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**SEROPREVALENCE AND PUBLIC HEALTH SIGNIFICANCE OF BOVINE BRUCELLOSIS
AND ITS ASSOCIATED RISK FACTORS IN DAIRY FARMS OF DEBREBIRHAN TOWN
AND ITS SURROUNDING, NORTH SHEWA ZONE, AMHARA REGION, ETHIOPIA**

MVSc. THESIS IN VETERINARY PUBLIC HEALTH

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A Thesis Submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University, in
partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary
Public Health

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As MVSc research advisors, we here certify that we have read and evaluated the Thesis prepared by Gashaw Adane Erkyihun, under our guidance, entitled: “Seroprevalence and public health significance of bovine brucellosis and its associated risk factors in dairy farms of Debrebirhan town and its surrounding, North Shewa Zone, Amhara Region, Ethiopia”; and we recommend that it can be submitted as fulfilling the MVSc Thesis requirement.

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I declare that this thesis is my original work and all sources of material used have been acknowledged. The thesis has been submitted in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Public Health at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I declare also that this Thesis is not submitted to any other institution for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
AI	Artificial Insemination
AMOS PCR	Abortus-Melitensis-Ovis-Suis PCR
BCS	Body Condition Score
BPAT	Buffered antigen Plate Agglutination Test
C-ELISA	Competitive Enzyme Linked Immunosorbent Assay
CFT	Complement Fixation Test
CSA	Central Statistical Agency
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
I-ELISA	Indirect-Enzyme Linked Immunosorbent Assay
KAP	Knowledge, Attitude and Practice
KM	Kilo meters
LPS	Lipopolysaccharide
MOA	Ministry of Agriculture
MSL	Meter above Sea Level
MZN	Modified Ziehl-Neelsen
N A H D I C	National Animal Health Diagnostic and Investigation Center.
NBTWG	National Brucellosis Technical Working Group
OIE	Office International des Epizooties
OR	Odds Ratio
PCR	Polymerase chain reaction
RBPT	Rose Bengal Plate Test
RES	Reticuloendothelial systems
RFM	Retained Fetal Membrane
SNNP	South Nations Nationalities and People
US CDC	United States Centre of Disease Communication
USA	United States of America
W H O	World Health Organization
WAHIS	World Animal Health Information System

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ABSTRACT

Bovine brucellosis is a zoonotic disease that causes serious public health hazard in human and economic crises in dairy cattle production in affected countries. A cross sectional study was conducted between November 2021 to 2022 in Debrebirhan town and its surrounding of North Shewa zone, Amhara Regional State, Ethiopia, to estimate seroprevalence of brucellosis in dairy cattle, identify associated risk factors and assess the knowledge, attitude and practice of dairy cattle owners on zoonotic brucellosis. Blood samples from dairy cattle were collected and performed the serological tests and risk factors associated with *Brucella* seropositivity as well as knowledge attitude and practice of respondents was collected based on questionnaire format and analyzed using univariable and multivariable logistic regression. Using purposive sampling method, a total of 426 blood samples were collected from dairy cattle for serological test. The collected serum samples were screened initially with Rose Bengal Plate Test and positive samples for the initial test were confirmed by Complement Fixation Test. The overall seroprevalence of bovine brucellosis in the study areas was 1.4% (95% CI: 0.28%-2.53%) and 0.47 % (95% CI: -0.182-1.121) using RBPT and CFT tests respectively at individual animal level and 13% (95%CI: -0.39% -26.48%) and 4.43% (95%CI: -1.77% - 10.47%) using RBPT and CFT tests respectively at herd level. Statistical analysis of risk factors by using multivariable logistic regression indicates that buying stock replacement (OR=33.83, p=0.0003), last trimester abortion history (OR=5.90, p=0.005) and bull mating service (OR=6.43, p=0.013) were found significantly associated with the seropositivity of *Brucella* infection in the dairy cattle. Knowledge, attitude and practice assessment of respondents revealed that the majority of interviewees have no knowledge about brucellosis and other zoonotic diseases and their way of transmission. Because of this, drinking of milk with out boiling and contact with aborted and parturition materials with barehand are highly practiced in the study areas which clearly shows the presence of high risk for human *Brucella* infection. The result of this study revealed that the overall seroprevalence of bovine brucellosis was estimated to be very low in the study areas. However, the presence of positive serological reactors with statistical significant association of risk factors indicate previous infection of brucellosis in the cattle population of study areas. More over, the presence of one or more positive reactors in the herd is a predictor for the presence of infection. Hence, culling of *Brucella* antibody reactor cows, carried out stock replacement through *brucella* testing before introduction in to the farms, improving of community awareness, further studies on isolation and molecular characterization of the causative agent in livestock and epidemiological study of human brucellosis and launch national control and elimination campaign through One Health approach are recommended.

Key Words: Amhara region, Brucellosis, Dairy farms, Debrebirhan, Public health, Seroprevalence

1. INTRODUCTION

Ethiopia has a total of 70,291,776 cattle with 22, 598,088 million are dairy animals which constitute 32.15% out of total cattle population (CSA, 2021). This huge livestock population has considerable potential for economic growth of the country through production of milk and meat fulfilling the domestic and export market demand. With this regard, the livestock production is challenged by different problems such as poor husbandry management, shortage of feed and water and diseases like brucellosis (Geletu *et al.*, 2021). More than 80% of Ethiopians are depending on agriculture with very close relationship to their livestock. Hence, the country has been threatening by the spread of different zoonotic diseases among its population (Pieracci *et al.*, 2016 ; Yilma *et al.*, 2016). Large number of zoonotic diseases has endemically occurs and both the livestock and human population are at risk by these diseases. Brucellosis, one of the top five listed priority zoonotic diseases in Ethiopia, is the one with potential public health and economic significance in the country (Pieracci *et al.*, 2016; Erkyihun *et al.*, 2022).

The disease brucellosis, particularly bovine brucellosis, is characterized by serious economic crises in dairy cattle of affected countries by causing indirect loss (mortality and morbidity, decrease fertility and milk production, decrease sale value of infected cows, trade limitation on animals and animal products and an impediment to movement of animals) and direct loss (neonatal death, abortion, replacement cost, treatment cost, emergency slaughtering of the infected animals and stillbirths) (Corbel *et al.*, 2006; Scholz *et al.*, 2010; Aparicio, 2013; Yilma *et al.*, 2016).

Countries in which their human population depends on their livestock are also characterized by the presence of strong correlation between poverty and burden of zoonotic diseases due to a high risk disease transmission and reemerging and emerging pandemic threats (Murphy *et al.*, 2019, Erkyihun *et al.*, 2022). Bovine brucellosis is caused by *B. abortus* and occasionally by *B. melitensis* and *B.suis* (OIE, 2018) and one of the most serious diseases that are recognized as major problem for cattle productivity and public health in Ethiopia (Ibrahim *et al.*, 2010;Yilma *et al.*, 2016). Specifically, the habit of the people living in close proximity to their animals, raw animal product consumption, contact regularly between people and their animals and improper handling of raw livestock byproducts at risk groups are the escalating factors and these factors, together with the widespread nature, make the disease to be listed among the top prioritized public health threatening zoonosis in the country (Tegegn & Feleke, 2016).

In Ethiopia, several seroprevalence studies has been conducted between 2010 to 2021 and reported different prevalence ranging from 0.4% to 11.9% for individual animal level (Megerssa *et al.*, 2012 ; Bulcha *et al.*, 2020; Negash & Dubie, 2021) and 12% to 63.6% for herd level (Amenu *et al.*, 2010; Mekonnen & Hoekstra, 2010; Ararsa *et al.*, 2021). Most of these previous studies that have been carried out in central Ethiopia are only on privately and institutionally owned commercial large and medium scale dairy farms in surrounding Addis Ababa (Beruktayet & Mersha, 2016).

Research studies done in dairy farm of Debrebirhan and its surrounding of North Shewa Zone of Amhara Region, particularly in small holder dairy farms regarding seroprevalence and risk factors for infection and public health importance of brucellosis are very limited. Due to this, there is still inadequate epidemiological picture of the disease in traditional management systems and/or smallholder dairy farms of Debrebirhan and its surroundings specifically in Debrebirhan town, Basona Werana and Angolla Tara districts of North Shewa zone, Amhara Regional State, Ethiopia. Hence, in order to understand the epidemiological status of brucellosis in dairy farms, identify the associated risk factors and its public health significance of the diseases in human population of Debrebirhan and its surrounding, in which the result used as base for future designing of a control measure, this study was designed and conducted with the following objectives.

General Objective

- ✓ To determine the prevalence and risk factors of brucellosis in dairy cattle and assess its public health importance in Debrebirhan, Basona werana and Angolalla Tara Districts of North Shewa zone, Amhara Regional State, Ethiopia.

Specific Objectives

- ✓ To estimate the seroprevalence of brucellosis in dairy cattle of Debrebirhan town and its surroundings.
- ✓ To identify associated risk factors of bovine brucellosis in the study areas.
- ✓ To assess the knowledge, attitude and practice of dairy cattle owners on brucellosis and its transmission in the study areas

2. LITRATURE REVIEW

2.1. Background of Brucellosis

Brucellosis is caused by gram-negative, facultative intracellular bacterium of genus *Brucella* (Pappas *et al.*, 2006). The knowledge of the brucellosis has been mostly started eighteen centuries later when Sir David Bruce isolated *Micrococcus melitensis* (now *B. melitensis*) from the spleen of a British soldier who died from febrile illness (Malta fever). Zoonotic brucellosis was clearly demonstrated in 1905 after the isolation of *B. melitensis* from goats milk in Malta (Godfroid *et al.*, 2005). This disease is one of the neglected zoonotic diseases of humans and animals in Sub-Saharan African countries including Ethiopia (Edao *et al.*, 2020). It is a highly contagious and considered (by WHO, FAO and OIE) as the most serious zoonotic with a high public importance in the world (Schelling *et al.*, 2004). Brucellosis has been ranked the second dangerous zoonotic diseases in the world next to rabies. It has synonyms in which these associated with its causative agent researcher and clinical symptoms as Malta fever, Undulant fever, Mediterranean fever, Rock Fever of Gibraltar and Gastric Fever in human while Bangs disease, enzootic abortion, epizootic abortion contagious abortion, Slinking of calf and Ram epididymitis in animals (Forbes & Tessaro, 1996; Mantur *et al.*, 2007).

2.2. Etiology of Brucellosis

Brucellosis is caused by a gram negative, facultative intracellular *which* infects a wide range of animal species and humans. *Brucellae* are classically grouped into different species as *B. abortus* (cattle, buffalo, elk, yaks, and camels, biovars 1–6, and 9), *B. melitensis* (goats, sheep, biovars 1–3), *B. suis* (pigs, reindeer and hares, biovars 1–5), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (desert wood rats). *B. ceti* (dolphins), *B. pinnipedialis* (seals), *B. microti* (voles) and *B. inopinata* (reservoir undetermined) are the new members included recently (Foster *et al.*, 2007). *B. inopinata*, the only species not isolated from animal reservoir, was also isolated from a breast implant infection in a woman (Scholz *et al.*, 2010).

Brucella melitensis (the most common cause of human brucellosis), *B. abortus* (the second), *B. suis* and *B. canis* are the most pathogenic to human (Godfroid *et al.*, 2005) (Table1). *Brucella* species are small (0.6 x 0.6 to 1.5 µm), non-motile, coccobacilli, gram-negative and modified Ziehl-Neelsen (MZN) positive bacteria. The bacterium is oxidase, catalase and urease positive and sensitive to heat and most disinfectants but can survive in the environment up to two years. Survivability of the bacteria is

prolonged at low temperatures and will remain viable for many years in frozen tissues. *Brucellae* in aqueous suspensions are readily killed by most disinfectants. A 10g/l solution of phenol will kill *Brucellae* when exposed at 37°C for 15 minutes. Formaldehyde solution is the most effective disinfectant provided that the ambient temperature is above 15°C (WHO, 1986; Corbel *et al.*, 2006).

2.3.Epidemiology of Brucellosis

Brucellosis has been controlled and/or eradicated in most developed countries due to they have conducted extensive control programs. However, it remains an important economic and human- animal health problem in developing countries; where large people are relying mainly on their livestock. This is mainly because of resources lacking and coordinated prevention and control programs are not yet started (Aworh *et al.*, 2013). Zoonotic brucellosis is accounting more than 500,000 reported cases annually with a high prevalence has been recorded in African, Asian and Latin American countries. Bovine brucellosis has been controlled and/or eradicated in most developed countries due to they have conducted extensive control programs but still have high incidence in the areas of human brucellosis widely distributed (Figure1). However, the disease remains an important economic and human- animal health challenge in developing countries. This is mainly because of resources lacking, under reporting and misdiagnosis of the disease and prevention and control or eradication programs are not started (Aworh *et al.*, 2013; Ducrotoy *et al.*, 2014).

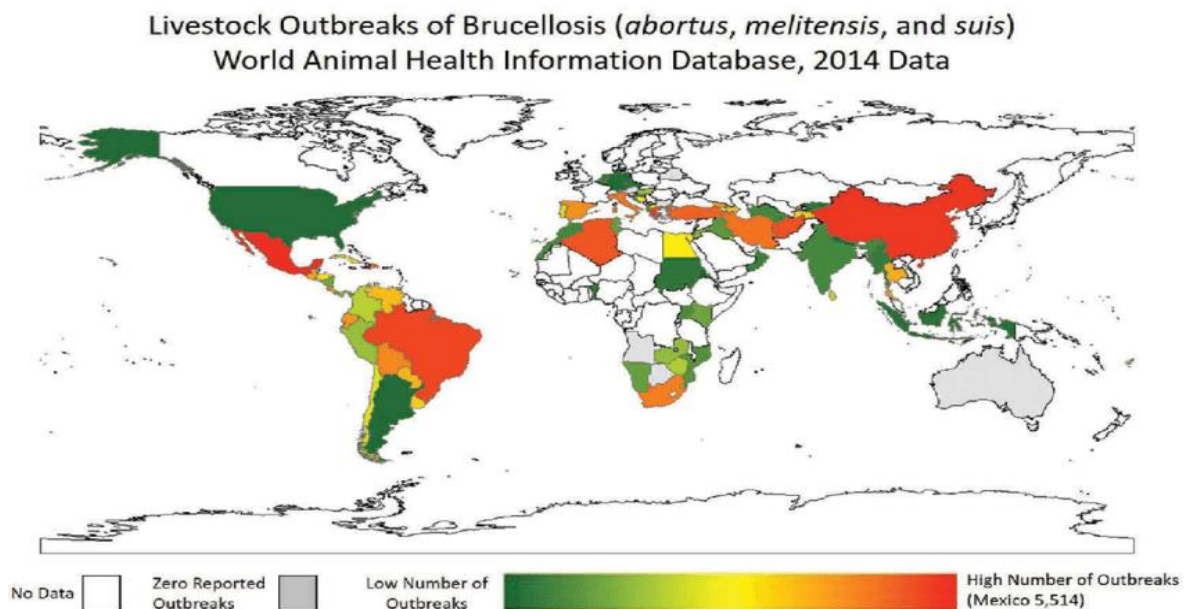


Figure 1: Heat Map of Brucellosis outbreaks in livestock - WAHIS 2014 (Hull & Schumaker, 2018).

