually low. It is rapid, safe and cost effective, the only real problems being some uncertainties regarding specificity (Padilla Poester *et al*., 2010). In addition to the commonly used PCR assays, a new Multiplex-PCR assay was developed that specifically identified *B. neotomae*, *B. pinnipedialis*, *B. ceti*, and *B. microti*. Furthermore, it differentiated *B. abortus* biovars 1, 2, 4 from biovars 3, 5, 6, 9, as well as between *B. suis* biovar 1, biovars 3, 4, and biovars 2 and 5 (Huber *et al*., 2009).

Molecular techniques are important tools for diagnosis and epidemiologic studies, providing relevant information for identification of species and biotypes of *Brucella* spp., allowing differentiation between virulent and vaccine strains (Lopez-Goni *et al*., 2008). Molecular detection of *Brucella* species can be done directly on clinical samples without previous isolation of the organism. In addition, these techniques can be used to complement results obtained from phenotypic tests (Bricker and Ewalt, 2005).

In order to avoid difficulties of bacteriological testing the molecular biological techniques, often based on the polymerase chain reaction (PCR) amplification, are successfully used for *Brucella* identification and typing (Yu and Nielsen, 2010). Recently a number of real-time PCR methods for *Brucella* detection in clinical samples were developed. The advantages of real-time PCR are speed (since there is no need to analyze the PCR products by agarose gel electrophoresis), high sensitivity in comparison to the conventional PCR, and reduced samples contamination. Various samples can be analyzed by this method, including cell culture, blood, serum, and tissues (Kattar *et al.*, 2007).

2.5.3. Indirect methods for diagnosis of brucellosis

Rose Bengal Plate Test (RBPT): It is a spot agglutination technique. It does not need special laboratory facilities and is simple and easy to perform. It is used to screen sera for *Brucella* antibodies. The test detects specific antibodies of the IgM and IgG type. Although the low PH (3.6) of the antigen enhances the specificity of the test and temperature of the antigen and the

ambient temperature at which the reaction takes place may influence the sensitivity and specificity of the test (Foulongne *et al*., 2000).

RBPT is a rapid, slide-type agglutination assay performed with a stained *B. abortus* suspension at pH of 3.6 - 3.7 and plain serum. Its simplicity made it an ideal screening test for small laboratories with limited resources. The drawbacks of RBPT include: low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozones make strongly positive sera appear negative in RBPT (Starr *et al*., 2008). The overall sensitivity is 92.9%, so the use of RBPT individuals exposed to brucellosis and those having history of *Brucella* infection. Rose Bengal plate test [RBPT] is an agglutination test that is based on reactivity of antibodies against smooth lipopolysaccharide (LPS). As sensitivity is high, false negative results are rarely encountered. To increase specificity, the test may be applied to a serial dilution (1:2 through 1:64) of the serum samples (Rhyan, 2000). The present World Health Organization (WHO) guidelines recommend the confirmation of the RBPT by other assays such as serum agglutination tests (SAT) (Rhyan, 2000; Rhyan *et al*., 2001).

Complement Fixation Test (CFT): Due to its high accuracy, complement fixation is used as confirmatory test for *B. abortus, B. melitensis*, and *B. ovis* infections and it is the reference test recommended by the OIE for international transit of animals (Navarro-Martinez *et al*., 2001; Ohishi *et al*., 2003). However, this method has some disadvantages such as high cost, complexity for implementation, and requirement for special equipment and trained laboratory personnel. In addition, the test presents limitations with hemolysed serum samples or serum with anti-complement activity of some sera, and the occurrence of prozone phenomena (Moriyona *et al.*, 2004). Sensitivity of complement fixation ranges from 77.1 to 100% and its specificity from 65 to 100% (Paton *et al*., 2001; Ohishi *et al*., 2003).

Enzyme Linked Immunosorbent Assay (ELISA): Enzyme linked immunosorbent assay (Navarro-Martinez *et al*., 2001) is an excellent method for screening large populations for *Brucella* antibodies and for differentiation between acute and chronic phases of the disease (Araj, 2010). It is the test of choice for complicated, local or chronic cases particularly when other tests are negative while the case is under high clinical suspicion. It can reveal total and individual specific

immunoglobulins (IgG, IgA and IgM) within 4-6 hours with high sensitivity and specificity. In addition to the detection of immunoglobulin classes, ELISA can also detect *Brucella*-specific IgG subclasses and other *Brucella* immunoglobulins such as IgE (Araj, 2010).

ELISAs are divided into two categories, the indirect ELISA (iELISAs) and the competitive ELISA (cELISAs). Most iELISAs use purified smooth LPS as antigen but a good deal of variation exists in the anti-bovine Ig conjugate used (Saegerman *et al*., 2004). Most iELISAs detect mainly IgGs or IgG sub-classes. Their main quality is their high sensitivity but they are also more vulnerable to non-specific reactions, notably those due to Yersinia enterocolitica serotype O9 (YO9) infection. These cross-reactions seen in iELISAs motivated the development of cELISAs. The O-chain of the smooth LPS of *Brucella* contains specific epitopes that are not shared with the LPS of YO9. Therefore, by using monoclonal antibodies directed against specific epitopes of the *Brucella* LPS, the development of more specific cELISAs has been possible. These tests are more specific, but less sensitive, than iELISAs (Weynants *et al*., 1996; Nielsen *et al*., 2008). The OIE considers these tests "prescribed tests for trade" (Office International des Epizooties (Paris, 1997).

2.6. Treatment

Generally, treatment of infected livestock is not attempted because of the high rates of treatment failure, cost, (Nicoletti. 2013) and potential problems of residues to public safety when high doses of antimicrobials are used as chemotherapy. Man can be treated with a combination therapy of Doxycycline and Rifampicin antimicrobials, however, relapses may occur (Corbel, 2006).

2.7. Impact of brucellosis on production and public health

Brucellosis is a 'multiple burdens' disease with economic impacts attributable to human, livestock and wildlife disease (Perry and Grace 2009). Losses in animal production due to brucellosis can be of major importance, primarily because of the decreased milk production by aborting dairy animals; the common sequel of infertility increases the period between lactation,

and in an infected herd the average inter calving period may be prolonged by several months. This is of greatest importance in beef herds where the calves represent the sole source of income. A high incidence of temporary and permanent infertility results in heavy culling of valuable and some deaths occur as the result of acute metritis following retention of the placenta (Radostits *et al*., 2000). Studies on the economic production losses of bovine brucellosis are reasonably consistent across a range of production systems in Africa, with losses estimated at 6% to 10% of the income per animal (Mangen, 2002). At the end of the last century, economic losses for Argentina were estimated at US$60 million per year or US$1.20 per bovine when the prevalence was around 5% (Samartino, 2002).

International travel and the importation of different dairy products into *Brucella* free regions contribute to the ever-increasing concern over human brucellosis. Human brucellosis can be a very debilitating disease, although the case fatality rate is generally low; it often becomes sub-clinical or chronic, especially if not recognized early and treated promptly. All ages of human beings are susceptible, and even congenital cases have been recorded (FAO *et al.*, 2006). High risk groups include those exposed through occupation in contexts where animal infection occurs, such as slaughterhouse workers, hunters, farmers and veterinarians. In human the disease cost of treatment and absenteeism from work brings many economical impacts (FAO, 2003).

