ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

SERO-PREVALENCE OF BRUCELLOSIS AND ASSOCIATED RISK FACTORS IN DAIRY CATTLE IN SULULTA TOWN, CENTRAL ETHIOPIA

MVSc THESIS

BY

NAOL MULUGETA

DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

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SERO-PREVALENCE OF BRUCELLOSIS AND ASSOCIATED RISK FACTORS IN DAIRY CATTLE IN SULULTA TOWN, CENTRAL ETHIOPIA

MVSc thesis submitted to the College of Veterinary Medicine and Agriculture of Adiss Ababa University in partial fulfillment of the requirments for the degree of Masters of Science in Veterinary Microbiology

By

Naol Mulugeta

Academic Advisors:

Hika Waktole (BSc, MSc, Assist Professor)

Gezahgne Mamo (DVM, MSc, PhD, Assoc. Professor)

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College of Veterinary Medicine and Agriculture

Department of Veterinary Microbiology, Immunology and Public health

As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by: **Naol Mulugeta** Entitled: **Sero-prevalence of brucellosis and associated risk factors in dairy cattle in Sululta town, Central Ethiopia**. And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

Mr. Takele Beyene	_____	_____
Chairman	Signature	Date
Dr. Kebede Amenu	_____	_____
Internal Examiner	Signature	Date
Dr. Hunduma Dinka	_____	_____
External Examiner	Signature	Date
Mr. Hika Waktole	_____	_____
Major advisor	Signature	Date
Dr. Gezahgne Mamo	_____	_____
Co advisor	Signature	Date
Dr. Gezahgne Mamo	_____	_____
Department chairperson	Signature	Date

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Name: Naol Mulugeta
College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia

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LIST OF ABBREVIATIONS

Ab	Antibodies
Ag	Antigen
AMOS	*Abortus*, *Meltineses*,*Ovis*,Suis
APCs	Antigen-Presenting Cells
BCV	Brucella-containing vacuole
cELISA	competitive Enzyme Linked Immunoabasorbant Assay
CFT	Complment Fixation Test
CI	Confidence Interval
COVID 19	Coronavirus Disease 2019
CSA	Central Statistics Agency
ELISA	Enzyme-Linked Immunosorbent Assay
FAO	Food Agricultural Organization
FPA	Fluorescence Polarization Assay
G+C	Guanine + Cytosine
iELISA	Indirect Enzyme-Linked Immunosorbent Assay
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
Masl	Meter Above Sea Level
Mbp	Mega Base Pair
MHC II	Major Histocompatibility Complex type II
MRT	Milk Ring Test
MZN	Modified Ziehl-Nielsen
NAHDIC	National Animal Health Diagnostic and Investigation Center
OMP	Outer membrane Protein
PRR	Pattern Recognition Receptors
PCR	Polymerase Chain Reaction
q-PCR	Real Time Polymerase Chain Reaction
RBPT	Rose Bengal Plate Test
rRNA	Ribosomal Ribonucleic Acid
SAT	Standard Agglutination Test

S-LPS	Smooth Lipopolysaccharide
TCR	T-cell receptor
Th1	T helper type 1
Th2	T helper type 2
TNF-α	Tumor Necrosis Factor alpha
TLR	Toll-like recptor

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ABSTRACT

A crosssectional study was conducted between November 2019 to May 2020 to estimate the seroprevalence of bovine brucellosis and associated risk factors for infection in dairy cattle in Sululta Town, Central Ethiopia. A total of 436 dairy cattle that composed of 110 males and 326 females greater than six months of age were sampled and tested for *Brucella* antibodies. The serum samples collected were firstly screened by using the Rose Bengal Plate test and confirmed by the Complement Fixation Test. The animal level seroprevalence was calculated as the number of seropositive samples divided by the total number of samples tested. Herd prevalence was calculated as dividing the number of seropositive herds in Complement Fixation Test with the number of herds tested. Thus the animal level seroprevalence of brucellosis in dairy cattle was 0.23% (95% CI: -0.2-0.7%) and herd-level was found to be 0.8% (95% CI: -0.08- 0.2%) by Complement Fixation Test. A Chi-square computed statistical analysis indicated that large herd size (> 20 animals) have a significantly association with the seroprevalence of bovine brucellosis as compared to small herd size and medium herd size ($\chi 2=17.20$; $P<0.05$). At herd level, herd size and history of abortion in the herd had a statistically significant association with herd-level brucellosis positivity ($P<0.05$). In conclusion, low prevalence was recorded at the study area and risk factors were present. Therefore, further isolation and molecular characterization of *Brucella* should be conducted to distinguish which *Brucella* species is circulating in the study area and awareness creation among the dairy farming community should be conducted.

Key words: *Bovine and Brucella*

1. INTRODUCTION

Ethiopia is believed to have the largest livestock population in Africa with total cattle population for the country is estimated to be about 60.39 million. Out of this dairy animals of the country is estimated to be 19.5 million (CSA, 2017). Even though Ethiopia has such a huge livestock population transboundary and zoonotic diseases such as bovine brucellosis is affecting the dairy production sector of the country and affecting livelihoods by the impact on animal health and milk production.

Bovine brucellosis is a main zoonotic disease with having worldwide occurence. Most developed countries has eradicated the disease however, it is still endemic in countries such as Africa, Asia and Latin America (Moreno, 2014). In cattle, the main causative agent is *Brucella abortus*. However it can also caused occasionally by *Brucella melitensis*, when infected sheep or goat kept together with cattle (Asgedom *et al*., 2016; Edao *et al*., 2018).

The disease is primarily reproductive disease characterized by causing abortion, impaired fertility, metritis, orchitis and epididymitis (Radostits *et al*., 2007; Seleem *et al*., 2010). Large amount of infectious bacteria are found in aborted fetuses and fluids that have a key role for transmission of disease in herds. Furthermore infected animals can shed lower amount of bacteria in milk which have impact for transmission to calves (McDermott and Arimi 2002; Adugna *et al*., 2013).

Humans can acquire the disease mainly through direct contact with infected animal fluids and tissues, inhalation of the bacteria and drinking of contaminated unpasteurized dairy products (Doganay and Aygen 2003). Dairy farmers, veterinarians, breeders and abattoir workers have higher chance for acquiring the disease because they have direct contact with cattle as compared to others (Corbel, 2006).

Diagnosis of brucellosis plays a key role for controlling and eradication program. It can be done by directly demonstration of the causative organism using culture, or polymerase chain reaction (PCR) or indirectly by demonstration of antibodies using serological techniques (Seleem *et al*., 2010).

In Ethiopia, brucellosis in livestock is known to be endemic in different production system, species and breeds of domestic animals since its first report in the 1970s (Domenech, 1977). A number of serological studies have been conducted from different parts of Ethiopia with prevalnce ranging from 0.05- 15.2% in extensive production systems (Hunduma and Regassa 2009; Degefa *et al*., 2011). Where as the in intensive production system the prevalence rate ranges from 0-11.0 % (Kebede *et al*., 2008; Lakew *et al*., 2019).

Although much work has done in different parts of Ethiopia, there is limited or no studies done on seroprevalence of bovine brucellosis and associated risk factors for infection in dairy cattle in Sululta Town, Central Ethiopia. Therefore, the study was designed

General objective

- To determine the seroprevalence of bovine brucellosis and associated risk factors for infection in dairy cattle in Sululta Town, Central Ethiopia.

Specific objectives

- To estimate the seroprevalence of bovine brucellosis in Sululta Town.
- To determine associated risk factors for infection based on RBPT and CFT in dairy cattle in Sululta Town.

2. LITERATURE REVIEW

2.1. Etiology

2.1.1. Brucella taxonomy and species with host preference

Brucella is taxonomically classified under the family of *Brucellaceae*, order of *Rhizobiales* and class *Alphaproteobacteria* (OIE, 2018). Presently, the genus contains twelve species in which most of them are highly host-specific. The main species that affect terrestrial animals are *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, and *B. microti* (Scholz *et al*., 2008). From these species *B. abortus* have seven biovars, *B. melitensis* have three biovars, *B. suis* have five biovars. Moreover, six species were also added to the genus known as *B. ceti*, *B. pinnipedialis, B. inopinata B. papionis* and *B. vulpis* (Whatmore *et al*., 2014; Scholz *et al*., 2016).

Table 1: List of *Brucella* species and host preference

Species	**Host preference**	**Zoonotic importance**
B. abortus	Cattle	High
B. melitensis	Sheep and goats	High
B. suis	Swine	Moderate
B. canis	Dogs	Absent
B. ovis	Sheep	Mild
B. ceti	Dolphins	Mild
B. neotomae	Dessert wood rats	Absent
B. pinnipedialis	Seals	Mild
B. microti	Voles	Absent
B. inopinata	Unknown	Mild
B. papionis	Red fox	Unknown
B. vulpis	Baboon	Unknown

Source: Godfroid *et al*. (2013); Scholz *et al*. (2016)

2.1.2 Brucella structure and morphology

Brucella are aerobic, slow-growing and facultative intracellular bacteria that can invade, persist and replicate inside in macrophages, placental trophoblast, epithelial and dendritic cells. Structurally they do not have neither capsule nor flagella. They measure from 0.6-1.5 micrometer long and from 0.5-0.7 micro-meter wide (Gorvel, 2008). In gram staining they appear as gram-negative coccobacilli. In modified Ziehl-Neelsen (MZN) stained smear of body fluids or tissues, they appear as clusters of red coccobacilli and are known as to be MZN– positive since they are not decolorized by 0.5% acetic acid (Quinn *et al*., 2011).

