



**ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY
MEDICINE AND AGRICULTURE**

**SEROEPIDEMIOLOGY OF BOVINE BRUCELLOSIS IN HORRO GUDURU
ANIMAL PRODUCTION AND RESEARCH CENTER, AND ITS SURROUNDINGS
WESTERN ETHIOPIA**

MSc THESIS

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**JUNE 2018
BISHOFTU, ETHIOPIA**

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ANIMAL PRODUCTION AND RESEARCH CENTER, AND ITS SURROUNDINGS
WESTERN ETHIOPIA**

MSc thesis submitted to the College of Veterinary Medicine and Agriculture of
Addis Ababa University in partial fulfillment of the requirements for the
degree of Master of Science in Veterinary Microbiology

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Date of Submission: June 15, 2018

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ACKNOWLEDGEMENTS

Above all, I praise the Almighty God for giving me the courage and strength in my life.

I would like to express my deepest gratitude to my academic advisors Dr Bedaso Mammo and Dr Gezahegne Mammo for their overall guidance and advices, sharing their knowledge and support from the beginning to the end of my thesis work.

My very great thanks go to my friend Dr Gemechu Gure and his wife W/ro Hilina Shimelis Mr Bikila Abdisa and his family, and Mr Yadesa Chala for their great support in all situations from the beginning to the end of my education. My great thanks also go to my colleagues those working with me in Wollega University Horro Guduru Animal Production and Research Center for their unreserved supports and advices.

It is also my pleasure to extend my gratitude to Finchaa and Guduru Veterinarian staff members for their technical and material support in realization of this study. Especially, I am highly indebted to Dr Hirpha Legese and Dr Kasahun Gurmessa Wollega University staff for their technical support from the beginning to the end of my education.

Much of the acknowledgment goes to my families, especially my elder brother obbo Adugna Garoma that he gave me chance to learn by losing his own and help me to learn from elementary to post graduate studies.

ABBREVIATIONS

AMOS	<i>Abortus, Melitensis, Ovis, Suis</i>
BCV	<i>Brucella</i> Containing Vacuole
CFT	Complement Fixation Test
DCs	Dendrite Cells
ELISA	Enzyme linked Immuno Sorbent Assay
FAO	Food and Agriculture Organization
G+C	Guanine and Cytocine
HG	Horroo Guduru
IFN γ	Interferon gamma
IL	Interleukin
kDa	kilo Delta
Mb	Mega bases (nucleotides)
OD	Optic Density
OIE	Office for International de Epizootic
OMP	Outer Membrane Protein
PCR	Polymerase Chain Reaction
POR	Prevalence Odds Ratio
RBPT	Rose Bengal Plate Test
S/R-LPS	Smooth/ Rough Lipopolysaccharide
SAT	Slighed Agultination Test
T4SS	Type four Secretary System
WHO	World Health Organization

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ABSTRACT

In Ethiopia, a number of zoonotic diseases are known to have public health and economic importance both in humans and animals. The present study has particularly given emphasis to brucellosis because of its morbidity and significance to the public health and the economy of the country. A cross-sectional study has been conducted in Horro Guduru Animal Production and Research Center and its surrounding, Western Ethiopia to estimate the seroprevalence of bovine brucellosis and its associated risk factors from December 2017 to March 2018. A total of 812 cattle from three stations (districts) were randomly selected for serology finding and questionnaire surveys from 102 respondents were collected using questionnaire format. All sample sera collected were screened by Rose Bengal Plate Test and positive samples were finally confirmed by competitive-Enzyme Linked Immuno Sorbet Assay and Complement Fixation Test. Out of 812 samples tested, an overall seroprevalence of 0.73 (95% CI: 0.241-3.461) was recorded. The higher seroprevalence, 4.41% (95% CI: 0.028-3.473) was observed around Fincha district as compared to Horro Guduru Animal production center (0.31%) and Guduru district (0.99%). A Chi-square computed statistical analysis indicated that origin ($\chi^2=7.951$; $P<0.05$), abortion history ($\chi^2=8.217$; $P<0.050$), retained fetal membrane ($\chi^2=36.47$; $P<0.001$) and abortion time ($\chi^2=9.756$; $P<0.05$) were the associate with *Brucella* infection in the study areas. Moreover, retained fetal membrane was statistically identified as a common risk factor by logistic regression (OR=30.47 ,95%CI) for brucellosis to observed in cattle. The respondents, indicated that only 38%, 18.18% and 30% of the farm owners in small, medium and large herd sizes responded, as they were aware of brucellosis, respectively. The risk assessed indicates that using raw milk for human consumption has significant zoonotic importance. In conclusion, the *Brucella* seropositivity detected in present study was low and risk factors for obtaining *Brucella* infection were present. Therefore, the result suggested that the need for further investigation and identification of the disease to take proactive measures, to protect the *Brucella* infection from economic effect and the risk of zoonotic infection in exposed human population in the study areas.

Keywords: *Bovine, Brucellosis, Horro Guduru, Risk Factor, Seroprevalence*

1. INTRODUCTION

Bacteria of the genus *Brucella* is recognized as one of the most wide spread and important pathogenic to animal and human in the world (Schelling *et al.*, 2003). In addition, it is the oldest and most known zoonotic, economically important disease of livestock causing reproductive wastage through infertility, delayed heat, loss of calves, reduced meat and milk production, culling and economic losses from international trade bans in tropics and subtropics (Mangen *et al.*, 2002).

Bovine brucellosis is a highly contagious, zoonotic and economically important disease, which usually caused by *Brucella abortus*; occasionally by *Brucella melitensis* and *Brucella suis*, and characterized by late term abortion, infertility and reduced milk production as a result of retained placenta and secondary endometritis and excretion of the organisms in uterine discharges and milk. Full-term calves may die soon after birth. In fully susceptible herds, abortion rates may vary from 30- 80% (Anonymous, 2007).

The genus *Brucella* consists of commonly known six classically recognized species (nomen species) based on antigenic/biochemical characteristics and primary host species: *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (swine, cattle, rodents, wild ungulates), *B. ovis* (sheep), *B. canis* from dogs and *B. neotomae* of rodents. More recently, other species have been recognized: *B. ceti* (cetaceans), *B. pinnipedialis* from seals, *B. microti* isolated from soil and *B. inopinata* single isolate from a human (Foster *et al.*, 2007; Scholz *et al.*, 2009).

Brucella species are facultative intracellular, Gram negative, non-spore-forming and partially acid-fast coccobacilli that lack capsules, endospores or native plasmids. They survive freezing and thawing but most disinfectants active against Gram-negative bacteria kill *Brucella*. Pasturization effectively kills *Brucella* in milk. The bacterium is of 0.5-0.7 μ in diameter and 0.6-1.5 μ in length. They are oxidase, catalase and urease positive. Although

Brucella species are described as non-motile, they carry all the genes except the chemotactic system necessary to assemble a functional flagellum (Fretin *et al.*, 2005).

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

Brucellae display strong tissue tropism and replicate within vacuoles of macrophages, dendritic cells (DCs) and placental trophoblasts. However, the pathogen has the ability to replicate in a wide variety of mammalian cell types, including microglia, fibroblasts, epithelial cells and endothelial cells (Allen *et al.*, 1998).

Brucellosis is recognized as a significant public health challenge, with major economic and financial burdens in countries where the disease remains endemic. In livestock, the disease typically manifests as reproductive failure, often through abortion or the birth of weak, infected off-spring. Complications in the genital organs and other parts of the body can also occur. Human clinical disease is characterized by severe flu-like illness, with a high fever that comes and goes (hence the name “undulating fever”), which may progress to a more chronic form with serious complications in joints (arthritis) or internal organs (heart failure). In this chronic, recurring form, humans can be so debilitated that they are no longer able to work and they become a health care burden on their families (Pappas *et al.*, 2006).

In cattle and other *Bovidae*, the mode of transmission is usually from animal to animal by contact following an abortion. Pasture or animal barn may be contaminated and the organisms are most frequently acquired by ingestion but also inhalation, conjunctival inoculation, skin contamination and udder inoculation from infected milking cups are other possibilities. The use of pooled colostrums for feeding newborn calves may also transmit infection. Sexual transmission usually plays little role in the epidemiology of bovine

brucellosis. However, artificial insemination can transmit the disease and semen must only be collected from animals known to be free of infection (Acha and Szyfers, 2001).

In Ethiopia, starting from 1970s brucellosis has been known as endemic disease and reported by different authors (Ibrahim *et al.*, 2010; Asgedom *et al.*, 2016 and Geresu *et al.*, 2016). The disease is highly prevalent in cattle, camels and small ruminants in lowland areas (Yimer. *et al.*, 2008). Also a large number of studies on bovine have been reporting individual brucellosis seroprevalence ranging from 1.1% to 22.6% in intensive livestock management systems (Tolosa *et al.*, 2010; Tesfaye *et al.*, 2011) and 0.05% -15.2% in extensive management systems (Degefa *et al.*, 2011; Megersa *et al.*, 2011). Isolation and identification of *Brucella* spp. in dairy cattle at farm level in the country reported recently indicate the importance of brucellosis in dairy cattle industry and potential public health implication for human population (Geresu *et al.*, 2016). Although livestock play an important vital role in the rural livestock keepers of western Ethiopia, mainly in present study area, research investigation that indicate the status of bovine brucellosis have not been done accordingly. This study was designed with the:

General Objective:

- To Investigate Seroepidemiology of bovine brucellosis in Horro Guduru Animal Production and Research Center, and Its surroundings Western Ethiopia

Specific Objective:

- To estimate the seropositive prevalence of bovine brucellosis in study area
- To assess risk factors associated with bovine brucellosis and its public health importance in study area.

2. LITERATURE REVIEW

2.1. Etiology

2.1.1. Taxonomy of *Brucella*

The genus *Brucella* resides within the family *Brucellaceae* (familyIII) with *Mycoplana* and *Ochrobactrum*, of the order *Rhizobiales* in the class *Alphaproteobacteria* of the phylum *Proteobacteria*. Analysis of 16S rRNA sequences places *Brucella* spp. as members of the alpha-2 subdivision of the *Proteobacteria*, along with *Ochrobactrum*, *Rhizobium*, *Rhodobacter*, *Agrobacterium*, *Bartonella*, and *Rickettsia* (Yanagi and Yamasato, 1993). Ten genome sequences representing five species of *Brucella* (*B. melitensis*, *B. suis*, *B. abortus*, *B. ovis*, and *B. canis*) are available and about 25 additional *Brucella* strains/species are being sequenced (Sriranganathan *et al.*, 2009).

2.1.2. *Brucella* genome and its biochemical characteristics

The genomes of the members of *Brucella* are very similar in size and gene make up. Each species within the genus has an average genome size of approximately 3.29Mb and consists of two circular chromosomes (Chromosome I, 2.11 Mb and Chromosome II, 1.18 Mb). The G + C content of all *Brucella* genome is 57.2% for Chromosome I and 57.3% for Chromosome II (Halling *et al.*, 2005). No *Brucella* species have been found to harbor plasmids naturally although they readily accept broad-host-range plasmid or genomic islands that relate to pathogenicity within its genome (Seleem *et al.*, 2008). In addition to lacking these two features, the genome also lacks many other genes that code for common virulence factors including capsules, fimbriae, exotoxins, cytolysins, resistance forms, antigenic variation, plasmids or lysogenic phages (DeIVecchio *et al.*, 2000).

The *Brucella* cell envelope is a three-layered structures namely an inner or cytoplasmic membrane, a periplasmic space and an outer membrane can be differentiated (Lapaque *et*

al., 2005). The outer cell membrane closely resembles that of other Gram-negative bacilli with a dominant lipopolysaccharide (LPS) component and three main groups of proteins. It contains lipopolysaccharide (LPS), proteins, and phospholipids. The major *Brucella* OMPs are group 2 porin proteins having 36-38kDa (Paulsen *et al.*, 2002); group 3 proteins contain 25-27 kD molecular mass and a lipoprotein covalently linked to peptidoglycan. Group 1 minor proteins have a molecular mass of 88 to 94 kDa (DeIVecchio *et al.*, 2000).

The species and biovars were classically differentiated on the basis of distinct host specificity and differential tests based on phenotypic characterization of lipopolysaccharide (LPS) antigens, phage typing, dye sensitivity, requirement for CO₂, H₂S production and metabolic properties. The most virulent zoonotic species, *B. melitensis*, *B. abortus* and *B. suis*, all have a smooth lipopolysaccharide (S-LPS) on their outer cell membrane. The S-LPS contains an O15 polysaccharide (OPS) that is chemically defined as a homopolymer of 4, 6-dideoxy-4-formamide- α -D-mannose, linked via 1, 2-glycosidic linkages (Nielson, 2002).