3. MATERIALS AND METHODS

3.1. Study Areas

The study was carried out from October 2017 to May 2018 in purposively selected three administrative zones and five towns of Oromia Region, central Ethiopia, namely North Shoa zone, Fiche town; East Shoa zone, Adama town; Arsi Zone, Asela town; Bale Zone, Bale Robe town and Goba town. The study areas were selected based on the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes Inc., 2010).

Fiche is a town in the central Ethiopia. It is the administrative centre of the North Shewa Zone of Oromia Region, which is located 112 km north of Addis Ababa. It is located along the main road of Addis Ababa to Bahir Dar. Fiche town has a latitude and longitude of 9°48′N 38°44′E and an elevation between 2,738 and 2,782 meters above sea level. Farmers in the vicinity of Fitche town use a mixed crop and livestock farming system. Moreover, Fitche and its surrounding have variable and yet representative agro-ecologies of the country. These agro-climatic zones are inhabited with different plant and animal species (Conway and McKenzie, 2007). A total of 194 dairy cattle from 22 small scale, one medium size and one large herd size dairy cattle of Holestein Friesian cross and local zebu breeds were sampled.

Adama city is located in Oromia Region, East Shewa Zone at a distance of 100 km from Addis Ababa. Its astronomical location is 8º44' North Latitude and 39º04' East Longitude at an elevation of 1712 meters above sea level. The city sits between the base of an escarpment to the west, and the Great Rift Valley to the east. From urban and peri-urban areas of Adama town, 26 smallholder intensive dairy farms consisting of 154 individual animals, where 106 animals from intensive and 48 animals from semi-intensive dairy farms were randomly selected for sampling. According to Woreda Livestock Development and Fishery Office data (unpublished data, 2017), the total livestock population is estimated as 119,650 cattle, 33,250 sheep, 17,295 goat, 2290 horses, 380 mules, 14650 donkey, 72270 chicken and 1915 bee hives.

Asella town is located in south east of Addis Ababa in the Arsi Zone of the Oromia Region which is about 175 kilometers from Addis Ababa. This town has a latitude and longitude of 7°57′N 39°7′E, with an elevation of 2,430 meters above sea level and a mean annual temperature of 22.5^{o}C (Arsi Zone Livestock Development and Fishery Office,2017, unpublished data). In Tiyo district there are 105,498 cattle population (Tiyo District Livestock Development and Fishery Office, 2018, unpublished data). Among 183 individual animals sampled, 127 cattle were from intensive and 56 were from semi-intensive farms. Accordingly, 9 small scale farms, 5 medium size farms and 1 large scale dairy farm were incorporated in the study.

Robe, more commonly known as **Bale Robe** (in order to differentiate it from other towns in Ethiopia which are also called Robe), is a town and separate woreda in south-central Ethiopia. Located in the Bale Zone of the Oromia Region, this town has a latitude and longitude of 7°7′N 40°0′E with an elevation of 2,492 meters above sea level. It is located about 430 kilometers by road from Ethiopia's capital Addis Ababa. The mean annual temperature of the district is 16.5°C whereas the minimum and maximum are 9 and 23°C, respectively. The mean annual rainfall is 1105 mm whereas the minimum and maximum are 1060 and 1150 mm, respectively (SDFED, 2006). This district has two rainy seasons where the main rainy season extends from July to October whereas; the short rainy season extends from February to April. Crop and livestock productions were interdependent framing systems in the district. The district has two crop growing seasons and is among the first four districts of the zone, which has large cattle population (BZFED, 2007). From urban and peri-urban areas of this town a total of 111 individual dairy cattle (29 from intensive and 82 from semi-intensive farms) from 20 small scale herds were randomly selected.

Goba town is located in the Bale Zone of the Oromia region in south-central Ethiopia and is approximately 446 km south east of Addis Ababa. This town has a latitude and longitude of 7°0′N 39°59′E and an elevation of 2,743 meters above sea level. According to Woreda Agriculture Office (WAO-unpublished source) data, the total cattle population of Goba district is estimated to be 49,841. From urban and peri-urban areas of Goba town 24 farms consisting of 161 individual dairy cattle of Holestein Friesian cross and local breeds were randomly selected

from 22 intensive and 2 semi-intensive farms. The livestock population in the district is 340,702 of which cattle is 210,445.

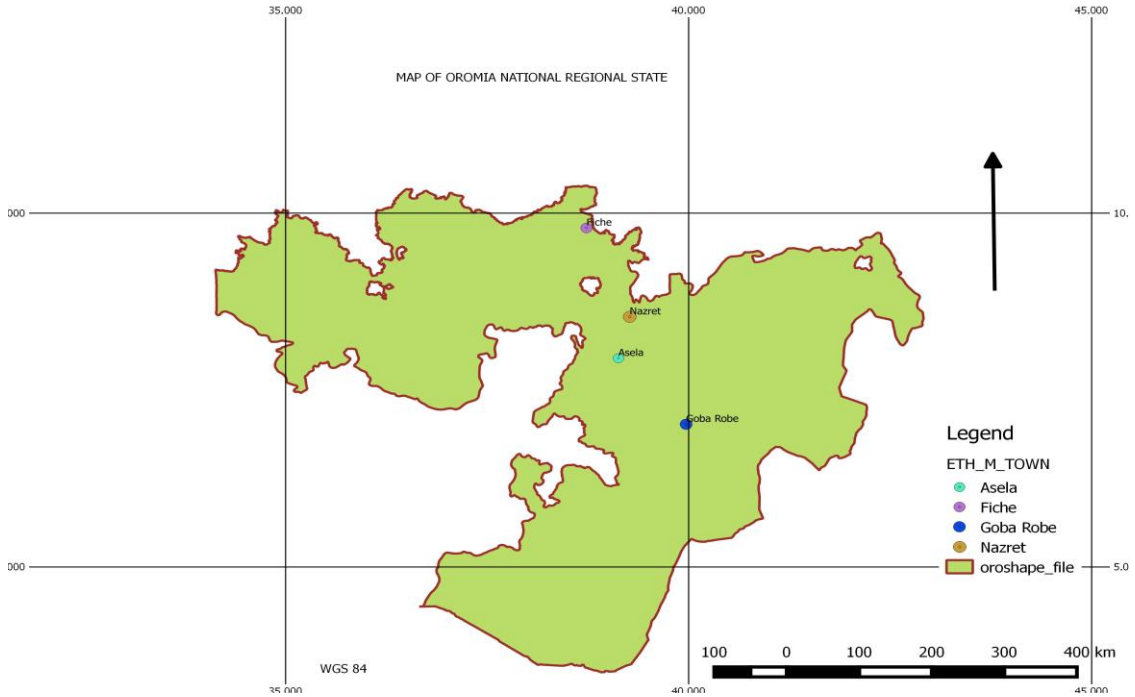

Figure 1. Map of the study areas

3.2. Study population, study design and sample size determination

The target populations were both smallholder, and commercial farms in urban and peri-urban areas of Fiche, Adama, Asella, Bale-Robe and Goba towns which were composed of Holstein Friesian crosses with local breeds and pure local zebu breeds established in the major milk sheds of the country (Asmare *et al*., 2013).

A cross-sectional study was conducted to determine the seroprevalence of brucellosis in dairy cattle using RBPT and c-ELISA, and to identify putative risk factors precipitating the

disease in the study areas. Relevant individual animal and farm level information were collected using a semi-structured questionnaire. The towns were purposively selected based on the abundance of dairy farms that constituting the known milk-sheds in the country (Land O'lakes Inc., 2010; Asmare *et al*., 2013). The sampling frame was prepared and a sampling unit was divided into groups, or strata based on herd size into three groups: small herd size (5-10), medium herd size (11-20) and large herd size (>20).