2.4. Risk Factors of Brucellosis

2.4.1. Host Related Factors

Animals of all species are comparatively susceptible and so far no specific breed is resistance to brucellosis. Age, sex and reproductive status are the main host factors and *Brucella* infection occurs in all ages with most commonly persisting in sexually mature animals (Radostits *et al*, 2000). The organism can localizes itself in the regional lymph nodes and persists there without provoking antibody production until the animal is conceived and start secreting erythritol(Dermott & Arimi, 2002). The reason for this is that sex hormones and meso-erythritol in male testicles and seminal vesicles and erythritol in female allantois fluid stimulate the growth and multiplication of *Brucella* organisms and tend to increase the concentration with age and sexual maturity (Megersa *et al.*, 2012). Once aborted, infected cows usually develop immunity but remains carrier, and excrete huge numbers of *Brucella* in the fetal fluids (Langoni *et al.*, 2000).

2.4.2. Herd Management

Free movement of animals from infected herd to non-affected one is the major means for the spread of the disease. Purchasing animals from infected source is also potential for disease transmission. Poor management of abortion and parturition materials is the main source of infection. In lactating cows, milk is an important source of infection, and bacteria are excreted in milk throughout the lactation period. Contaminated environment and equipment used for milking and artificial insemination are also main sources of infection too (Corbel *et al.*, 2006).

2.4.3. Agent Factors

Brucella is facultative intracellular pathogen capable of multiplying and surviving within host phagocytes and macrophages. This is because of the organism has an outer-membrane lipopolysaccharide (LPS) and phagosome-lysosome inhibition characteristics. This virulent and inhibition factors are used as a protective mechanism for its intracellular survival and preventing itself from antibiotics. The other mechanism used by *Brucella* is the presence of stress protein, production of catalase and superoxide dismutase which help the bacteria to resist oxidative killing (Radostits *et al*, 2000).

2.4.4. Environmental and Climatic Factors

Brucella may retain for several months in water, aborted fetuses and fetal membranes, faces and liquid manure, wool, hay, buildings, equipment and clothes. It can also able to withstand drying and will persist viable in dust and soil. This survival characteristic of the organism plays a vital role in the transmission and epidemiology of the disease. Temperature, humidity and pH influence the organism's ability to survive in the environment. *Brucella* is sensitive to direct sunlight, disinfectant and pasteurization (Coelho *et al.*, 2015; Beruktayet & Mersha, 2016).

2.4.5. Human Related Factors

Age and Sex: Adults are more exposed to *Brucella* infection in the chronic form. Brucellosis is considered as pediatric problem due to children are usually account for high proportion of acute cases. The disease is highly occupational and the majority of cases are observed in males between the ages of 20 and 45 years (Radostits *et al*, 2000).

Cultural Habit : The highest prevalence reports shows living in close proximity to livestock, habit of raw animal product consumption and contact with aborted materials are the main risk factor for human brucellosis (Wakene, 2017). Blood transfusion, organ transplantation, parenteral drug administration and sexual intercourse can be considered as risk factors for brucellosis in rare cases (Coelho *et al.*, 2015).

Occupation: Laboratory workers and other risk groups who handling *Brucella* cultures and cases are at high risk of acquiring brucellosis through aerosolizing (Bislimovska *et al.*, 2010). Though *Brucella* has low concentration in muscle tissue of animals, consumption of under cooked meat can transmit brucellosis mainly due to contamination with blood and other secretions. However, *Brucella* has higher concentration in liver, kidney, spleen, udder and testis and consumption of these organs and those tissues undercooked have very high risk (Greenfield *et al.*, 2002).

2.5. Transmission and Source of Infection

2.5.1. In Animals

Infection in animal is transmitted through ingestion or inhalation of organisms from different sources like aborted fetus, fetal fluids and vaginal excretions of contaminated pasture or water (Corbel *et al.*, 2006). It can be also transmitted when animals from an affected herd mingle with brucellosis-free herds. Aborted and other materials are the main source of infection and large numbers of organisms

are shed at the time of parturition (Bedore & Mustefa, 2019). Horizontal transmission via ingestion and inhalation of contaminated feed, skin penetration, conjunctiva and licking of discharge from newborn are possible means of transmissions. Venereal and AI infection has also been reported (Corbel *et al.*, 2006).

2.5.2. In Humans

Unpasteurized milk, raw blood and meat, contact with infected animal discharges during and in the post parturition period are the main sources of infection. As occupational disease, it is occurring often in risk group workers (Sewell & Brocklesby, 1990). Inhalation of contaminated dust or aerosol and inoculation of live vaccine like *B. abortus* Strain 19 and *B. melitensis* Rev.1 can cause infection (Robinson *et al.*, 2003). Blood transfusion, tissue transplantation and trans-placental transmission are possible in humans (Corbel *et al.*, 2006).

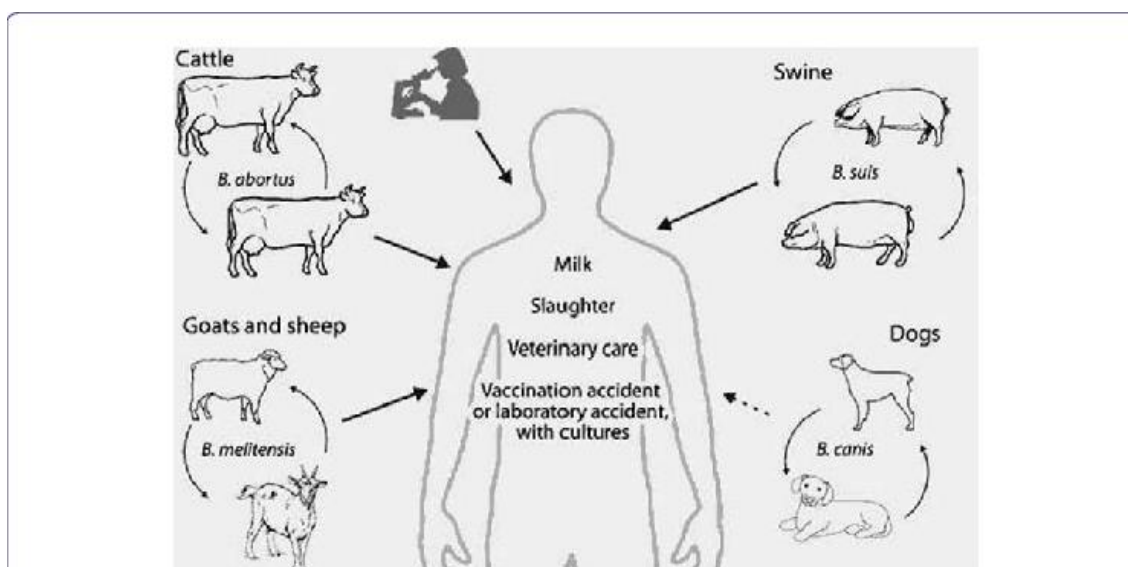


Figure 2: Transmission of *Brucella* to humans (Gadaga, 2013).

2.6. Pathogenesis of Brucellosis

The main predilection site for *Brucella* species are Macrophages, dendritic cells and trophoblasts. It can invade the epithelial cells of the host and use M cells as a portal of entry and then sequestered in monocytes and macrophages of Reticulo-Endothelial Systems such as lymph node, liver, spleen and bone marrow (Ackermann *et al.*, 1988). Once invaded the epithelial cell, *Brucellae* are capable of surviving intracellularly and then the organism spread through hematogenous route in females to

reaches the placenta and the fetus (Carvalho *et al.*, 2010). Finally, the damage to placental tissue together with fetal infection and/or fetal stress will inducing maternal hormonal changes that cause abortion (Gul & Khan, 2007).

2.7. Clinical Signs of Brucellosis

The disease brucellosis is considered as latent infection in animals and clinical manifestation is relating to reproductive tract. In females, third trimester abortion, neonatal weakness, retained placenta, endometritis and reduced milk yield are main signs. In non-vaccinated susceptible pregnant cattle, abortion after the fifth month of pregnancy is cardinal sign of the disease. Orchitis, epididymitis and infertility in males, hygroma in chronic cases are some of the main signs in cattle (Radostits *et al.*, 2000). Leathery and necrotizing placentas in females and palpable testicular alterations and prostatitis in male are also suggested signs (Mohammed *et al.*, 2017).

On the other hand, human brucellosis is clinically characterized by confusing with malaria and influenza and progressing to chronically debilitating ones. Abortion, irregular fever, sweating, anorexia, malaise, weight loss, depression, headache, joint pains and epididymo-orchitis are common signs. Abortion and intrauterine transmission spontaneously may happen during the early trimesters of pregnancy. In the chronic form, it may result in serious complication with musculoskeletal, cardiovascular and central nervous systems affected (Liu *et al.*, 2017).

2.8. Diagnosis of Brucellosis

It is impossible to identify *Brucella* by single test, so that definitive identification should be achieved by combination of growth characteristics, serological, bacteriological or molecular methods (Alton *et al.*, 1988; FAO, 2003).

2.8.1. Bacteriological methods

These are the gold standard method of brucellosis diagnosis mainly for isolation of the agent because of they are specific and used for bio typing (Dahouk *et al.*, 2003). *Brucella* isolation requires high number of viable bacteria, proper storage, quick delivery to the laboratory and BSL3 facilities (Hadush & Pal, 2013). Due to contamination of clinical samples is a major problem for *Brucella* isolation, the use of nutrient-rich media supplemented with antibiotics is used to inhibit the growth of

contaminants (Miguel *et al.*, 2011). The main techniques under this method are staining, culture and biochemical test.

Staining: *Brucella species* are not truly acid fast and they are resistant to decolonization by weak acids and stain red against a blue background with Modified Ziehl-Neelsen method. The presence of aggregates of intracellular and weakly acid-fast organisms with *Brucella* morphology, is presumptive evidence of brucellosis (Alton *et al.*, 1988).

Culture: Isolation of bacteria is required for the bio typing of strains. Direct isolation and culture of *Brucella* are usually performed on solid media which is most satisfactory method. Identification is based on morphology, staining and metabolic profile (catalase, oxidase, and urease) (Alton *et al.*, 1988; Corbel *et al.*, 2006).

Biochemical test: Different biochemical tests like oxidase activity, urease activity, H₂S production, Dye tolerance (basic fuchsin and thionin) and sero-agglutination are used to identify *Brucella* strains. For rapid identification of *Brucella*, Gram stain morphology and MZN staining coupled with the urease test can be recommended (Mantur *et al.*, 2007).

2.8.2. Serological Techniques

They are the most crucial and important for laboratory diagnosis because of easily accessible material and the tests are cheap and sensitive so that control and eradication programs depend on them. They can be classified as screening tests (e.g. buffered antigen plate agglutination - BPAT), monitoring and surveillance tests (e.g. milk ring test), complementary or confirmatory tests (complement fixation, ELISAs, Fluorescence polarization assays) (Poester *et al.*, 2014). If brucellosis is suspected in livestock or in wildlife because of positive serological results, isolation of the organism is mandatory (Godfroid *et al.*, 2010). The main techniques under this method include:

Rose Bengal Plate Test: It is a rapid test method applied with a stained *B. abortus* suspension at pH of 3.6-3.7 and plain serum. It is simple and an ideal screening test for small laboratories with limited resources. RBPT is an agglutination test that is based on reactivity of antibodies against smooth LPS. RBPT is based on the detection of specific antibodies of the IgM and IgG types with more effective in detecting antibodies of the IgG1 type than the IgG2 and IgM types (Díaz *et al.*, 2011).

Complement Fixation Test: The test which has accuracy characteristics that used as confirmatory diagnosis for *Brucella* infections. It is highly specific but it is laborious and requires highly trained personnel as well as suitable laboratory facilities. This test is the reference test recommended by OIE for international transit of animals (Meri & von Bonsdorff, 1998). CFT measures more antibodies of IgG1 than IgM type, as the latter are partially destroyed during inactivation. Since antibodies of the IgG1 type usually appear after antibodies of the IgM type, control and surveillance for brucellosis is best done by CFT. Has sensitivity 77.1 to 100% and specificity of 65 to 100% (Perrett *et al.*, 2010)

Serum Agglutination Test: It measures the total quantity of agglutinating antibodies IgM and IgG . The differentiation in the type of antibody is 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 & Faber, 1980).