The colony morphology of *Brucella* is different among the species. Most have a smooth colony morphology; a characteristic related with the expression of the lipopolysaccharide (LPS) O-side-chain. While, other *Brucella* species such as *B. ovis* and *B. canis* have rough colony morphologies because they do not express the LPS O-side-chain. Smooth colonies appear round, shining and blue-green in color and do not take up crystal violet stain. Whereas, rough colonies have dry, granular appearance, yellowish-white in color and takes up crystal violet stain (Scott *et al*., 2013).

2.1.3 Genome of Brucella organism

The genome of *Brucella* is different from among veterinary pathogenic bacteria since it is made of two round chromosomes except from *B. suis biovar* 3, that has a only one chromosome. The two round chromosome is known as Chromosome I and Chromosome II having an average genome size of around 3.29 Mbp. Their individual genome size of Chromosome I and Chromosome II is about 2.11 Mbp and 1.18 Mbp respectively (Halling *et al*., 2005; Quinn *et al*., 2011). The G+C ratio for Chromosome I and Chromosome II are 57.2% and 57.3% respectively (Quinn *et al*., 2002). There is vital difference between two chromosomes. The large chromosome (Chr I) is classic bacterial chromosomes like and most of the essential genes are located on Chr I, incontrast the small chromosome (Chr II) is plasmid like (Halling *et al*., 2005).

2.1.4 Growth requirement and biochemical characteristics

Growth of *Brucella* is enhanced in an atmosphere condition of 5-10% CO_2. *Brucella* species require an optimal temperature of 37^0C and optimal pH from 6.6-7.4 for growth. However they can also grow under temperatures ranging from 20-40^0C (Alton *et al*., 1988). Growth of the other *Brucella* such as *B. ovis and B. abortus biotype* 2 is enhanced enriched media with blood or serum. However, they can also grow on nutrient media (Quinn *et al*., 2011; Quinn *et al*., 2016).

The biochemical characteristics of most *Brucella* species are catalase-positive, indole negative, oxidase-positive (excluding *B. ovis* and *B. neotmae),* and urease- positive (excluding *B. ovis).* Furthermore they have an ability to reduce nitrate to nitrite and Methyl Voges proskar negative (Ananthanarayn and Paniker's, 2005; Quinn *et al*., 2011; Quinn *et al*., 2016).

Table 2: Characteristics of *Brucella* species of veterinary importance.

Brucella species	No of biotypes	CO_2 requirement	Production of H_2S	Urease Activity	Growth in media containing	
					Thionin (20 µ g/ml)	Basic fuchsin (20 µ g/ml)
B.abortus	7	V	V	+	V	V
B. melitensis	3	-	-	V	+	+
B. suis	5	-	V	+	+	V
B. ovis	1	+	-	-	+	-
B. canis	1	-	-	V	+	-

V: variable reactions related to different biotypes.

Source: Quinn *et al*. (2011)

2.2 Epidemiology

2.2.1 Source of infection and transmission mode

Aborted fetuses, fetal membranes, vaginal discharges and milk from diseased animals are the main sources of infection (Tolosa *et al*., 2010; Geresu *et al*., 2016). Bovine brucellosis can be transmitted through direct contact with aborted discharges and through indirect contact with contaminated fomites. Ingestion of contaminated pasture, feed, fodder, and water can act as secondary role for transmitting the disease (Robinson, 2003). Born calves can aquire the disease vertically from milk and reproductive discharge of chronically infected cattle (McDermott and Arimi 2002; Adugna *et al*., 2013). Semen collected from infected bulls for artificial insemination has a great potential of transmitting the disease (Eshetu *et al*., 2005).

2.2.2 Risk factor

The risk factors which can contribute for animal brucellosis can be classified into those related with characteristics susceptible animal population, management system and the organism biology (McDermott and Arimi 2002; Radostits *et al*., 2007).

The risk factor related with organism biology: *B. abortus* is a facultative intracellular organism that has ability to multiply and survive inside host phagocytes. Polymorph nuclear leukocytes phagoctyes the organisms. Organisms, which has survived, are transported to lymphoid tissues and fetal organs. The organism have unconventional non-endotoxin lipopolysaccharide. This lipopolysacride helps the bacterium to survive within macrophages and replicate in phagolysosome. Moreover it helps the organisms to reverses resistance to antimicrobial attacks and controls the host immune response (Lapaque *et al*., 2005).

The risk factor related with host: The risk factor related with characteristics disposed animal population are age, sex and reproductive status of the animal that effect the vulnerability to bovine brucellosis. Based on age, the vulnerability increases when stage of growth increases. For example, younger animals are resistant as in contrast to sexually mature animals (Colibaliy *et al.,* 2000; Zee and Dwight, 2003). Regarding the

sex, for instance bulls are comparatively resistant as compared to sexually mature heifers while less resistant than young heifers (Godfroid *et al*., 2010).

The risk factor related with management: The management-related risk factors which contribute to spread of infection include the type and compostion of herd, hygiene status of the farm and regular contact with diseased herds (McDermott and Arimi 2002; Radostits *et al*., 2010).

2.2.3 Host range

Brucellosis is infectious disease caused by the genus *Brucella*. Bovine brucellosis is mainly caused by *B. abortus*, frequently by *B. melitensis*, and seldomly by *B. suis*. Other livestock animals can occasionally infected with *B. abortus* when they are herded together with infected animals (Kebede *et al*., 2008). *B. melitensis* is the main species affecting sheep and goats and *B. ovis* is alternative species that infect sheep (Corbel, 2006; Lilenbaum *et al*., 2007). The primary source of *Canine* brucellosis is *B. canis* however, other *Brucella* species such as *B .abortus*, *B. melitensis B. suis* can cause sporadic cases (Acha and Szyfres 2003).

2.2.4. Occurrence

Brucellosis is one of the world's main zoonotic disease having worldwide occurrence with the exception of developed countries that had been eliminated (Gul and Khan, 2007). But, the disease is still prevalent in counries such as Europe, Asia, Africa, India, Near East countries, Central and South parts of America (Refai, 2002).

In African countries, it has been an important livestock disease. The disease was firstly reported in countries such as Zimbabwe, Kenya and South Africa respectively (Chukwu, 1985). Currently brucellosis is prevalent in most African countries for instance in Western parts of Africa, Central Africa (Dean *et al*., 2012; Sanogo *et al*., 2013; Boukary *et al*., 2013) and East Africa (Asmare *et al*., 2010; Muendo *et al*., 2012). The prevalence and the epidemiology of the disease in livestock and humans are not still known. Moreover the appropriate preventive methods are not well identified and such information is inadequate mainly in sub-Saharan Africa with the exclusion of

brucellosis structured eradication programs found in southern Africa (McDermot and Arimi 2002; Racloz *et al*., 2013; Pappas *et al.,* 2006).

2.2.5 Occurence of bovine brucellosis in Ethiopia

In Ethiopia, there is no recognized information when and how the disease was entered and established. However, it was first reported in the 1970s (Domenech, 1977; Meyer, 1980). Bovine brucellosis has reported from different areas of the country with seroprevalence ranging from 0-11.0 % in intensive production system (Kebede *et al*., 2008; Lakew *et al*., 2019) and 0.05-11.2% in extensive management systems using different serological tests (Hunduma and Regassa 2009; Degefa *et al*., 2011). The results of seroprevalence studies of bovine brucellosis in the both production systems are summarized in (Table 3).

Table 3: Seroprevalence of cattle brucellosis in different areas of Ethiopia.

Place	**Tests**	**Prevalence(%)**	**Reference**
Tigray	RBPT, CFT	3.2	Berhe *et al*. (2007).
Central Ethiopia	RBPT, CFT	11.0	Kebede *et al*. (2008).
East Showa Zone	RBPT	11.2	Hunduma and Regassa (2009).
Sidamo zone	RBPT, CFT	1.66	Asmare *et al*. (2010).
Western Tigray	RBPT,CFT	4.9	Haileselassie *et al*. (2010).
Arsi Oromia	RBPT, CFT	0.05%	Degefa *et al*. (2011).
Jijjiga	RBPT, CFT	1.38	Hailu *et al*. (2011).
Western Tigray	RBPT, CFT	6.1	Mekonnen *et al*. (2011).
East Wollega	RBPT, CFT	1.97	Yohannes *et al*. (2012).
Ethiopia(N,W,E,C)	RBPT, CFT	1.9	Asmare *et al*. (2013).
Benishangul	RBPT, CFT	1.21	Adugna *et al*. (2013).
Southeast	RBPT, ELISA	1.4	Gumi *et al*. (2013).
Debrebirhan,Ambo	RBPT, CFT	0.2	Bashitu *et al*. (2015).
Asela	RBPT, CFT	2.6	Tsegaye *et al*. (2016).
Alage, Oromia	RBPT, ELISA	2.2	Asgedom *et al*. (2016).
Bishoftu and Arsi	RBPT, CFT, ELISA	1.4	Geresu *et al*. (2016).
North Shewa	RBPT, CFT	0	Pal *et al*. (2016)
Bishoftu	RBPT, CFT	3	Waktole *et al*. (2018).
Western Ethiopia,	RBPT, CFT, ELISA	0.73	Garoma (2018).
Addis Ababa	RBPT, CFT, ELISA	0.06	Edao *et al*. (2018).
Hawassa, Town	RBPT, CFT	2.7	Abera *et al*. (2019).
Somalia Fafan	RBPT, CFT	0	Lakew *et al*. (2019).
West Shewa	RBPT, ELISA	1.04	Tadesse *et al*. (2019).

Seroprevalnce of bovine brucellosis in intensive production sytsem

Based on available data lower prevalence rate of bovine brucellosis was documented in the intensively production system incontrast to the extensive production system.