The number of farms to be sampled was calculated based on the formula described by Thrusfield, (2005), using exepected herd level prevalance of 10.6% by Asmare *et al*.(2013) and 95%CI and 5% desired precesion.

$$n = \frac{1.96^2 P_{exp}(1 - P_{exp})}{d^2},$$

Where; n= required sample size (number of farms); Pexp = expected prevalence and
d = desired absolute precision

Using the above formula the number of farms to be sampled was 145. However, the estimated number of farms was adjusted by the formula described by Thrusfield, (2005) because the population of farms from which the sample is to be drawn is small.

$$n_{adj} = \frac{N \times n}{N + n},$$

Where, **N** is the total number of farms in the population; **n** is calculated farms; $\mathbf{n_{adj}}$ is adjusted number of farms. Thus, n_{adj} = 258x145/258+145=93, but the number of farms sampled was increased to 110 in order to increase precision (Thrusfield, 2005). As a result all the farms were rondomly selected which was 99(38.4%) small scale; 13(5%) medium scale and 2(0.8%) large scale frms, respectivly. In oreder to caluclate animal level seroprevalance of brucellosis a total of 803 dairy cattle were sampled based on the willingness of the owners but individual animals

within the herds were selected using simple random sampling method except those cows with history of abortion or active case of abortion which were included purposively.

Lists of dairy farms were prepared for each of the 5 towns in collaboration with the respective district livestock health departments. Of the total animals (803) sampled, 87% were female and 13% male dairy cattle above six months of age were picked at random using individual farm based list.

Before sample collection, members of the target population were identified by constructing a list (the sampling frame). Thus a total list of 258 farms consisting of 55, 57, 53, 43 and 50 dairy farms from Fiche, Adama, Asela, Bale Robe and Goba towns respectively, were generated. Then, the study population was divided into strata based on different ranges of herd size (Thrusfield, 2007). There were 55 farms in Fiche town (51, 2, and 2 were from small, medium and large farms respectively). In Adama and Bale Robe towns there were 57 and 43 small farms, respectively. Among 53 farms in Asela town, 34, 16 and 3, small, medium and large farms were existed respectively. In Goba town also there were 42 small and 8 medium farms.

In the total 258 dairy farms; there were, 227, 26 and 5 dairy farms consisting of small, medium and large farms respectively. From the total 258 listed farms, 110 farms and 803 dairy cattle were randomly selected for this study.

The sampled farms were as follows: Fiche (23, 1, 1; small, medium and large farms respectively); Adama (25 small farms); Asela (6, 8, 1; small, medium and large farms respectively); Bale Robe (20 small farms) and Goba (21 small and 4 medium farms). Then individual dairy cattle were also selected randomly from each stratum of the selected dairy farms.

3.3. Questionnaire for risk factors assessment

Information regarding herd management practices and individual animals history for a survey of the potential risk factors at herd and individual animal level were extracted by face to face interviewing of the owners using semi structured questionnaire (Annex: 3). The type of

management system were categorized into intensive and semi-intensive, herd size was categorized into 5-10 animals (small herd size); 11 - 20 animals (medium herd size) and > 20 animals (large herd size) (Alehegn *et al*., 2016). Presence or absence of separate calving pen; replacement source; retained fetal membrane and aborted fetus disposal methods, use of personal protective equipment (PPE) during handling aborted cows retained fetal membrane and awareness of farmers on diseases causing abortion in late pregnancy (knowledge on brucellosis) were the risk factors considered at the herd level. While categories of sex, age, breed and occurrence of previous abortion and abortion stage (classified as first trimester, second trimester or third trimester); retained placentas of the relevant animal were the risk and clinical factors considered at animal level. Strategy of breeding was characterized by service types as artificial insemination (AI) or natural mating (bull) or both (natural mating and AI service), the replacement stock of each farm was defined as buy in or raise own replacement or both.

3.4. Sample collection and laboratory test

3.4.1. Serological blood sample collection

Blood samples of 10 ml were collected from the jugular vein of each animal, using sterile needles and plain vacutainer tubes. The blood samples were allowed to stand overnight at room temperature and centrifuged at 1500 × rpm for 10 minute to obtain the serum. The sera were decanted into cryovials, labeled and transported to Asella Regional Veterinary Laboratory (ARVL) stored at −20 $^{\circ}$C until been screened for antibodies against natural *Brucella* exposure using serological analysis, according to OIE, (2009).

3.4.2. Serological laboratory techniques

Rose Bengal plate test (RBPT): An antigen prepared from *Brucella abortus* (S99) stained with Rose Bengal dye and suspended in acid buffer (pH 3.65), was used to detect *Brucella* antibodies in serum -using plate agglutination test. It detects antibodies against *B.abortus, B.melitensis* and *B.suis* in serum samples. All sera samples collected were initially screened by RBPT using RBPT

antigen (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom), together with positive and negative sera were used according to Alton *et al.* (1975) procedures (Annex 1). Briefly, sera and antigen were taken from refrigerator and left at room temperature for half an hour before the test to maintain to room temperature and processed following the recommended procedures. Serum of 30µl was mixed with 30µl of antigen on a white tile or enamel plate to produce a zone approximately equal to 2 cm in diameter. The mixture was rocked gently for four minutes at ambient temperature and then observed for agglutination. Any visible reaction was graded positive and otherwise negative. The test was conducted in Asella Regional Veterinary Laboratory (ARVL), according to the procedure of OIE (2004). An animal was classified as positive if both the RBPT and the c-ELISA were positive. RBPT is a simple screening test, and its sensitivity and specificity values were 97.6% and 77.6%, respectively (Blasco *et al*., 1994).

Competitive ELISA: All RBPT positive sera were further tested using the SvanovirTM Brucella-Ab c-ELISA test kits (Svanova Biotech, Uppsala, Sweden) at NAHDIC. The test was performed according to the manufacturer's instructions. The test was conducted in 96-well polystyrene plate (Nalge Nunc, Denmark) that had been pre-coated with *Brucella* species lipopolysaccharide (LPS) antigen. Serum diluted 1:10 was added to each well followed by equal volume of pre-diluted mouse monoclonal antibodies specific for a common epitope of the *O*-polysaccharide of the smooth LPS molecule. The reactivity of the mouse monoclonal antibody was detected using goat antibody to mouse IgG that was conjugated to horseradish peroxidase. Hydrogen peroxidase substrate and ABTS chromagen was developed for 10 min. The reaction is then stopped using 1 M H_2SO_4. Optical densities read at 450 nm using Titertek Multiscan® PLUS reader (Flow laboratories, UK). The threshold for determining seropositivity was based upon the manufacturer's recommendations (>30 %), with antibody titers recorded as percentage inhibition as defined by the c- ELISA kit supplier.

3.5. Ethical clearance

All procedures were carried out according to the experimental practice and standards approved by the Animal Welfare and Research Ethics Committee at Addis Ababa University, College of Veterinary Medicine and Agriculture, that is in accordance with the international guidelines for animal welfare. A seven page request for explanation of the purpose of carrying out the studies and all possible care planned to reduce animal suffering due to sampling was given to the committee. After the committee evaluated the significance of this research, approval was given by *Ref No.VM/ERC/13/05/10/2018*).