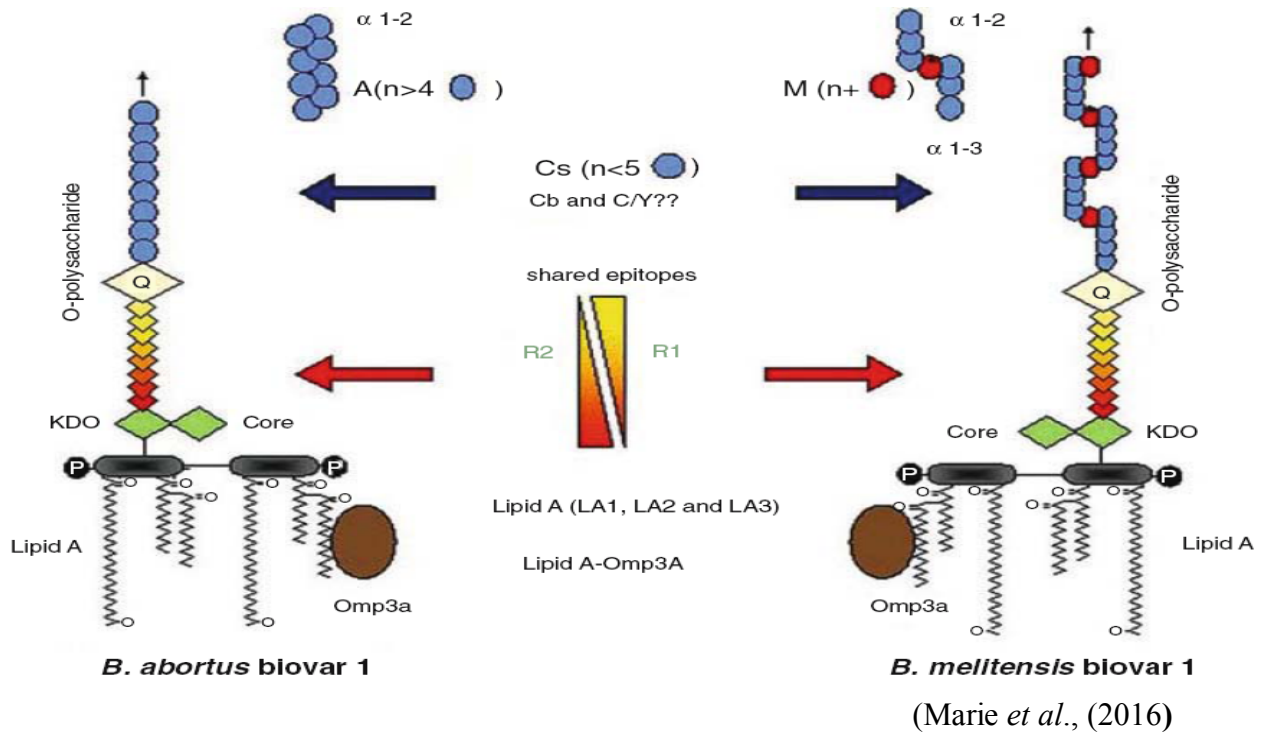


Figure 1: Simplified epitopic structure variation of the smooth LPS of *B. abortus* and *B. melitensis*.

2.1.3. Morphology and staining

Brucellae are coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is constant, except in old cultures where pleomorphic forms may be evident. *Brucella* spp are nonmotile, nonspores and no true flagella, pili or true capsules is produced. They are Gram negative and usually do not show bipolar staining, not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method (ROOP II. *et al.*, 1987).

The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of brucellosis. However, these methods have a low sensitivity in milk and dairy products where *Brucella* is often present in small numbers, and interpretation is frequently impeded by the presence of fat globules (OIE, 2016).

2.1.4. Growth requirement and cultural characteristics

All culture media should be subject to quality control and should support the growth of *Brucella* strains from small inocula or fastidious strains, such as *B. abortus* biovar 2. On suitable solid media, *Brucella* colonies can be visible after a 2–3-day incubation period. After 4 days' incubation, *Brucella* colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium (OIE, 2016).

Smooth (S) *Brucella* cultures have a tendency to undergo variation during growth, especially with subcultures, and to dissociate to rough (R) forms. Colonies are then much less transparent, have a more granular, dull surface, and range in colour from matt white to brown in reflected or transmitted light. Checking for dissociation is easily tested by crystal violet staining: rough colonies stain red/violet and smooth colonies do not uptake dye or stain pale yellow (OIE, 2016)

Table 1: Classification of *Brucella* species and *biovars*

Species	Biovar	CO ₂ Requiremen t	H ₂ S Productio n	Growth on Dyes ^a		Agglutination with Monospecific Sera		
				Thionin	Basic Fuchsin	A	M	R
<i>B. melitensis</i>	1	–	–	+d	+	–	+	–
	2	–	–	+	+	+	–	–
	3	–	–	+	+	+	+	–
<i>B. abortus</i>	1	+b	+	–	+	+	–	–
	2	+b	+	–	–	+	–	–
	3	+b	+	+	+	+	–	–
	4	+b	+	–	+c	–	+	–
	5	–	–	+	+	–	+	–
	6	–	–	+	+	+	–	–
	9	+ or –	+	+	+	–	+	–
<i>B. suis</i>	1	–	+	+	–e	+	–	–
	2	–	–	+	–	+	–	–
	3	–	–	+	+	+	–	–
	4	–	–	+	–f	+	+	–
	5	–	–		–	–	+	–
<i>B. neotomae</i>	–	–	+	–g	–	+	–	–
<i>B. ovis</i>	–	+	–	+	–f	–	–	+
<i>B. canis</i>	–	–	–	+	–f	–	–	+
<i>B. ceti</i>	–	–	–	+d	+	+	–f	–
<i>B. pinnipedi</i>	–	+	–	+	+	+	–f	–
<i>B. microti</i>	–	–	–	+	+	–	+	–
<i>B. inopinata</i>	-	-	+	+	+	-	+	-

^aDye concentration in serum dextrose medium:

^bUsually positive on primary isolation.

^cSome basic fuchsin-sensitive strains .

^dSome strains are inhibited by dyes.

^eSome basic fuchsin-resistant strains

^fNegative for most strains.

^gGrowth at a concentration

(Menachem B and Michael, 2010).

2.2. Pathogenesis of Brucellosis

2.2.1. Infection

The ability of *Brucella* spp. to cause disease requires a few critical steps during infection. *Brucella* spp. can invade epithelial cells of the host, allowing infection through mucosal surfaces: M- cells in the intestine have been identified as a portal of entry for *Brucella* spp. Once *Brucella* spp. has invaded, usually through the digestive or respiratory tract, they are capable of surviving intracellularly within phagocytic or non-phagocytic host cells. Then replicate within the phagocyte, release to circulation and colonization of the bacteria in multiple tissues, like lymphoid tissues, mammary gland and reproductive tract (Carvalho *et al.*, 2010).

2.2.2. Virulence

Brucella are intracellular organisms and their virulence appears to be related to their ability to survive and multiply within the macrophages. It eludes the bactericidal activities of macrophages by escaping from fused phagolysosomes into non-fused vacuoles in the cytoplasm. In addition to these survival mechanism is an important aspect of pathogenicity of *Brucella* is their ability to subvert the protective immune response. A characteristic feature of virulent strains of *Brucellae* (*B.melitensis*, *B.abortus* and *B.suis*) have smooth LPS, which inhibits the phagosome–lysosome fusion. In addition, S-LPS confer resistance to nitric oxide, free radicals and lysozyme, which are important antimicrobial mechanisms of macrophages and neutrophils (Franco *et al.*, 2007).

2.3. Immunity against *Brucella* infection

2.3.1. Humoral immune response

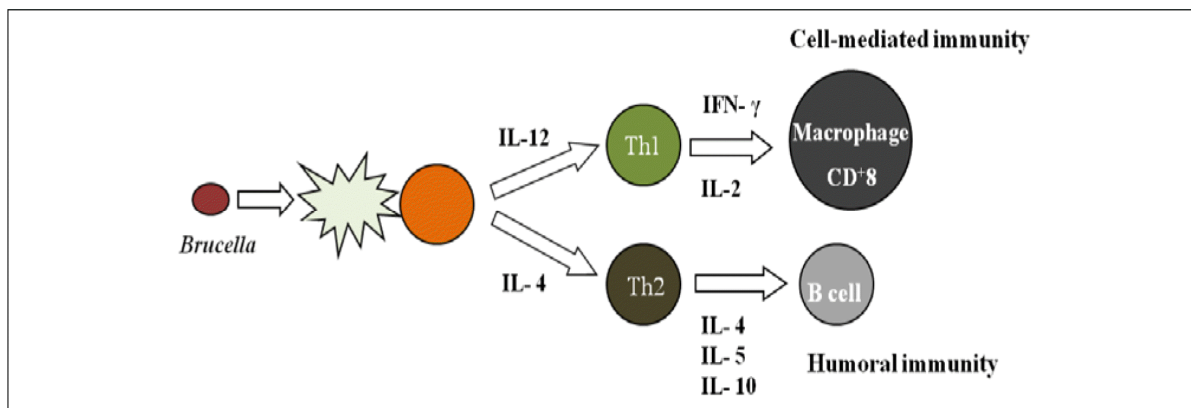
Naturally infected animals and those vaccinated as adults with strain 19 remain positive to the serum and other agglutination tests for long periods. The serum of infected cattle contains high levels of IgG1, IgG2, IgM and IgA isotypes of antibody. Similar isotypes at different relative concentrations occur in milk, although most of the IgA is present in secretory form. The first isotype produced after an initial heavy infection or strain 19 immunization is IgM and is soon followed by IgG antibody. IgG1 immunoglobulin is the most abundant in serum and exceeds the concentration of IgG2. The magnitude and duration of the antibody response following immunization is directly related to the age at immunization and the number of organisms administered (Tegegne and Crawford, 2000).

Following immunization with a standard dose of strain 19 during calf hood, IgG antibody decline to diagnostically insignificant levels over 3-6 months. Residual antibody if present, is usually predominantly of the IgM class. Following exposure to virulent *B. abortus*, antibody may appear in 4-10 weeks or longer, depending on the size and route of entry of the inoculum and the stage of pregnancy of the animal. Antibodies of IgM, IgG1 and IgG isotypes can all react in the tube agglutination but those of the IgM class are by far the most efficient (Tegegne and Crawford, 2000, and Alem and Solomon, 2002).

2.3.2. Cellular immune response

Brucella species are facultative intracellular pathogens. They are readily phagocytised by macrophages and polymorph nuclear leukocytes and, in the case of virulent strains, are capable of surviving within these cells and phagocytosis is promoted by antibody. However, since virulent *Brucella* can survive within normal macrophages for long periods, recovery from infection is likely to be dependent acquisition of increased bactericidal activity by phagocytic cells. Macrophage activation occurs when T-lymphocytes of the appropriate subset are stimulated to release lymphokines (interleukins) (Meyer, 1980).

The release of these activating factors is dependent upon recognition of the appropriate antigen by the T-lymphocyte and is subject to regulation through the major histocompatibility complex. Live organisms capable of establishing persistent intracellular infection and certain types of antigen, with or without adjuvant, are the most effective inducers of cell-mediated immunity. The role of cytotoxic cells, including cytotoxic T-lymphocytes, natural killer (NK) and killer (K) cells, in the cell-mediated immune response to *Brucella* has not been elucidated (Bekele *et al.*, 2000).



(Tegege and Crawford, 2000)

Figure 2: Humoral and cellular immune response of host body to *Brucellae*

2.4. Epidemiology of Bovine Brucellosis

2.4.1. Occurrence

The disease occurs worldwide, except in those countries where bovine brucellosis (*B. abortus*) has been eradicated. It remains endemic among Mediterranean countries of Europe, Northern and Eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America (FAO, 2003). Furthermore, brucellosis is also considered as a re-emerging problem in many countries such as Israel, Kuwait, Saudi Arabia, Brazil and Colombia, where there is an increasing incidence of *B. melitensis* or *B. suis* biovar 1 infection in cattle (Cutler *et al.*, 2005).

Brucellosis is endemic in many developing countries and is caused by *Brucella* species that affect man, domestic and some wild animals, and marine mammals (Geresu *et al.*, 2016). An estimated 500,000 new human *Brucella* cases were reported annually worldwide. Brucellosis is the second most important zoonosis after rabies and has gained prominence over the years since its discovery on the island of Malta (Seleem *et al.*, 2010 and Abubakar *et al.*, 2012).

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

2.4.2. Source of infection and mode of transmission

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

2.4.3. Risk factors

The risk factors can be categorized into those associated with characteristics of animal populations, management and the parasite biology (OIE, 2009).

2.4.3.1. Risk factors associated *Brucella* spp (Agent)

***Brucella* spp:** *B. abortus* is an important risk for the maintenance of the agent in the animal population with special importance in areas where wildlife and cattle rearing occur together. Moreover, infections in wildlife can hinder eradication efforts in cattle. *B. abortus* is still a human pathogen and outbreaks associated from infected cattle and also from ingesting contaminated dairy products represent an important risk of infection (Godfroid, 2002).

B. melitensis is the main etiological agent of brucellosis in small ruminants, although sheep can be also infected by *B. ovis*. Sporadic cases of brucellosis have been described in sheep and goats as *B. abortus* and *B. suis*. The dogs that guard the herds and flocks can also be infected (OIE, 2009).

In domestic pigs, risk factors associated with infection are ingestion of aborted fetuses, foetal membranes, abortion products and uterine discharges, or contaminated foodstuffs. Transmission during copulation is very common. Artificial insemination with contaminated semen or conjunctival mucosal should also be considered a risk. Other risk factors could be attributed to transmission of the disease by mechanical vectors due to contamination of vehicles, holding equipment or utensils and also to the introduction of infected offal (placenta and afterbirths) (AHAW, 2009).

2.4.3.2. Risk factors associated with host

Age: it has been referred to as one of the intrinsic factors associated with brucellosis. Higher sero-prevalence of brucellosis has been observed in older animals. Brucellosis has traditionally been considered a disease of adult animals since susceptibility increases after sexual maturity and pregnancy. However, variations in the age of sexual maturity among breeds could present differences between age and brucellosis positivity (Bekele *et al.*, 2011).