Kebede *et al*. (2008) conducted a study in the intensive cattle production systems that found that an overall prevalence of 11.0 % in Central parts of Ethiopia. As compared to the above study, Bashitu *et al*. (2015) recorded lower prevalence of 0.2 % in Debrebirhan and Ambo town and Waktole *et al*. (2018) recorded 3% overall prevalence in Bishoftu by using RBPT and CFT. The authors explained that the low prevalence of bovine brucellosis in the study areas was because of good sanitary practices such as cleaning and disinfection activities, separation of cows during parturition or use of maternity pen for animals in parturation, culling of *Brucella* infected animals, replacing animals from own herds and farm owners awareness of brucellosis in these intensive farms.

Similarly another study conducted in dairy farms of Addis Ababa found a low prevalence of 0.06% after sampling of 1550 cattles (Edao *et al*., 2018). According to the authors, the low prevalence was due to informal culling practices such as culling of cows having repeated abortion history, eliminating seropositive animals from the herd for economic reasons and absence of infectious agent in and around the study areas.

In contrast to the above reports, Pal *et al*. (2016) failed to find seropositive cattle in Northern Showa after screening 384 cows by using RBPT and CFT despite revealing that abortion and retained fetal membrane case in the study region.

Seroprevalence of bovine brucellosis in extensive production system

In Ethiopia, the majority of cattle are raised under extensive production systems. Based on the reports so far the seroprevalence of bovine brucellosis is high in the extensive cattle production systems in contrast with intensive production systems. For example, Hunduma and Regassa (2009) documented a high prevalence rate of 11.6 % in the Eastern Showa Zone. The author's reason for high prevalence in the study area was due to the sharing of feed and water with other herds that gives a higher chance for getting the disease from other potentially infected herds during their movement into the

different areas. Another study conducted in Western Tigray recorded an overall prevalence in female cattle 6.1% after screening of 1,354 cattle (Mekonnen *et al*., 2011)

Another seroepidemiological study done out in the extensive production system of Tigray Region documented an overall prevalence of 3.19 % after a sampling 816 cattle (Berhe *et al*. 2007). Another study conducted by Asmare *et al*. (2010) in the extensive system of Southern Sidama Zone recorded a low prevalence rate of 1.66% after selection 1627 cattle. In contrast to the above reports, Lakew *et al*. (2019) unsuccessful find seropositive cattle in Somalia after screening 268 cattle.

2.3. Clinical sign

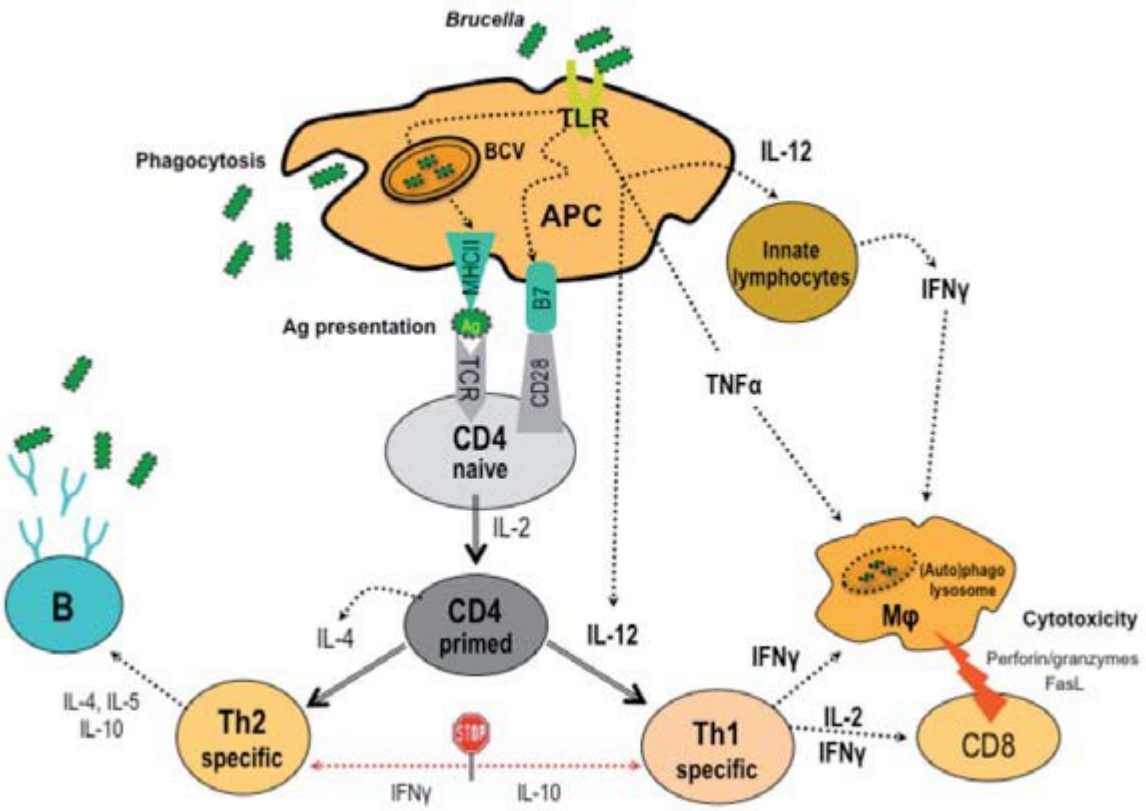
Brucellosis mainly affects sexually mature animals and localizes in the reproductive system. Young animals and non-pregnant females are also affected by the disease without showing the clinical sign of the disease. Brucellosis is characterized by causing placentitis and metritis followed by abortion in pregnant female during the last third trimester of pregnancy (Acha and Szyfres 2003; Corbel, 2006; Xavier *et al*., 2009). Abundant excretion of the organism can occur mainly in the fetal fluids, placenta and vaginal discharges even in the absence of abortion. Other organs such as lymph nodes and mammary gland could be infected by the disease. Later pregnancies are usually carried to term, but uterine and mammary infection relapses, with lower amount of organisms in afterbirth products and milk. In acute infections, the organism is present in most major body lymph nodes (OIE, 2018).

In bulls, *Brucella* confine in the testicles and adjacent genital glands. When the clinical disease is obvious, one or both testicles may become enlarged, with decreased libido and infertility. Occasionally a testicle may atrophy due to adhesions and fibrosis. Seminal vesiculitis and ampullitis are common in the infected bulls. Sometimes, hygromas and arthritis are observed in cattle (Acha and Szyfres 2003).

2.4. Immunity

Both the humoral immunity and cellular immunity are involved in *Brucella* infection; however, extent varies with the virulence of the organism and immune status of the animal (FAO/WHO, 1986). Phagocytic cells play a crucial role in starting T-cells through processing and presenting the antigens. Chronic granulomatous lesions develop in infected tissue where macrophage, neutrophils and lymphocytes respond to *Brucella* antigens. *Brucella* triggers Antigen Presenting Cells (APCs) to release interleukin (IL-12), which causes T helper cells to differentiate into T helper type 1 (Th1) cells, which secrete interferon gamma (IFN-γ) or IL-2. The T helper type 1 (Th1) cytokines increase anti-*Brucella* mechanism of macrophage or induce the $CD8^+$ cytotoxicity. Macrophages readily ingest *Brucella* when opsonized with either complement or specific antibodies. Both $CD4^+$ and $CD8^+$ subsets are involved in cell-mediated protection in part because they secrete interferon-gamma (IFN-γ) for the activation of bactericidal functions in macrophages (Ko *et al*., 2003).

The T helper type 2 (Th2) cytokines inhibit the action of T helper type 1 (Th1) cytokines and activate B cell for antibody production, enabling the phagocytosis of *Brucella* through opsonization (Skendros and Boura 2013). The survival of the organisms in macrophages may result from a failure of phagosome-lysosome fusion and resistance to oxidative killing by producing superoxide dismutase and catalase (Quinn *et al*., 2002). The O-antigen of intact LPS has a vital role in protection against intracellular killing; rough forms of *Brucella* cannot prevent fusion of lysosomes with *Brucella*-containing vacuole. Thus, inhibition of phagosome–lysosome function is the major mechanism for intracellular survival and an important determinant of bacterial virulence (Quinn *et al.,* 2016). The O-chain specific antibodies play a main role in protecting immunity however, they do not eliminate the organisms as they are protected being intracellular. This shows that a lack of relationship between protection and high antibody level (Walker, 1999; Benkirane *et al*., 2014).



Source: Skendros and Boura (2013).

Figure 1: Simplified representation of immune response against *Brucella*

2.5. Pathogenesis

Brucella species can enter the body through the lungs, the digestive tract, mucous membranes, and intact skin. After they enter the body, the organisms are phagocytized by neutrophils and macrophages. However, they have the capability to survive and replicate in the macrophages of different mammalian cells, which helps to prevent exposure of the organism to the innate immune response (Martirosyan and Gorvel 2013). They survive within the phagocytic cell and live in a vacuole named the *Brucella*-containing vacuole (BCV), in which they control the internal environment and transform the vacuole into a brucellosome (Arenas *et al*., 2000; Köhler *et al*., 2002).

After replication, the bacteria are released by prompting cell lysis then move towards regional lymph nodes where they multiply, cause lymphadenitis and subsequently enter the circulation to result in bacteremia (Gorvel and Moreno, 2002; Hirsh *et al*., 2004).

Once they are in the bloodstream, the organism spreads to multiple organs, there by displaying an affinity for reticuloendothelial tissues, such as the liver, spleen, the skeletal, hematopoietic system and both male and female reproductive tracts, where it causes localized infection (Greenfield *et al.,* 2002). The main reason for the localization of *Brucella* species in the reproductive tract is due to the presence of steroid hormones and erythritol. Erythritol is a polyhydric alcohol that aids as a growth factor for *Brucella*, which is present in high concentrations in allantoic fluids by stimulating the replication of *Brucella* (Xavier *et al*., 2009; Quinn *et al.,* 2016).