3.6. Data analysis

Data generated from questionnaire survey and laboratory investigations were recorded and coded using Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). The seroprevalence was calculated as the number of seropositive samples divided by the total number of samples tested. To identify association of seropositivity with the potential risk factors (sex, age, breed type, herd size, separate parturition, abortion history, abortion period, parity, replacement source, breeding type, history of retained fetal membrane, RFM and aborted fetal disposal, practice of using personal protective equipment (PPE) and farmers awareness on brucellosis) was computed by Pearson's Chi-square test, univariable and multivariable logistic regression analysis. The 95% confidence interval and a significance level, at P= 0.05 was used.

4. RESULTS

4.1. Questionnaire survey

4.1.1. Socio-demographic characteristics of the respondents

The majority of the respondents' ages categorized between 30-50 years which accounts for about 50 % of the respondents who were participated in the study. The Interview result indicated that half of the respondents (50%) were uneducated, while the remaining 50% of the respondents were educated at different levels (Table 2). From a total 6 positive herds 83.3% (5/6) were owned by uneducated dairy farmers, but there was insignificant association (P>0.05) with the disease. The socio-demographic characteristics such as educational level and gender did not showed significant association with herd seroprevalence of bovine brucellosis (P>0.05), where as the age groups <30 years had herd seroprevalence of 66.7% having significant association (P=0.04) with brucellosis.

Table 2: Socio- demographic characteristic of the respondents using chi square analysis

Variables	Groups	Proportion of respondents		No. of positive herds		X^2	P- value
		n=110	%	n=6	%		
Educational level	Uneducated	55	50.0	5	83.3	7.3157	0.120
	Primary school	28	25.5	0	0.0		
	Secondary school	10	9.1	0	0.0		
	Vocational						
Age	<30	37	33.6	4	66.7	6.3483	0.042
	30-50	55	50.0	0	0.0		
	>50	18	16.4	2	33.3		
Gender	Male	65	59.1	2	33.3	1.7417	0.187
	Female	45	40.9	4	66.7		

4.1.2. Occupational risk and awareness among dairy cattle farmers about brucellosis

Among the total 110 farm owners, 85.5% (94/110) had no awareness on brucellosis with insignificant association with brucellosis. Few numbers of the farm attendants, 58.2% (64/110) seemed to be aware of the risk of zoonotic brucellosis transmission through consuming raw milk and meat, however 41.4% (46/110) of the respondents consume raw milk and meat, but did not have significant association with *Brucella* infection (P>0.05).

Though respondents practice of disposing retained fetal membrane and aborted fetus had no significant association (P>0.05) with brucellosis, nevertheless a small number of the small farm owners15.5% (17/110) bury fetal and aborted materials, while the majority, 85.9% (93/110) dispose the aborted fetus and fetal membrane to open environment or feed to dogs.

Some of participants were conducting parturition process [51.8% (57/110)] in their farms, however it had no significant association with *Brucella* infection (P>0.05), on the other hand 68.2% (75/110) of these farmers did not use basic personal protective equipments when assisting parturition procedure which had high significant association with brucellosis (P=0.001). Some of the respondents [31.8% (35/110)], handle aborted fetus and other aborted materials without precautions and protective clothes and it had significant association with brucellosis (P=0.001), see details on Table 3.

Table 3: Occupational risk and awareness among dairy cattle farmers about brucellosis

Variables	Groups	Small farms (n=99)	Medium farms (n=9)	Large farms (n=2)	X^2	P-value
Awareness about zoonotic brucellosis						
	Yes	17	4	1	4.9811	0.083
	No	88	5	1		
Habit of drinking raw milk or eating raw meat						
	No	58	4	2	2.1422	0.343
	Yes	41	5	0		
Use of personal protective equipment						
	Yes	26	7	2	14.4571	0.001
	No	73	2	0		
Assisted animals giving birth						
	No	50	3	0	2.8684	0.238
	Yes	49	6	2		
Handling of fetal and aborted material						
	Yes	26	7	2	14.4571	0.001
	No	73	2	0		
RFM and after birth disposal						
	Buried/ burn	14	3	0	2.6980	0.259
	Thrown or feed to dogs	85	6	2		

n=number of interviewed respondents

4.1.3. Association of herd level risk factors with bovine brucellosis seropositivity

According to interviewed respondents, majority of dairy farmers 90 % (99/110) used to replace with their stock from their own farms and 12.7% (14/110) of them used to substitute the stock from the market, with significant association with brucellosis ($P<0.05$). When breeding practices considered, 13.6% (15/110) of the farm owners engaged both natural mating and AI options with insignificant association with *Brucella* infection. Artificial insemination [(78.2%, (86/110)] was used for breeding in herds with Holstein cross breeds and bull service [8.2% (9/110)] was used in herds with local breeds with insignificant association with brucellosis ($P>0.05$).

Large herd sized farms were regarded as clean with good farm management practices, with insignificant association (P>0.05). Survey result of risk factors such as presence of maternity pens; cleaning of calving pen; and management systems of the farms are indicated in Table 4.

Table 3: Association of herd level risk factors with brucellosis

Variables	Groups	Small farms (n=99)	Medium farms (n=9)	Large farms (n=2)	X^2	P-value
Replacement source	Raise own replacement	35	0	0	9.8179	0.044
	Bought	14	0	0		
	Both	50	9	2		
Breeding strategy	AI	78	6	2	1.3161	0.859
	Bull	8	1	0		
	Both	13	2	0		
Maternity pen	No	86	6	1	4.4374	0.109
	Yes	13	3	1		
Cleaning of calving pen	Flushing with water	42	4	1	1.2394	0.872
	Disinfect with detergent	25	3	0		
	No sanitation service	32	2	1		

4.2. Seroprevalence of dairy cattle Brucellosis by c-ELISA test

4.2.1. Brucella seropositivity at animal level and associated risk factors

From a total of 803 cattle sera samples tested by RBPT, 1.6% (13/803) of the animals was seropositive. The RBPT positive sera samples were further tested using c-ELISA, accordingly, 1.4 % (11/803) of the animals were confirmed to be seropositive. From the 5 study areas, the highest brucellosis prevalence 0.9% (7/803) at individual animal level was recorded in Asela town. Consequently seroprevalence 0.3% (2/803) and 0.1 % (1/803) was recorded in Fiche and Adama towns respectively. The lowest prevalence 0% (0/111) was recorded in Bale Robe town, while 0.1% (1/803) was recorded in Goba town. Age showed statistically significant associations (P<0.05) with seropositivity of brucellosis. From tested animals, all seropositive were adult

females with an age of ≥ 17 months and 1.4% (11/803) prevalence. Higher *Brucella* seropositivity was observed in cross breeds 1 % (8/803) than local breeds 0.4% (3/803). It is observed that the prevalence of brucellosis 1.1 %(9/803) in dairy cows with parity (≥ 3) was higher than those with (≤ 2) parity 0.3 % (2/803) showing significant association (P<0.05). Cases with history of abortion has higher prevalence 0.9% (7/803)] than those without abortion cases 0.5% (4/803) and significant association with brucellosis infection is recorded (P=0.000). Brucellosis in late abortion cases (>5 month) was more prevalent 0.8% (6/803) than those with early abortion cases (<5 month) [0.6% (5/803), moreover cases with retained fetal membrane (RFM) were more prevalent than those without RFM with significant association (P<0.05).