Brucella spp. presented a tropism to the reproductive tract due to the production of erythritol, a 4-carbon sugar produced in the foetal tissues of ruminants that stimulates the growth of *Brucella*. Thus, it may also explain the higher prevalence in adult animals than in young. On the other hand, a higher prevalence of brucellosis in adults has also been associated with longer contact with infected animals or with the environment. This potential risk may be significant in those herds without culling of positive animals (Bekele *et al.*, 2011).

Sex: Female ruminants presented a higher odd of brucellosis infection, the same has been observed in female dogs compared to male dogs. It could be associated with the intrinsic biology of the microorganisms and its tropism to the foetal tissue. Since brucellosis infection in males presented clinical signs such as epididymitis and orchitis, the prevalence in males could be lower than females because they may be culled faster. On the other hand, the absence of clinical signs such as abortion or metritis in non-pregnant infected females or

the absence of observation/ identification/ of abortions in extensive herds may also explain the higher prevalence in females (Coelho *et al.*, 2013).

Species and Breed: the prevalence of brucellosis in farm animals seems to be lower in small ruminants than large ruminants and lower in sheep than in goats. Transmission of brucellosis occurs in ruminants through the excretion of contaminated materials from the female genital tract, which constitutes the main form of transmission to other animals and humans. In most of the circumstances, the main route of spread is the placenta, foetal fluids and vaginal discharges expelled after delivery or abortion. At that time, large numbers of *Brucellae* are released (OIE, 2008).

The vaginal excretion of *Brucella* spp. in goats is greater and more prolonged than sheep, lasting for 2-3 months. In sheep, it is generally lower and normally ceases within 3 weeks after birth or abortion. The excretion of *Brucella* in milk is generally intermittent and usually only appears 6 to 12 days after the abortion. In goats, the excretion is more abundant and more prolonged, so there is an increased risk of infection via the consumption of milk from this species (Coelho *et al.*, 2013).

Host Range and *Brucella* Diversity: different *Brucella* species can affect the same livestock species and human. The principal strain that infects cattle is *B. abortus*, but also become transiently infected by *B. suis* and more commonly by *B. melitensis* when they share pasture or facilities with infected pigs, goats and sheep. *B. melitensis* and *B. suis* can be transmitted by cow's milk and cause a serious public health threat (Acha and Szyfres, 2003). The main etiologic agent of brucellosis in goats is *B. melitensis*. However in certain case where there is no *B. melitensis*, goats can get infected with *B. abortus* (Lilenbaum *et al.*, 2007).

Camels can be infected by *B. abortus* and *B. melitensis* when they are pastured together with infected sheep, goats and cattle. The main etiologic agent for dog brucellosis is *B. canis*, but sporadic cases of brucellosis in dogs caused by *B. abortus*, *B. suis* and *B. melitensis* have been reported (Acha and Szyfres, 2003) Table 2

Table 2: Risk Factors identified for epidemiology of brucellosis

Sr. no.	Factors	Reference
1	Age	Magona <i>et al.</i> ,2009; Bekele <i>et al.</i> , 2011
2	Breed	Jergefa <i>et al.</i> ,2009;Yohannes <i>et al.</i> ,2012
3	Parity	Musa, <i>et al.</i> ,1990
4	Cleaning and disinfection	Asgedom. <i>et al</i> , 2016
5	Agro-ecology/Climate	Muñoz. <i>et al.</i> ,2010
8	Contact with wildlife	Asgedom <i>et al.</i> ,2016
9	Education	Díez <i>et al.</i> ,2013
10	Handling of aborted material	Díez <i>et al.</i> ,2013
11	Management systems	Jergefa <i>et al.</i> , 2009; Asmare <i>et al.</i> , 2010. Regasa. <i>et al.</i> , 2004; Megersa <i>et al.</i> , 2011
12	Herd size	Crawford <i>et al.</i> ,1990,
13	Production system (beef /dairy)	Stringer. <i>et al.</i> ,2008
14	Milking procedures	Bekele, <i>et al.</i> ,2011
15	New entrance of animals	Mikolon, <i>et al.</i> ,1998:
16	Sex	Radostits <i>et al.</i> , 2007; Asgedom,2016
17	Specie	Acha and Szyfres, 2003; Lilenbaum <i>et al.</i> , 2007
18	Transhumance	Muna, <i>et al.</i> , 2007
19	Veterinary services	Muna, <i>e tal.</i> , 2007; Kabagambe. <i>et al.</i> 2001,
20	Water sources	Coelho. <i>et al.</i> ,2007; Aguiar. <i>et al.</i> , 2007
21	Culture	Omoro <i>et al.</i> ,1999
22	Stage of parturition	Haileselassie., 2008; Coetzer and Tustin, 2004 Radostits <i>et al.</i> , 2007.

2.5. Status of Bovine Brucellosis in Ethiopia

Ethiopia, located in Eastern Africa, is predominantly an agrarian country with over 85% of its population engaged in agricultural activity. Since the first report of brucellosis in the 1970s in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Ibrahim *et al.*, 2010; Kebede *et al.*, 2008 and Geresu *et al.*, 2016). A large number of studies on bovine have been reporting individual brucellosis seroprevalence ranging from 1.1% to 22.6% in intensive livestock management systems (Tolosa *et al.*, 2010 and Tesfaye *et al.*, 2011) and 0.05% -15.2% in extensive management systems (Degefa *et al.*, 2011 and Megersa *et al.*, 2011).

These prevalence studies in animals and human were largely confined to serological surveys and commonly targeted on bovine brucellosis, occasionally sheep and goats and rarely camels. Higher individual bovine brucellosis seroprevalence has been recorded in extensive managed cattle herds as compared to those in the intensively management system. In Borena Zone of Oromia Region, the highest seroprevalence (50%) was documented using ELISA in Didituyura Ranch (Alem and Solomon, 2002). A seroprevalence of 39% was also recorded at the Institute of Agricultural Research in Western Ethiopia (Meyer, 1980), 22% in dairy farm in Northeastern Ethiopia (Tariku, 1994), 11 to 15% in dairy farms and ranches in Southeastern Ethiopia (Bekele *et al.*, 2000), 8.2% in Arsi area (Molla, 1989), 8.1% in dairy farms in and around Addis Ababa (Asfaw *et al.*, 1998) and 7.7% in Tigray region (Haileselassie *et al.*, 2010).

Relatively low individual animal seroprevalence were recorded in some intensive farms in different parts of the country. Kassahun *et al.* (2007) documented 2.46% in Sidama Zone of Southern Ethiopia, Mussie *et al.* (2007) reported a prevalence of 4.63% in Northwestern part of Amhara Regional State. According to these authors, the reasons for the low prevalence of bovine brucellosis in these study areas were explained by better hygienic practices, use of maternity pen and/or separation of cows during parturition, cleaning and disinfection activities, culling of infected animals, depending on own herds for replacing stock and farm owners knowledge of brucellosis in these intensive farms.

In contrast to the above reports, Alem and Solomon (2002) and Belihu (2002) failed to find any seroreactive cattle after screening 564 animals in Eastern and Western Showa zones of central Ethiopia using rosebengal plate test (RBPT), serum agglutination test (SAT) and complement fixation test (CFT). Similarly, Belihu (2002) could not find positive reactors in intensive dairy farms in Addis Ababa area. Short summary in table (3) below.

Table 3: Seroprevalence of bovine brucellosis based on survey studies published in Ethiopia

Study Area	DiagnosticTests	Authors	Prevalence
Jimma zone	RBPT,CFT,	Tolosa, <i>et al.</i> ,2008	0.77
Ambo and Debrebrian	RBPT,CFT	Bashitu <i>et al.</i> ,2015	0.2
Arsi Negele	RBPT, CFT	Amenu <i>et al.</i> ,2010	2.6
Addis Ababa	RBT,CFT, SAT	Alem and Solomon, 2002	No +ve
Borena	RBPT,CFT	Alem and Solomon, 2002	50
Wuchale-jida	RBPT,CFT	Kebede <i>et al.</i> ,2008	11.0
Hawassa	RBPT,CFT,	Abebe <i>et al</i> 2010	3.9
Sidamo	RBPT,CFT,	Asmare <i>et al.</i> ,2010	1.66
Tigray	RBPT,CFT	Haileselassie <i>et al.</i> ,2010	7.7
Pastoral and mixed farming	RBPT CFT	Megersa <i>et al.</i> ,2011	26.1
East Wollega zone	RBPT,CFT	Yohannes <i>et al.</i> ,2012	1.97
Benishangul Gumuz	RBPT,CFT	Adugna <i>et al.</i> ,2013	1
Somali and Oromia	RBPT,CFT	Gumi <i>et al.</i> ,2013	0.9
Central Ethiopia	RBPT,CFT	Alemu <i>et at.</i> ,2014	2
Adami Tulu	RBPT,CFT	Tibesso <i>et al.</i> ,2014	4.3
East showa	RBPT,CFT	Geresu <i>et al.</i> ,2016	1.4

2.6. Zoonotic Importance of Bovine Brucellosis

Brucellosis remains amongst the most normally disregarded zoonotic diseases worldwide. The true incidence of brucellosis in human and animals worldwide is obscure and the occurrence is expanding in low and middle income nations like Ethiopia. The bacterial pathogen is considered by US Centers for Disease Control and Prevention (CDC) as a category (B) pathogen that has potential for improvement as a bio-terrorism weapon with a capability of airborne transmission (Seleem *et al.*, 2010).

Brucellosis primarily affects livestock, but can be transmitted to humans by ingestion, close contact, inhalation or accidental inoculation. The prevalence of human brucellosis differs between areas and has been reported to vary with standards of personal and environmental hygiene, animal husbandry practices, species of the causative agent and local methods of food processing (Chugh, 2008).

The brucellosis 2003 International Research Conference estimated that 500,000 human infections occur per year worldwide, with incidences ranging from less than one case per 100,000 population in UK, USA and Australia, through 20 to 30 cases per 100,000 in southern European countries such as Greece and Spain, to more than 70 cases per 100,000 in Middle Eastern States such as Kuwait and Saudi Arabia (Cutler and Whatmore, 2003; and Pappas *et al.*, 2006).

As compared to study of animal brucellosis, study of human brucellosis in Ethiopia is sparse with even less information on risk factors for human infection. For instance, out of 56 cases with fever of unknown origin, two (3.6%) were reported to be positive for *B. abortus* antibodies by RBPT and CFT (Tolosa *et al.*, 2007). A study conducted in traditional pastoral communities by Regassa *et al.* (2009) using *B. abortus* antigen revealed that 34.1% patients with febrile illness from Borena, 29.4% patients from Hammer and 3% patients from Metema areas were tested positive using *Brucella* IgM/IgG Lateral Flow Assay.

The seroprevalence studies conducted in high risk group such as farmers, veterinary professionals, meat inspectors and artificial insemination technicians were reported 5.30%, (Mussie *et al.*, 2007), 3.78% and 4.8% by Kasahun *et al.*(2007) and (2006) in different region of Ethiopia from individuals humans.

Table 4: Summary of humans tested for brucellosis in Ethiopia based on literature

Study Area	Prevalence	Reference
Jimma zone	3.6	Tolosa, <i>et al.</i> , 2007
Hawasa	3.78	Kasahun <i>et al.</i> , 2007
Addis Ababa	4.8	Kasahun <i>et al.</i> ,2006
Borena	34.1	Regasa <i>et al.</i> ,2009
Amhara region	5.3	Mussie., <i>et al.</i> 2007
Oromiya	2.15	Tibeso <i>etal.</i> ,2014
South Gonder	3.0	Regasa <i>et al.</i> , 2009
Yabello oromiya	10.0	Yohannies 2012
Hammer	29.4	Regasa <i>et al.</i> ,2009
Jimma zone	2.1	Bashahun <i>et al.</i> ,2016

2.7. Economic Impact of Bovine Brucellosis

Food and Agriculture Organization of the United Nations (FAO) and the Organization of Animal Health (OIE) consider brucellosis not only as direct public health implications, but also poses a barrier to trade of animals and animal products and has a wide socioeconomic impacts especially in countries where people in rural areas rely to a large extent on livestock breeding and dairy products as a source of income (Fitch, 2003).

The economic loss from brucellosis in developed countries arises from the slaughter of cattle herds that are infected with brucellosis and all the cost of eradication and control program. In developing countries farmers suffer from the actual abortion of calves and the decreased in milk yield, birth of weak calves that die soon after birth, retention of placenta, impaired fertility and sometimes arthritis or bursitis and all the cost of tests and samples, death may occur as a result of acute metritis (Radostits, 2000).

The estimation of the financial loss caused by brucellosis depends mainly on the type of cattle farming, herd size, and loss in reproduction in meat and milk due to abortion. The infected non aborting dairy cows produced 10% below potential and the aborted ones at 20%. The percentage of abortion in infected cows annually is 10-35% (FAO, 2003).

The economic impact of brucellosis varies from country to country and from region to region. In Latin America annual losses were estimated at \$600 million and the losses for Argentina were estimated at US\$ 60 million per year or US\$1.20 per bovine considering prevalence around 5%. In the U.S.A. the cost of abortion and reduced milk production in 1952 alone were put at \$400 million and in Nigeria losses were estimated at US\$ 575,605 per year or US\$3.16 per bovine based of prevalence rate ranging between 7% - 12% (Seleem, 2010).