2.5 Diagnosis

The diagnostic tests can be categorized into two types such as those that demonstrate the presence of the organisms (direct) and those that detect an immune response to its antigens (indirect) (Corbel, 2006).

2.5.1 Staining and microscopy of Brucella organisms

Brucella can be microscopically examined by staining from smears of vaginal discharge, or placental or fetal tissue by using Koster or modified Ziehl-Neelsen (mZN) procedures. They are not actually acid-fast and can not decolorized through weak acids, hence looks as red alongside a blue background. They are coccobacilli or short rods, usually arranged singly but sometimes-in pairs or small groups. However, they can be miss diagnosed because they resemble with other infectious agents such as *Coxiella burnetii* or *Chlamydia* (Alton *et al*., 1988; Corbel, 2006).

2.5.2 Bacteriological diagnostic method

The gold standard diagnostic method for brucellosis is through isolation of the organism because it helps for differentiating the biotypes of *Brucella* with high specificity (Al Dahouk *et al*., 2003; Bricker, 2002). However, isolation of *Brucella* is challenging due to requirement of advanced skills to handle the organism and Bio-Safely Level 3 (BSL-3) laboratory facilities (Ahasan *et al*., 2017). Moreover, culturing of the bacteria requires a large amount live bacteria in samples, appropriate storage and quickly

transportion by cold chain to the laboratory (Hadush and Pal, 2013; Seleem *et al*., 2008).

The most important samples required for culturing of *Brucella* includes vaginal swabs, fetal membrane, milk, and semen. While organs such as spleen, bronchial lymph nodes,lung, and liver can used as other secondary choice of samples from abattor (Poester *et al*., 2006).

The Farrell medium is the most commonly used medium for the isolation of *Brucella* species that contains antibiotics such as bacitracin (25mg), polymyxin B sulphate (5mg), nalidixic acid (5mg), nystatin (100,000 units), vancomycin (20mg), natamycin (50mg). However, the Farrell´s medium has a drawback of growing some *B*. *abortus*, *B*. *melitensis*, and *B*. *ovis* biotypes. Therefore, a modified Thayer-Martin medium may be used together with Farrell's in order to grow biotypes. modified Thayer-Martin can be prepared with GC medium as basal medium supplemented with 1% hemoglobin and antibiotics containing per liter of medium, colistin methanesulphonate (7.5mg), amphotericin B (2.5mg), vancomycin (3mg), nitrofurantoin (10mg) and nystatin (100,000 units) (Poester *et al*., 2010; Miguel *et al*., 2011).

Another recommended solid media for primary isolation of *Brucella* includes dextrose agar, tryptose agar, and trypticase soy agar. However, in order to improve the growth of *B. ovis* and *B. canis* it require the addition of 5-10% of sterile bovine or equine serum to these media. In the case of blood or milk, biphasic media such as Castaneda's medium can be used since it improve sensitivity from this samples (Poester *et al*., 2010).

Brucella species can be biochemically differntiated using different tests. Some of them are by agglutination tests with antibodies against the A or M epitopes of O chain lipopolysaccharides (O-LPS), phage typing, growth on CO_2 supplement in culture, H_2S production, growth in the presence of basal fuchsine or thionine and crystal violet or acriflavine test (Alton *et al*., 1988).

2.5.3 Molecular diagnostic methods

Molecular diagnostic methods are used for diagnosis and epidemiologic studies, since they provide significant information for identification of species and biotypes of

Brucella by permitting differentiation between field and vaccine strains (Le Flèche *et al*., 2006; López-Goñi *et al*., 2008). They are used most broadly for diagnosis of brucellosis because they give high sensitivity than blood culture and more specificity than serological tests. They can be used directly on clinical samples or with previous isolation of the organism to pair results obtained from bacteriological method (Bricker, 2002; Al Dahouk *et al*., 2013).

Polymerase chain reaction (PCR)**:** is one of technique that allows for rapid and accurate diagnosis of brucellosis without the restrictions of conventional methodology (Baddour, 2012). The principle is based on amplifying using primers specific genomic sequences such as the 16S rRNA and the *Brucella* Cell Surface 31 kDa Protein (bcsp31) genes that are highly conserved in the genus *Brucella* (Baily *et al*., 1992; Romero *et al*., 1995; Da Costa *et al*., 1996; O'Leary *et al*., 2006)

Multiplex PCR: Different type of multiplex PCRs exists which can identify *Brucella* at genus or species level and relatively at the biovars level by using different primers combinations. The first multiplex PCR is called AMOS PCR assay (AMOS is an abbreviation for "*abortus-melitensis-ovis- suis*"), that is composed of five oligonucleotide primers for the differatation of selected biovars of four species of *Brucella*. The principle of this AMOS PCR is relies on the polymorphism arising from species specific localization of the insertion sequence IS711 found in chromosome of *Brucella* (Bricker and Halling 1994). However, it has its own disadvantage such as having a difficulty of identifying some species like *B. canis* and *B. neotomae* and some biovars within a given species gave negative results (Scholz and Vergnaud 2013).

Another type of new multiplex PCR assay is known as the Bruce-ladder. This assay quick and can identify *Brucella* species with one-step. The major benefit of this assay as compared to other PCRs is that it can identify and differentiate *Brucella* species as well as the vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev.1 in a single step. In contrast to other PCRs, the Bruce-ladder is also able to detect *B. abortus biovars* 3, 5, 6, 7, 9, *B. suis biovars* 2, 3, 4, 5, *B. neotomae*, *B. pinnipedialis* and *B ceti*. However, the Bruce-ladder have some drawback in differentiation of some *B. canis* strains from *B. suis* (López *et al*., 2011).

Real-time PCR (q-PCR): Real-time PCR is more quick and sensitive assay as compared to convention PCR. It is advantageous since it does not require post amplification handling of PCR products; reduce risk of laboratory contamination and false positive results. It is used to detect *Brucella* from urine, blood, and paraffin-embedded tissues samples (Redkar *et al*., 2001; Kattar *et al*., 2007). Real-time PCR is an important technique in quantification of nucleic acids in individual blood samples. Real time PCR using the IS711 based insertion element assay has been shown to be the most sensitive, specific, efficient, and reproducible method to detect *Brucella* species (Wei and Klaus 2010).

2.5.4 Serological diagnostic method

The most commonly used serological diagnostic methods relies on the recognition of antibodies against smooth surface lipopolysaccharide (LPS). Some of the indirect serological tests used for diagnosis of brucellosis includes Rose Bengal Plate Test, Complement Fixation Tests (CFT), Milk Ring Test (MRT), primary binding immunoassays precipitation tests (Poester *et al*., 2010).

Rose Bengal plate test (RBPT) is one of serological tests called as the buffered *Brucella* antigen tests that depend on the principle that the ability of IgM antibodies to bind to antigen in significantly reduced low PH (Corbel, 2006). It is an agglutination test where drops of stained antigen and serum are mixed on a plate to identify resulting agglutination that signifies a positive reaction. It is regularly used as quick screening test having very high sensitivity reaching at 99% while the specificity is low as 68.8% (Barroso *et al*., 2002).

Complement Fixation Test (CFT): is another serological test that detects specific antibodies of the immunglobuin M and immunglobuin G1 to fix the complement. The tests measures more of immunglobuin G1 antibodies as compared to immunglobuin M. This selogical test is highly specific however, it requires highly trained staffs with the appropriate laboratory facilities (Georgios *et al.,* 2005).

Milk Ring Test (MRT): is inexpensive, easy, simple and quick test that detects lacteal anti-*Brucella* IgM and fat globules from milk. Positive milk samples will form red ring However, false positive reaction occurs when milk that contains colostrum, milk at the end of the lactation period, milk from cows suffering from abnormal disorder or mastitis is used. In contrast milk containing low concentration of lacteal IgM, IgA or lack the fat clustering factors, tests can give false negative. The reason is due reduction of lacteal antibodies after abortion or parturition. Due to the above reason the reliability of the test by using 1ml milk to detect *Brucella* antibodies is highly reduced (Nielson *et al*., 2001).

Enzyme linked immune sorbent Assays (ELISA): is another serological test having higher sensitivity and specificity while being robust, easy to perform with a minimum of equipment and easly obtainable from a different commercial sources. Currently ELISA is used for analysis of a many animal and human diseases because it is more comfortable as compared to Complment fixataion Test use in small laboratories and (Asnake *et al*., 2017).

They are two types of ELISAs that can be used for diagnosis of brucellosis which are indirect ELISA (iELISA) and the competitive ELISA (cELISA). The iELISA uses a purified smooth LPS as antigen but there is difference in the anti-bovine Ig conjugate used in different labratories (Saegerman *et al*., 2004). The competitive ELISA (cELISA) is advantageous to use because it eliminates the problems rising from residual vaccine antibody and from cross-reacting antibodies. This assay is highly specific and detects all antibody isotypes (IgM, IgG1, and IgG2 and IgA) (Nielson *et al*., 2001). The principle behind the assay is by selecting a monoclonal antibody with a slightly higher affinity for the antigen than most of the vaccine/cross-reacting antibody, but with lower affinity than antibody arising from infection (Munoz *et al*., 2005; OIE, 2009; Poester *et al*., 2010).

Fluorescence Polarization Assay: uses *B.abortus* polysaccharide obtained from S-LPS labelled with fluorescein and measurements can be performed in a few minutes. It measures the degree of depolarization in milli-polarization units is based on a physical principle how fastly a molecule goes in a liquid medium reagarding its mass. Small size

molecules depolarize the light beam more since they rotate quicker as compared to larger molecules where rotate more slowly (Godfroid *et al.,* 2010). When there is an antibody present in testing serum, blood or milk, the rate of rotation of the labelled antigen will be reduced at a rate which is proportional to the amount of antibody present (Muma *et al.,* 2007).