Enzyme Linked Immunosorbent Assay

It is the test of choice for complicated cases and standard test for serological diagnosis of brucellosis and measures IgG, IgA and IgM antibodies with more suitable than the CFT for use in smaller laboratories. It is best for screening of large population for *Brucella* antibodies and for differentiation between acute and chronic phases of the disease (Agasthya *et al.*, 2012). ELISA can be divided into indirect ELISA and competitive ELISA and can be used as supplementary test CFT. However, ELISAs are more sensitive than RBPT, sometimes they do not detect RBPT positive infected (McGivern & McGivern, 2013).

i. Indirect ELISA (i-ELISA)

It is based on the specific binding of antibodies present in the test sample with immobilized antigen and the binding event is visualized using chemically or enzymatically derived fluorescent, luminescent or colorimetric reaction (Poester. *et al.*, 2014). It is sensitive and specific for *B.abortus* or *B.melitensis*, but not capable of differentiating antibodies induced by the vaccine strains S19 or Rev1. Sensitivity is 96 to 100%, and its specificity reach 93.8% to 100% (Gall *et al.*, 2003).

ii. Competitive ELISA (c-ELISA)

Are used for differentiate vaccine antibody response from actual infections and its sensitivity varies from 92 to 100% while its specificity ranges from 90 and 99%. It can also be used both for screening and confirmatory tests (Febo *et al.*, 2012).

2.8.3. Molecular Methods

Molecular methods, often based on the PCR amplification, can provide relevant information for identification and bio typing of *Brucella* species and allowing differentiation between virulent and vaccine strains (Goñi *et al.*, 2008). PCR techniques as listed hereunder are the most widely used for brucellosis diagnosis (Ewalt & Bricker, 2000).

Multiplex PCR: is the most reliable method of *Brucella* diagnosis which identify at the species and biovars level. The first method is AMOS PCR (Abortus-Melitensis-Ovis-Suis-PCR) which identifies *B. abortus* *B. melitensis*, *B. ovis* and *B. Suis* (Ewalt & Bricker, 2000).

Real-time PCR: This is the technique which has been recommended recently to test *Brucella* in cells, urine, blood and tissue that embedded by paraffin. It has the advantage of rapidness and sensitivity than conventional PCR. There are three real-time PCR assays to identify *Brucella* spp. These are primers of IS711, reverse primer and probes (Kattar *et al.*, 2007).

2.9. Economic and Public Health Significance of Brucellosis

2.9.1. Economic Significance

Brucellosis is causing serious economic loss in animals by causing infertility, decrease milk production and abortion storm in the herd. The primary impact of brucellosis, both in intensive and extensive farming systems, is loss of production particularly decreasing of milk production by 20%. Generally, loss of calves and lambs decrease milk production, cull and condemnation due to failure of breeding, medical and research cost, restriction of trade and hit productive man power are common consequences (Radostits *et al*, 2000).

2.9.2. Public Health Significance

The disease is an occupational which is occurring often in risk group workers. It may be difficult to detect the outbreak of brucellosis in human because of the initial symptoms are confused with those of flu (Chain *et al.*, 2005). In dairy products pasteurized areas of the world, the disease brucellosis is mainly affects persons who have close contact with animal products (Pappas *et al.*, 2006). Zoonotic brucellosis affect productivity by causing prolonged illness that resulted in loss of strength, income and working time with long treatment costs (Nicoletti, 2010). Generally, lack of vaccine for human, the

disease requiring long treatment time and costs, bioterrorist ability of the agent and due to it has been affecting a half million people annually make the disease Brucellosis a global health threat (Yang *et al.*, 2013).

2.10. Control and Prevention for Brucellosis

2.10.1. In Animals

The aim of controlling the disease in animals is to reduce the impact of the disease on human health and economic crises. As explained by (Gwida *et al.*, 2010) and other scholars, the most important principles of control measures are :

Test and isolation and/or slaughter: It is an identifying of immunologically responded animals to a *Brucella* infection and subsequent culling of the reactors. But, immediate slaughter of test-positive animals requires animal owner cooperation due to compensation is usually necessary under poor economic settings. Due to this, it is not affordable in developing countries (Benkirane *et al.*, 2014).

Hygiene: It can be applied to reduce the exposure of susceptible animals to source of infection and includes isolation of calving animals' in separate disinfected (with 2.5 % formalin) calving pens, disposal of aborted fetuses, fetal membranes and discharges (preferably by incineration), isolation of all animals brought to the farm for 30 days followed by retesting, isolate advanced pregnancy cow until and after parturition (Moti *et al.*, 2013).

Restriction of animal movement: Unauthorized sale or movement of animals from an infected area to free areas should be forbidden to limit the spread of brucellosis (Corbel *et al.*, 2006).

Vaccination: is the leading method to prevent and control brucellosis in animals. Live *B. abortus* strain 19, Live *B. abortus* strain RB51 vaccine and attenuated strain of *B. melitensis* strain Rev.1 are some the most important vaccines (Cheville *et al.*, 1996).

2.10.2. In Humans

Pasteurization of milk and other milk products, cooking meat, take care of handling abortion materials , wear protective clothes at risk areas and extreme care while using S19, RB51 or Rev 1 *Brucella* vaccine and raising community awareness are important measures (Corbel *et al.*, 2006). So far, no vaccine is invented for human (Yang *et al.*, 2013).

3. STATUS OF BOVINE AND HUMAN BRUCELLOSIS IN ETHIOPIA

Several serological studies have been conducted and reported (in the last two decades) from different localities, agro ecology and production system both in human and animal and showed that it's endemic and widespread characteristics (Geresu *et al.*, 2016; Edao *et al.*, 2018). Bovine brucellosis is still the main problem and due to this it has prioritized and/or listed as one of the top zoonotic disease, in Ethiopia. The government of Ethiopia has launched NBTWG to organize prevention and control of the disease through One Health approach (Pieracci *et al.*, 2016; Erkyihun *et al.*, 2022)

3.1.1. Status of Bovine Brucellosis

The disease bovine brucellosis endemic in low land and high lands of the country and has been causing high economic losses with becoming major public health threats (Asmare *et al.*, 2007; Negash & Dubie, 2021; Getahun *et al.*, 2021). According to the studies conducted so far, the distribution and prevalence of Brucellosis in Ethiopia is varied from place to place and time to time (Bulcha *et al.*, 2020; Edao *et al.*, 2020). The disease has high zoonotic and economic implication for the rural communities. Hence, knowledge of the disease has a very high importance in order to reduce economic and public health impacts (Getahun *et al.*, 2021).

Sero-prevalence of bovine brucellosis: For the last decades, bovine brucellosis seroprevalence has been reported by different scholars with the endemic nature of the disease with varying evidence observed from farm to farm (Edao *et al.*, 2020; Negash & Dubie, 2021)(Table 1). Accordingly, a very recent study in Afar reported prevalence of 7.2% and 6.2% in Afar and Somali respectively (Tschopp *et al.*, 2021). Getahun has reported 0.6% prevalence from dairy cows with recent abortion history (Getahun *et al.*, 2021). A cross sectional study from Jimma zone has reported animal and herd level prevalence of 16.4% and 4.3% respectively (Robi *et al.*, 2021). A studies by (Negash & Dubie, 2021) and (Ararsa *et al.*, 2021) were also reported 11.9% and 22.5% from Afar and East respectively .

In 2020, prevalence of 3.2% from Borena, Southern Ethiopia, 9.5% from Adama 0.4% Sendafa (Oromia Region) were recorded by different scholars (Edao *et al.*, 2020; Bulcha *et al.*, 2020; Bifo *et al.*, 2020). Prevalence report was recorded as 2.7% and 25.8% at individual and farm level by (Abera *et al.*, 2019). Tadesse was reported from South West Shewa of Oromia Region 5.48% herd level 1.04% animal level prevalence (Tadesse *et al.*, 2019). In 2018, 0.06% and 0.8% of animal and herd level prevalence was reported in intensive dairy farms of Addis Ababa (Edao *et al.*, 2018).

Prevalence reports were also recorded as 2.81%, 1.3% & 6.8% and 9.87 in dairy farms of central high lands of Ethiopia (Geresu *et al.*, 2016; Terefe *et al.*, 2017). In Alage and its around, a researcher reported 2.4% animal level and 45.9% herd level prevalence (Asgedom *et al.*, 2016). From Debrebirhan and Ambo towns 0.7% and 0.2% for RBPT and CFT based prevalence report were recorded respectively (Bashitu, 2015). 1.7% animal and 35% herd level prevalence reported from Arsi (Tschopp *et al.*, 2013). Seroprevalence of 8.0% and 51.7% have recorded at animal and herd level respectively from pastoral region of Borena (Megersa *et al.*, 2012). An overall seroprevalence of 3.5%, and 26.1% at animal and herd level respectively from Southern and Eastern Ethiopia was also reported (Megersa *et al.*, 2011). Seroprevalence report from Arsi Negele of Southern Ethiopia was showed 2.6% and 12% (Amenu *et al.*, 2010). A prevalence of 11% and 63.6% was also reported from smallholder farms of Tigray Region (Mekonnen *et al.*, 2010).

Table 1 : Bovine Brucellosis sero-prevalence reports, in Ethiopia (2010- 2021)

Study Area		Year Public ation	Number of sample		Type of Test	Prevalence and 95% CI based on CFT & ELISA test			Reference
Region	Specific location		IL	HL		CFT	in %	95% CI	
IL	HL	IL							
Afar	Dubti,Asyta,Chifr	2021	420	-	RBPT,CFT	5.7	-	3.8-7.94	(Negash & Dubie, 2021)
Afar	(in 7Districts)	2021	488		ELISA	7.2		5-10.2	(Tschopp <i>et al.</i> , 2021)
Somalia	(in 6Districts)	2021	116		ELISA	6.9		3.5-13	(Tschopp <i>et al.</i> , 2021)
AA	C H	2021	352		RBPT,CFT	0,6		0.016-2	(Getahun <i>et al.</i> , 2021)
Oromia	Jimma	2021	80		CFT	22.5			(Ararsa <i>et al.</i> , 2021)
Oromia	Borena	2020	750		RBPT,ELISA	2.4	-	1.4-3.7	(Edao <i>et al.</i> , 2020)
Oromia	Adama	2020	384		CFT	1.04	9.5	0.02,2.6	(Bulcha <i>et al.</i> , 2020)
Oromia	Around AA	2020	503		RBPT,CFT	0.4	-		(Bifo <i>et al.</i> , 2020)
SNNPR	Awassa	2019	370		RBPT,CFT	2.7	25.8		(Abera <i>et al.</i> , 2019)
Oromia	Becho	2019	384		RBPT,ELISA	1.04	5.48		(Tadesse <i>et al.</i> , 2019)
AA	AA	2018	1550		CFT	0.06	0.8		(Edao <i>et al.</i> , 2018)
SNNPR	NNP	2018	268	50	RBPT,CFT	9.7	32	6.4413.8	(Chaka <i>et al.</i> , 2018)
Oromia	Asela	2018	304		RBPT,CFT	9.87			(Eticha <i>et al.</i> , 2018)
E/E	E/E	2017	967		RBPT,CFT	1.3	6.8	0.72,2.2	(Terefe <i>et al.</i> , 2017)
Oromia	Asela & Bisho.	2016	570		RBPT,CFT	1.4		0.24,3.46	(Geresu <i>et al.</i> , 2016)
Oromia	West Arsi	2016	421	33	RBPT,CFT	3.3	42.4		(Asgedom <i>et al.</i> , 2016)
Oromia &Amara	DB &Ambo	2015	415		RBPT,CFT	0.7			(Bashitu, 2015)
Oromia &Afar	Pastoral areas	2013			RBPT,CFT	1.7	35		(Tschopp <i>et al.</i> , 2013)
Ethiopia	Farm level	2013	2334		RBPT,CFT	1.9	10.6	1.2-2.6	(Asmare <i>et al.</i> , 2013)
Amhara	West Gojjam	2013	1152	164	RBPT,CFT	1	4.9	0.5-1.7	(Adugna <i>et al.</i> , 2013)
Somali& Oromia	SE/Ethiopia	2013	862	59	RBPT,CFT	1.4	17	0.8-2.6	(Gumi, 2013)
Oromia	Borena	2012	575	50	RBPT,CFT	8	51.7	6-10	(Megerssa <i>et al.</i> , 2012)
Somalia	Jigjiga	2011	435	-	RBPT,CFT	1.38	-	1.35- 1.41	(Degefu <i>et al.</i> , 2011)
Oromia	Borena	2011	271	16	RBPT,CFT	4.7	68.6	2.1-7.3	(Megersa <i>et al.</i> , 2011)
SNNPR	South Ommo	2011	180	12	RBPT,CFT	3.4	33	0.9-6.1	(Megersa <i>et al.</i> , 2011)
Somalia	Shinle	2011	210	15	RBPT,CFT	6.6	40	3.1-10.1	(Megersa <i>et al.</i> , 2011)
Somalia	Jigjiga	2011	62	4	RBPT,CFT	3	50	1.7-7.1	(Megersa <i>et al.</i> , 2011)
SNNPR	Sidama	2010	1627	27	RBPT,CFT	1.66	13.7		(Amenu <i>et al.</i> , 2010)
Tigray	North West	2010	848	210	RBPT,CFT	1.2	3.3		(Mekonnen <i>et al.</i> , 2010)

AA (Addis Ababa), CFT (Complement fixation test), CH (Central Highland), CI (Confident Interval), EE (Eastern Ethiopia) ELISA (Enzyme linked Immunosorbent assay), HL (Herd Level), IL (Individual Level), NNP (Nechisar National Park), SNNPR (Southern Nations Nationalities People Region)

3.1.2. Human Brucellosis in Ethiopia

In Ethiopia, brucellosis has a widespread prevalence, endemic characteristics and one of the public health and economic problems among the rural and urban areas (Getahun *et al.*, 2021). This is due to the determination of associated risk factors and intervention of health are not regularly exercised, lack of effective diagnostic facilities, common close contact between humans and livestock and the country has not yet started prevention and control program (Genene *et al.*, 2009). Human brucellosis seroprevalence between the mid-2000^s to 2021 were reported from different localities of Ethiopia (Table 2) and showed a record of prevalence ranging from 2.15% to 48.3% (Tibesso *et al.*, 2014; Tschopp *et al.*, 2021). A high prevalence was recorded in pastoral communities due to the habit of unpasteurized dairy products consumption, living close proximity with animals and often have poor access to health services (Genene *et al.*, 2009; Edao *et al.*, 2020 ; Tschopp *et al.*, 2021). Isolation of *Brucella* from human cases is not achieved in Ethiopia so far.