Generally the economic losses of bovine brucellosis to be: losses due to abortion in the affected animal population, diminished milk production, *Brucella mastitis* and contamination of milk, cull and condemnation of infected animals due to breeding failure,

endangering animal export trade of a nation, human brucellosis causing reduced work capacity through sickness government costs on research and eradication schemes and losses of financial investments (FAO, 2002).

2.8. Diagnosis of Brucellosis

There is no single test by which a bacterium can be identified unequivocally as *Brucella*. Accordingly, for a definitive identification, a combination of growth characteristics, serological, bacteriological or molecular methods is required (Alton *et al.*, 1988 and FAO, 2002). 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 and Zinstag *et al.*, 2005).

Isolation of *Brucella* from the animal or human host and identification of the species offers a definitive diagnosis of brucellosis. However, culture is not performed regularly due to the risks it poses to laboratory personnel, the difficulty in growing the organism and the low bacterial load in the collected specimens. To circumvent these difficulties, various serological tests have been developed to identify antibodies against *Brucella* species. Although, there are still some limitations to the use of serological assays. All serological assays for brucellosis show variable levels of cross-reactivity with other Gram-negative bacteria such as *Yersinia enterocolitica*, *E coli*O:157, *Franciscella tularensis*, etc. This is because some of the structural elements within the immuno dominant *Brucella* oligopolysaccharide antigen are shared with these other bacterial species (Al-Dauho *et al.*, 2013).

2.8.1. Direct detection of the causative 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 (Alton *et al.*, 1988).

Cultural examination: For the diagnosis of animal brucellosis by cultural examination, the choice of samples usually depends on the clinical signs observed. The most valuable samples include vaginal secretions (swabs), aborted fetuses (stomach contents, spleen and lung), fetal membranes, and milk, semen and arthritis or hygroma fluids. From animal carcasses, the preferred tissues for culture are those of the reticulo-endothelial system (i.e. head, mammary and genital lymph nodes and spleen), the pregnant or early post-parturient uterus, and the udder. Growth normally appears after 3–4 days, but cultures should not be discarded as negative until 7–10 days have elapsed (OIE, 2016).

Growth and Colony Morphology on Culture Media: *Brucella* spp Culture requires a bio-safety level three laboratory (World Organization for Animal Health, 2008), which makes the ability to culture samples prohibitive. Direct isolation and culture of *Brucella* are usually performed on solid media which is most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. A wide range of commercial dehydrated basal media is available, such Tryptose Soy Agar (TSA), blood agar base (Oxoid) or Columbia agar serum–dextrose agar (SDA) or glycerol dextrose agar, can be used (Alton *et al.*, 1988).

A nonselective, biphasic medium, known as Castañeda's medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is usually advised. Castañeda's medium is used because *brucellae* tend to dissociate in broth medium, and this interferes with biotyping by conventional bacteriological techniques (Angus & Barton, 1984).

The most widely used selective medium is the Farrell's medium, which is prepared by the addition of antibiotics to a basal medium. Farrell's medium, have inhibitory effect on some *B. abortus* and *B. melitensis* strains. Therefore, the sensitivity of culture increases significantly by the simultaneous use of both Farrell's and the modified Thayer–Martin medium (OIE, 2016).

All culture media should be subject to quality control and should support the growth of *Brucella* strains from small inocula or fastidious strains, such as *B. abortus* biovar 2. On suitable solid media, *Brucella* colonies can be visible after a 2–3-day incubation period. After 4 days' incubation, *Brucella* colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium (OIE, 2016).

Smooth (S) *Brucella* cultures have a tendency to undergo variation during growth, especially with subcultures, and to dissociate to rough (R) forms. Colonies are then much less transparent, have a more granular, dull surface, and range in colour from matt white to brown in reflected or transmitted light. Checking for dissociation has been easily tested by crystal violet staining: rough colonies stain red/violet and smooth colonies do not uptake dye or stain pale yellow (OIE, 2016).

Staining Property of *Brucella* spp: *Brucellae* is coccobacilli or short rods measuring from 0.6 to 1.5 μm long and from 0.5 to 0.7 μm wide. They are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. *Brucella* spp are nonmotile, nonspores and no true flagella, pili or true capsules is produced. They are Gram negative and usually do not show bipolar staining, not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method (ROOP II. *et al.*, 1987).

The presence of intracellular, weakly acid-fast organisms of *Brucella* morphology or immuno-specifically stained organisms is presumptive evidence of *brucellosis*. However, these methods have a low sensitivity in milk and dairy products where *Brucella* is often present in small numbers, and interpretation has been frequently impeded by the presence of fat globules (OIE, 2016).

Biochemical test: Identification of *Brucella* strains using different biochemical tests like oxidase activity, urease activity, H₂S production, Dye tolerance (basic fuchsin and thionin) and sero-agglutination. 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 (Mantur *et al.*, 2006).

2.8.1.1. Molecular identification (Biotyping) of *Brucella* spp

Traditional methods of bacterial identification rely on phenotypic identification of the causative organism using Gram staining, culture and biochemical methods. However, these methods of bacterial identification suffer from two major drawbacks. First, they can be used only for organisms that can be cultivated in vitro. Second, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species. In the past decade or so, molecular techniques have proven beneficial in overcoming some limitations of traditional phenotypic procedures for the detection and characterization of bacterial phenotypes. Several non-culture based methods have emerged in the past 15 years (OIE, 2008).

Polymerase Chain Reaction (PCR) techniques are also very useful tools for differentiating *Brucella* spp, especially follow-up testing of unusual phenotypic results of *Brucella* isolates. This technique is fast and can be performed on any clinical specimen. A number of nucleic acid sequences have been targeted for the development of *Brucella* genus-specific PCR assays, including 16S rRNA, the 16S-23S (Navarro *et al.*, 2002).

Multiplex PCR typing: Several multiplex PCRs which identify the genus *Brucella* at the species level and partly at the biovar level using different primer combinations. The first multiplex PCR, called AMOS PCR assay (AMOS is an acronym from “*Abortus-Melitensis-Ovis-Suis*”), comprised five oligonucleotide primers for the identification of selected biovars of four species of *Brucella*. The assay exploited the polymorphism arising from species-specific localization of the genetic element IS711 in the *Brucella* chromosome.

Identity was determined by the size of the product amplified from primers hybridizing at various distances from the element. This method could identify three biovars (Ashford *et al.*, 2004) of *B. abortus*, all three biovars of *B. melitensis*, all *B. ovis* biovars and biovar 1 of *B. suis*. An abbreviated multiplex AMOS PCR assay based on three additional primers was developed to differentiate *B. abortus* vaccine strains S19 and RB51 from field strains (OIE, 2008).

Real-Time PCR: Real-time PCR is more rapid and more sensitive than conventional PCR. It does not require post amplification handling of PCR products, thereby reducing the risk of laboratory contamination and false-positive results. Real-time PCR assays have been recently described in order to test *Brucella* cells, urine, blood, and paraffin-embedded tissues. Three separate real-time PCRs were developed to specifically identify seven biovars of *B. abortus*, three biovars of *B. melitensis* and biovar one of *B. suis* using fluorescence resonance energy transfer. The upstream primers used in these real-time PCRs derived from the insertion element, IS711 whereas the reverse primer and FRET probes are selected from unique species or biovar-specific chromosomal loci (Nielsen, 2002).

High Resolution Melt: The development of a molecular technique which utilizes real-time PCR followed by High-Resolution Melt (HRM) curve analysis to reliably type members of this genus has been described by Winchell *et al.*, (2010). The assay targeted discriminating loci within the genomes of *Brucella* spp and through the dissociation curve analysis allowing the accurately identification of *Brucella* isolates at the species level and of unusual. This assay also proved successful for discriminating *B. suis* from *B. canis*, but was unable to accurately differentiate a *B. suis* bv4 from *B. canis*. However, this particular *B. suis* biovar has previously been reported to exhibit a genotypic pattern identical to *B. canis*, and it is still debated as to whether this is truly a unique biovar of *B. suis* (Whatmore *et al.*, 2007 and Huynh *et al.*, 2008).

Restriction Fraction Length Polymorphism (RFLP) based approaches: Recently, PCR-RFLP has provided evidence of polymorphism in a number of genes including the outer membrane protein 2 (omp2), the heat shock protein, and the erythrose-1-phosphate

dehydrogenase gene. Particularly, the DNA polymorphism in omp2a, omp2b, omp25 and omp31 has been found to be useful for the differentiation between the *Brucella* species and their biovars, including the marine mammal *Brucella* isolates. Results of PCR-RFLP allowed to identify in omp25 a marker for *B. melitensis* in the form of absence of an EcoRV site though *B. suis* biovars 3 and 4 and *B. canis* could still not be distinguished. Other omp genes examined include omp31, known to be deleted in *B. abortus*, but which has markers for *B. canis*, *B. suis* biovar 2 and *B. ovis* (Garcia *et al.*, 2005).

Single Nucleotide Polymorphisms (SNPs) typing: Single nucleotide polymorphisms (SNPs) represent powerful markers that allow accurately describing the phylogenetic framework of a species, particularly in a genetically conserved group as *Brucella*. The approach is based on a series of discrimination assays interrogating SNPs that shown to be specific to a particular *Brucella* species. A new SNP signature for the rapid identification and biovar characterization of *B. suis* was described by Fretin *et al.*, (2008). An advancement of this method has represented by a novel SNP-based typing platform that, incorporating targets that define the three *Brucella* vaccine strains, allows the differentiation of the live *Brucella* vaccine strains from field isolates (Gopaul *et al.*, 2010).

Tandem Repeat Based Typing: The availability of microbial genome sequences has facilitated the development of multilocus sequence-based typing approaches such as multiple locus VNTR (variable number of tandem repeats) analysis (MLVA). The VNTR, allelic hyper variability related to variation in the number of tandemly repeated sequences observed at several genomic loci in the *Brucella* genomes, were used for the discrimination of bacterial species that display very little genomic diversity. The first application of VNTR based typing to *Brucella* was the HOOOF-Prints scheme (Hyper variable Octomeric Oligonucleotide Finger-Prints) published by (Bricker, 2003). The approach was based on a comparison of the newly completed genome sequences of *B. suis* and *B. melitensis* along with a draft *B. abortus* sequence which identified an eight base pair tandem repeat sequence at nine distinct genomic loci (Whatmore, 2009).

2.8.3. Detection of Host Body Response to Brucellosis

Antibodies usually begin to appear in the blood at the end of the first week of the disease, IgM appearing first followed by IgG. The serological tests like Rose Bengal Plate Agglutination Test (RBPT), Standard Tube Agglutination Test (SAT), Coombs Test, Immune Capture Agglutination Test, Complement Fixation Test, ELISA, Lateral Flow Assay-a simplified version of ELISA, and Milk Ring Test (MRG) are commonly used tests in the diagnosis of brucellosis (Lucero *et al.*, 2003).

Rose Bengal Plate Test (RBPT): Often used as a rapid screening test; the sensitivity is very high (>99%) but the specificity is disappointingly as low as 68.8% (Barrsol *et al.*, 2002). RBPT is a rapid, slide-type agglutination assay performed on serum. The general principle of this test is the agglutination of serum antibodies with Rose Bengal dye-stained *B. abortus* whole cells buffered at a pH of 3.65 to inhibit nonspecific agglutinins. Due to its simplicity and low cost, it is the most common test used for brucellosis screening purposes, especially in laboratories with limited resources. However, this is of value as a screening test in high risk rural areas where it is not always possible to perform the other tests (Manture *et al.*, 2006).

This approach may result in a lower sensitivity. Whenever possible, a serum that gives a positive result should be confirmed by a more specific test. The RBPT is also of value in the rapid confirmation of neurobrucellosis, arthritis, epididymo-orchitis, hydrocele due to *Brucella* if the neat is positive in CSF, synovial fluid, testicular fluid /semen and hydrocele fluid respectively (Manture *et al.*, 2006).

Complement Fixation Test (CFT): This test detects specific antibodies of the IgM and IgG1 type that fix complement. The CFT is highly specific but it is laborious and requires highly trained personnel as well as suitable laboratory facilities that makes less suitable for use in developing countries. Although it specify is very important for the control and eradication of brucellosis, it may test false negative when antibodies of the IgG2 type hinder complement fixation. The CFT measures more antibodies of the IgG1 than antibodies of the

IgM type, Since it usually appear after antibodies of the IgM type, control and surveillance for brucellosis is best done by CFT (OIE, 2016).

Serum Agglutination Test (SAT): measures the total quantity of agglutinating antibodies IgM and IgG. The quantity of specific IgG is determined by treatment of the serum with 0.05M 2-mercaptoethanol (2ME), which inactivates the agglutinability of IgM. SAT titers above 1:160 have been considered diagnostic in conjunction with a compatible clinical presentation. However, in areas of endemic disease, using a titer of 1:320 as cut off may make the test more specific. The differentiation in the type of antibody is also important, as IgG antibodies are considered a better indicator of active infection than IgM and the rapid fall in the level of IgG antibodies is said to be prognostic of successful therapy (Buchanan and Faber, 1980).

Sero-conversion can be attributed to the performance of tests early in the course of infection, the presence of blocking antibodies, or the so-called “prozone” phenomenon i.e., the inhibition of agglutination at low dilutions due to an excess of antibodies or to nonspecific serum factors. Some of these shortcomings can be overcome by modifications such as the addition of EDTA, 2-mercaptoethanol, or antihuman globulin (Almunneef and Memish, 2003).