Brucella allergen skin test: is an alternative serological test with very high specificity. It is mainly used for testing unvaccinated animals by using purified (free sLPS) and standardized antigen preparation. However, not all infected animals react to the test and false positive reaction occur due to infection with cross-reacting bacteria, especially in brucellosis-free areas. Due to the above reasons, the test alone is not recommended for diagnostic test purposes for international trade (OIE, 2009).

2.7 Zoonotic implications

Brucellosis is an important zoonosis causing undulant fever in humans. Humans can be infected with *B. abortus*, *B. suis*, *B. melitensis* and, rarely*, B. canis* (Doganay and Aygen 2003; Quinn *et al*., 2016). Humans acquire the infection through ingestion or inhalation, accidental inoculation and direct handling of contaminated materials from infected animals such as aborted fetuses, fetal membranes and vaginal secretion (Smits and Kadri 2005; Corbel, 2006; Dasari *et al*., 2013).

The risk factors related for human brucellosis include the handling of infected animals, ingestion of contaminated animal products such as unpasteurized milk and milk products, raw meat, history of travel to endemic areas and improper handling of cultures of *Brucella* species in laboratories. Laboratory workers handling *Brucella* cultures have the high risk for acquiring the disease by accidental injection, inhalation and not sufficient laboratory procedures. Furthermore, abattoir workers, farmers and veterinarians have the high chance for acquiring the disease (Chain *et al*., 2005; Corbel, 2006).

In humans, brucellosis is an acute febrile illness that can progress to chronic forms with complications involving musculoskeletal, central nervous and cardio-vascular systems (Regassa *et al*., 2009). Acute form of illness is characterized by mild flu like symptoms,

or abdominal pain, back pain, chills, excessive sweating, fatigue, intermittent fever, (so called "undulant" fever because the fever rises and falls in waves, Malta Fever, Mediterranean Fever and Rock fever) where high fever spikes usually occur every afternoon, headache, joint pain, depression, anorexia, weakness, weight loss and generalized aching, localized.

In chronic form, it can produce a serious complication affecting the musculoskeletal, cardiovascular and central nervous system. Complications affecting the bones and joints are common (they occur in 20–60% of cases) with Sacroilitis occurring most frequently. Other symptoms that may occur with this disease include muscle pain and swollen glands. Ultimately, the illness may become chronic and last for years (Franco *et al*., 2007; Lopes *et al*., 2010).

Even though brucellosis can be treated with antibiotics, the extended time for treatment influences compliance and the absence of vaccines for humans will make the disease a global health threat (Xinghong *et al*., 2013). The lack of a human brucellosis vaccine remains challenging due to the risk of *Brucella* as a possible bio-terrorist agent, and because brucellosis remains a global health problem affecting at least 500,000 humans annually as shown in (Figure 2) (Gwida *et al*, 2010).

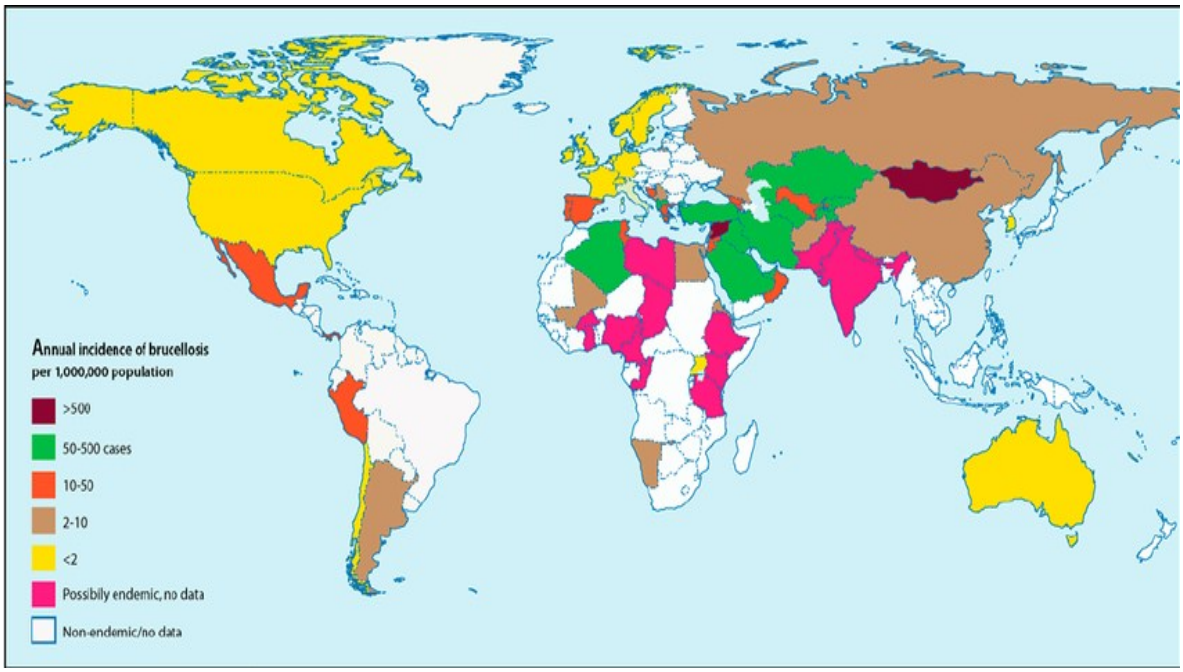


Figure 2: Global distribution of human brucellosis

Source: Pappas *et al*. (2006).

Status of human brucellosis in Ethiopia

The seroprevalnce of human brucellosis in Ethiopia differs between areas and species causing the disease, hygiene of the environment, with ocuupation of individual, herd practices (Chugh, 2008).

Human brucellosis studies in Ethiopia are scarce in contrast to the studies of animal brucellosis with few information on risk factors that contributes for human infection. For example, Regassa *et al*. (2009) showed 34.1%, 29.4% and 3% prevalence of human brucellosis in Borena, Hammer and Metema in pastoral groups having febrile cases of by using *Brucella* IgM/IgG Lateral Flow Assay. The authors mentioned that the key risks factors for acquiring brucellosis in the pastoral groups was living nearby to livestocks and consuming raw milk or products of infected animals.

Mekonnen *et al*. (2011) recorded low prevalence with an overall prevalence of 1.2% in the western Tigray region among human risk groups, all of which were herdsmen. According to the authors, the variation of prevalence herdsmen as compared to other in the study area was due to the majority of the herding practice is carried out by males, which might have contributed to high exposure to the infection.

Another study conducted by Tolosa *et al*., (2007) record a prevalence of 3.6% from two among 56 febrile cases using RBPT and CFT in the selected site of Jimma zone. Seroprevalence of human brucellosis in the farmers, veterinary professionals, abattoir workers and artificial inseminators study conducted by Mussie *et al*. (2007) and Kassahun *et al*. (2006) reported 5.30% Amhara region and 4.8% in Addis Ababa respectively. The summary of seroprevalnce studies of human brucellosis in Ethiopia are presented in Table 3

Table 4: Seroprevalence of human brucellosis in different parts of Ethiopia

Place	Tests	Prevalence(%)	Reference
Addis Ababa	RBPT/2-MET	4.8	Kassahun *et al*. (2006).
Jimma zone	RBPT/CFT	3.6	Tolosa, *et al*. (2007).
Amhara region	RBPT/CFT	5.3	Mussie *et al*. (2007).
Metema	Lateral flow device	3.0	Regassa *et al*. (2009).
Borena	Lateral flow device	34.1	Regassa *et al*. (2009).
Western Tigray	RBPT, CFT	1.0	Mekonnen *et al*. (2011).
Afar region	RBPT, CFT	15-16	Mekonnen and Sisay (2012).
Adami tulu	RBPT, CFT	2.2	Tibesso *et al*. (2014).
Jimma zone	RBPT, CFT	2.0	Bashahun *et al*. (2016).
Central Ethiopia	RBPT, CFT	1.3	Tsegay *et al*. (2017).
Southern Ethiopia	SAT	11	Workalemahu *et al*. (2017).
Somalia Fafan zone	RBPT, CFT	0.4	Lakew *et al*. (2019).

2.8 Economic impact

Brucellosis has worldwide occurrence in livestock, as well as humans, causing a serious economic problem for the intensive and extensive livestock production systems (Bshop *et al*., 1994). Heavy economic losses occur from brucellosis such as reduced milk production, losses of calves due to abortion and stillbirth, contamination of milk, obstructing animal export trade of a nation, cull and condemnation of infected animals by breeding failerity. Furthermore, human brucellosis causes reduced work capacity due to sickness of the affected people through losses of person-hours, therapeutic costs and government costs funded for research and eradication program (Chukwu, 1987; Georgios *et al.,* 2005).

The economic impact of brucellosis is different from country to country as well as from region to region. According to available literature data south America Official, estimations showed annual losses from bovine brucellosis was approximately US$ 600 million (WHO, 2001). Even though brucellosis eradication programs money spent can be very expensive, they are able to save US$7 for each 1 dollar spent on eradication. The USA national brucellosis eradication program done in between 1934 and 1997 year costed 3.5 billion dollar while the cost of reduced milk production and abortion in 1952 only costed 400 million dollars (Sriranganathan *et al*., 2009).

In Africa, a study conducted in Nigeria estimated economic loss occurs from brucellosis is around 3.60 dollar per cattle with 7 to 12% prevalence (McDermott *et al*., 2013). While in Ethiopia, there is limited information on economic losses due to brucellosis in different production systems with the exception of report by Tariku (1994) in which he reported that an annual loss around 88,941.96 Ethiopian Birr ($5231) from reduction of milk production and abortions among 193 cattle.