Table 2: Sero-prevalence of human brucellosis in Ethiopia (2006 -2021)

Location of study	Year of study	Study subjects	Type of test	prevalence in %	Reference
Afar	2021	Pastoralists	ELISA	48.3	(Tschopp <i>et al.</i> , 2021)
Somalia	2021	Pastoralists	ELISA	34.9	(Tschopp <i>et al.</i> , 2021)
Afar	2021	Patient	ELISA	31.5	(Mehari <i>et al.</i> , 2021)
SNNPR(Borena)	2020	LS owners	RBPT, ELISA	2.6	(Edao <i>et al.</i> , 2020)
Afar	2018	Patients	CFT	4.4	(Zerfu <i>et al.</i> , 2018)
SNNPR	2017	Blood donors	NS	10.6	(Workalemahu <i>et al.</i> , 2017)
Oromia/ Adamitulu	2014	LS owners	RBPT, CFT	2.15	(Tibesso <i>et al.</i> , 2014)
Afar	2012	Community	RBPT, CFT	16	(Zewolda & Wereta, 2012)
Amhara(West Gojjam)	2009	Patients	RBPT	2.6	(Animut <i>et al.</i> , 2009)
Oromia(Borena)	2009	Patients	LFA	34.1	(Genene <i>et al.</i> , 2009)
SNNP/Hamer	2009	Patients	LFA	29.4	(Genene <i>et al.</i> , 2009)
Oromia/Jimma	2008	Patients	RBPT, CFT	3.6	(Tolosa <i>et al.</i> , 2008)
Amhara/Bahirdar	2007	HRG	RBPT, CFT	5.3	(Mussie <i>et al.</i> , 2007)
Sidama	2007	HRG	RBPT, CFT	3.78	(Asmare <i>et al.</i> , 2007)
Addis Ababa	2006	HRG	RBPT, CFT	4.8	(Kassahun <i>et al.</i> , 2006)

HRG (High Risk Group), LS (Livestock), LFA (Lateral Flow Assay)

4. MATERIALS AND METHODS

4.1. Description of study Areas

The study was conducted in Debrebirhan town, Basona Werana and Angolalla Tara districts of North Shewa Administrative Zone of Amhara Regional State (Ethiopia) between November 2021 and April 2022. North Shewa is one of the 10 Zones of Amhara Region bordered on the south and the west by, the Oromia Region, on the north by South Wolo Zone, on the north east by special zone of Oromo of Amhara Region and on the east by the Afar Region (figure3). It has 23 districts which are Mida Woremo, Merhabete, Ensaro, Moretna Jiru, Menz Gera Midir, Geshe, Antsokiya Gemza, Efratana Gidim, Menz Mama- Midir, Tarmaber, Mojana Wdera, Kewet, Angolalla Tara, Asagirt, Ankober, Hagere Mariamna Kesem, Berehet, Minjarna Shenkora, Basona Werana, Debrebirhan(town), Menz Keya Gebreal, Menz Lalo Midir and Siyadebrna Wayu (Wikiwand, 2020). The highest point in the Zone is Mount Abuye Meda with 4012 MSL which is parts of central highlands of Ethiopia. North Shewa Zone is classified into agro-climatic zones of lowland - "Kola" (< 1500 m. a. s. l.); midland - "Weynadega" (1500 - 2400 m. a. s. l.) and highland - "Dega" (> 2400 m. a. s. l.). Geographically, it cover longitudinal and latitudinal ranges of 39° 29' 59.99" East and 10° 00' 0.00" North with mean annual rainfall and temperature between 800-1000 mm and 20.1-25°C respectively (Latitude, 2022).

The specific study areas were Angolalla Tara, Basona Werana and Debre-Birhan town (three of the 23 North Shewa Zone districts and parts of Ethiopian central highlands). Debrebirhan town is the capital of North Shewa and located 130 Km away from Addis Ababa in northern direction and geographically it lays at 39° 75' 81.44" Longitudes East and 9° 93' 61.44" latitude North and lay approximately at 3000m altitude ASL (Eshetu *et al.*, 2019). It has an average mean annual rainfall of 1100mm and annual temperature recorded between 16°C and 31°C (Bashitu, 2015). This study area was characterized by occasional frost occurs between October and January. According to the 2007 population and housing census, Debrebirhan has a total of 65,231 (Male= 31,669 and female=33,563) human population (CSA, 2020). Total cattle population of the study area is estimated to be 15, 864 (Tahir, 2018). Dairy cows constitute 10,221 from total cattle population and the dairy production system in Debrebirhan town is mostly dominated by cross breed- animals in which the house holds use them for production of milk to sell for urban communities, private milk collectors and milk cooperatives. Basona Werana and Angolalla Tara are two of the rural districts of North Shewa Zone which are located at 130 and 113 Km away from Addis Ababa by circling and South of Debre-Birhan town

respectively. It is estimated that both are geographically lays at similar location with Debre-Birhan town so that shares its climatic condition characteristic. Their human population number is estimated as 120,930 (Male= 61,924 and female= 59,006) and 82,349 (Male= 41,849 and female= 40,500) respectively (CSA, 2020). According to the Districts Livestock Development Offices (DLDO), the total cattle population of these study areas is estimated to 156,652 for Basona Werana and 168,113 for Angolalla Tara. Cattle, in all study areas, are kept under intensive, semi-intensive and extensive systems and the feeding practice is characterized by crop residue, hay, green fodder and some improved feeds. In Ethiopia, dairy production system is classified in to commercial, urban and peri-urban smallholder, rural smallholder(mixed crop-livestock production) and pastoral and agro-pastoral (Asmare *et al.*, 2013). This study focuses on commercial, urban and peri-urban and rural smallholder dairy production systems that are dominated by cross breed animals and some local zebus. As livestock brucellosis control intervention by immunization has never been attempted in Ethiopia, there is no history of vaccination against brucellosis in the study areas.

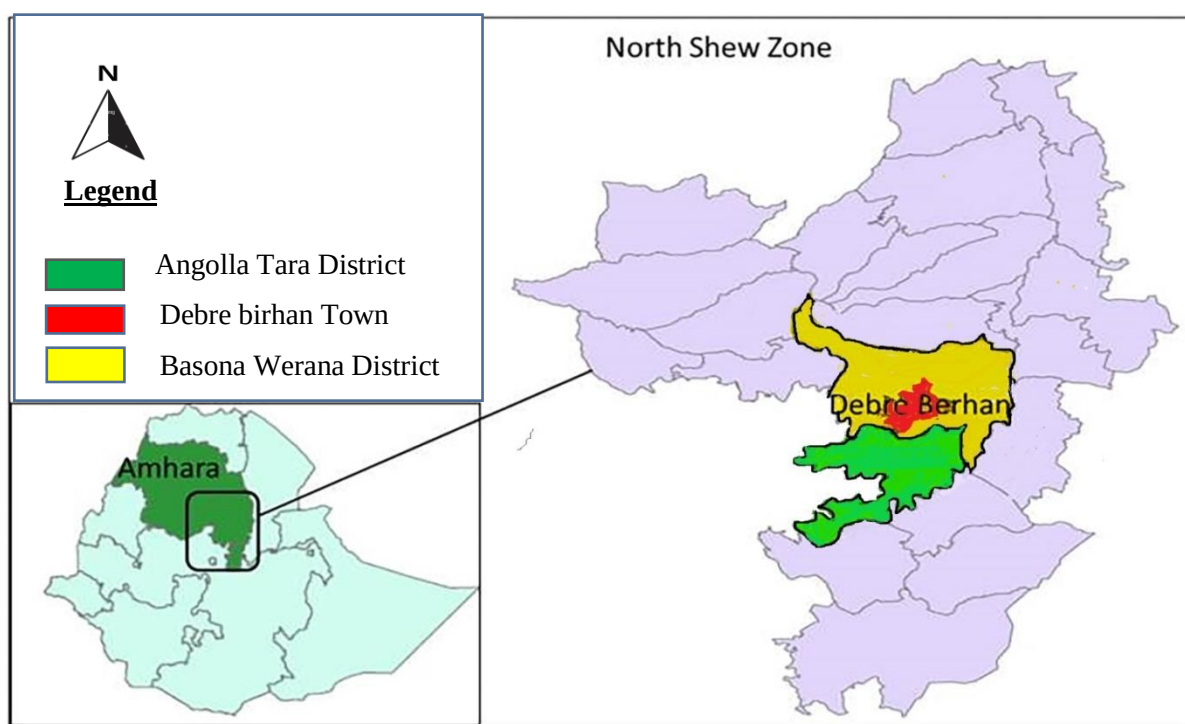


Figure 3: Map of the study areas, Source: (Deressa *et al.*, 2018) and Projected by the author.

4.2. Study Populations

The target populations were small holder dairy cattle and their owners in Debre-Birhan town, Basona Werana and Angolalla Tara districts of North Shewa Zone, Amhara Regional State, Ethiopia. Dairy cattle above six month of age and both lactating crossbred and indigenous small holder dairy cows that are reared under intensive, semi-intensive and extensive management systems were included in the study. The breed of this study areas were cross of local breeds (Zebu-Holstein Friesian and Zebu-Jersey cross breeds). Dairy cows were classified into three age groups below 4 years, 4-8 years and > 8 years as young, adult and old respectively (Morgan *et al.*, 2006). The body condition score (BCS) were subjectively estimated as poor, medium and good based on the guides published by (Grimm, 1997). None of the tested animals were vaccinated against brucellosis.

4.3. Study Design

A cross sectional study was carried out from November 2021 to April 2022 to determine the seroprevalence of brucellosis in dairy cattle and assess associated potential risk factors and to assess KAP of dairy cattle owners on zoonotic brucellosis and its transmission in selected commercial and small holder dairy farms of Debre-Birhan town, Basona Werana and Angolalla Tara districts of North Shewa Zone, Amhara Regional State, Ethiopia.

4.4. Sampling method and sample size determination

Purposive sampling technique was used to select districts, Kebelles, small holder dairy farmers and farms. Accordingly, a total of three districts, seven kebelles and five commercial dairy farms (four Kebelles from Debre-Birhan town, two kebelles of Basona Werana and one Kebele from Angolalla Tara) were selected purposively based on accessibility, owner's willingness and number of dairy farms. A partial list of dairy farms and rural smallholder dairy farmers was obtained from Debre-Birhan town, Basona Werana and Angolalla Tara District Livestock Development Offices. Commercial dairy farms were classified based on herd size and management in to small-scale (≤ 15 heads of cattle), medium-scale ($\geq 16-29$ heads of cattle) and large-scale (≥ 30 heads of cattle) farms depending on number of animals (Asgedom *et al.*, 2016). Holding size of cattle for small holder farmers was defined as small (3-4 head), medium (5-9 head) and large (10-19 head) (CSA, 2021). Cattle that were owned by a dairy farmer and housed in the same barns were considered as one herd (Tolosa *et al.*, 2008; Asgedom *et al.*, 2016) and all animals in each selected herd were sampled. Since there is no previous study in

Debrebirhan town and its surrounding, the sample size for dairy cattle was calculated based on the formula described by (Thrusfield, 2007) with 5% defined precision, 95% level of confidence interval and 50% expected prevalence. Hence, the sample size was calculated using the formula

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

n = Required sample size,

P_{exp} = Expected prevalence (50%)

d^2 = Desired absolute precision (5%)

$$\text{Therefore, the sample size is estimated as } n = \frac{3.84 \times 0.5(1-0.5)}{(0.05)^2} = 384$$

Accordingly, the calculated sample size for the study was 384 dairy cattle. To increase the precision, I have been increased the sample size to 426. Accordingly, a total of **426** dairy cattle were sampled from 3 districts, seven kebelles and five commercial dairy farms.

The sample size for questionnaire survey was calculated based on the formula recommended by (Taherdoost H, 2018); $n = \frac{p(100-p)Z^2}{E^2}$, Where:

N =the required sample size

p = the proportion of desired condition

E = the percentage maximum error required (0.05 precision /5%).

Z = the value corresponding to level of confidence required (95% level of confidence interval)

$$\text{Accordingly, } n = \frac{0.25(100-0.25) (1.96)^2}{(0.05)^2} = 108$$

Hence, the required sample size for questionnaire survey was 108. However, only 97 volunteers were included.

4.5. Sample Collection

4.5.1. Blood Sample Collection

After animals were restrained by handlers, approximately 5-8 ml of blood was collected from jugular vein using vacutainer tube and sterile needles based on aseptic techniques principles (shaving and disinfecting the site). Each sample (vacutainer tube) was labeled with permanent marker and the case number of the respective study subject was recorded and coded. According to OIE, the blood samples were kept in slanting position at room temperature (4⁰c) for 24 hours in order to separate the serum. Then, serum was harvested using 1.8 ml cryovials and each cryovials containing the serum was labeled and stored at (4⁰c) in ice box and transported to NAHDIC laboratory to be stored at -20°C until screened and/or tested RBPT and Compliment Fixation Test (CFT)). All serum samples collected from animals were screened for RBPT and positives were tested for CFT in serology laboratory of NAHDIC, Sebeta-Ethiopia.

4.6. Questionnaires Survey

Personal demography information like sex, age, educational status and KAP towards brucellosis, milk drinking practice and others was collected on the developed questionnaire format. Farmer's awareness about knowledge of brucellosis, knowledge of zoonotic disease, handling of aborted material was recorded as bare hand, wear glove, and others, while hand washing practice was recorded as Yes or No. The explanatory variables which were hypothesized to be risk factors were assessed at individual animal level. Herd information and herd level parameters including herd size was extracted from herd records and farm owners or managers interview using pretested structured questionnaires. Questionnaire data was collected and recorded on traits of study animals such as about the breed, species, age, sex, body condition score, lactation, reproductive status and history of abortion and retained fetal membrane, stage of abortion (first trimester, second trimester and third trimester) and breeding systems. The presence of reproductive problems, such as abortion, still births and retention of fetal membranes were recorded too. Breeding methods (as bull service, AI or both), hygiene status of farm (as poor, medium and good) and stocking method (as buying, own replacement or both) were recorded at farm or herd level. Purposive sampling technique was used to select the key informants and the questionnaire was administered by the principal investigator.