Table 5: *Brucella* seropositivity at animal level and associated risk factors

Variables	Group	n	Positive	(%)	X^2	P-value
	Fiche	194	2	0.3	11.1315	0.000
Town	Adama	154	1	0.1		
	Asela	183	7	0.9		
	Bale Robe	111	0	0.0		
	Goba	161	1	0.1		
Age	Young	289	0	0.0	6.2707	0.012
	Adult	514	11	1.4		
Gender	Male	103	0	0.0	1.6411	0.200
	Female	700	11	1.4		
Breed type	Cross	734	8	1.0	4.9547	0.026
	Local	69	3	0.4		
Parity	No parity	333	0	0.0	23.3844	0.000
	1 – 2	294	2	0.3		
	≥ 3	176	9	1.9		
Abortion history	Absent	762	4	0.5	78.8569	0.000
	Present	41	7	0.9		
	No abortion	763	0	0.0		
Stage of abortion	< 5 month	24	5	0.6	148.0076	
	>5 month	17	6	0.8		0.000
RFM	No	777	5	0.6		
	Yes	26	6	0.8		

4.2.2. Brucella seropositivity at farm level and associated risk factors

The c-ELISA test showed that the overall farm level seroprevalence was 5.4% (6/110). *Brucella* seropositive farms in respective towns were, 1.8% (2/110) in Fiche, 0.9% (1/110) in Adama, 1.8% (2/110) in Asela, 0% (0/110) in Bale Robe and 0.9% (1/110) in Goba towns has been recorded, with high significant association (P=0.000).

The chi square analysis risk factors such as, study area, farm type and herd size were significantly associated (P<0.05) with bovine brucellosis, while farm location (urban and peri-urban),

replacement sources (homebred, purchased and both) and breeding strategy were not significantly associated (P>0.05) with dairy cattle brucellosis. *Brucella* infection was more prevalent [5.4% (6/110)] in intensive farms than semi-intensive [0% (0/110)] ones.

The seroprevalence variation in small, medium and large herd sizes was 0.9% (1/110), 2.7% (3/110) and 1.8% (2/110), respectively. By chi square analysis, farm location and source of replacement herds have no significant association (P>0.05) with brucellosis. For farms kept under urban and peri-urban production systems there was 2.7% seroprevalence in each farms, while home bred herds mixed with purchased animals without brucellosis pretest were more prevalent than those home bred herds. The highest herd level seroprevalence of 1.8% was recorded in Fiche and Asela towns, while the lowest 0%, prevalence was recorded in Bale Robe town (Table 6). Sero-positivity for *Brucella* infection in AI service used cows (3.6%) was prevalent than those natural (bull) mating (0%) and both AI and natural mating cows (1.8%).

Table 6: *Brucella* seropositivity at herd level and associated risk factors

Variables	Groups	No. of herds (n)	No. positive herds (n)	Prevalence	X^2	P-value
Origin	Fiche	26	2	1.8	37.5336	0.000
	Adama	25	1	0.9		
	Asela	15	2	1.8		
	Bale Robe	20	0	0.0		
	Goba	24	1	0.9		
Farm type	Intensive	53	6	5.4	6.8251	0.009
	Semi-intensive	57	0	0.0		
Farm location	Urban	75	3	2.7	0.9670	0.325
	Peri-urban	35	3	2.7		
Herd size	5-10	99	1	0.9	52.0228	0.000
	11-20	9	3	2.7		
	>20	2	2	1.8		
Replacement source	Home bred	35	0	0.0	5.2885	0.071
	Purchased	15	0	0.0		
	Both	60	6	5.4		
Breeding strategy						
	AI	86	4	3.6	2.4324	0.296
	Bull	9	0	0.0		
	Both	15	2	1.8		

4.3. Univariable logistic regression analysis of the variables associated with animal and herd level sero-positivity

The odds of large herd sizes were 29.3 times at higher risk (P=0.000) than small and medium herd sizes. There was no significant variation (P>0.05) between semi-intensive and intensive management systems. The odds of local breeds were 4.1 times at high risk of an infection compared to cross breeds. Bovine brucellosis was not significantly associated (p> 0.05) with AI serviced cows and naturally mated cows. There was a higher sero-prevalence (1.9%) in those older cows calved above or equal to three (≥3) calves than those younger cows calved less than or equal to two times (0.3%), with high significant association (P=000). The odds of older cows calved greater than or equal to three (≥ 3 calved) were about 18 times at higher risk to *Brucella* infection than those younger cows calved less than or equal to two (≤2 calved), with significant

association (P<0.05**),** details on Table 5. Seroprevalence of Bovine brucellosis was higher in animals with a history of abortion (0.9%) and retained fetal membrane (0.8%) than those without history of abortion (0.5%) and retained fetal membrane (0.6%), with a high significant association (P=0.000). The odds of animals with history of abortion were 39 times more likely to be seropositive than those without such history. Similarly, the odds of animals that had suffered from retained fetal membrane were 46.3 times at higher risk to *Brucella* infection than those without such history (Table 7).

Table **4:** Association between animal and herd characteristics and seropositivity of brucellosis assessed by univariate logistic regression analysis

Factor	Group	n	Positive	%	Univariate			
					OR	CI (95%)		P-value
Herd size	5-10	99	1	0.9	Ref.			
	11-20	9	3	2.7	5.59633	.780031	40.15086	0.087
	>20	2	2	1.8	29.24658	5.963508	143.432	0.000
Breed type	Cross	734	8	1.0	Ref.			
	Local	69	3	0.4	4.125	1.068707	15.92169	0.040
Parity								
	1 ≤ 2	294	2	0.4	1.133106	.0705599	18.19631	0.930
	≥ 3	176	9	1.1	17.89222	2.24798	142.4085	0.006
Abortion history	Absent	762	4	0.5	Ref.			
	Present	41	7	0.9	39.01471	10.8952	139.708	0.000
Stage of abortion	< 5 month	24	5	20.8	Ref.			
	>5 month	17	6	35.3	82.69091	21.9199	311.9442	0.000
RFM	No	777	5	0.6	Ref.			
	Yes	26	6	0.8	46.32	13.04569	164.4637	0.000

4.4. Multivariable logistic regression analysis of risk factors for *Brucella* seropositivity

Variables with a p-value <0.05 from univariable analysis were included in the final multivariable logistic regression model. Variables such as herd size breed type, parity, abortion history, stage of abortion and retained fetal membrane were subjected to the multivariable logistic regression model. Thus further selection of variables in the final model was based on stepwise backward elimination procedure. The final multivariable logistic regression model (Table 8) showed that the odds of large herd sizes were 21 times at higher risk exposed to *Brucella* infections than [(OR=21, 95% CI =3.5, 127.5)] small and medium herd sizes, with significant association (P<0.05). The odds of local breeds were 10.5 times at higher risk (OR=10.5, 95%CI=1.4, 76.5**)** than cross breeds for *Brucella* infection, with significant association (P<0.05). Association between Bovine brucellosis and abortion history of the cows was statistically significant (P=0.000) (Table 6) and the odds of presence of abortion history were 29.2 times at higher risk than those without history of abortion.

Table 5: Multivariable logistic regression model of risk factors for *Brucella* seropositivity in dairy cattle at individual (n =803) and farm (n=110) levels.