2.9 Treatment

Generally, antibiotic treatment of infected animals or those that are potentially exposed to brucellosis is not recommended. Since it is ineffective due to intracellular hiding of the organisms in lymph nodes, the mammary gland, and reproductive organs (Corbel, 2006; Radostits *et al*., 2007).

Humans brucellosis can be treated with administration of Tetracycline (500 mg every six hours orally) for at least six weeks, Doxycycline (a long-acting tetracycline analog) in a dose of 100gm every 12 hours orally with amino-glycoside for the first two to three weeks of therapy. Furthermore, other antibiotics such as Streptomycin, Gentamicin, Rifampicin, Fluoroquinolones, and Trimethoprim/sulfamethoxazole in combination with other antibiotics such as doxycycline, rifampicin or streptomycin can be used for treatment brucellosis; however, relapses may occur (Corbel, 2006).

2.10 Prevention and control

Prevention and control of brucellosis can achieved accurately through understanding of countries and national differences in animal husbandry practices, community customs, infrastructures and epidemiological distribution of the disease. The methods used to control brucellosis in livestock includes quarantine of replacement stock purchased from market before introducing to own herd, proper disposal of aborted fetuses and discharges by disinfection of contaminated area (Bshop *et al*., 1994). Hygiene methods is a standard technique to the control of brucellosis in order reduce of exposure of susceptible animals infected animals and to avoid contact of healthy animals with the discharges and tissues of infected animals. Features such as the methods of animal husbandry (e.g. commingling of herds or flocks), replacement source, prevalence of clinical signs, type of facilities and degree of assurance of the livestock owners will determine the success of control of brucellosis. Awareness creation among livestock owners should be conducted about disease transmission and recommendations, such as separation of parturient animals (Corbel, 2006).

Replacement stock should be purchased from a herd free of brucellosis, and decide for or against immunization of negative animals. Furthermore test and slaughter can be applied if it is feasible (economically and logistically) (Godfroid *et al*. 2004; Olsen and Tatum 2010). In general, test and slaughter of infected animals are only successful in decrease the occurrence if the herd prevalence is very low (Corbel, 2006).

Prevention and control of brucellosis in livestock can be achieved successful by vaccination of uninfected animals since it reduces the number of infected animals and permits disease control. Currently two types of vaccines are available for cattle that are *B. abortus* strain 19 and approved strain RB51 (Dwight, 1999; Moriyon *et al*., 2004; Radostits *et al*., 2007).

The *B. abortus* strain RB51 live attenuated *Brucella* vaccine, lacks S-LPS, has much lower pathogenicity in vaccinates, is considered only mildly infectious and is thought to have lower pathogenicity in humans in response to accidental exposure. Furthermore, the *B abortus* RB51 vaccine has a considerable advantage in the livestock control programs since it does not produce an antibody response against S-LPS and does not interfere with results of the serological test (Blasco, 2006).

Human brucellosis can mostly prevented by controlling and eradicating of the disease in livestock sector. Furthermore awareness creation among livestock owners how to handle and disposing of aborted fetus, fetal membrane and discharges infected animals and well as not to drink unpasteurized milk should be made. Abattoir workers should also be educated in transmission of infection particularly through skin cut (Acha and Szyfres 2003).

3. MATERIALS AND METHODS

3.1. Study area

Sululta town is a special zone of Oromia region, Central Ethiopia and found at distance of 23 km northwest from Addis Ababa. Geographically the town lies at 09º17'84" North latitude and 38º75'79" East longitude. The altitude of town ranges from 2851 to 3700 meters above sea level (m.a.sl), annual temperatures ranges from 10-25^{0}C and annual rainfall ranging from 834-1447 mm (CSA, 2015). The total cattle population of the the town is predicted to be 224,600 and in which 15% are crossbreed (SDAO, 2012).

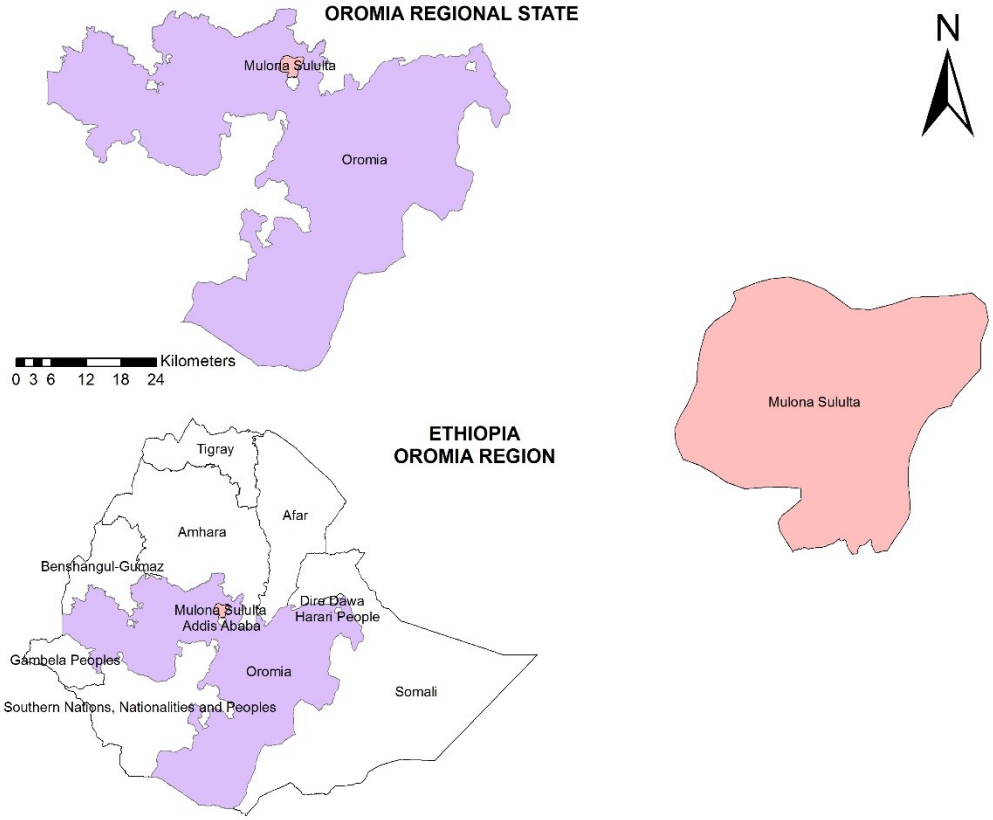


Figure 3: Map of the study area (Sululta).

3.2. Definition

In Ethiopia dairy cattle production systems are catagorized into commercial dairy systems, urban and peri-urban smallholder dairy, rural smallholder, the pastoral and agro-pastoral (Asmare *et al*., 2013). The urban and peri-urban smallholder farms hold

mainly exotic breeds or cross and local zebu breeds, while the rural systems contains largely zebu breeds. The study focuses both on commercial, urban and peri-urban smallholder farms, that produce milk for home use and sale. The main source of milk and products for the capital city are provided by these system (Geresu *et al*., 2016).

3.3. Study population

The study populations were dairy cattle found in both commercial and smallholder dairy farms of Sululta Town. The herds were categorized into 5-10 animals (small herd size); 11 - 20 animals (medium herd size) and > 20 animals (large herd size) based on Alehegn *et al*. (2016). Dairy cattle age were classified as Young (6-17 months) or Adult (equal or more than 17 months), the breed were classified as an exotic breed (crossbreed) and local zebu.

3.4. Study design

A crossectional study was conducted between November 2019 up to May 2020 to determine the Seroprevalence of bovine brucellosis and associated risk factors for infection in dairy cattle in Sululta Town, Central Ethiopia.

3.5. Sample size determination and sampling method

The sample size for sampling of dairy herd was calculated by using a herd level prevalence of 2.4 % based on (Asmare *et al*., 2013) by using 95% confidence interval (CI) and 5% required precision. According to Thrusfield (2018) formula, the sample size was determined as follows:

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

Where: n- required dairy herd to be sampled from the area

Z- relablity coefficient (1.96)

Pexp- expected herd prevalence (2.4%)

d- desired absolute precision (5%)

$$n = \frac{(1.96)^2 \times 0.024\ (1-0.024)}{(0.05)^2} = 36$$

Therefore, the total number of the dairy herds to be sampled from the study area was 36 dairy farms. To increase precision it was multiplied by 3 fold which was 108 dairy farms. However, due to available resource 119 dairy farms were sampled form the study area.

Before sample collection the list of the dairy farms was obtained from Sululta town animal health departments. Then farms were divided into three groups based on herd size: small herd size (of 5-10 dairy animals), medium herd size (11-20 dairy animals) and large herd size (more than 20 dairy animals). Stratified sampling methdology was employed to sample herds. Sululta town has 150 dairy farms. Out of this 119 dairy farms comprised of 77 small herd size (173 dairy animals), 38 medium herd size (236 dairy animals) and 4 large herd size (24 dairy animlas) were sampled.

3.6. Sample and Data collection

3.6.1 Blood sample collection

About 7-10ml of the blood sample was collected through jugular vein puncture of each selected dairy animal by using plain vacutainer tubes and needles. Information concerning the characteristics of individual animal biodata such as breed, sex, age, presence/absence of reproductive problems such as abortion, retained placenta, was obatained during sample collection (Annex 1). Then the samples were kept in an icebox and transported to the National Animal Health Diagnosis and Investigation Centre (NAHDIC). The collected blood sample was putted at room temperature for 24 hours and serum was harvested by centrifuging at 2500 rpm for 5 minutes and transferred to cryovials. Each serum was properly labeled and kept at -20°C until tested.