4.7. Laboratory Tests

4.7.1. Serological Tests

The two common serological tests, the Rose Bengal Plate and the Complement Fixation tests were used as a screening and confirmatory test respectively for the detection of *Brucella* antibody at NAHDIC, Sebeta-Ethiopia.

4.7.1.1. Rose Bengal Plate Test

All samples were screened using RBPT based on the procedure described and specifically recommended by (“*Joint FAO/WHO Expert Committee on Brucellosis.*,” 1986; Alton *et al.*, 1988) and manufacturers instruction . It was Rose Bengal antigens which constitute a suspension of *Brucella antigen* (Annex 4). Accordingly, the antigen and sera were taken from refrigerator and left at room temperature for half an hour and then a drop (30 µl) of serum was taken by micropipette on a test plate and the antigen bottle was shaken well to ensure homogenous suspension and one drop (30 µl) of Rose Bengal *Brucella* antigen was added on a test plate. The serum and the antigen were mixed thoroughly with spreader and then the plate was rocked for four minutes using rocker machine. The results was read and interpreted according to the presence and degree of agglutination (clamping) of bacterial suspension. Finally, the presence of agglutination was considered as positive reaction while the absence was negative. Agglutinations were recorded as 0, +, ++ and +++ scores which indicates as absence of agglutination, barely visible agglutination, fine agglutination and coarse agglutination (clamping) respectively.

4.7.1.2. Complement Fixation Test

All sera tested positive for RBPT were further tested using CFT for confirmation. CFT is a test in which a sample of serum is exposed to a particular antigen and complement in order to determine whether or not antibodies are present to that particular antigen or serum. The nature of complement is to react in combination with antigen-antibody complex. The test mainly measures IgG antibodies. CFT has two components. The first is complementary antigen and protein - test serum and complement obtained from Guinea pig are mixed and incubated. The second is indicator (hemolytic) system - which consist of 3% SRBC and an amboceptor (hemolysine serum) which binds to erythrocyte and sensitizes red blood cells to the action of the complements. Preparation of the reagent was evaluated by titration

and performed according to protocol recommended by OIE, 2009 (Annex 4). The *Brucella* antigen and control sera (positive and negative) used during the test was produced by Cenogenics Corporation, USA. The *Brucella* antigen, complement and 3% sensitized sheep red blood cell were added after the sera were serially diluted (1:5, 1:10, 1:20, and 1:40) in microliter pipette. Then the plates were incubated at 37⁰c for 30 min. It was considered that the validity of the test as complete hemolysis in negative control and positive control shows inhibition of hemolysis.

To define the cases, animals were considered as positive to brucellosis when they tested positive on RBPT/CFT. When at least one animal in a herd or farm tested positive, the herd is considered as positive.

4.8. Ethical Consideration

It was obtained the ethical clearance from animal research ethical review committee CVMA of Addis Ababa University with Ref. No. VM/ERC/03/01/14/2022, Minute No. and Date of review VM/ERC/01/14/22, 22/02/2022 (Annex 2) for study animal sample collection and support letter taken from North Shewa Zone officials. After clearly explained about the objectives of the study, oral and written consents were obtained from dairy farm owners to collect blood samples from their animals and information about their knowledge, attitude and practice towards brucellosis. Based on this, ethical principles were implemented.

4.9. Data Analysis

The generated data from laboratory investigation and questionnaire survey were recorded and coded using Microsoft Excel spread sheet of Microsoft Corporation for Window 2010 and transferred to and/or analyzed using **R software** version 4.3. The units of analysis were individual animal and herd on the basis of RBPT and CFT positivity. Descriptive statistics were used for farm and demographic characteristics including KAP's relating to bovine brucellosis. Prevalence of animal level was calculated as number of *Brucella* reactors divided by the total number of tested animals and for herd level seroprevalence the number positive herds was divided to the total number herds tested. Association between outcome (*Brucella* sero-positivity) and explanatory (risk factors) variables for all units of analysis were investigated by multivariable logistic regression. A multivariable logistic regression model was used to identify risk factors associated with *Brucella* infection.

5. RESULTS

5.1. Questionnaire Surveys results

5.1.1. Demographic Characteristics of KAP Respondents

A total of 97 volunteer participants (59 from Debre-birhan town, 32 from Basona Werana and 6 from Angolalla Tara districts) were interviewed to assess their knowledge, attitude and practice towards brucellosis and 87.6% of them were married. The vast majority of interviewees (88.6%) were male and the remaining was female. The age category of majority respondents (40%) was 31-45 years followed by <30 years (27%). Educational status of respondents were recorded as 21.6% read and write, 42.3% primary school, 19.6% high school, 10.3% college graduates and 6.2% university graduates. When we see the animal ownership status percentage of the respondents, 15.4% farm employees, 79.4% smallholder farmers, 2.1% small scale farm owners, 2.1% percent Medium scale farm owners and 1% large scale farm owners (Table:3).

Table 3: Demographic Characteristics of KAP Respondents

Demographic Variables	Categories	Frequency	Percentage (%)
Sex	Male	85	87.63
	Female	12	12.37
Age categories	< 30 Years	27	27.8
	31-45 Years	40	41.3
	46-60 Years	20	20.6
	> '60 Years	10	10.3
Marital status	Married	85	87.6
	Single	12	12.4
Educational status	Read and write	21	21.6
	Primary school	41	42.3
	High school	19	19.6
	College	10	10.3
	University	6	6.2
Address	Debre birhan	59	60.8
	Basona Werana	32	33
	Angolalla Tara	6	6.2
Animal ownership status	Farm employer	15	15.4
	Smallholder farmers	77	79.4
	Small scale farm owner	2	2.1
	Medium scale farm owner	2	2.1
	Large scale farm owner	1	1

5.1.2. Characteristics of Dairy Farms in the Study Areas

Out of 46 surveyed farms, 89.1 % (41) were small holder. Off which, 51.2 %(21) have been followed extensive farming system. From all surveyed farms, about 10.9% (5) were commercial dairy farms with scale proportion of 2 small intensive, 1 medium (semi-intensive) and 2 large intensive farms. The majority of small holder farms (51.2%) were used bull service strategy while 31.7% small holder farms use both buying and own stock replacement system. From commercial dairy farms, there are two large scale farms (Misale Agro-Industry PLC and Esayas Dairy Farms in Angolalla Tara) and follows buying stock replacement and bull service systems. Of the 41 small holder dairy farms, 80.5 %(33) have not parturition pen, 83 %(34) farms follows open dump disposal system of RFM and aborted materials. 44 % of small holder farms have continuous contact with other herds. The majority of farms were surveyed from Debre-birhan town (35) followed by Basona Werana district (9) (Table 4)

Table 4: Characteristics of Surveyed Dairy Farms in the Study Areas

Farming Variables	Categories	Dairy farm type and frequency (%)						
		Small holder dairy farms				Commercial dairy farms		
		Very Small	Small	Medium	Large	Small scale	Medium scale	Large scale
		(n=8)	(n=15)	(n=15)	(n= 3)	(n=2)	(n=1)	(n=2)
Farming system	Extensive	2 (25)	8 (53.3)	9 (60)	2 (66.7)	0 (0)	0 (0)	0 (0)
	Semi-intensive	5 (62.5)	3 (20)	4 (26.7)	0 (0)	0 (0)	1 (100)	0 (0)
	Intensive	1 (12.5)	4 (26.7)	2 (13.3)	1 (33.3)	2(100)	0 (0)	2(100)
Stock replacement	Buying	4 (50)	7 (46.7)	3 (20)	1 (33.3)	0 (0)	0 (0)	1(50)
	Own replaced	0 (0)	1(6.6)	4 (26.7)	0 (0)	0 (0)	0 (0)	0 (0)
	Both	4 (50)	7 (46.7)	8 (53.3)	2 (66.7)	2(100)	1 (100)	1(50)
Type of service	AI	2 (25)	2 (13.3)	2 (13.3)	1 (33.33)	1 (50)	0 (0)	0 (0)
	Bull	2 (25)	9 (60)	9 (60)	1 (33.33)	1 (50)	1 (100)	2(100)
	Both	4 (50)	4 (26.6)	4 (26.7)	1 (33.33)	0 (0)	0 (0)	0 (0)
Hygiene of barn	good	2 (25)	1(6.7)	4 (26.7)	1 (33.33)	0 (0)	0 (0)	1(50)
	medium	2 (25)	12 (80)	6 (40)	1 (33.33)	1 (50)	1(100)	1(50)
	poor	4 (50)	2 (13.3)	5 (33.3)	1 (33.33)	0 (0)	0 (0)	0 (0)
Parturition pen	Yes	0 (0)	4 (26.6)	3 (20)	1 (33.3)	2(100)	1(100)	1(50)
	No	8 (100)	11(73.4)	12 (80)	2 (66.7)	0 (0)	0 (0)	1(50)
Reproductive problem	Yes	3 (37.5)	5 (33.3)	9 (60)	2 (66.7)	2(100)	1 (100)	2(100)
	No	5 (62.5)	10 (66.7)	6 (40)	1 (33.3)	0 (0)	0 (0)	0 (0)
Disposal of Abo.& RFM	Burning	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Burying	1 (12.5)	5 (33.3)	1(6.7)	0 (0)	1 (50)	0(0)	1(50)
	Open dump	7 (87.5)	10 (66.7)	14 (93.3)	3 (100)	1 (50)	1 (50)	1(50)
Contact with other herd	Yes	2 (25)	5 (33.3)	9 (60)	2 (66.7)	0 (0)	0 (0)	0 (0)
	No	6 (75)	10 (66.7)	6 (40)	1 (33.3)	2(100)	1(100)	2(100)
Address	Debre-birhan	5 (62.5)	13 (86.7)	13 (86.7)	2 (66.7)	1 (50)	1 (100)	0 (0)
	Basona Werana	3 (37.5)	2 (13.3)	2 (13.3)	1 ((33.3)	1 (50)	0 (0)	0 (0)
	Angolalla Tara	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	2(100)

5.1.3. Characteristics of Sampled (Individual) Animal

A total of 426 dairy animals were sampled and the vast majority (95.5%) was cross breed of indigenous zebu with Holstein Frisian and Jersey breeds. Of all sampled dairy animals, 87.3% (372) of them were female and of which 77.4 % (288) of cows were Pluriparous while only 2.95 % (11) of cows were Primiparous. The sources of sampled dairy animals were from local market (2.8%), own replaced

(49.1%) and 48.1% of animals have a history of both own replacement and market system. The reproductive performance percentage of the study animals was recorded as 51.88% milking cows, 14.79% non-milking cows, 4.7% pregnant cows, 15.96% heifers and 12.67% bulls. Out of 299 female adult study animals, 40 cows have history reproductive problems (19 abortions and 21 RFM). About 203 study animals (47.6%) were sampled from Angolalla Tara and the other 38 and 185 cattle head were from Basona Werana district and Debre birhan town (Table 5).

Table 5: Characteristics of Sampled Animals in the Study Areas

Demographic Variables	Categories	Frequency	Percentage (%)
Sex	Female	372	87.3
	Male	54	12.7
Age Categories	Young	100	23.5
	Adult	277	65
	Old	49	11.5
Breed	Cross	407	95.5
	Local	19	4.5
Stock replacement	Own replaced	209	49.1
	Bought	12	2.8
	Both	205	48.1
Parity	Primiparous	11	2.95
	Pluriparous	288	77.4
	No	73	19.6
Reproductive performance	Heifer	68	15.96
	Milking Cow	221	51.88
	Non-milking Cow	63	14.79
	Pregnant Cow	20	4.7
	Bull	54	12.67
Body condition score	Poor	105	24.65
	Medium	252	59.15
	Good	69	16.2
History of Reproductive Problem	Abortion	19	5.1
	Still birth	13	3.5
	RFM	21	5.65
	No	319	85.75
Address/District	Debrebirhan	185	43.4
	Basona Werana	38	9
	Angolalla Tara	203	47.6

5.1.4. Knowledge, Attitude and Practice (KAP) of Respondents about Brucellosis

In this study, 87.6% (84) of KAP respondents had no knowledge on brucellosis while 12.4 % (12) of the interviewees had information about zoonotic brucellosis. More than 54.6 % (53) of those dairy animal owners were consumed raw milk (not boiled milk) and about 45.4% of them had reported that they were consumed boiled milk. All 97 respondents (100 %) of this study reported their common contact practice with afterbirth, aborted & retained fetal membrane materials. The respondent's ratio for ways of contact and/or handling these materials were recorded as 27(27.8%) with bare hand, 15 (15.46%) with glove and 55 (56.70%) handled with others local materials (plastic, wood, shovel, etc.). Hand washing with soap after contact with afterbirth, aborted & retained fetal membrane materials were not practiced by 35 % (36) of respondents while 64% were done the issue (Table 7).

The majority of interviewed dairy animal owners (72 (74.22%)) had no knowledge about what zoonotic disease means and its way of transmission. Only 25, out of 97 respondents, had the knowledge on zoonotic disease and its way of transmission (like consumption of uncooked milk and meat, improper handling of afterbirth, aborted and retained fetal membrane materials). Based on the present study, the housing status of the dairy animal owners (respondents) in relation to their livestock was reported as 85 (87.63%) of respondents had separate while 12 (12.37%) of respondents shares common house with their animals (Table 6).

Table 6: Knowledge, Attitude and Practice (KAP) of Respondents about Brucellosis

Variables	Categories	Frequency	Percentage (%)
Housing with animals	Separate	85	87.63
	Common	12	12.37
Drinking of raw milk	Yes	53	54.6
	No	44	45.4
Contact with afterbirth, aborted & retained fetal membrane materials	Yes	97	100
	No	0	0
Way of Contact with afterbirth, aborted & retained fetal membrane materials	Bare hand	27	27.84
	Glove	15	15.46
	Others	55	56.70
Method of disposal for afterbirth, aborted & retained fetal membrane materials	Burning	0	0
	Burying	21	21.65
	Open dump	76	78.35
Hand washing with soap after contact with afterbirth, aborted & retained fetal membrane materials	Yes	62	64
	No	35	36
Knowledge on Zoonotic Disease & its way of Transmission	Yes	25	25.78
	No	72	74.22
Knowledge on Brucellosis	Yes	12	12.4
	No	85	87.6

Out of 97 respondents, about 69 % (67) of them were from small holder farmers while only 31% of them were from commercial dairy farms. Accordingly, the total number of those who have a read and write educational status was 21 and 90.5% of them were from small holder dairy farmers. This shows those who have low level educational status, particularly small holder dairy farmers could highly exposed for *Brucella* and/or other zoonotic diseases infection than who have high level education status. In addition to this, all respondent who shared common house with their dairy animals were also from smallholder farms. The majority of respondents in the study areas drink raw milk with the highest proportion accounts for small holder farms and this shows the presence of high risk for human *Brucella* infection.