Variable	Level	Odds Ratio	[95% Conf. Interval]		P-value
Herd size	5-10	Ref.			
	11-20	11.69367	1.334015	102.504	0.026
	>20	20.99048	3.454907	127.5288	0.001
Breed	Cross	Ref.			
	Local	10.45457	1.429493	76.4594	0.021
Abortion history	Absent	Ref.			
	Present	29.19381	6.609939	128.939	0.000

5. DISCUSSION

The present study showed that the overall individual animal level seroprevalence of Bovine brucellosis determined with RBPT and c-ELISA were 1.6% and 1.4% respectively. The finding is comparable with the earlier reports of Asmare *et al.*, 2013 (1.9%) status of bovine brucellosis in Ethiopia with special emphasis on exotic and cross bred cattle in dairy and breeding farms; Tesfaye *et al.*, 2011 (1.5%) reported in Addis Ababa; Hailu *et al*., 2011 (1.4%); Asmare *et.al*., 2013 reported (1.9%) in Ethiopia, Geresu *et al.*, 2016 reported (1.4%) in Asela and Bishoftu towns and Meles and Kibeb, (2018) reported (1.0%) in Chencha district of Gomogofa zone. On the other hand, there were reports with a relatively higher seroprevalence of bovine brucellosis in other parts of the country, Berhe *et al*., 2007 (3.2%); Kebede *et al*., 2008 (11%); Hunduma and Regassa, 2009 (11.2%); Ibrahim *et al*., 2010 (3.1%); Hailesillasie *et al*., 2011(4.9%); Megersa *et al*., 2012 (8.0%); Tibesso *et al*., 2014 (4.3%); Asgedom *et al*., 2016 (2.4%) and Alehegn *et al*., 2016 (4.9%). The difference observed in prevalence could be due to variation in production systems and animal management. Since most of the previous authors who reported higher prevalence were studied in extensively managed herds, where cattle from several areas mingle at grazing or areas could be explained by better hygienic practices, separation of cows during parturition, cleaning and disinfection activities, culling of infected animals and/or the prevailing management differences between the intensive, semi-intensive and extensive production systems (McDermott and Arimi, 2002; Matope *et al*., 2011). There were lower reports of negative results by Alem and Solomon, 2002 who failed to find any positive reactors in intensive dairy farms in Addis Ababa area. Similarly Belihu, (2002) could not find positive reactors in intensive dairy farms in Addis Ababa area (n=747). There were also reports with a relatively lower seroprevalence of bovine brucellosis than the present finding in other parts of the country by Bashaitu *et al*., 2015 (0.2%) in Ambo and Debrebirhan and Pal *et al*., 2016 (0.8%) in small-scale dairy farms of North Shewa zone. Variations in the seroprevalence findings might be seen due to relatively better farm managements in these study areas than the present study.

The present univariable logistic regression analysis revealed that agro-ecology of the dairy cattle was not significantly associated ($p>0.05$) with brucellosis in dairy cattle; even though higher individual animal level seroprevalence result has been found at high land agroecological areas (1.5%) than the mid highland area (0.7%). The reasons for the variations in brucellosis seroprevalence among the study areas might be related to the difference in management practice performed in the two agro ecological study sites. At the onset of the dairy schemes in high lands, especially in Asella town, farm owners purchased *Bos taurus* cattle from market, but the screening of bought female breeder animals for brucellosis was not done due to limited availability of veterinary diagnostic services, however there is better practice of screening brucellosis before purchasing cattle in most parts of Adama dairy farms. According to the report of different studies, purchasing of cattle from commercial farms without screening for brucellosis increases the chances of contact with infected herds (Omer *et al.*, 2000; Reviriego *et al*., 2000; Muma *et al*., 2007b). This finding is in accordance with the findings of Berhe *et al.*, 2007 (3.2%) in which agro climate was significantly associated with brucellosis seropositivity in which low seroprevalence was reported in low land areas. Similarly, literature witnessed that the seroprevalence of brucellosis is lower in low land agro climate which is unsuitable for survival of *Brucella* microorganisms compared to high lands (Radostits *et al.*, 1994). But, the observation was not in agreement with those of the previous findings such as that of Jergefa *et al*., 2009; Geresu *et al*., 2016, in which the agro ecology was not significantly associated with seropositivity.

The final multivariable logistic regression model (Table 8) showed that animals kept in large (OR = 21, 95% CI = 3.5, 127.5) and medium (OR = 11.7, 95% CI =1.3, 102.5) herd sizes were more likely to be exposed to *Brucella* infection than those maintained in small herds. This finding was in line with that of Berhe *et al*., 2007; Muma *et al*., 2007a, b; Mekonnen *et al*., 2010; Megersa *et al*., 2011b; Geresu *et al*., 2016, indicated that herds of larger and medium sizes had higher seroprevalence of bovine brucellosis as compared to smaller herds. This can be explained by the fact that an increase in herd size is usually accompanied by an increase in stocking density, one of the determinants for exposure to *Brucella* infection especially following abortion or calving,

(Crawford *et al*., 1990). However, in contrary to this Kebede *et al*. (2008); Jergefa *et al*, (2009); Asmare *et al*. (2013) reported that the risks of seropositivity were independent of herd size.

In the present study even though there was no statistically significant association between the age groups at animal level seropositivity to brucellosis, seroprevalence of 1.4% was found among adult age groups whereas no *Brucella* seropositivity was observed in young age groups might be due to similar proper management across the herds irrespective of the ages in the study areas. Several previous reports have been indicated that higher seroprevalence of brucellosis in adult age group of cattle (Megersa *et al.*, 2011, Amenu *et al*., 2010; Geresu *et al*., 2016; Meles and Kibeb *et al***.,** 2018) which is similar to the findings of this study. Similarly, statistically significant difference of a higher seroprevalence in the adult age groups has been reported by many researchers (Kebede *et al*., 2008; Megersa *et al*., 2011b; Asmare *et al*., 2013). This could be explained by sexual maturity and pregnancy due to the influence of sex hormones and placental erythritol on the pathogenesis of brucellosis (Radostits *et al*., 2007). Besides, higher prevalence of brucellosis in older cattle can be attributed to the constant exposure of cattle over time to the agent. On the other hand, younger animals tend to be more resistant to infection and frequently clear infections, although latent infections could occur (Walker, 1999).

Among the potential risk factors considered in the present study, the breed of cattle was shown to have a significant effect on the serological prevalence rate of bovine brucellosis [(OR=10.5, 95%CI=1.4-76.5)]. In this study, breed prevalence of 1% (8/803) of the seropositive animals were cross-breeds while 0.4 % (3/803) were local breeds. This finding was contradicting with higher prevalence rates reported in indigenous cattle in Nigeria (32.2%), Junaidu *et al*. (2008). On the similar way, Kebede *et al*. (2008); Amenu *et al*. (2010); Yohannes *et al*. (2012); Asmare *et al*. (2013); Geresu *et al*. (2016), have been reported that seropositivity of *Brucella* infection was independent of the breed. Moreover, Radostits *et al.* (2000) reported as there is no association between the breed of cattle and the seroprevalence of bovine brucellosis. At the onset of the dairy schemes in Asella, large farm owner purchased local zebu breeds from market for breed improvement by AI, without screening test for brucellosis due to limited veterinary laboratory service. The practice of purchasing cattle from market without screening for

brucellosis together with other agro-ecological factors could partly explain the observed higher sero prevalence of local breed dairy cattle brucellosis as compared to cross breeds in Asella town. According to the report of different studies, purchasing of cattle from commercial farms or market without screening for brucellosis increases the chances of contact with infected herds (Omer *et al.*, 2000; Reviriego *et al*., 2000; Muma *et al*., 2007b).