3.6.2 Questionnaire survey

A pre-tested structured questionnaire was used to gather information about possible factors associated with herd seroprevalnce of brucellosis (Annex 2). The questionnaire

used to gather farm characteristics and ways of disease exposure. Some of the questions includes the type of breeding used, source of replacement stock, type of breed raised, herd size, type of management system.

3.7. Serlogical Labratory analysis

3.7.1 Rose Bengal Plate Test (RBPT)

Rose Bengal Plate Test were used as screening test and performed at the National Animal Health Diagnostic and Investigation Center (NAHDIC) by using RBPT antigen (Moniplier SAS, 326 rue de la Galera, 3490 Moniplier France) based on Nielsen (2002) (Annex 3).

3.7.2 Complement Fixation Test (CFT)

Rose Bengal Plate Test positive serum sample was further confirmed with CFT by using *B. abortus* antigen S99 (Surrey KT15 3NB, United Kingdom,Veterinary Laboratories Agency) in the National Animal Health Diagnostic and Investigation Center (NAHDIC) based on (OIE, 2009).

3.8. Ethical consideration

All the procedures were carried out based on the experimental standards and approved by the animal welfare and research ethical committee in Addis Ababa University College of Veterinary Medicine and Agriculture.

3.9. Data management and analysis

Data generated from serological laboratory analysis was entered into a Microsoft Excel spreadsheet and statistically analyzed using (STATA version 11.0, a computer based software program for Windows (StataCorp. 2009. Stata Statistical Software: College Station, TX, USA).

Descriptive statistics was used farm characteristics concerning to bovine brucellosis. Individual animal level prevalence was calculated as the number of seropositive

samples divided by the total number of samples tested. Herd level prevalence was calculated by dividing the number of positive herds in Complment Fixation test with the number of herds tested. Associations of risk factors with *Brucella* seropositivity was analyzed using Chi Square test for animal and herd level.

4. RESULTS

4.1 Seroprevalence survey

4.1.1 Seroprevalence of bovine brucellosis at an animal level

A total of 436 cattle, 110 male and 326 females above six months of age were sampled and examined for *Brucella* antibodies. Of this 1 animal was found to be positive by the Rose Bengal plate Test. The serum sample was also found to be positive when confirmed with Complement Fixation Test. Thus the overall seroprevalence brucellosis in dairy cattle was 0.23% (95% CI: 0.2-0.7%) by Complement Fixation Test. The prevalence of bovine brucellosis was significantly higher in animals included in herd size greater than 20 ($P<0.00$). However, there were no significant differences between Kebele, Sex, Age, and breed as shown in ($P >0.05$) (Table 5).

Table 5: Association of risk factor at animal level with bovine brucellosis by using Pearson's Chi-square test.

Variables	Categories	Number tested	Number positive	χ2-value	*P-value*
Kebele	Nunu munbuchu	109	0(0%)	3.24	0.356
	Sululta 01	103	0(0%)		
	Qaso wasrbi	131	1(0.8)		
	Wale lube	100	0(0%)		
Herd type	Small	173	0(0%)	17.20	0.00*
	Medium	239	0(0%)		
	Large	24	1(4.1%)		
Age	Young	170	1(0.6%)	1.56	0.210
	Adult	266	0(0%)		
Sex	Female	326	0(0%)	2.97	0.085
	Male	110	1(0.2%)		
Breed	Local	79	0(0%)	0.22	0.63
	Exotic	359	1(0.2%)		
Parity	Primaparus	97	0(0%)	-	
	Pluriparious	229	0(0%)		
Abortion history	Yes	16	0(0%)	-	
	No	310	0(0%)		
Abortion stage	1st trimester	0	0(0%)	-	
	2nd trimester	11	0(0%)		
	3rd trimester	5	0(0%)		
History of RFM	Yes	7	0(0%)	-	
	No	319	0(0%)		
Total		436	1(0.23%)		

χ2: Chi-Square, *significant RFM: Retained Fetal Membrane

4.1.2 Seroprevalence of bovine brucellosis at herd-level

The herd-level prevalence of bovine brucellosis was found to be 0.8% (95% CI: -0.08-0.2%) with Complement Fixation Test. From the study herd, there was a history of retained fetal membranes and abortion in 27 (22.5%) and 22 (18.3%) herds respectively. The presence of abortion history was statistically significant with herd-level seropositivity to brucellosis ($P<0.05$). However, there were no significant variations of the seroprevalence among retained fetal membranes other factors such as replacement stock, mating type and production system as shown in (Table 6).

Table 6: Association of herd-level with bovine brucellosis *by using Pearson's Chi-square test.*

Variables	Categories	No of tested herd (%)	No of positive herd (%)	χ2- value	P-value
Management system	Intensive	55(46.2)	1(1.8%)	1.2	0.2
	Semi-intensive	64(53.7)	0(0%)		
Herd type	Small	77(64.7)	0(0%)	17.20	0.00*
	Medium	38(31.9)	0(0%)		
	Large	4(3.3)	1(25%)		
Breed reared	Local	30(25.2)	0(0%)	0.33	0.56
	Exotic	89(74.7)	1(1.12)		
Replacement stock	Own	81(68.0)	1(1.23%)	0.4	0.78
	Market	9(7.5)	0(0%)		
	Both	29(24.3)	0(0%)		
Breeding strategy	AI	19(15.9)	0(0%)	0.22	0.89
	Bull	97(81.5)	1(1.03%)		
	Both	3(2.5)	0(0%)		
Contact with other herds	Yes	56(47.05)	0(0%)	0.89	0.34
	No	63(52.94)	1(1.6%)		
Separate parturition pen	Yes	47(39.4)	0(0%)	0.65	0.41
	No	72(60.5)	1(1.4%)		
Isolate RFM cases in a separate pen	Yes	23(19.3)	0(0%)	0.24	0.6
	No	96(80.6)	1(1.0%)		
History of abortion	Yes	22(18.4)	0 (0%)	4.46	0.03*
	No	97(81.5)	1 (1.0%)		
History of RFM	Yes	27(22.6)	0(0%)	0.29	0.58
	No	92(77.3)	1(1.0%)		
Aborted fetus disposal	Burying	19(15.9)	0(0%)	2.23	0.2
	Open dump	37(3)	1(2.7%)		
	Feed to dog	63(52.9)	0(0%)		
Farm hygiene	Good	19(15.9)	0(0%)	2.23	0.3
	Fair	37(31)	1(2.7%)		
	Poor	63(52.9)	0(0%)		

χ2: Chi-Square, *significant RFM: Retained Fetal Membrane

4.2 Questionary survey

4.2.1 Farm characteristics and management practice of dairy farms

Most of the farm owners 64.7% (n=77) have a herd size less than 10 heads of cattle, while others 31.9% (n=38) had a herd size between 10-20 heads of cattle and few of them 3.3% (n=4) had more than 20 heads of cattle. The majority of respondents practice semi-intensive management system 53.8 % (n=64) while, 46.2% (n=55) are from the intensive management system. According to the respondents 7.5% (n=9) of them use replacement stock from market and 68 % (n=81) raise their own while 24.2% (n=29) of them use both option. Based on the questioner result that no farms test replacement animals purchasing from the market before entering the farm. The majority of farms 81.5% (n=97) use bull for breeding in herds and 15.9% (n=19) use artificial insemination service while, 2.5% (n=3) of them use both bull and artificial insemination service. Around 47.5% (n=57) use a communal bull with other farms. Of the farms interviewed 47.06% (n=56) of the farms had frequent contact with other herds. Around 83 % of respondents dispose of aborted material or after birth in the open dump (Table 9). The presence of separate parturition pen was 32.4 % for small farms while 25 % in large farms.

Table 7:Farm characteristics and herd management practice

Variables	Categories	Small (n=77) (%)	Medium (n=38) (%)	Large (n=4) (%)
Management system	Intensive	36(46.7)	15(39.4)	4(100)
	Semi-intensive	41(53.2)	23(76.3)	0(0)
Replacement stock	Market	9(11.6)	0(0)	0
	Own	51(66.2)	28(73.6)	3(75)
	Both	17(22.7)	11(28.94)	1(25)
Breeding strategy	AI	10(12.9)	8(21.05)	1(25)
	Bull	65(84.1)	29(76.3)	3(75)
	Both	2(2.5)	1(2.63)0	0(0)
Contact with other herds	Yes	36(46.7)	20(52.63)	0(0)
	No	41(53.2)	18(47.3)	4(100)
Separate parturition pen	Yes	25(32.4)	21(55.2)	1(25)
	No	52(67.5)	17(44.7)	3(75)
Cleaning calving parturition	Flush with water	22(28.5)	20(52.6)	0(0)
	Disinfect with detergent	2(2.59%)	0(0)	0(0)
	Both	1(1.2%)	1(2.63)	1(100)
Aborted fetus disposal	Burying	9(11.6%)	8(21.05)	2(50)
	Open dump	25(32.4%)	11(28.9)	1(25)
	Feed to dog	43(55.8)	19(50)	1(25)
Farm hygiene	Good	9(11.6)	8(21.05)	2(50)
	Fair	25(32.4)	11(28.94)	1(25)
	Poor	43(55.8)	19(50)	1(25)

n= number of herds

4.3 Limitation of study

The limitation of the study was swab sample was collected from seropositive animal and processed for microbiological investigation and molecular analysis at NADHIC, however, due to COVID-19 pandemic situation as well as NAHDIC were assigned as laboratory to investigate the COVID-19 cases it was not completed.