The study showed that respondents' way of contact with aborted, retained fetal membrane and parturition material was important risk point for smallholder farms due to 81% of respondents used their bare hand to manage aborted, retained fetal membrane and parturition material in the study areas. About 72 (74.22%) dairy cattle owners (80.5% of them from small holder farms and 19.5% from commercial dairy farms) have no Knowledge about zoonotic disease and its way of transmission, which was showed the presence of high risk for human *Brucella* infection. The vast majority (87.62%) of dairy cattle owners of the study areas were not aware of the zoonotic brucellosis. Of which, 63 (74.1%) were small holder dairy farmers while 22 (25.9%) were commercial dairy farm owners and animal attendants.

Generally, low level educational status, sharing common house with their animals, drinking of raw milk, and way of contact with aborted and parturition material, lack of knowledge for Zoonotic disease and its way of transmission and lack of awareness about zoonotic Brucellosis were negatively responded parameter by the majority of respondents and showed the presence of high risk for human *Brucella* infection in the study areas.

5.2. Seroprevalence of Brucellosis in Dairy Cattle

5.2.1. *Brucella* Seropositivity in Dairy Cattle

A total of 426 dairy cattle (407 cross and 19 local) were tested with RBPT and 6 (1.4 % with 95%CI: 0.28% - 2.53%) of them were positive for this test. All the 6 RBPT test positive serum samples were further tested using CFT and only two animals were confirmed seropositive for bovine brucellosis in the study areas. Hence, the overall seroprevalence of bovine brucellosis in the study areas was 1.4% (95% CI: 0.28% - 2.53%) and 0.47 % (95% CI: 0.182-1.121) using RBPT and CFT tests respectively at individual animal level and 13% (95% CI: 0.39% -26.48%) and 4.34% (95% CI: 1.77% - 10.47%)

using RBPT and CFT tests respectively at herd level. Paired t-test of RBPT and CFT result showed that p-value = 0.04537, which is statistically that much not significant.

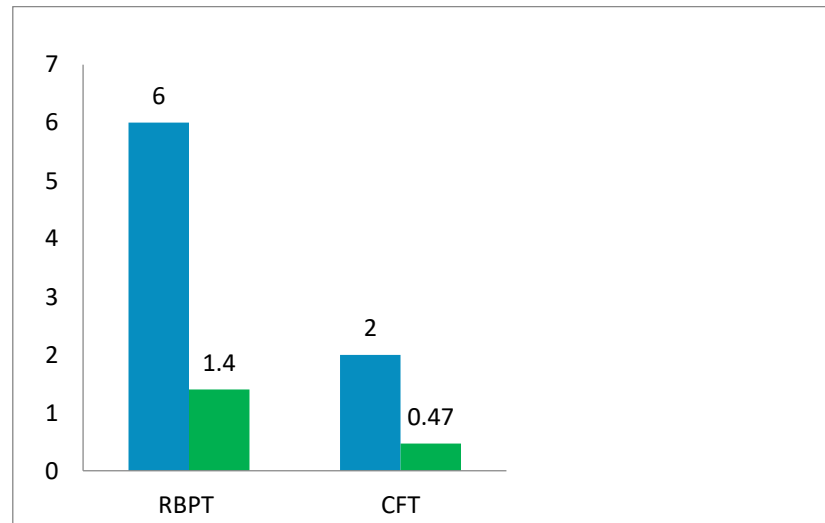


Figure 4 : The overall sero prevalence of dairy cattle brucellosis using RBPT and CFT tests

Based on the CFT test, the animal, herd and Kebelle levels prevalence distribution is recorded as 0.47 % (2/426), 4.34% (2/46) and 28.5% (2/7) respectively. As far as concerning farm level seroprevalence, it was 2.44% (1/41) in small holder dairy farms and 20% (1/5) in commercial dairy farms. The distribution of *Brucella* infection seroprevalence at animal, herd and Kebelle levels of the study areas has been presented by Table 7. The high prevalence of Kebelle level *Brucella* infection is may be due to the clustering effects and this shows that Kebeles would be more appropriate unit for the study.

Table 7: Distribution of *Brucella* Infection Seropositivity in Dairy Cattle of the Study Areas.

Study areas		Individual Animal level		Farm level	
District	Kebelle	Sample	Positive	Sample	Positive
Debrebirhan	1	19	0(0)	4	0(0)
	2	29	0(0)	7	0(0)
	6	104	1(0.96%)	20	1(5%)
	10	33	0(0)	2	0(0)
	Sub total	185	1(0.54%)	35	1(2.85)
Basona Werana	Saria	19	0(0)	5	0(0)
	Angolalla	19	0(0)	4	0(0)
	Sub total	38	0(0)	9	0(0)
Angolalla Tara	Chaki	203	1(0.5%)	2	1(50%)
	Sub total	203	1(0.5%)	2	1(50%)
Over all total		426	2(0.47%)	46	2(4.34%)

5.2.2. Association of risk factors with bovine brucellosis seropositivity

Univariable logistic regression analysis: For the evaluation of the association between risk factors and brucellosis seropositivity, univariable logistic regression analysis was computed. Out of 426 serologically screened dairy cattle, 4 (0.94%) animals from two large scale commercial farms in Angolalla Tara and 2(0.47%) animals from small holder dairy farm of one Kebele in Debrebirhan were positive using RBPT. Of the 6 RBPT positive samples, 2(0.47%) animals (1 from one of the large scale commercial farms of Angolalla Tara (Esayas dairy farm) and 1 from one of the small holder dairy farm of Debrebirhan (Kebele 6) were confirmed as positive using CFT. According to the analysis, there was no statistically significant association between bovine brucellosis and district of animals in the study areas ($P>0.05$) (Table 8).

The seroprevalence of bovine brucellosis in buying stock replacement ($OR = 6.041$, $P=0.0437$), history of RFM ($OR= 17.49$, $P=0.0457$), Primiparous parity ($OR=23.91$, $P=0.028$), abortion history ($OR=19.55$, $P=0.0382$), bull service ($OR=9.26$, $p=0.0186$) and open dump disposal of aborted material ($OR=0.04$, $P=0.02770$) were statistically significant for *Brucella* seropositivity while other risk factors were not statistically significant ($P >0.05$) (Table 8).

The result recorded that positive animals in intensive (0.38%) and extensive production systems (1.03%), while no reactor was recorded in semi-intensive systems. In terms of herd size, lower seroprevalence of brucellosis was observed on large scale farms (0.5%) than small and medium scale farms (0.72%). Positive animal were kept at good hygiene barn small holder farms and poor hygiene of large scale commercial farm.

Table 8: Univariable logistic regression analysis of risk factors associated with bovine brucellosis seropositivity with combined test CFT

Variables	Sampled animals	Positives (in %)	OR/95 % CI	P-Value
Districts				
Angolalla Tara	203	1(0.5)	1.00	
Debre-Birhan	185	1(0.54)	1.1(0.04-2.78)	0.948
Basona Werana	38	0(0.0)	0.00	0.996
Herd Size				
Large Scale farm	203	1(0.5)	1.00	
Medium scale	138	1(0.72)	3,866(0.055 -10.832)	0.804
Small scale	85	0	15.63(0.12 -254.9)	0.996
Age				
Adult	277	1(0.36)	1.00	
Old	49	1(2.04)	15.63(0.22-5.99)	0.219
Young	100	0	3.77(NA -212.24)	0.996
Source of Stock replacement				
Buying	12	1(8.33)	6.041 (0.70-4.90)	0.043*
Own replacement	209	0	0.78(NA-46.76)	0.995
Both	205	1(0.48)	1.00	
Farming System				
Extensive	97	1(1.03)	1.00	
Intensive	266	1(0.38)	8.84(0.014- 25.06)	0.474
Semi-Intensive	63	0	3.234(NA-269.5)	0.996
Hygiene of Farm				
Poor	59	1(1.7)	5.155(0.07-14.19)	0.653
Medium	256	0	3.26(0.57-23.55)	0.995
Good	111	1(0.9)	1.00	
Stage of abortion				
1st trimester	2	1(50)	1.00	
2nd trimester	8	0		0.996
3rd trimester	9	1(11.11)	2.39(0.002-4.79)	0.239
History RFM				
No	351	1(0.28)	1.00	
Yes	21	1(4.76)	17.49(0.67-53.5)	0.045 *
Contact with other Herd				
Yes	81	1(1.23)	4.29(0.16-109.5)	0.304
No	345	1(0.29)	1.00	
Parity				
Pluriparous	288	1(0.35)	0	
Primiparous	11	1(9.1)	23.91(0.9 - 630.04)	0.028 *
Abortion history				
No	353	1(0.28)	1.00	
Yes	19	1(5.26)	19.55(0.75-50.20)	0.038 *
Mating Service				
AI	305	1(0.38)	1.00	
Bull	11	1(9.09)	9.26(1.14-23.94)	0.018 *
Both	110	0	3.44 (NA-215.11)	0.995
Disposal method of aborted material				
Burying	16	1(6.25)	1.00	
Open dump	356	1(0.28)	0.04(0.001 -1.09)	0.027 *

Multivariable logistic regression analysis: Association of predictor variable with bovine brucellosis b seropositivity was also showed by multivariable logistic regression analysis. Using this analysis, buying stock replacement, abortion history and bull mating service were included in the final model. It was recorded that all these risk factors were significantly associated with bovine brucellosis seropositivity (Table 9). Hence, dairy animals with buying stock replacement, having abortion history and bull mating service were 33.83, 5.90, 6.43 times more likely to be seropositive to *Brucella* infection than own replacement, have not abortion history and both services (AI and bull) respectively.

Table 9: Multivariable logistic regression analysis of risk factors associated with bovine brucellosis seropositivity with CFT.

Variables	Sampled animals	No. of Positives (in %)	OR/95 % CI	P-Value
Source of Stock replacement				
Buying replacement	12	1(8.33)	33.83(5.03-83.10)	0.0003 ***
Own replacement	209	0	0.48(0.02-5.13)	0.559
Both replacement	205	1(0.48)	1.00	
Abortion history				
No	353	1(0.28)	1.00	
Yes	19	1(5.26)	5.90(2.29-6.71)	0.005 **
Mating Service				
AI	305	1(0.38)	1.00	
Bull	11	1(9.1)	6.43(1.66-8.69)	0.013 *
Both	110	1	2.40(14.5-58.4)	0.995

6. DISCUSSION

The disease brucellosis, particularly bovine brucellosis, is one of the top five prioritized zoonoses diseases and characterized by exerting high public and economic impact in Ethiopia. Even though several studies have been conducted in intensive dairy cattle of some major cities of the country, little attention has been paid in small holder dairy farms, animal owners and farm workers of Debrebirhan and its surrounding of North Shewa zone of Amhara Region, Ethiopia. Due to this, there is still inadequate epidemiological picture of the disease in the traditional dairy cattle management systems of North Shewa Zone of Amhara Region, Ethiopia. Therefore, availability of reliable and up to date information on the occurrence and distribution of the disease for the effective control was needed.

The study revealed that the seroprevalence of *Brucella* antibodies in dairy cattle of small holder and commercial dairy farms of Debrebirhan town, Basona Werana and Angolalla Tara districts of North Shewa Zone of Amhara Region was 0.47% using CFT test. Since CFT has considered as a confirmatory test for brucellosis (Perrett *et al.*, 2010), the overall seroprevalence of brucellosis in the study areas was 0.47%.

Similar to this study, 0.4 % seroprevalence of bovine brucellosis has been reported from dairy cattle of Sendafa, one of the districts of Oromia Special Zone that surrounds Addis Ababa, Ethiopia (Bifo *et al.*, 2020). In addition to this, the findings of the present study was consistent to the prevalence report of Getahun and his colleagues who were reported 0.6% from central high land of Ethiopia (Getahun *et al.*, 2021). The study was also consistent to Bashitu's research that was reported 0.2% prevalence based on a study conducted in Ambo and Debrebirhan towns of Oromia region and Amhara Region, Ethiopia (Bashitu, 2015).

Other studies that were found in close agreement with this study result are ELISA based 0.8% herd level result in Addis Ababa dairy cattle (Edao *et al.*, 2018), 0.3 % CFT result of a study conducted in selected districts of Jimma Zone (Dirar *et al.*, 2015), 0.2% of CFT based result in selected districts of Benadir Region of Somalia (Keten, 2017), 0.9% of CFT result from south east Ethiopia pastoral areas (Gumi, 2013) 0.77% CFT based result in Jimma Zone (Tolosa *et al.*, 2008), 1.04% in Adama-Oromia (Bulcha *et al.*, 2020), 0.49% in West Shewa (Union & Africaine, 2011) and 1.04% I-ELISA based result in Becho District of South West Shewa Zone of Oromia Regional State, Ethiopia (Tadese *et al.*, 2019) within the country and 0.26% in small-holder crop livestock production system of Kenya

(Fèvre *et al.*, 2017), 0.44% in goat, 0.79% in cattle and 0.13 in buffalo in Egypt (Hegazy *et al.*, 2011) and 0.32% in 2002 and 0.91% in 2012 in Brazil (Baumgartem *et al.*, 2016) from abroad.