Enzyme-Linked Immune Sorbent Assay (ELISA): it measures IgM, IgG and IgA, which allows for a better interpretation of the clinical situation. A comparison with the SAT, ELISA yields higher sensitivity and specificity. ELISA is also reported to be the most sensitive test for the diagnosis of central nervous system brucellosis. Among the newer serologic tests, the ELISA appears to be the most sensitive; however, more experience is needed before it replaces the SAT as the test of choice for brucellosis (Almunneef and Memish, 2003).

Generally, all told, antibody profiles do not have specific clinical correlations, and titers often remain high for a protracted period. The asymptomatic patient with an isolated positive titer of class G and A immunoglobulins, or A immunoglobulin only, has not been

adequately studied. Variations of ELISA exist, such as competitive ELISA and sandwich ELISA, which may prove useful as a follow-up tool ((Almunneef and Memish, 2003).

Fluorescence Polarisation Assay: is a simple technique and a prescribed test for international trade. Used for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid. The mechanism of the assay is based on random rotation of molecules in solution (Nielsen and Gall, 2001).

Molecular size is the main factor influencing the rate of rotation, which is inversely related. Thus a small molecule rotates faster than a large molecule. If a molecule is labelled with a fluorochrome, the time of rotation through an angle of 68.5° can be determined by measuring polarised light intensity in vertical and horizontal planes. A large molecule emits more light in a single plane (more polarised) than a small molecule rotating faster and emitting more depolarised light. For most FPAs, an antigen of small molecular weight, less than 50 kD, is labelled with a fluorochrome and added to serum or other fluid to be tested for the presence of antibody. If antibody is present, attachment to the labelled antigen will cause its rotational rate to decrease and this decrease can be measured (Christopher *et al.*, 2010).

Brucellin Skin Test: An alternative immunological test is the brucellin skin test, which can be used for screening unvaccinated herds, provided that a purified (free of s-LPS) and standardized antigen preparation like brucellin INRA. The brucellin skin test has a very high specificity, such that serologically negative unvaccinated animals that are positive reactors to the brucellin test should be regarded as infected animals. Also, results of this test may aid the interpretation of serological reactions thought to be FPSR due to infection with cross reacting bacteria, especially in brucellosis-free areas (Lucero *et al.*, 2003).

Milk Ring Test The milk ring test is based on agglutination of antibodies secreted into the milk. This test allows screening of large number of cattle by using milk samples from tanks or pools from several cows. This test is useful for monitoring cattle herds or areas free of

brucellosis so it is classified as surveillance or monitoring test. Importantly, the number of false positive results is proportional to the number of cows secreting acidic milk due to colostrums or mastitis. A positive result indicates the presence of infected cattle in the herd so the test should be followed by individual serological test in the entire herd (OIE, 2009).

2-Mercaptoethanol: is a confirmatory test that allows selective quantification of IgG anti-*Brucella* due to inactivation of IgM in the test sample. Production of IgG is usually associated with chronic infection, and therefore, a positive result with this test is a strong indicator of brucellosis. However, this test has some drawbacks including the toxicity of mercaptoethanol, which requires a fume hood for its manipulation, and the possibility of IgG degradation caused by the 2-mercaptoethanol, which may result in false negative results. Sensitivity of the 2-mercaptoethanol test varies from 88.4 and 99.6%, and its specificity from 91.5 and 99.8% (Nielsen *et al.*, 2004).

Agar Gel Immunodiffusion Test: The agar gel immunodiffusion test is based on precipitation of the antigen-antibody complex. This method is often used for the diagnosis of *B. ovis* infection. This test has a low cost, it is easily performed and it has sensitivity levels that are comparable to complement fixation. However, it has some disadvantages such as a marked decrease in sensitivity in chronic infections and high variability of the quality of commercially available antigens. Therefore, it is highly advisable to perform complementary diagnostic techniques such as PCR. Sensitivity of the agar gel immunodiffusion test varies from 50 to 92.7% and the specificity from 94.3 and 100% (Estein *et al.*, 2002).

Coombs Test: This is the most suitable and sensitive test for confirmation of relapsing patients with persistent disease. It is an extension of the SAT test i.e., if the SAT test yields negative results due to the presence of blocking antibodies, Coombs test may be used instead. Agglutination can be determined visually, as for SAT, by using an agglutino-scope or a drop on a slide examined under the microscope. Coombs test is used for detection of incomplete, blocking or no agglutinating IgG. It is time consuming, technically difficult, requires skilled personnel and not routinely performed in clinical laboratories. It is good for

complicated and chronic cases but misses about 7% of cases compared with ELISA (Gall and Nielsen, 2004).

2.9. Treatment of Bovine Brucellosis

Due to the intracellular localization of *Brucella* and its ability to adapt to the environmental conditions encountered in its replicative niche e.g. macrophage (Seleem *et al.*, 2008), treatment of domestic animals with antibiotics is not usually successful. Even though, treatment failure and relapse rates are also high in humans, treatment depend on the drug combination of doxycycline with streptomycin which is currently the best therapeutic option with less side effects and less relapses, especially in cases of acute and localized forms of brucellosis (Seleem *et al.*, 2009).

Neither streptomycin nor doxycycline alone can prevent multiplication of intracellular *Brucella* (Shasha *et al.*, 1994). Although the doxycycline-streptomycine regimen is considered as the golden standard treatment, it is less practical because the streptomycin must be administered parentally for 3 weeks. A combination of doxycycline treatment (6 weeks duration) with parentally-administered gentamicin (5 mg/kg) for 7 days is also considered an acceptable alternate regimen (Seleem *et al.*, 2009)

2.10. Control and Prevention Brucellosis

The general strategies proposed in FAO,2002 by the WHO including Mediterranean Zoonoses Control Program to eradicate animal brucellosis were the: prevention of spread between animals and monitoring of brucellosis-free herds and zones, elimination of infected animals by test and slaughter programs to obtain brucellosis-free herds and regions, and vaccination to reduce the prevalence (FAO,2002).

2.10.1. Immunization

One of the most successful methods for prevention and control of livestock brucellosis is through vaccination. In different parts of the world both live vaccines, such as *B. abortus* S-19, *B. melitensis* Rev-1, *B. suis* S-2, rough *B. melitensis* strain M111 and *B. abortus* strain RB51 and killed vaccines, such as *B. abortus* 45/20 and *B. melitensis* H.38 are available. Each vaccine has been reported to have its own advantages and disadvantages with protection following localized persistence of live vaccines preferred by most and showing efficacy in small ruminants (Smits, 2012)

Use of the RB51 attenuated live vaccine has recently gained popularity for control of brucellosis in cattle. But on a cautionary note, the failure of this strain to induce serological reactivity, coupled with its inherent resistance to *rifampicin*, might complicate detection and management of zoonotic infection spilling into humans with occupational risk factors for acquiring brucellosis. Currently, despite huge research efforts, no vaccine has been approved for the prevention of human brucellosis. Treatment regimes for human brucellosis require combination of antibiotics. These have recently been compared using meta-analysis (Marzetti *et al.*, 2013).

Currently, vaccination against animal brucellosis has yet to be explored in Ethiopia. As a prerequisite, *Brucella* species identification should be undertaken to inform selection of the most appropriate vaccine, for example, *B. melitensis* has recently been found infecting cattle

in Kenya (Muendo *et al.*, 2012) and to enable differentiation of vaccine and wild-type strains.

Vaccination is generally recommended for seroprevalence rates between 2 and 10%. Whether a strategy of test and segregation alone for high seroprevalence rates is sufficient may depend on the farming conditions. This might be appropriate for farms in conjunction with appropriate hygienic measures, but supplementation with vaccination may be required to control the disease in extensive livestock conditions (Marzetti *et al.*, 2013).

Adaptive immune response against brucellosis through production of IFN by CD4+, CD8+, and $\gamma\delta$ T cells that activates the bactericidal action of the macrophages to hamper the intracellular survival of *Brucella*, the cytotoxic action of the CD8+ and $\gamma\delta$ T cells kills infected macrophages and Th1 antibody IgG2a isotypes opsonize the bacteria to facilitate effective phagocytosis (Perkins *et al.*, 2010).

An ideal vaccine for use either in humans or in animals should be effective and avirulent, and induce long-lasting protection. Subunit vaccines, like recombinant proteins, are promising vaccine candidates because they are less biohazardous, well defined, avirulent, noninfectious, and nonviable (Pasquevich *et al.*, 2011).

Recombinant Proteins: Selecting the optimal antigens represents the cornerstone in vaccine design. Depending on the desired response, the antigenic proteins should contain appropriate epitopes to *B-cell* receptors and can be recognized by the *T-cell* receptor in a complex with MHC molecules. Several *Brucella* immunogenic antigens have been found in the outer membrane of this Gram-negative pathogen. Bacterial cell surface antigens are prime candidates as they represent the initial point of contact between the pathogen and its host (Delvecchio *et al.*, 2006).

Vectored Vaccines: Antigen delivery systems become necessary when antigens are not efficiently transported to the appropriate sites or presented to the immune system. Rapid degradation can result in weak or virtual lack of responses to otherwise immunogenic antigens. Recently, a vectored vaccine has been developed based on protein BP26 or Omp16 and Omp31; that is, *E. coli* expressing *B. melitensis* BP26 induced lymphocyte proliferation, IFN- γ production, and protection in mice (Perkins *et al.*, 2010).

Lactococcus lactis expressing Cu-Zn SOD has also been used as a delivery system in mouse model and induced protection against a *B. abortus* challenge. Semliki Forest Virus (SFV) was packed with RNA encoding the *B. abortus* Cu-Zn SOD and was able to induce protection in mice against *B. abortus* (Delvecchio *et al.*, 2006).

DNA Vaccines: DNA vaccines are able to induce both humoral and cellular immune responses. However, it is generally perceived that they induce less potent immune responses than protein vaccines. Nevertheless, this may not be the case for brucellosis; for example, a DNA vaccine expressing Omp31 appears to elicit similar levels of protection as the recombinant protein combined with IFA. Moreover, a BSL-DNA vaccine was more effective than the same recombinant protein against *B. abortus* challenge (Velikovsky *et al.*, 2002).

One advantage of DNA vaccines is that multiple antigens can be expressed and induction of a diverse immune response led to different levels of protection. Brucellosis DNA vaccines is more effective through the modulation of the immune response by the co-expression of cytokines as adjuvants. When genes encoding IL-2 or IL-18 were fused to SOD and expressed in a single DNA vaccine improved protection was observed compared to a SOD DNA vaccine alone (Singha, *et al.*, 2008).

Outer Membrane Vesicles: Outer Membrane Vesicles (OMVs) from the Gram-negative bacteria have been implicated in many processes including the release of virulence factors to the host as well as in the delivery of toxins, modulation of the immune system, trafficking of

signaling molecules between bacterial cells, and biofilm formation (Mashburn and Whiteley, 2006).

Recently, the use of *Brucella* OMVs as a potential vaccine has been explored. Purified OMVs from both *B. melitensis* strains 16 M (smooth strain) and VTRM1 (rough lacking O-side chain) by differential centrifugation were used to immunize mice. When *Brucella* OMVs were administered by an intramuscular route, OMVs from both strains induced similar levels of protection against virulent *B. melitensis* challenge compared with the live vaccine *B. melitensis* Rev. 1. In contrast, rough OMVs induced better levels of IgG2a antibodies than OMVs from smooth and live vaccine strain (Avila, *et al.*, 2012).

Compared to recombinant proteins, DNA vaccine, or vectored vaccines, OMVs could be less expensive in terms of production and purification. Because the genome sequences of many *Brucella* species are available, this makes it possible to genetically modify the content of OMVs using recombinant DNA technology (Avila *et al.*, 2012)

3. MATERIALS AND METHODS

3.1. Study area and Population

The study was conducted in Horro Guduru Animal Production Center which is found in Horro Guduru Wollega Zone, Oromiya National Regional state, of Western Ethiopia. It is located at distance 275Km West of Addis Ababa along Gedo-Fincha Sugar factory high way; between 09⁰29' North altitude and 37⁰26' East longitudes. Its average altitude is about 2296meters above sea level. Horro Guduru Animal Breeding Center was established in 1994 E.C. for the purpose of improving the production of Horro cattle breed and to provide the farmers of the area Horo-Jersey cross bred cattle adapted to the natural habitat of Western part of the country. Mixed crop-livestock farming is common practiced in zone districts. Livestock production plays an important role in the rural economy of farmers. Cows are hand milked twice daily and suckled before and after milking. The main breeding strategies of the center is naturally mating of pure Horro female with pure Horo bull, and the second one is cross breeding program between pure Horro female cattle with 100% pure exotic Jersey/Holstein Friesian breed through artificial insemination to get 50% F1 cross breed. Horo-jersey cross breed give an average 10-15 liter of milk, and tolerate and adapted with agrio ecology of the area. The center distributes improved cross breed heifers and bulls for the surrounding community, Woredas, Zones, Research Organizations, Universities and Investors. Daily milk produced from the center around 250-350 liter purchased by surrounding community for consumption, cafeterias and hotels. small holder dairy farms and dairy cows owned by farmers the surrounding the center represent the study population of this study. Cattle above 6 months of age including milking, none milking, replacement heifers and bulls were included in the study.