5. DISCUSSION

The current study recorded an overall seroprevalence of bovine brucellosis at animal level was 0.23% (95% CI: -0.2-0.7%) and the herd-level prevalence of bovine brucellosis was found to be 0.8% (95% CI: -0.08- 0.2%). This low prevalence was in agreement with previous reports conducted in different parts of countries such as by Degefa *et al*. (2011) with (0.05%) prevalence in Arsi Zone, Adugna *et al*. (2013) (0.5%) in Western Ethiopia, Bashitu *et al*. (2015) (0.2%) in Ambo and Debre Birhan, Dirar *et al.* (2015) (0.3%) in the selected district of Jimma zone, Pal *et al*. (2016) (0%) in North Shewa, Ethiopia, Tesfaye (2018) (0%) in Lemu Bilbilo District, Oromia, Edao *et al*. (2018) (0.06%) in Addis Ababa.

As compared to the above mentioned reports there was higher seroprevalence of bovine brucellosis reports in other different parts of the country such as Kebede *et al*. (2008) reported prevalence of (11.1%) in Central Ethiopia, Jergefa *et al*. (2009) reported (2.9%) in three agro-ecological areas of Central Ethiopia, Ibrahim *et al*. (2010) (3.1%) in Jimma zone of Oromia Region, Mekonnen *et al*. (2011) recorded a prevalence of (6.1%) in Tigray, Tesfaye *et al*. (2011) that reported 1.5% prevalence rate in dairy farms of Addis Ababa, Asmare *et al*. (2013) (1.9%) in different part of Ethiopia, Geresu *et al*. (2016) (1.4%) in Asella and Bishoftu, Terefe *et al*. (2017) (1.3%) in Eastern Ethiopia, Waktole *et al*. (2018) (3%) in dairy farms of Bishoftu town, Wubishet *et al*. (2019) (4.7%) in Borena Zone. The reason for this low prevalence in the study area as compared to other studies conducted in different parts of the country is due to the variation of the production system, sample size and sampling methodlogy used in the study area.

Among the risk factors assessed at animal level such as kebele, sex, and age did not show a statistical association with seroprevalence of brucellosis at animal level ($P>0.05$). In Addition history of abortion and retained fetal membranes was not positively associated with individual animal seroprevalence of bovine brucellosis ($P>0.05$). In contrast, previous studies conducted in different parts of country such as Bashitu *et al*. (2015), Abera *et al*. (2019) showed a statistical association with animals that have abortion history ($P<0.05$). But, the questionnaire identfied major reproductive problems was abortion and retained fetal membrane cases in the study area. From 119

herds assessed by questionary there were 27 (22.5%) and 22 (18.3%) herds that have the occurrence of abortion and RFM in this study area. In contrast, Tesfaye *et al*. (2011) stated 4.55% and 9.5% abortion and retention of fetal membranes in dairy farms of Addis Ababa.

One of the other risk factor that affects the occurrence of brucellosis is herd size (Yohannes *et al*., 2012). The result revealed that the herd size of dairy cattle was statistically significantly associated with the animal level and herd-level seroprevalence of brucellosis in dairy cattle ($P<0.05$). Higher individual animal seroprevalence was observed in large herd size (4.1%), as compared to small and medium herd sized in which no positive reactors animal were recorded. The result in line with previous studies conducted by Berhe *et al*. (2007), Geresu *et al*. (2016), Mekonnen *et al*. (2010), Tadesse *et al*. (2019) showed a higher seroprevalence of dairy cattle brucellosis in large herd size as compared to small and medium-sized. In larger herd sizes, brucellosis spreads by numerous modes, such as through direct contact with infected cattle discharges to its fetus (Radostits *et al*., 2007). However, contrary to this other authors showed that the risk of seropositivity was not depend on herd size (Kebede *et al*., 2008; Jergefa *et al*., 2009; Asmare *et al*., 2013; Abera *et al*., 2019).

Despite there was no statistically significant association between the management system ($P>0.05$) the study showed a slightly high seroprevalence rate of brucellosis in intensive management system (1.8%) whereas no positive animals (0%) were found in semi-intensive production systems. The reason for high prevalence in intensive production systems were due to a great chance of contact between infected and healthy animals as compared to other production system and additionally since most farmers do not follow hygienic practices as also suggested by Jergefa *et al*. (2009) and Alehegn *et al*. (2016).

6. CONCLUSION AND RECCOMENDATIONS

The present study showed that the overall prevalence of bovine brucellosis at animal and herd level was 0.23% and 0.08% respectively in dairy cattle in Sululta Town, Central Ethiopia. In the current study, low prevalence were recorded at both and herd level. At herd level, herd size and history of abortion in the herd had a statistically significant association with herd-level brucellosis positivity ($P<0.05$). Despite there were no significant association between risk factors such as feeding aborted fetus to dogs and dispose to open dump, do not isolate retained placenta cases until treated and absence of separate parturition were commonly practice by the community that can contribute to the spread of bovine brucellosis between the dairy farms.

- Awareness creation among the dairy farming community should be conducted.
- Replacement animals should be tested before introducing to the farm.
- Separation of animal during parturition in separate calving pen and proper disposal of aborted should be practiced.
- Sharing of communal bull in the community should be avoided.
- Further isolation and molecular characterization of *Brucella* should be conducted to distinguish which *Brucella* species is circulating on the study area.

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8. ANNEXES

Annex 1: Questioner format used for individual animal sample collection

Farm code	Animal Id no	Kebele	Age	Sex	Breed	Parity	History of abortion	History of RFM

Annex 2: Questionary used to collect general characteristics, management, ways of disease exposure and introduction to the farms

1. Herd size:
2. Type of farm:

   Small (<10) ☐ Medium Farm (10-20) ☐ Large Farm (>20)

3. Farm location:
4. What type of farm management system do you have?

Intensive ☐ Semi-intensive ☐ Extensive ☐

5. What type of breed of cattle you reared at your farm?

Local breed ☐ Exotic (Cross) breed

6. Where do you get the replacement stock?

Market ☐ Raise own replacement ☐ both ☐

6.1 Do you test (brucellosis) replacement animals before entering to the farm?

Yes ☐ No ☐

7. What type of mating service do you use for your animals?

AI ☐

Bull ☐, specify circle (A. use own bull; B. use communal bull with other farms)

Both (AI and Bull) ☐

8. Is there frequent contact between your animals with other herds (for semintensive/extensive)?

a. Yes b. No

9. Do you have separate parturition pen for cow to give birth?

Yes ☐ No ☐

10. If yes, for how long does the cow remain in separated calving pen after parturition?

_____days

11. If yes, what kind of method do you use to clean the calving pen after parturition?

☐ Flushing with water ☐ disinfect with detergents ☐ both

12. Does any abortion case happened in the farm with in the last 12 months?

Yes ☐ NO ☐

12.1. If yes, in which of the cows does the abortion and at what stage?

Cow identification	Parity status	Abortion stage (1^{st}, 2^{nd}, 3^{rd} trimester)

13. Does any Presence of retained fetal membranes in the last 12 months

Yes☐ No ☐ Unknown ☐

13.1 No. of cows with retained fetal membranes cases in the last 12 months_____

14. If yes which cow?

Cow id no	

15. Do you isolate cows with Retain Placenta cases in separate isolation pen until treated/get well?

Yes ☐ No ☐

16. Ways of disposal of aborted fetus and RP at farm level

☐ Burying ☐ Open dump ☐ feed to dog

☐ Others (specify) _____

17. What is the level of your herd/farm hygiene? (Based on respondents judgment)

Good ☐ Fair ☐ Poor ☐

Annex 3: Rose Bengal Plate Laboratory Test Procedures

Sera (control and test sera) and antigen for use was brought to room temperature for half an hour before testing,

1. 30 µl serum were placed and mixed with 30 µl of antigen on a white enamel plate to produce a zone approximately 2 cm in diameter.

2. The antigen and serum was mixed thoroughly using an applicator stick (a stick which is used only once for each sample).

3. Plate was rocked by hand for about 4 minutes.

4. It was examined in a bright light for presence of agglutination.

Interpretation

Positive: a positive result is indicate by agglutination (clumping) of bacterial suspension.

Negative: A negative result is indicate by absence of agglutination

Annex 4: Complement Fixation Laboratory Test Procedures

1. First the sera including positive and negative control was deactivated by using hot water bath at 58^0 C (+/- 20C) for 30 minutes.

2. Then a U- shaped 96-well micro titer plate was prepared.

3. Dilute the test serum 1:2 (100µl test serum in 100µl VCM)

4. Use each well of row "A" for anti-complementary control.

5. Dispense 25µl of VCM by using a hand held 12 channel micropipette into the wells of rows A, C, D, E, F, G, and H.

6. Dispense 25µl of diluted sera (1:2) in wells of rows A, B, C and homogenize wells of row C and pick up 25µl from row C of the test plate and deliver to the wells of row D Continue this serial dilution to row H (column 1-12) from which after homogenization 25µl is picked up and discarded.

7. Add 25µl of diluted antigen in to all the wells of rows B, C, D, E, F, G, H wells and to the antigen and positive and negative control wells.

8. Add 25µl diluted complement in the wells of rows A, B, C, D, E, F, G, H and all control wells except hemolytic system.

9. The plate was covered with sealer and incubated at $+37^0$ C under constant agitation on incubator shaker for 30 minutes.

10. Add 25µl hemolytic system to all wells including control wells.

11. Then the plate was finally covered with sealer and incubated at $+37^0$ C under constant agitation on incubator shaker for 30 minutes.

12. Reading: Centrifuge at 2500 rpm for five minute or put in a refrigerator at +4ºC overnight to let non-hemolysized SRBCs sediment. Then read the result for degree of sedimentation or hemolysis.

Interpretation and principle of the test

Positive reaction- If there is antibodies of *Brucella* in test serum an antigen-antibody complex will be formed and the complement is used up, so it cannot react in the hemolytic system. Therefore, sedimentation of SRBC will occur.

Negative reaction- If there is no *Brucella* antibodies in the test serum the complement will not be fixed and Hemolysin cause lyses of SRBC due to presence of free complement.