Parity in this study was not found to be significantly associated with *Brucella* seropositivity in multivariable logistic regression analysis (Table 8). Though there is insignificant association between parity and brucellosis seropositivity, in the current study high seropositivity was observed in cows which gave birth above 2 calves. The seroprevalence of brucellosis in the pluriparous cattle of this study was in line with (Adugna *et al*., 2013; Asmare *et al.*, 2013) correspondingly parity status was not associated with animal level *Brucella* seropositivity.

Individual animal prevalence of abortion records were 35.3% and 20.8% after 5 month of pregnancy and before 5th month of pregnancy respectively. A previous history of abortion after 5th month of pregnancy was, as expected, significantly associated with sero-positivity ($p<0.05$). Many studies also reported supporting the above finding (Adugna *et al*., 2013; Alemu *et al*., 2014; Bashitu *et al*., 2015; Alehegn *et al*., 2016; Geresu *et al*., 2016; Yohanes *et al*., 2016; Meles and Kibeb, 2018). This could be explained by the fact that, erythritol sugar in the placenta and fetal fluid is elevated during gestation period. This stimulates the growth and multiplication of the bacteria in the reproductive organs (Walker, 1999). The bacterial load often reduced in months following calving and abortion until the next pregnancy (Coetzer *et al*., 2004; Radostits *et al*., 2007).

In the present study, a previous history of abortions and retained fetal membrane were significantly associated ($P<0.001$) with animal level brucellosis sero positivity (Tab 6, 7, 8). Animal level seroprevalence of abortion history, 0.9% and retained fetal membrane (RFM), 0.8% was recorded for the occurrence of previous abortion and RFM in the studied areas based on questionnaire survey. This could be explained by the fact that abortions or/and RFM are typical outcomes of brucellosis (Radostits *et al*., 1994). Other studies also showed a significant association between seropositivity and history of abortion and RFM (Berhe *et al*. 2007; Ibrahim

et al. 2010; Megersa *et al.* 2011b; Adugna *et al*. 2013; Alemu *et al*. 2014). In contrary to this finding, Kebede *et al*., 2008; Asmare *et al*. 2013 reported that *Brucella* infection is not associated with abortion history and RFM.

Different risk factors were studied in the present study, such as source of replacement stock, study area, farm type, age, parity, retained fetal membrane, knowledge/awareness and practices of the respondents on brucellosis, but none of these risk factors showed significant association with occurrence of bovine brucellosis. The positive reactor animals in this study were all females, but this does not mean that male animals were not tested. It had been reported that males are usually more resistant than female cattle (Bayemi *et al*. 2009; Muma *et al*. 2007). Even though statistically significant difference was not shown between these factors with *Brucella* seropositivity, lack of maternity pen enhances risk of exposure and maintenance of *Brucella* infection within the farm. Knowledge of diseases is a crucial step in the development of prevention and control measures (WHO, 2006). Thus based on information gathered from the respondents, very large gape was seen. Concerning knowledge on brucellosis (Tab. 2), most of the respondents from semi-intensive and extensive dairy farms were relatively did not understand the root of zoonotic disease transmission including brucellosis. Farmers lack of awareness about brucellosis, improper handling of aborted materials or after birth discharges might contributed to further spread of brucellosis in their livestock and expose the community to public health hazard (Megersa *et al*., 2011b). This low awareness is a limiting factor if control strategies are to be implemented, lack of knowledge on the causative agent, mode of transmission and prevention measures against brucellosis can be detrimental. It is therefore important to establish additional campaign in the study areas to enlighten the communities on the disease, risk factors and preventive measures, as well as control strategies particularly in both livestock and humans against brucellosis can be detrimental. The respondents were practiced calling veterinarians for their dairy cattle when they faced reproductive problems than others problems. This could be best practice due to its protective effectiveness but best segregation is more preferred for further protection of the herds.

Brucellosis is continues to be a major public and animal health problem in Ethiopia. However, there is no national control scheme in place because of economic reasons and lack of information about the disease status at national level (Megersa *et al*. 2011; Yohannes 2013). Therefore, it is necessary to devise and implement an integrated disease control approach involving inter-sectorial collaborative strategies between animal and human health sectors, nongovernmental and governmental institutions to minimize the burden of the disease.

The false-positive results in the RBPT are due to cross-reactions with other bacteria since none of them have been vaccinated against brucellosis. RBPT is highly sensitive test and could easily apply in field conditions whereas, c-ELISA is highly specific usually used as a confirmatory test method (Samui *et al*. 2007).

In the present study risk factors related to respondents' education level, age and sex were studied to see their effect on herd prevalence of brucellosis. It was noted that the overall effect of education level and gender groups were not significantly associated with the herd seroprevalence of brucellosis ($P>0.05$). Good knowledge about brucellosis has been shown to have a protective effect towards animal and human infections in Kyrgyzstan (Kozukeev 2006), however in the present study herd prevalence was not showed to have significant variation across the level of education of the respondents ($P>0.05$) however, age groups had a significant association with herd seroprevalence of bovine brucellosis ($P<0.05$). It might be reasoned out that young age groups have the energy to manage (supplying feed, watering, sanitation, e.t.c.) and can easily accept and exercise new technologies and provide veterinary services to their animals. The adult age groups have also indigenous knowledge and the experience to manage and respond to problems encountering their animals.

These factors combined with the poor cleaning practice by the owners could pose a great risk of spread of the disease to unaffected animals (Tolosa, 2004). The occurrence of brucellosis in humans is associated with contact with domestic animals exposure to aborted animals and assisting animal parturition (Kozukeev *et al*. 2006). In this study, some of the participants in [(small, 41.4% (41/99) and medium, 55.6% (5/9)] types of farms have the habit of drinking raw

milk or eating raw meat. Prevalence of brucellosis in humans is attributed to the culture and tradition of consuming raw milk and milk products (Omore *et al*. 1999).

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that the overall seroprevalence of bovine brucellosis at animal and herd level was 1.4% and 5.5% respectively in dairy cattle of Fiche, Adama, Asela, Bale Robe and Goba towns of central Ethiopia. In this study, we found a very low level seroprevalence at individual animal level. However, a high herd level prevalence was recorded in one intensive farm; indicating the low spread distribution of the disease in the area. Herd characteristics such as herd size, history of abortion and intensification were found to be important risk factors associated with occurrence of bovine brucellosis. Local zebu breeds were at high risk of acquiring *Brucella* infection as compared to cross breed animals. Lack of maternity/calving pen and poor awareness of the communities were important gaps observed during this study. Questionnaire survey to assess the farmers' knowledge, attitude and practices indicated that there were potential risk factors for acquiring the disease in humans such as handling of aborted fetuses and afterbirths, assisting during calving, and consumption of raw milk and milk products and meat capitalizing high risk of public health.

Therefore based on the present findings, the following recommendations are forwarded:

- The study result warrants the need of integrated intervention strategies to minimize the spread of the disease in animals and reduce the risk of transmission to humans.
- Strict pre-purchase test policy has to be advocated among the dairy farmers to prevent the entry of brucellosis into the disease free herds.
- Public education to improve the awareness, skill and attitudes among the farmers, farm keepers and the animal product consumers is important.
- Further studies on isolation and strain characterization should be undertaken and coordinated intensive epidemiological study has to be conducted to determine the disease status in other domestic animals and humans in the study areas.