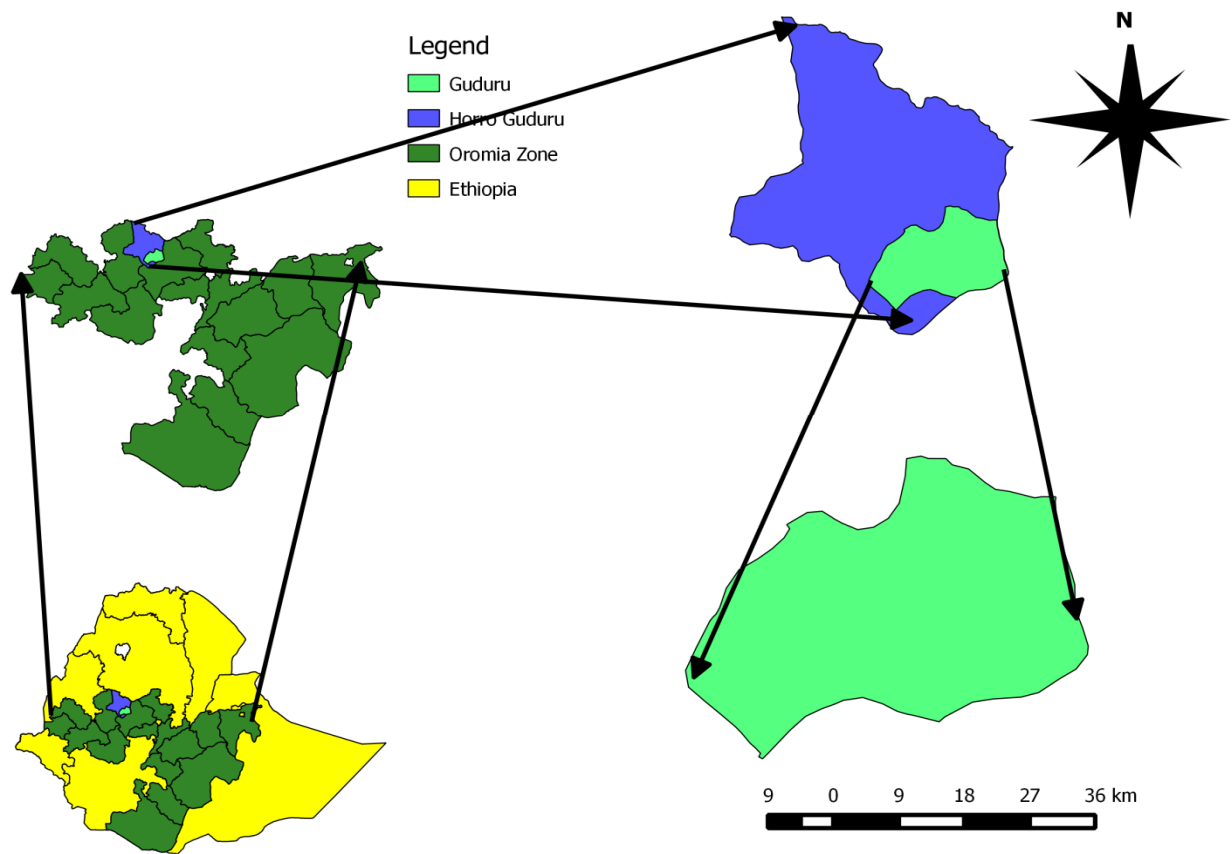


Figure 3: Diagram of Study Area

3.2. Study Design and Sample Size Determination

A cross-sectional study design was conducted to determine the sero-positivity of *Brucella* infection in cattle in the selected study area and to identify the potential risk factors associated with the sero-positivity. The study was conducted from October 2017 to May, 2018. Cattle above six months of age were selected for this study. Relevant individual animal bio-data and farm level information were collected using a structured questionnaire. The sampling was performed using systematic selecting individual animals, systematically inside the farm and clinics.

The sample size for sero-screening of cattle in this farm were calculated on 50% expected prevalence, 95% confidence interval and 5% required precision bases, resulting in a sample size(n) based on the formula given by (Thrusfield, 2007) as given below.

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

where n=required sample size

Z=reliability coefficient (1.96 at d=0.05 or 95% CI)

P_{exp}=expected prevalence (50%)

d= desired absolute precision (95% CI)

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

Since there was no previous study carried out on bovine brucellosis in study area, 50% expected prevalence was used in formula. Accordingly, the total number of animal to be sampled from the center were 384, this can be reasonably increased to 812 animal samples to obtain a desired precision.

3.3. Sample Collection

3.3.1. Questionnaire survey

A structured questionnaire was prepared and applied to all concerned workers of the farm and house holders in the study area. Information related to personal demography like age, sex, educational background and knowledge attitude and practice toward brucellosis were collected on format developed (annex 1). The presence of abortion, still births, retention of fetal membranes, separate parturition/maternity pen, contact between animals with other herds were categorized as yes or no variables. Breeding was characterized by service types (artificial insemination (AI), bull or both). The method of after birth disposal (placenta, aborted material and dead fetus) was also categorized into burying/burning, or thrown to open dump. The culling criterion of animals from the farm was categorized as reproductive problems, non-reproductive problems or both variables. The replacement stock of each farm

was defined as purchased in, raise own replacement or both. Individual animals were categorized as young (≤ 3 years) and adult (>3 years), as cross or local, as male or female. Site of each individual animal was defined as either Fincha, Guduru vet clinic or Horro Guduru A/P/R/Center(HG ranch). Parity of the animals in the farm were categorized as primiparous, pluriparous or not applicable variables whereas the abortion stage was classified as first trimester, second trimester or third trimester.

3.3.2. Blood sample collection

Blood samples (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 $1500 \times g$ for 10 min to obtain the serum. Sera were decanted into cryovials, identified and transported to the Addis Ababa University College Veterinary Medicine Bishoftu, Ethiopia in ice packs and stored at -20°C until screened for antibodies against natural *Brucella* exposure using serological analysis.

3.4. Serological Laboratory Techniques

3.4.1. Rose bengal plate test (RBPT)

All sera samples collected were initially screened in Addis Ababa University College of Veterinary Medicine and Agriculture Veterinary Microbiology Laboratory by RBPT using *Brucella* antigen coated commercially prepared (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) according to OIE (2016) procedures. Briefly, sera and antigen (30 μl) 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.

3.4.2. competitive enzyme linked immuno-sorbent assay (c-ELISA)

For further laboratory analysis, RBPT seropositive sera samples were transported to Addis Ababa University, Akililu Lemma Institute of Pathobiology (AAU-ALIPB), and c-ELISA was performed in using a commercial c-ELISA kit (BRUCELISA(160+400), (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom) to detect circulating antibodies of *Brucella* in cattle serum sample, and the protocol provided by the developers was followed precisely. When shortly summarize the test, the positive and negative controls were used at a dilution of 1/40 as has been indicated by the manufacturer. Following the addition of the conjugate and substrate-chromogen mixture at a recommended strength, the plate was incubated and examined for the intensity of reaction with an automated ELISA reader at 405 nm.

Color development within a well indicates that the tested serum has antibodies to *Brucella*. A positive/ negative cut-off was calculated as 10% of the mean of the optical density (OD) of the eight positive control wells. And then test sera were analyzed at a final dilution of 1/200. Finally, any test serum with an OD value equal to or above this value was considered as positive.

3.4.3. Complement fixation test (CFT)

Sera that tested positive to the RBPT were further tested using CFT confirmation using standard *B. abortus* antigen S99 (Veterinary Laboratories Agency, New Haw, Addlestone, and Surrey KT15 3NB, United Kingdom) at National Veterinary Institute (NVI) Bishoftu, Ethiopia. Standard *B. abortus* antigen for CFT was used to detect the presence of *anti-Brucella* antibody in the sera. Preparation of the reagent was evaluated by titration and performed according to protocols recommended by World Organization for Animal Health (OIE, 2009). Sera with a strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of complement (2+) at a dilution of 1:10 and above were classified as positive and lack of fixation/complete hemolysis was considered as negative.

3.6. Case Definition

Animals were considered as positive to brucellosis when they tested positive on either test RBPT / c-ELISA or RBPT/CFT in parallel interpretation. Since there is no history of vaccination of brucellosis in Ethiopia, seropositive observed in this study was considered as to be due to natural infection.

3.7. Data Management and Analysis

Data generated from the questionnaire survey and laboratory investigations were recorded and coded using a Microsoft Excel spreadsheet (Microsoft Corporation) and then imported to the Statistical Package for Social Sciences (SPSS) for Windows® version 20.0 (SPSS Inc., Chicago, Illinois) for conducting appropriate statistical analyses.

The seroprevalence was calculated as the number of seropositive samples divided by the total number of samples tested. To identify association of *Brucella* seropositivity with the risk factors (origin, age, farm system, breed type, herd size, separate parturition, abortion history, abortion period and parity) were computed by Pearson's Chi-square test. After the association of exposure variables with *Brucella* seropositivity was analyzed at individual animal level by the Chi-square test, those variables significantly associated with *Brucella* seropositivity (origin, source of stock replacement, abortion history, abortion time, and previous history of retain fetal membrane) were further analyzed by multivariable logistic regression. A multivariable logistic regression model was used to identify the potential risk factors associated with *Brucella* infection in animal and variables with a p-value lower than or equal to 0.05 (in Chi-square analysis) were included in the multivariable logistic regression model.

3.8. Ethical Consideration

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 for ethical approval.

4. RESULTS

4.1. Questionnaires Survey Result

4.1.1. Demographic characteristics of the respondents

A total of 102 participants were interviewed to assess their knowledge, attitude and practices towards brucellosis. The majority (84.3%) of farms were run by male members of the households. Overall, 60.7% of respondents had primary education, while 16.6% informal and the remain one completed high school or above. Around half (50%) of respondent population of the participants were in the 35-45 age group. In regard to primary role of the participants in management of the herd, 60(58.8%) were herd owner, 25 (24%) were herders and 17 (16.6%) were involved mostly in milking animals in the herd. Also 76 (74.5%) of the participants were married and 26 (25.5%) were single table (5)

Table 5: Demographic characteristics of the respondents

Demographic variables	category	No.	%
Gender	male	86.	84.3
	female	16	15.7
Age	18-34	15	14.7
	35-45	52	50
	>45	35	34.3
Marital status	married	76	74.6
	singe	26	25.4
Education level	informal	17	16.6
	primary	62	60.9
	secondary	7	6.8
	College/university	16	15.7
Responciblity in farm	Owner	60	58.8
	Herder/feder	25	24.6
	Milker	17	16.7

4.1.2. Knowledge and herd management practices regarding brucellosis

In current study, the majority of participants from small (33.%) medium (37.5%) and large (29.1) scale farm owners totally (70.5%) had not heard of brucellosis in human and animal. From this only (29.5%) of participants having heard about brucellosis table (6). It revealed that most of the livestock owners (38.23%) had a herd size of ten animals or fewer, while most of the farmers (32.35%) had a herd size between 10-20 and some of them (29%) had more than 20 heads of cattle.

Table 6: Knowledge, Attitudes and Practices (KAP) of farm owner's regarding brucellosis different herd size

Variable	category	No	%
Knowing of Brucellosis	No	72	70.6
	Yes	30	29.4
After birth dispose	Protective glove	13	12.7
	Without p/glove	89	87.3
Action to uterus discharge	Fed dogs	35	34.3
	Burying	30	29.4
	Through over field	37	36.3
Drinking raw milk	No	9	8.8
	yes	93	91.2
Assisting parturition	Yes	40	39.2
	No	62	60.8

Most participants 88 (86.2%) reported that they do not have separate space or shed for parturition, and the space was shared with other animals. Only 34% of farmers properly disposed the farm wastage table (7).

Table 7: Farm characteristics in three districts of study area

Variables	Categories	Proportion of Respondents (n %)		
		HG Ranch (25)	Fincha (40)	Guduru (37)
Having parturition pen	No	11(12.83)	40(45.45)	37(42.04)
	Yes	14	-	-
Having Disposal Area	No	15(22.38)	30(44.77)	22(32.83)
	Yes	10(28.57)	10(28.57)	15(42.85)
	Very good	0	1(100)	0
Hygiene of the house	Good	3(33.3)	4(44.)	2(2)
	Satisfactory	11(23.9)	19((41.3)	16(34.7)
	Poor	11(23.9)	16(34.7)	19(41.3)
Service type	AI	0	6(31.57)	13(68.42)
	Bull	0	34(58.6)	24(41.37)
	Both	25(100)	0	0
Source of stock	Raise /growing	20(24.40	33(40.2)	29(35.3)
	purchased	5(25)	7(35)	8(40)

HG=Horro Guduru, n=number, %=percent

4.1.3. Occupational risk and awareness among farm owners and works regarding brucellosis

In study area only around one fourth of the participants in intensive (26.9%) farms were aware of brucellosis. Up to 73.1 % of the respondents were regarded as having poor knowledge about brucellosis. Besides, 92.3% of the participants in intensive farms and up to

90.7 % those in extensive farms responded that they have habit of drinking raw milk. The level of activities was significantly lower ($P < 0.05$) as shown in table (8).