On the other hand, higher seroprevalence findings than this study were reported from different parts of the country by several scholars. Some of these are 5.7% in Afar (Negash & Dubie, 2021), 7.2% and 6.9% in Afar and Somali respectively (Tschopp *et al.*, 2021), 2.4% in Borena- Oromia (Edao *et al.*, 2020), 2.7% in Awassa (Abera *et al.*, 2019), 9.7 % in Nechisar National Park (Chaka *et al.*, 2018), 1.3% in east Ethiopia (Terefe *et al.*, 2017), 1.4% in Asela and Bishoftu (Geresu *et al.*, 2016), 3.3% in West Arsi (Asgedom *et al.*, 2016), 1.7% in Oromia (Tschopp *et al.*, 2013), 1.9% at farm level (Asmare *et al.*, 2013), 1.38% in Jigjiga (Degefu *et al.*, 2011), 4.7% in Borena Zone (Megersa *et al.*, 2011), 1.66% in Sidama (Amenu *et al.*, 2010) and 1.2% in Jigjiga (Mekonnen *et al.*, 2010). In addition to this, relatively higher bovine seroprevalence records were reported from other countries as 8.2% in Eritrea (Omer *et al.*, 2000), 3.4% in Kenya (Ogola *et al.*, 2014), 31% in South Sudan (Madut *et al.*, 2018), 9.5% in Somalia Republic (Hassan-Kadle, 2015), 3.5% in Nigeria (Ayoola *et al.*, 2017), 24.9% in Sudan (Angara *et al.*, 2004), 17.22% in Egypt (Laskin, 1976), 10.1% in Jordan (Majali *et al.*, 2009), 1.5% in Saudi Arabia (Radwan *et al.*, 1983), 1.9% in India (Isloor *et al.*, 1998) and 2.8% in Tibet, China (Zeng *et al.*, 2017). The observed difference in the seroprevalence might be due to the difference in management and husbandry system, age and sex of animals, sample size, parity of the animals and herd size among the dairy farms. It has been known that susceptibility of cattle to *B.abortus* infection is influenced by age of an individual animal (Radostits *et al.*, 2000).

In this study, only 2 (0.47%) sero-reactor animals to *Brucella* antibody were found in the cattle population of Debrebirhan town and its around. The reason for the low prevalence of bovine brucellosis in the study area could possibly be due to better farm hygiene practice, low contact between herds, low proximity between animals and herd size and less interaction between animals in grazing and watering system. The confirmed cases were one from extensively managed small holder farm of Debrebirhan town and one from intensively managed commercial dairy farm of Angolalla Tara District. According to the individual animal history, one of the sero-reactor animal is from commercial dairy farm of Angolalla Tara District and was purchased from outside North Shewa Zone (from Shashemene district of Oromia region) and had third trimester abortion and retained fetal membrane history. The other sero-reactor animal from extensively managed Debrebirhan town small holder dairy farmer had first trimester abortion history and herd stock replacement history of both buying and own

replacement. This situation clearly proves that the considering of bovine brucellosis as the world's most common bacterial zoonosis and being one of the top five prioritized zoonotic diseases in Ethiopia.

Even though the standing hypotheses assumed brucellosis highly endemic in dairy cattle of study areas, it was estimated to be very low. In this study, the seropositivity for *Brucella* species was found in a low percentage by CFT test, risk factors like buying stock replacement, abortion history and bull mating service were significantly present according to the farm characteristics analysis. The study also assessed different risk factors that associated with the occurrence of the disease in the study areas. Buying stock replacement had significantly associated with the seropositivity of bovine brucellosis in the study area. This was in agreement with previous study of Edao *et al*, 2020. This could be the presence of *Brucella* infected foci around the cows' original area like her herd and/or the surrounding. Other possible explanation might be the animal had get contact with infected animals during transportation to the new stock. The other risk factor which significantly associated with bovine *Brucella* seropositivity in the study area was abortion history. There was statistically significant association ($p < 0.05$) between third trimester abortion and seropositivity of *Brucella* in the present study. This could be due to the higher prevalence in cow and because of the high number of brucella in the uterus in which allantioic fluid and erythritol increase at this time which could stimulate the growth and multiplication of the organism (Radostits *et al*, 2000). This association result was in agreement with findings of other authors (Geresu *et al.*, 2016), Abera *et al.*, 2019; Getahun *et al.*, 2021).

In the present study, there was also statistically significant association ($p < 0.05$) between mating or breeding service and seropositivity of *Brucella*. There was a prevalence record both in AI and bull service. But, the prevalence rate was higher ($p = 0.013 *$) in bull service. This might be due to the frequent contact of the bull to different cows in the herd. Even though not confirmed in the multi variable model analysis, RFM was statistically associated ($p = 0.0457 *$) with the seropositivity. Retention of placenta and inflammation of the wall of the uterus and/or metritis are common sequelae to abortion because of brucellosis. *Brucella* infected cows were expected to abort 3 to 4 times more than unexposed cows (Aparicio, 2013). It could be explained due to retained fetal membrane is typical outcome of brucellosis. Studies showed significant association between *Brucella* sero positivity and retained fetal membrane (Tolosa *et al.*, 2008). Primiparous parity was found at low level as risk factors for *Brucella* seropositivity in the study areas. This may be possible due to the reproductive maturity of the animal in which the allantioic fluid and erythritol are increased which in turn could increase the chance of being infected by *Brucella* organism. An other explanation could be due to the fact that the

cows had get a great chance of being exposed to carrier animals in other herds followed by infected with *Brucella*.

There was no significant association ($p>0.05$) between herd size and *Brucella* seropositivity in the study area, though higher prevalence was recorded in large herd size. Possible explanation might be the majority of farms were characterized by small herd size which possibly decreases stocking density, one of the determinant factors for exposure to *Brucella* infection, particularly following abortion and parturition. Based on this study, there was no statistically significance difference among age group to *Brucella* seropositivity. The ages of positive cows were found one as an adult and the other as old, whereas no confirmed *Brucella* seropositivity was observed in young age group in the study areas. Other similar findings to this result were reported by different scholars (Geresu *et al.*, 2016; Getahun *et al.*, 2021). The prevalence of brucellosis among adult and old cows might be because of the higher number of *Brucella* in adult cows and the susceptibility of old cow. In addition to this, it may be related to the advanced age as the organism remains latent or chronic for unspecified period of time before clinical manifestation of the disease.

As compare to the overall percentage of seropositivity, the finding of this study reveals that the presence of prevalence in intensive farms, though the finding was not statistically significant ($p>0.05$), could be explained by the fact that due to poor hygienic management system of the farms. The presence of prevalence in extensive farming also could be explained by the fact that due to poor management system of the extensive farm and due to communal grazing practice which is the main feeding system in extensive of management. Under such condition, cattle of unknown disease status might be mingle and often graze together followed by the spreading and transmission of disease among free herds. About 89.13% of farms in the study area are small holder farms and this shows the free grazing system that allows unrestricted contact between animals which have great contribution to the spread of brucellosis in extensive farming systems (Langoni *et al.*, 2000). Brucellosis is labeled as a disease of poor hygiene condition that can be resulted in exposure animals to aborted fetus, placentas, vaginal discharge or to new born from the infected cows. Similarly, the use of maternal pen at calving is proved to be an association with a decrease in prevalence infection, possibly due to decreasing exposure of infected and susceptible animals (Radostits *et al*, 2000)

In the study area, all positives are female animals. This might be due to the fact that female animals can maintained in the herd for over long period of time which permits ample time for exposure to the pathogen *Brucella* and being the source of infection for other animals Therefore, practicing culling of

cow with positive *Brucella* reactors, reduced reproductive performance and old age is mandatory which might reduce the risk of spreading zoonotic brucellosis in animals and humans.

In Ethiopia, despite the wide spread of brucellosis in animals and very exposure for humans, published informations regarding human brucellosis are very small. It is expected that people would get infection with brucellosis in an area where there are infected animals with brucellosis, since the seroprevalence of brucellosis in human is largely influenced by the seroprevalence of the disease in animals (Omer *et al.*, 2000). Hence, it is possible to say that there might be human infection with *Brucella* species in the study areas. Even though it lacks testing of the serological picture of brucellosis in human due to the high risk of COVID-19 pandemic transmission in the study area, this study was critically assessed on KAPs of respondents. Accordingly, a total of 97 respondents (59 from Debrebirhan, 32 from Basona Werana and 6 from Angolella Tara) were interviewed to assess their knowledge and/or awareness level regarding brucellosis, occupational risks and dairy cattle management risks using structured interview question. Based on their response to my interview, parameters like educational status, human housing relating to animals, drinking of raw milk, way of contact with aborted and parturition material, hand washing after aborted and parturition material contact, knowledge of zoonotic disease and its way of transmission and knowledge of the disease brucellosis were assessed.

The overall awareness level of the disease brucellosis and general knowledge of zoonotic diseases and their way of transmission in the study area is very low both in small holder farmers and commercial dairy farm owners and animal attendants, based on the interviewee response. Most of the respondents (87.6%) were not wear protective glove while handling aborted animal and aborted, fetal membrane and parturition materials, which means they were handled with their hand and other materials (plastics, shovels, woods). Other studies in agreement with this study were reported (Getahun *et al.*, 2021). This might be because of their low awareness level since most of respondents (74.22%) responded to the interviewer as they have not knowledge about brucellosis and other zoonotic diseases and their way of transmission. Lack of knowledge about the disease brucellosis and other zoonotic diseases, regular exposure to infected materials (aborted, carcass, visera, blood and urine) through aerosol and contact are considered as fertile ground for the transmission of diseases in human. Regarding the issue, very little has been done about awareness creation of brucellosis in the community, not only in the study area but also as a whole in Ethiopia.

The knowledge, attitude and practice assessment of this study revealed that brucellosis was not well known by the vast majority of the community, since 87.63% of respondents in the study area had no knowledge about brucellosis. This explanation is very similar to the findings of previous study done by a researcher which was reported 93.3% of the study area respondents had not information about brucellosis (Getahun *et al.*, 2021). Regarding the knowledge of zoonotic diseases and their way of transmission, 86.56% small holder farmers have not awareness. Farmers lack of awareness about brucellosis, improper handling of aborted materials and habit of consuming unpasteurized milk might be contribute for further transmission of the disease in their livestock and expose the community to public health hazard (Megersa *et al.*, 2011). In addition to this, low awareness about the disease, lack of knowledge on causative agent, mode of transmission and preventive measures are major limiting factors for the implementation of control and prevention strategies of the disease. Therefore, it is so important to launch an awareness campaign not only in the study areas but also through out the country (Ethiopia) in order to enlighten the community about the disease and its risk factors including the national prevention and control strategy which can be resulted in the actual implementation of the 2030 elimination goal.

The use of personal protective equipments is the most important measure against brucellosis (Radostits *et al.*, 2000). About 100% of respondents have contact with aborted, retained fetal membrane and parturition materials and only 15.46 % of them use glove. 28% the interviewees in the were handled with out using personal protective equipments. From all respondents, 36% of them were not practiced hand washing with soap after contact of aborted, retained fetal membrane and parturition materials. This two factors could possibly pose a great risk for the spread of brucellosis to non infected animals, in which the explanation is in agreement with previous studies (Tolosa *et al.*, 2008). In addition to this, the majority of interviewees (78.35%) in the study area were not practiced the burying method of aborted, retained fetal membrane and parturition materials. They dispose simply to open dump. In terms of milk drinking status, the majority of the respondents (63%) in all farms have the traditional habit of raw milk consuming. This condition can be contribute for the high transmission of zoonotic brucellosis in the study area.

Limitation of this Study

Testing of the seroprevalence of zoonotic brucellosis suggests both in animal and human simultaneously. However, due to the high risk of COVID-19 pandemic transmission in the study area, it was not possible to include the human blood sample in the study area

7. CONCLUSION AND RECOMMENDATIONS

This study revealed that the overall seroprevalence of bovine brucellosis was found to be very low (0.47%) in Deberbirhan and its surrounding of Noth Shewa Zone, Amhara Regional State, Ethiopia. The presence of positive serological reactors indicate previous infection of brucellosis in the cattle population of study areas. More over, the presence of one or more positive reactors in the herd is a likely risk of infection. In this study, buying stock replacement, last trimester abortion history and bull mating service were statistically significant risk factors which was associated with bovine brucellosis seropositivity in the study area. On the other hand, the overall awareness level of brucellosis and general knowledge of zoonotic diseases and their way of transmission in the study area was very poor both in small holder and commercial dairy farm owners and showed very little has been done about awareness creation of brucellosis in the community. Accordingly, human housing relating to animals, boiling of milk and way of contact with aborted and parturition materials were poorly practiced by the respondents. In addition to this, lack of knowledge about zoonotic disease and its way of transmission and specifically about zoonotic brucellosis were very low. Based on the above conclusion, the following recommendations are forwarded.

- ✓ Culling of *Brucella* antibody reactor cows in the study farms is recommended
- ✓ Stock replacement should be carried out through *brucella* testing before introduction in to the farms
- ✓ Using artificial insemination mating service is advisable rather than bull service method
- ✓ Improving of community awareness on brucellosis and other zoonotic disease, particularly on farmers and animal attendants is needed.
- ✓ An elimination campaign of the disease based on One Health approach is required.
- ✓ Further studies on isolation and molecular characterization of the causative agent in livestock and epidemiological study of human brucellosis in exposed risk groups including farmers should be carried.

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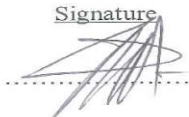
9. ANNEXES

Annex 1 Approval page for Research Proposal

Seroprevalence and Associated Risk Factors of Bovine Brucellosis in Small Holder Dairy Farms and its Public Health Significance in North Shewa Zone of Amhara Region, Ethiopia

MSc Thesis Research Proposal Submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University for the Course 'MVSc Research Proposal'-7272.