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8. ANNEXES

Annex: 1 Rose Bengal Test Antigen for the Serological Diagnosis of Brucellosis

An antigen prepared from *Brucella abortus* (S99) stained with Rose Bengal dye and suspended in acid buffer (ph 3.65), used to detect *Brucella* antibodies in serum – using plate agglutination test. It detects antibodies against *B.abortus*, *B.melitensis* and *B.suis* in serum samples. The test was undertaken at Asela Regional Veterinary Laboratory

Procedure

Sera (control and test sera) and antigen for use were left at room temperature for half an hour before testing, since active materials straight from the refrigerator react poorly

1. 30 µl serum was mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
2. The antigen and serum were mixed thoroughly using an applicator stick (a stick being used only once)
3. The plate was rocked by hand for about 4 minutes
4. The tests were read by examining for agglutination in a good light
5. Magnifying glass was used to detect micro agglutination when suspected

Note: In order to standardize the reading, it is better to introduce as control for each series of analysis a positive control serum for Brucellosis and a known negative serum sample

Interpretation of results:

- Check that the results of controls are in accordance with the expected results.
- Samples showing presence of agglutination (even slight) are considered positive samples (presence of anti-*Brucella* antibodies)
- Samples showing no agglutination are considered negative samples (absence of anti-*Brucella* antibodies)

Annex 2: Competitive ELISA

All RBPT positive sera were further tested using the SvanovirTM *Brucella*-Ab c-ELISA test kits (Svanova Bio tech, Uppsala, Sweden) at NAHDIC. The test was performed according to the manufacturer's instructions. The test was conducted in 96-well polystyrene plate (Nalge Nunc, Denmark) that was pre-coated with *Brucella* species lipopolysaccharide (LPS antigen. Serum diluted 1:10 was added to each well followed by equal volume of pre-diluted mouse monoclonal antibodies specific for a common epitope of the O-polysaccharide of the smooth LPS molecule. The reactivity of the mouse monoclonal antibody was detected using goat antibody to mouse IgG that was conjugated to horseradish peroxidase. Hydrogen peroxidase substrate and ABTS chromagen was developed for 10 min. The reaction was then stopped using 1 M H_2SO_4. Optical densities was read at 450 nm using Titertek Multiscan ® PLUS reader (Flow Laboratories, UK).The threshold for determining seropositivity was based upon the manufacturers Recommendations (>30 %), with antibody titers recorded as percentage inhibition as defined by the ELISA kit supplier.

Procedure

1. The c-ELISA involved the adsorption of *Brucella* S-LPS antigen diluted in 0.06 M sodium carbonate buffer (pH 9.6) to polystyrene plates (Nunc 2-69620; Nunc, Roskilde, Denmark) at 100 ml/well.
2. The plates were incubated overnight (18 h) at 4°C and then washed four times in 0.01 Mphosphate-buffered saline containing 0.05% Tween 20 (pH 7.2) (PBS/T).
3. This step was followed by the addition of 95 ml of prediluted ascetic fluid containing mouse monoclonal antibody (MAb), diluted in 0.015 M EDTA–0.015 M EGTA in PBS/T (pH 6.3), specific for an epitope on the O-polysaccharide portion of the immobilized antigen, and the addition of 5 ml of undiluted control or test serum; the mixture was mixed gently for 5 min in a microplate shaker and incubated for 30 min at room temperature.

4. After this incubation period, the plate was washed four times in PBS/T, and goat anti-mouse immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (diluted in PBS/T) was added;

5. The mixture mixed gently for 5 min in a shaker and incubated for 30 min at room temperature.

6. The plate was washed four times.

7. The final step was the addition of 100 ml of chromogenic substrate [4.0 mM hydrogen peroxide and 1.0 mM 2,2 9-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) diammonium salt in 0.05 M citrate buffer (pH 4.5)] per well.

8. After 10 min of continuous shaking, the optical density (OD) was measured photometrically (Lab systems Multiscan EX microplate reader with a 414-nm filter) by use of 100 ml of chromogenic substrate in a plate to serve as a control for the microplate reader.

Principle

In the absence of anti *Brucella* antibody in the test serum, the MAb binds, resulting in color development. If the test is positive, the test serum competes with the MAb for the epitope sites, and inhibition of MAb binding is inversely proportional to subsequent color development.

Annex: 3 Questionnaire format used for interview purpose

I. Socio demographic characteristics of the respondents

1. Owner Name (Respondent): ……
2. Gender of the respondent: a) Female…….. b) Male ……..
3. Age (years): .....
4. Marital status: a) Single b) Married c) Widowed d) Divorced
5. Respondent's position in the farm (with respect the head):
   a) Farm manager…… b) Husband……. c) Wife …….. d) Member of the family…… e) Farm laborer…….
6. What is the highest level of education attained by the head of the respondent?

a) Primary school…….. b) Secondary school……c) Vocational

school……d) College/University……. e) No formal education……..

II. General characteristics of the farms, farm management, ways of disease exposure and introduction to the farms

7. Herd size …….
8. Farm location:
   a) Urban…….. b) Peri-urban …….
9. What type of farm management system do you have?
   a) Intensive ……. b) Semi-intensive……. c) Extensive ……
10. What type of mating service do you have for your animals?
    a) AI ……. b) Bull …… c) Both ……
11. What type of breed of cattle you reared at your farm?
    a) Local breed…… b) Exotic breed …… c) Cross breed ……..
12. Where do you get the replacement stock?
    a) Market …….. b) Raise own replacement ……. c) Both ……..
13. What is the level of your herd/farm hygiene?
    a) Good …….. b) Fair ……. c) Poor ……..
14. What types of housing system of the farm you use?
    a) Loose ……. b) tying …….. c) Both ……..
15. Are there separate parturition pen?
    a) Yes ……. b) No …….

III. Knowledge, attitude and practice on herd management and brucellosis

16. Do you know any zoonotic disease transmitted from animal to human through handling of infected animals and its products? Yes …… b) No ……..
    a) Do you know any zoonotic diseases that transmit through drinking raw milk or eating raw meat? a) Yes …….. b) No …….

16. Do you know any diseases that transmit during handling of delivery or abortion?

a) Yes ……. b) No ……..

17. Have you had cases of abortion in your herd in the past years?

a) Yes……. b) No ……..

18. What do you think that cause abortion in cows?

a) Infectious disease …….. b) Non-infectious disease …….

19. When did abortion occur?

a) 1st trimester …….. b) 2nd trimester …….. c) 3rd trimester ……

20. Have you ever heard of brucellosis

a) Yes …… b) No ……

21. How do you handle aborted material?

a) Burying ……. b) Open dump ……. c) Feed to dog …….. d) Others ……..

22. Do you use PPE during handling aborted cows and RFM?

a) Yes …….. b) No ……..

23. Do you separate cows during parturition?

a) Yes …….. b) No ……

Annex: 4 Questionnaire formats for serum sample collection to individual cattle

Format: Serum sample collection format for individual cattle:

Date……..

Region ……. Zone……. District/Town ……...

PA…….Sample type …… Preservatives used ……

Geo. Ref.: Longitude…….. Latitude ……. Altitude …… m.a.s.l.

No.	Owners name	Sex	Age	Breed	Herd size	Parity	Abortion history	Stage of abortion	History of RFM