Table 8: Occupational risks and awareness among farm works and owners about brucellosis

Variables	Semi-intensive (%)	Extensive (%)	X ²	P -value
Knowing of Brucellosis				
Yes	7(23.3)	23(76.6)	0.104	0.474
No	19(26.38)	53(73.6)		
Drinking raw milk				
No	2(22.2)	7(77.7)	12.68	0.013*
Yes	24(25.8)	69(74.1)		
Human housing				
Common	3(17.6)	14(82.35)	0.661	0.316
Separate	23(27.05)	62(72.94)		
Assisting parturition				
Yes	10(33.3)	20(66.6)	0.08	0.55
No	16(25.80)	46(74.2)		

*-Significant

4.2. Serological Results

Among 102 bovine herd tested in study area 28(27.45%) 6(5.8%) and 6 (5.8%) were found to be serologically positive by RBPT, c-ELISA and CFT respectively. For individual seroprevalence, among the 812 cattle screened for *B. abortus* antibodies, 28 (3.4%) tested positive by RBT. From these, 6 animals (95% CI: 0.5-1.7) were confirmed positive by c-ELISA and CFT, giving an current seroprevalence of 0.73% in the study area table (9). Associations of the putative risk factors as indicated in table (9) were computed by Pearson's Chi-square test. The prevalence of bovine brucellosis in different origin (districts), abortion history, retain fetal membrane, source of stock replacement and abortion time were statistically significant ($\chi^2=7.951$; $P=0.019$, $\chi^2=8.217$, $P=0.04$, $df=1$; $X^2=36.47$, $P=0.001$, $X^2=8.436$, $P=0.015$, $X^2=9.756$, $P=0.02$) respectively table (9).

Table 9: Association of risk factors with overall seroprevalence of brucellosis in individual animal level

Variables	Group	No.	Prevalence rate		X ²	P-value
			+ve	%		
Origin/Site	HG Ranch	643	2	0.31	14.19	0.001*
	Fincha	68	3	4.41		
	Guduru	101	1	0.99		
Breed	local	718	5	0.6	0.15	0.69
	Cross	94	1	1.06		
Parity	primiparous	169	-		2.38	0.303
	Pluriparous	582	6	1.03		
	No parity	61	-			
Age	Young	45	-		00.35	0.55
	Adult	767	6	0.78		
Sex	Female	753	6	0.79	0.474	0.34
	Male	59	-			
Mating type	AI	149	-		1.88	0.39
	Bull	476	5	10.84		
	Both	178	1	0.56		
Herd size	Small	299	2	0.66	0.47	0.977
	Medium	272	2	0.73		
	Large	241	2	0.82		
Source of stock	Growing (Rasing)	693	5	0.72	8.436	0.015*
	Purchased	119	1	0.84		
Abortion history	Aborted	17	2	11.76	8.217	0.04*
	Non aborted	795	4	0.50		
Abortion Time	1 st trimister	4	0	-	42.17	0.000*
	2 nd trimister	8	0	-		
	Last trimister	12	2	16.6		
	No abortion	788	4	0.50		
Historyof RFM	Yes	30	3	10	36.47	0.001**
	No	782	3	0.38		
Farming System	Intensive	137	2	1.45	1.16	0.280
	extensive	675	4	0.59		

RFM= Retain Fetal Membrane, *:=Significant, No.=number of animals , X² =Chi-square

4.2.1. District level of seropositive prevalence of bovine brucellosis in present study

Out of 812 serologically screened cattle included in the study, around 79.18% (643 heads), 12.43%(101 heads) and the remaining 8.37 (68 heads) with prevalence rate (%) 0.31, 0.99 and 4.41 from three (3) districts (station) Horro Guduru Animal Production Center (HG Ranch), Guduru (Kombolcha) and Fincha district with statically significant ($\chi^2=7.951$, $P=0.019$, $df=2$) respectively as indicated in table (9).

4. 2.2. Host related seropositive prevalence of bovine brucellosis in present study

The breed category in study area 638 (78.57%) Horro indigenous breed and 174 (21.43%) cross breeds. The prevalence of antibrucella antibody is relatively higher in the case of cross breed (1.06%) than Horro pure breeds (0.6%) and this difference in prevalence was statistically insignificant ($P>0.05$).

Also a significantly higher sero-prevalence (2.17 %) of bovine brucellosis in animals with less than or equal to 3 years old compared to animals with greater/equal to 3 years old (0.51 %). Seropositivity in females (0.79 %) were indicated in study area where as no seropositive in males; this finding is in agreement with previous report by Adugna *et al.*(2013) that all seropositive animals were females were either pregnant or lactating, local or cross breed. This implies that female cattle were at higher risk than males. Besides, association between brucellosis sero-positivity and parity status of the cows and herd sizes, there were no significant differences table (9).

4.2.3. Herd/Farm management related seroprevalence of bovine brucellosis

The serology result of the apparent study also reveal more positive test was recorded in animals of semi-intensive production systems (1.45%) than in those of extensive systems (0.5%). Similarly, a higher seroprevalence of brucellosis was observed on farms that used bull (natulary) only for mating (10.84%), as opposed to those that used artificial inseminator and bull commonly (0.56%).

4.2.4. Seroprevalence of bovine brucellosis associated with cattle physiology

In apparent study of seroprevalence of bovine brucellosis in farm management variable group, there was association between abortion history and seropositivity to brucellosis ($P < 0.05$). The seropositivity of cows that have previous abortion history was compared with no abortion history cows which result in 11.76% and 0.50% respectively table (9).

4.2.5. Factors affecting the seroprevalence of Brucellosis in the study area

The results of logistic regression analysis for different risk factors were presented in table (9). Among the potential risk factors considered in the study area origin (district) abortion history and retain fetal membrane ($P < 0.05$), had a significant effect on over all seroprevalence of brucellosis. In addition duration of abortion and source of stock replacement also statistical significance. The compound effect on multivariable logistic regression analysis of putative risk factors with *Brucella* seropositivity using CFT result indicate that cattle those had history of retain fetal membrane were more likely to be infected (OR= 30.7, 95% CI: 4.4 -212) than others (Table 14).

Table 10 : Multivariable logistic regression analysis of putative risk factors with *Brucella* seropositivity using CFT

Variables	Crude estimate			Adjusted estimate		
	OR	CI	p-value	OR	CI	p-value
Abortion history	0.42	.023-7.43	0.014	0.065	-	.000
Retain Fetal Membrane	30.7	4.4-212	0.001	-	-	.000

OR:=Odds ratio; CI=Confidence interval

5. DISCUSSION

In Ethiopia, seropositive prevalence of bovine brucellosis recorded by different scholars using various sensitive and specific serological tests and very few direct detection of agent methods. Those findings were reconciled with the present research in Western Ethiopia. The present study recorded an overall cattle seroprevalence of 0.738% brucellosis in three selected districts of Horro Guduru Wollega Zone. This is quite low and remarkable observation signifying lower risk of acquiring brucellosis cattle around this area.

This confirmed seropositivity result record was consistence with earlier report of Tolosa *et al.* (2008) (0.77%) Jima Zone, and slightly higher individual serological prevalence rates by Gumi *et al.* (2013) (0.9%) in SE-Somalia and Oromiya; Adugna *et al.* (2013) (1%) around Metekel, Yilma *et al.* (2016) (1.04) Chench Gama Goffa Southern from Ethiopia and Kang'ethe *et al.* (2007) (1%) from Kenya.

There were also reports with a relatively lower seroprevalence of bovine brucellosis than the present find in other study area by Bashitu *et al.* (2015) (0.2%) in Ambo and Debrebrihan, and Alem and Solomon (2002) and Belihu (2002) failed to find any seroreactive cattle after screening 564 animals in Eastern and Western Showa zones of central Ethiopia using Rose-Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and Complement Fixation Test (CFT). Similarly, Belihu (2002) could not find positive reactors in intensive dairy farms in Addis Ababa area (n=747). Similarly, Asmare *et al.* (2013) could not found positive reactors in Adama, central Ethiopia (n=52) and northern Ethiopia (Mekele and Gonder)(n=252). Variation in the seroprevalence findings might be seen to due to relatively more farm management systems in these studies than present study.

On the other hand there were reports with higher seropositive of bovine brucellosis in other part of the country reported by Geresu *et al.* (2016) (1.40) Eastern shoa dairy farms, Asgedom *et al.* (2016) (2.4) Alagea districts; Degefu *et al.* (2011) (1.38%) in agro-pastoral areas of Somali Regional State, Asmare *et al.* (2007) reported (1.92%) in Southern Ethiopia, Tolosa, *et al.* (2012) (1.97%) in Jimma area and Tesfaye *et al.* (2011) (1.5%) in

Addis Ababa and Yohanis, *et al.*(2011) (1.97) East Wollega Zone, Ethiopia. In general, brucellosis in animals has been reported from different localities of country, with prevalence ranging from 0.0%-50% particularly associated with cattle in different farming system and agroecology (Alem and Solomon, 2002 and Belihu, 2002).

The sero reactor result of the current study in Western cattle population is remarkable as bovine brucellosis is considered the world's most common bacterial zoonosis(Pappas *et al.*, 2006) and listed among top five zoonotic diseases in Ethiopia (Pieracci *et al.*,2016). The hypothesis that brucellosis is endemic in Ethiopia also tested in present study area. The presence of less positive reactors in the herd is a reliable predictor of the presence of infection as indicated by Radostits *et al.* (2006).

The present seroprevalence of bovine brucellosis at the animal level is relatively low. This might be attributable to more extensive grazing conditions than semi-intensive management in this study as confirmed from farm characterization survey; these could reduce both animal-to-animal contact and the contamination of pastures under dry climatic conditions (Crawford *et al.*, 1990). Another explanation could be that in the area studied, mostly in Horro Guduru Animal Production center and other surrounding farmers replace their animals from their own stock instead of buying animals from markets. In addition to this the apparent study area is commonly known by mixed farming of crop cultivation and livestock production that might be reduce pasture contamination through use of land rotation for production. Similar low prevalences of bovine brucellosis, based on RBT and CFT, have been reported in other studies on indigenous cattle under extensive production systems (Adugna *et al.*, 2013; Asmare *et al.*, 2013 and Abebe *et al.*, 2010).

In epidemiological studies, the use of two tests applied serially has been recommended to maximize the accuracy of test results. A combination of RBT and c-ELISA or CFT tests is the most widely used serial testing scheme. RBT is highly sensitive test and could easily applied in field conditions where as, c-ELISA and CFT are highly specific usually used as a confirmatory test method (Godfroid *et al.* 2010). The combination of these tests in this study could therefore maximize the accuracy of the findings.

In addition to estimate of seroprevalence, this study was carried out to assess the risk factors associated with disease occurrence. In most studies including this finding, cattle production systems play a vital role to determine seropositivity of brucellosis in livestock. Higher prevalence recorded in present study in semi-intensive farm, which was in line with the previous researches (Kebede *et al.*, 2008). Even though, the finding procedure was based on the same diagnostic tests, a higher prevalence has been reported among cattle in semi-intensive production systems.

This variation could be explained by the fact that there is a greater chance of contact between infected and healthy animals in these systems, or between healthy animals and infectious materials, since most farmers do not follow hygienic practices. This increased risk of contact was described by Jergefa *et al.* (2009). In opposite side the higher prevalence in extensive management systems reported by Gebretsadik. (2005) could be due to the transhumant nature of cattle herding in northern Ethiopia, which can enhance the spread and distribution of infection.

The current seroprevalence study in three district areas were significantly associated with brucella positive in cattle ($P < 0.05$) and the results showed higher individual animal seroprevalence in Fincha (4.41%) when compared to Guduru(0.99) and HG ranch (0.31%). The reasons for the variations in brucellosis seroprevalence among the study areas might be related to the difference in management practice performed in the three study sites. In addition, different studies revealed that the seroprevalence of brucellosis is affected by different agroclimate conditions, which determine of survival of *Brucellae* organisms (Radostits *et al.*, 2007). Although the location of the present study area the districts were not more than 20km apart from each other. The close related of significant descriptive figure in cattle seroprevalence between the HG ranch and Guduru districts could be due to the similarity of management practices and common share of environmental condition since the HG ranch located in Guduru districts but independent administration.

In the present study, the herd size seroprevalence of bovine brucellosis was observed and result in insignificant association with univariable logistic regression. This study finding

was in not agree with that of Geresu *et al.* (2016) and Adugna *et al.* (2013), in which they found significant association between *Brucella* seropositivity and herd size. But in line with, Kebede *et al.* (2008) reported that the risk of *Brucella* seropositivity was independent of herd size in small holder farms from Wuchale Jida district of East Wollega zone of Ethiopia near with present study.

Even though sex was not significantly associated with *Brucella* seropositivity ($P > 0.05$), all the apparent seropositive cattle were females (0.79 %) among tested cattle table (9). This finding is in agreement with previous report by Adugna *et al.* (2013), Tolosa *et al.* (2008) and Kebede *et al.* (2008) that all seropositive animals were females either pregnant or lactating, local or cross breed. Incontrast to this research they report a significant association between sex and *Brucella* seropositive. This implies that female cattle were at higher risk than males. The explanation for this finding could be that male animals were kept for a shorter time than females and thus the chance of exposure is lower for males (Mangen *et al.*, 2002).

The parity status was not found to be a significant determinant of seroprevalence, which was a similar observation made by Berehe *et al.* (2007) Geresu *et al.* (2016). Though there is insignificant association between parity and brucellosis seropositivity, there is little bit higher seroprevalence was observed in pluriparous (0.89%) than primiparous cattle (0.57%) where no seropositive result in heifers and male animals in the study areas (table 9). The higher seroprevalence of brucellosis in the pluriparous cattle of this study was in line with Asmare *et al.* (2013), Geresu *et al.* (2016), and Adugna *et al.* (2013).

Like parity and others age was also not significantly associated with *Brucella* seropositivity ($P > 0.05$), a seroprevalence of 0.79% was found among the adult age group whereas no brucella seorpositivity was observed in the young age group of bovine in the study sites. Several previous reports have indicated that higher seroprevalence of Brucellosis in adult age group of cattle (Magona *et al.*, 2009) similar to the findings of this study. This could be explained by sexually mature cows are more susceptible to infection with *Brucellae* than sexually immature cattle of either sex. This has been attributed to the affinity of these

bacteria to the female uterus and to erythritol in fetal tissue, possibly also to steroid hormones (Radostits *et al.*, 2007).

Like origin of the cattle, there was an association between abortion history and seropositivity to brucellosis ($P < 0.05$). The seropositivity of cows that have previous abortion history was compared with no abortion history cows, which result in 11.76% and 0.50% respectively. This finding was an average record when compare with a relatively lower seroprevalence (6.1%) was reported by Tesfaye *et al.* (2001) in Mekele dairy cattle and 6.7% by Yayeh (2003) in North Gondar, and Geresu *et al.* (2016) and Tolosa (2004) who reported equivalently 17.6% in different selected areas in Ethiopia. This could be explained by the fact that abortions or stillbirths and retained placentas are typical outcomes of brucellosis (Radostits *et al.*, 2007).

In addition to this there was a highly significant association between history of retain fetal membrane and seropositivity test of Brucellosis in present study. These two cases of association were in agree with other studies have also shown a significant association between seropositivity and abortion time (Tolosa *et al.* 2008; Ibrahim *et al.*, 2010 and Adugna *et al.*, 2013). Retention of the placenta and inflammation of the wall of the uterus (metritis) are common sequelae to abortion due to brucellosis (Radostits *et al.*, 2007). Aparicio (2013) reported that *Brucella*-infected cows were expected to abort 3 to 4 times more than unexposed cows.

The present study also reveled that previous history of abortion time was statistically significant association ($X^2=36.42$; $P=0.001$) between abortion period and sero positivity of Brucellosis (10.0%). This was in agreement with previous reports of Geresu *et al.* (2016). This could be explained by the presence of higher seropositivity in cows in the last trimester which may be due to the preferential localization of *Brucella* in the uterus, in which allantoic fluid factors such as erythritol could stimulate the growth of *Brucella* and elevate in the placenta and fetal fluid from about the 5th month of gestation (Radostits *et al.*, 2007; Coetzer and Tustin, 2004).

In the present study area most farms used bull (58.6%) than Artificial insemination (18.3%) or both (23.%) for mating method. Although relatively higher seroprevalence rate (10.84%) in bull service, where as no seropositive record in AI mating method, it was not statistically significant ($p > 0.05$). But the sources of replacement stock ($p < 0.05$) were shown to significantly affect the prevalence of bovine brucellosis in study areas. Those animals purchased from other area were relatively free of brucellosis than cattle grown and replaced the stock. From these two variable factors, using artificial insemination for mating method and giving an attention for cattle purchased from other farm should be practiced in present study area to prevent brucellosis.

The Proportion of respondents was different in the seroprevalence rate observed in this study. This may oppose those recorded by previous researchers, that might be due to differences in herd size, different management systems, the presence or absence of infectious foci, such as *Brucella* infected farms or ranches in the surrounding areas, which are used as sources of replacement stock.

In areas like the present study, where the disease is less prevalent, for livestock seroprevalence of less than 1%, some reports (Yonanis *et al.*, 2013) may recommend test and cull policy with compensation. And for areas with high and moderate prevalence ($>5\%$) under well-organized farming systems, may recommend test and segregation policy by which animals with brucellosis will be isolated and products consumed after pasteurization, with animals being disposed of properly at the end of their productive life.

Although several risks have been identified by serological survey, the most important are related to farm management, animal management and knowledge about the disease. Thus, to benefit from proper risk identification of brucellosis, it is essential to put an effective and efficient brucellosis control programme into study area.

The present study, also aimed to assess brucellosis infection with significant importance for public health around the study area. Based on socio-demographic characteristics of participants, the cross-sectional study showed that brucellosis was not known by the general

community in the present study area, since around 70% (three quarters) of the study respondents had not heard of brucellosis. This is similar to findings of previous studies done in the Kenyan study, 30% of dairy respondents and 22% of non-dairy respondents knew of the existence of brucellosis whereas in Tajikistan 85% of the respondents had never heard of brucellosis (Lindahl *et al.*, 2015).

The survey also wants to identify risk factors related to age, educational background and practice with livestock. The report by Díez *et al.* (2013) was noted that knowledge ages equal to or older than 55 years was a protective factor for brucellosis prevention. This may be due to experience that the older farmers have more familiarity with recognizing the clinical signs of the disease or the main route of transmission and can be more aware of the importance of preventive measures.

Based on the questionnaire survey, most of the respondents were practice with uterus discharges without using protective cloth (87.20%) table (6). Even though using protective cloth are the most known measures against zoonotic brucellosis (Radostits *et al.*, 2007). These factors combined with the poor cleaning practice by the owners could pose a great risk of the spread of the disease to unaffected animals (Tolosa, 2004). In addition, most of the respondents in this study with the large herd size (70%) did not bury afterbirth (aborted fetus, still birth and retained foetal membrane), rather they left them on open field and fed dogs or wild carnivores.

Studies also assessed the occupational risks that, the majority of the participants in both types of farms have the habit of drinking raw milk. This was may be attributed to the culture and tradition of consuming raw milk and milk product (Omore *et al.*, 1999). The seroprevalence study research conducted in high risk group such as farmers, veterinary professionals, meat inspectors and artificial insemination technicians in Amhara Regional State (Mussie *et al.*, 2007b), Sidama Zone of Southern People Nations and Nationalities Sate (Kasahun *et al.*, 2007) and Addis Ababa (Kassahun *et al.*, 2006) found a seroprevalence of 5.30%, 3.78% and 4.8% by screening sera from 238, 38 and 336 individuals respectively.

6. CONCLUSIONS AND RECOMMENDATIONS

Eventhough this study of Western Ethiopia has shown that the prevalence of bovine brucellosis observed in cattle at the individual animal level is very low, based on three steps testing of sera collected, brucellosis is continues to be a major animal health problem in Ethiopia. In this finding selected district (origin) where sample collected, source of stock and source of replacement stock were an important risk factor. Other significant risk factors in study area were abortion history and retain fetal membrane. This study also provides important information on knowledge attitude and practice of livestock owners and occopetional workers towards of brucellosis that result in siginificant zoonotic importance of using raw milk for human consumption.

Based on the above conclusion the following recommendations are forwardedon the bases of finding results:

- The seropositive animals should be culled and public awareness about brucellosis economic and zoonotic importance
- Constant surveillence program should be designed and implemented nationally
- Further research on the isolation and characterizatioon of its caustive agent, *Brucellae species* should be conducted by using the level three-biosefty laboratory and by molecular technique in livestocks and human.

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8. ANNEXES

Annex 1: Questionnaire format

Seroepidemiology of Bovine Brucellosis in and around in Horro Guduru Animal Production and Research Center, and its surroundings Western Ethiopia

QUESTIONNAIRE FORMAT

Date

Code.....

I General Information of Respondent

1. Name of Respondent.....

1.1. Age: _____ 1.2. Sex: _____ 1.3 Address-----

1.3. Family Status: Single _____ Married _____ Divorced _____

1.4. Educational background a) Primary school _____ c) College/University _____

b) Secondary school _____ d) non academic/informal _____

II. Response of Respondent on Livestock Management

1. Type of farming; a) semi-intensive b) extensive

a) Dairy only b) beef c) crop-livestock mixed d) other

2. Herd size: a) <10 b) 10-20 c) >20

3. Breed: a) Local b) Cross breeds

4. Herd type: A) Dairy B) Beef C) other

5. Number of adult cows _____.

6. Number of adult bulls _____.

7. Number of adult bulls for service _____

8. Number of productive cows: _____

9. Number of unproductive females (cows)

10. Number of served heifers _____

11. Number of replacement heifers (weaning to service) _____

12. Number of calves (birth to weaning) _____

13. Why do you keep animals (cattle)? A) For dairy B) beef C) for drought D) for all

14. Source of replacement A) From local market B) From governmental farms

15. Which breed of cattle do you have/handle ?

Breed of cattle	number	sex	adult	Heifer	bull	calf
Local						
25% cross						
50% cross						
75% cross						
More than 75%cross						
100% exotic						

16. What is parity status and average calving interval of the dairy cows in the farm?

Identity of the cows	Parity status	Calving interval

17. What type of insemination is used in the farm? a) AI b) natural C) both

III. Response of Respondent on Husbandry Practice

1. Type of housing Barn a) separate b) common

Corral a) separate b) common

Open field a) separate b) common

2. Human housing A) Housed with family and livestock

B) Housed in family without livestock

3. General Hygiene of the House: A) Very good, B) Good C) Satisfactory D) Poor

4. Do you separate cows during parturition? Yes/ No

5. What are the commonly encountered disease affecting cattle in order of importance?

A..... C.....

B..... D.....

6. Do you know disease that cause abortion in cattle? Yes/No

7. What are the name of the disease locally? A).....

B).....

C).....

8. Was any event of abortion in your farm? yes/no

8.1. If yes which animals and what time(months) does occurred?

Cow identification	Time of abortion

8.2. was any event of still birth in your farm a)yes b) no

8.3. If yes which animals and what time(months) does occurred?

Cow identification	Time of still birth

8.4. Which animal mostly abort ? A) heifer B)cows C) equally

8.5. Did you encounter retain fetal membrane in your farm ? a) yes b) no

8.6. If yes from which animal? A) aborted B) still birth C) normal one

8.7. Do you know disease Brucellosis A) yes B) no

8.3.Where do you dispose the aborted/still/RFM birth or retain placenta?

A) Burying

B) Open dump

C) Fed to Dogs

D) Throughover the field

8.4. How many animals have aborted in your farm during last three years? _____

9. What do you do when your animals abort ?.A) Take to clinic

B) Dispose aborted fetus and others

C) Others

9.1.How do you dispose the aborted fetus? A) using protective B) by hand

9.2. Where do you dispose aborted fetus A) On field B) Deep ground

10. Do you keep other animals in the farm? Yes/ No If yes which animal

11. What are the reasons of culling in the farm? a)Disease b) Old age

c) Infertility poor production d) others

12. Do you drink raw milk and its products? a) No, b) Sometime , c) frequently

12.1. If no in what form do you drink milk? a) Boiled b) processed c) pasteurized

12.2. Has any member of the family felt sick and visited health institutes during last six months? Yes/no

If yes what was the health complaints and symptoms?

a)..... c).....

b)..... d).....

Annex 2: Blood Collection Format

Sr.no	Owner name	site	Animal ID	sex	Breed	age	Parity	Current p/stage	Abortion history	RFM	Mating type	Abortion time	RBPT	c-ELISA	CFT
1															
2															
3															
4															
5															
6															

Annex 3: Rose Bengal Plate Test

- Place the same volume (30µl) of neat serum (if possible non-inactivated) a
- Then antigen side by side on a plate
- Mix thoroughly and rapidly the serum and the antigen
- Shake lightly the plate for 4 minutes.
- After four minutes of rocking, any visible agglutination had been considered as positive result

Interpretation

- ❖ No agglutination: negative
- ❖ Any visible agglutination: positive
- ❖ Flocculates (false agglutination): un-interpretable or unreadable

Annex 4: competitive Enzyme Linked Immuno-Sorbent Assay (c-ELISA)

Using a commercial c-ELISA kit (BRUCELISA(160+400), (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom)

When shortly summerize the test,

- the positive and negative controls were used at a dilution of 1/40 as has been indicated by the manufacturer.
- Following the addition of the conjugate and substrate-chromogen mixture at a recommended strength,
- the plate was incubated and examined for the intensity of reaction with an automated ELISA reader at 405 nm.

Interpretation

Color development within a well indicates that the tested serum has antibodies to *Brucella*. A positive/ negative cut-off was calculated as 15% of the mean of the optical density (OD)

of the eight positive control wells. And then test sera were analyzed at a final dilution of 1/200. Finally any test serum with an OD value equal to or above this value was considered as positive.

Annex 5: Complement Fixation Test

The CFT performed according to the procedure test:

- The sera pre –diluted 1:25 and incubate at 25⁰c in water bath for 30 minutes in order to inactivate the complement.
- 25µl of diluted test was placed in wells of first and second rows of U-bottom plate, and 25µl of veronal buffer was added to all the wells except those of the first row.
- Serial doubling dilutions were made by transferring 25 µl volume of serum from the second row on wards continuing at least for seven dilutions.
- 25µl of antigen at working dilution was added to all the wells excluding those of the ant complementary controls, which receives 25µl VCM instead.
- 25µl of complement in working dilution was added to all the wells except control wells
- The plate are incubated at 37 °c for 30 minutes with agitation (warm fixation)
- 25µl of volume sensitized 2% sheep red blood cell suspension was added to each well. The plate are sealed and re-incubated at 37 °c for minutes with agitation.
- Results were taken on next day after the plates are kept overnight at 4 °c

Interpretation

Sera with a strong reaction more than 75% fixation of the complement (3+) at a dilution of 1:5 and with at least 50% fixation of the complement (2+) at dilutions of 1:10 and 1:20 were classified as positive (+), according to the guidelines of the OIE *Manual* (OIE, 2002).

Annex 6: Miscelinous pictures

Rose Bengal plate test positive result