I. Submitted by:

Name	Signature	Date
1. Gashaw Adane		Nov. 2021

II. Advisors

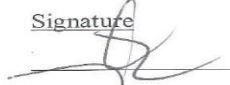
A. Major Advisor

Name	Signature	Date
1. Professor Gezahegne Mamo		Nov. 2021

B. Co- Advisors

Name	Signature	Date
1. Dr. Fikru Regassa		Nov. 2021
2. Mr. Teferi Benti		02/2021

III. Evaluator:

Name	Signature	Date
1. Dr. Bedaso Mamo Edao		Nov. 2021

IV. Final approval by principal advisor & Department chairperson

1. Professor Gezahegne Mamo		Nov. 2021
Name	Signature	Date

Annex 2: Certificate of Ethical Clearance

አዲስ አበባ ዩኒቨርሲቲ
የእንስሳት ሕክምናና
ግብርና ኮሌጅ
ቢሽፋቱ



ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/03/01/14/2022

Name of Applicant: **Gashaw Adane (BVSc, MSc fellow)**

Address: Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Seroprevalence, associated risk factors and public health significance of bovine brucellosis in dairy farms of Debre Birhan Town and its rounding in North Shewa Zone of Amhara Regional State, Ethiopia*

Date of application: **November, 2021**
Nature of the project: **Mildly invasive (little stress)**
Target animal species: **Dairy cattle**
Number of animals involved: **426**
Study area: **Debre Birhan, Ethiopia**

Minutes No. and date of review: **VM/ERC/01/14/022, 22/02/2022**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is ethically sound to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected, minor comments are corrected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee when deemed necessary
3. Separate clearance is required from relevant authorized body for works involving human subjects

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

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Please quote Our Ref. No. When replying

ፋክስ
Fax 251-11-4339933

ስልክ
Tel. +251 114338450

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College of Veterinary Medicine
Bishoftu, Ethiopia

Annex 3: Consent Letters from Large Farms

	Company Name: ምዳሌ አግሮ ኢንዱስትሪ ጋ. የተ. የግ. ማህበር MISALE AGRO-IND 0936554750 Tin No. 0045955294	Document N° OF/MAIF/ ቀን: _____
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ሰአቶ ጋሻው እርቅይሁን

ባለቤት

ጉዳዩ፡- ወደ ፋርማችን በመግባት አስፈላጊውን የጥናት መረጃና ናሙና ለመሰብሰብ እንዲችሉ የተፈቀደለዎት ስለመሆኑ

እርስዎ በአዲስ አበባ ዩኒቨርሲቲ እንስሳት ህክምና እና ግብርናኮሌጅ የሁለተኛ ዲግሪዎን የንድፈ- ሃሳብ ትምህርት አጠናቅቀው የመመረቁ ጽሁፍ "Seroprevalence, Associated Risk Factors and Public Health Significance of Bovine Brucellosis in Dairy Farms of North Shewa zone of Amhara Regional State, Ethiopia" በሚል ርዕስ ለመስራት በዩኒቨርሲቲው ጸድቆ ፈቃድ እንደተሰጠዎት እና ጉዳዩን ለሰሜን ሸዋ ዞን እና ለአንጎላላ ጠራ ወረዳ አመልክተው ፋርማችን በዚህ ጥናት እንዲደግፍ ወደ እኛ የተመሩ መሆኑን ገልፀው ወደ ፋርማችን በመግባት አስፈላጊውን መረጃና ናሙና ለመሰብሰብ ይችሉ ዘንድ እንዲፈቀድለዎት እና ትብብር እንዲደረግልኝ በማለት በ17/6/2014 ዓ.ም በተጻፈ ማመልከቻ ጠይቀዋል፡፡

በመሆኑም የምርምር ሥራው ለፋርማችንና ለአካባቢው ህብረተሰብ ያለውን ጠቀሜታ በመረዳት ወደ ፋርማችን በመግባት ከፋርሙ ማህበረሰብና የወተት ላሞች አስፈላጊውን የጥናት መረጃ እንዲሰበስቡ የተፈቀደለዎት መሆኑን እየገለጽን የጥናቱን ውጤትም ለፋርሙ /ለድርጅቱ/ እንዲያሳውቁ እናሳስባለን፡፡

"ከሰላምታ ጋር"



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Annex 4: Questionnaire Survey Format

Questionnaire format prepared to assess Knowledge, Attitude and Practice (KAP) of Smallholder and Commercial dairy farmers and workers towards Brucellosis in Debrebirhan and its Rounding

Dear participant:

This survey is an investigation of risk factors precipitating the transmission of bovine brucellosis to humans and assessment of knowledge, practice and attitude of smallholder dairy farmers and dairy farm workers towards bovine brucellosis. The result of this study will help human and animal health professionals and government institutions in deciding control strategies.

The questionnaire will be administered by the principal investigator and your responses will be kept confidential. You will in no way be personally linked to any of the results of the survey. There is no risk to you from participating in this questionnaire, and there is no anticipated direct benefit. Thank you for your participation!

I. Demographic Characteristics of Respondents

1. Owners Name-----Sex-----
2. Age : < 30 years..... 31-45..... 46-60..... >60 years
3. Address: District Kebele/Village.....
4. Marital Status: Married.....Single...../
5. Educational status:
Illiteracy.....Readandwrite/informal....Primaryschool.....College.....University.....
6. Are you practicing dairy as the only way of life? Yes----- No-----

II. KAP of respondents regarding brucellosis

1. Is there have been conducted disease research here around? Yes.....No.....
2. Do you and your family drink milk? Yes.....No..... If yes,
 - Is the milk boiled before drink?
 - Are you drinking without boiling?
3. Have you ever here about the disease caused because of unpasteurized milk?.....
4. Do you know the disease brucellosis?....If yes, what is the name in locality?.....
5. Family: Chronic headache.....knee pain..... Testicular swelling abortion.....
6. Do you know what ZD means and their way of transmission?
 - NO.....Yes..... If yes, what you mention?
7. Do you know the transmission of ZD by improper handling of disease infected animal products?
Yes.....No.....
8. Do you separate cows during parturition? Yes.....No.....

9. How do you dispose of the afterbirth?
 - Burning Burying dispose.....Open to field /thrown to the environment (open dump).....
10. Was your cow/bull is sick by reproductive problems? Yes.....No.....
 - If yes, explain.....
11. Have you observed abortion/still birth in your cow? Yes..... No.....
 - If yes, when did abortion occur? A, First trimester. B, Second trimester. C, Third trimester
12. How many abortions/still births or retained after birth have you encountered during the last 5 years? ----
-----abortion----- b. still birth----- c. retained fetal membrane-----
13. Your housing condition with dairy animal: Common.....Separate housing
14. How do you dispose of the afterbirth? Burning Burial disposes to open field /thrown to the environment
15. You contact with aborted materials? Yes.....NO..... If yes, Bare hand.....wear glove.....others..... Hand washing after contact: Yes..... No.....
16. What do you do with the known disease /*Brucella* infected animals?
 - a. Culling b. Slaughtering. C. Take to clinic

III. Herd Information

- 1) Type of farming: Intensive.....Semi-intensive..... Extensive.....
- 2) Herd size: Large scale ----- Medium scale.....small holder.....
- 3) Breed of the dairy cattle: Local Cross
- 4) Number of milking cows -----
- 5) Age: young.....Adult.....Old.....
- 6) From where you stock the cow? Buy.....Raise own replacement.....Both.....
- 7) What type of service do you have for your animals? AI..... Bull/natural mating.....Both.....
- 8) Hygiene of Barn: poor.....medium.....good.....
- 9) Is there a frequent contact between your animals and other herds? Yes.....No.....

Annex 5: Reagents, Materials and Procedures of Serological Tests

1. Rose Bengal Plate Test

The diagnostic kit which was used in the screening of the samples was Cenogenics Rose Bengal *Brucella* antigen, prepared by Cenogenics Corporation (Registered to ISO 13485, Morganville, NJ 07751, USA). Cenogenics Rose Bengal *Brucella* antigen is a bacterial suspension for use in slide agglutination test to detect the presence of bacterial agglutinins associated with *Brucella* infections or previous exposure.

Principles: When viable bacteria are introduced in to a susceptible host, an immune response generally occurs. This immune response results in the production of antibodies called agglutinin. The principle of the test is an immunological reaction (agglutination) between the antibodies produced to the viable bacteria (agglutinins) and the corresponding bacterial antigen.

Reagents:

- ✓ RBT *Brucella* antigen
- ✓ Positive control sera (from previously positive serum)
- ✓ Negative control sera (from previously negative serum)
- ✓ Test sera

Materials:

- ✓ Plain vacutainer tubes, needles and holders
- ✓ Cryovials serum collection and racks
- ✓ Vortex mixer
- ✓ Micro pipette of 30 µl and micro pipette tips for dispensing
- ✓ Plate and Mixing Sticks
- ✓ Rocker machine

Procedures:

- All reagents (Controls, test sera and antigen) were left at room temperature for half an hour before testing and the serum was shaken with vortex mixer in order to crate uniformity and/or homogenize it.
- Then 30µl of serum was mixed with 30 µl of antigen on a white enamel plate circle to produce a zone of agglutination (2cm diameter)

- The antigen and serum were mixed thoroughly using an applicator stick (a stick being used only once).
- Rock the plate by rocker machine for 4 minutes
- Observed (Examined) under bright light for the presence of agglutination.

Result:

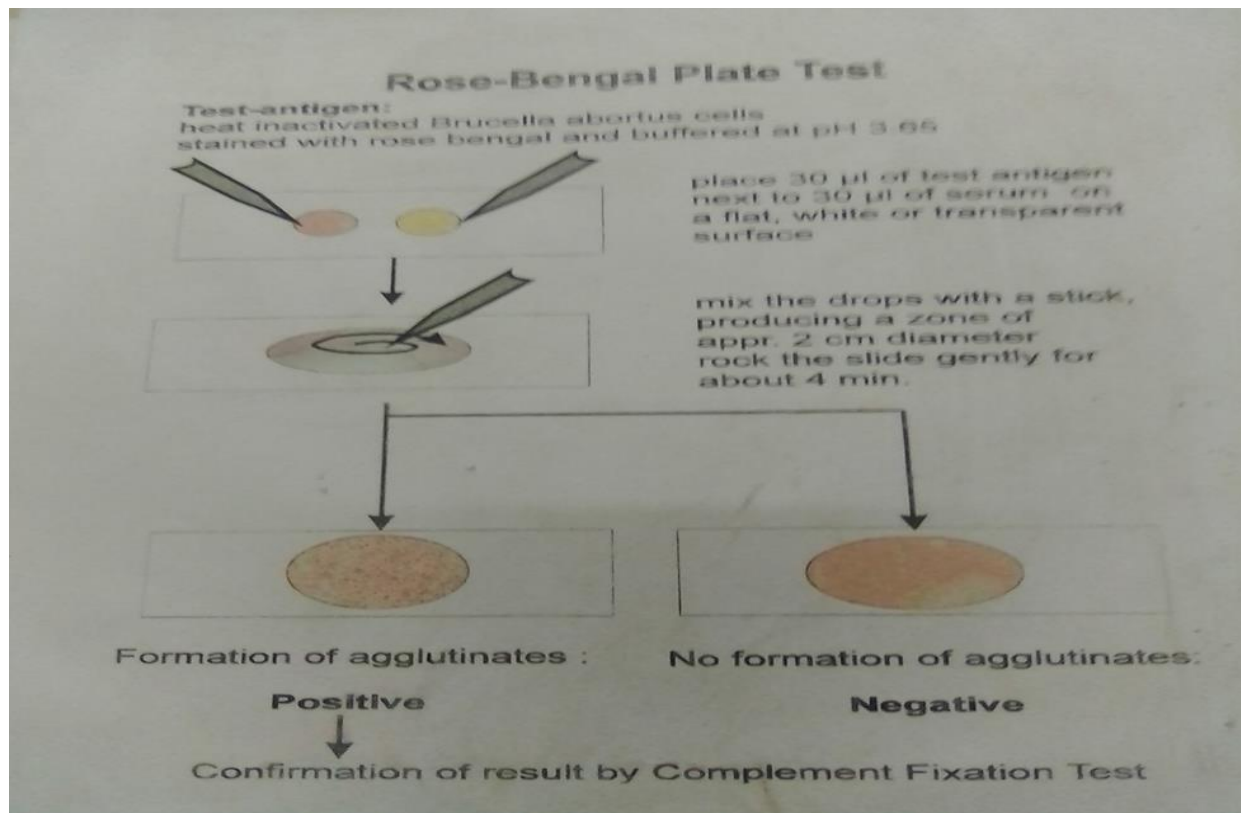
A **positive** result is indicated by agglutination (clumping) of the bacterial suspension.

A **negative** result indicated by absence of agglutination (clumping).

Interpretation:

- 0 = no agglutination
- + = barely perceptible
- ++ = fine agglutination, some clearing
- +++ = coarse clumping, definite clearing

General Principle of RBPT



Photos taken while screening the samples with RBPT



2. Complement Fixation Test Procedure

CFT is a test in which a sample of serum is exposed to a particular antigen and complement in order to determine whether or not antibodies are present to that particular antigen or serum. The nature of complement is to react in combination with antigen-antibody complex. The test mainly measures IgG antibodies. CFT has two components.

1. The complementary antigen and protein - test serum and complement obtained from Guinea pig are mixed and incubated.
2. Indicator system (hemolytic system) - which consists of 3% sheep red blood cells (SRBC) and an amboceptor (hemolysine serum) which binds to erythrocyte and sensitizes red blood cells to the action of the complements.

Reagents:

- ✓ **Veronal buffered diluent** (prepared by mixing 1 vial of the constituents into 1 liter of distilled water (PH 7.25). Veronal buffered diluent is used as a stabilizer
- ✓ **Working strength antigen and freeze dried Complement** (used to increase or improve the quality). The complement is a powder which can be diluted by 5 ml distilled water.
- ✓ **Amboceptor** and Alsever's solution. An amboceptor is an antibody which lyses an antigen in combination with complement.
- ✓ **3 % Sheep RBC (50%)** -
- ✓ **Positive and negative control sera** (from previously tested serum)

Materials:

- ✓ U-Shaped micro titer plate
- ✓ Shaker housed in an incubator (37 °C)
- ✓ 0-100 µl adjustable single channel pipette
- ✓ 25 µl multi-channel pipette and 1000 µl adjustable single channel pipette
- ✓ Pipette tips and plastic troughs and traditional glass pipette
- ✓ Centrifuge tubes, measuring cylinders, glass and plastic beakers
- ✓ Water bath (58 °C) , centrifuge, +4 refrigerator and racks

Preparation of working Solution**1. Preparation of SRBC for hemolytic system**

- ✓ Take the SRBC before 72 hours of CFT test (from *Brucella* infection free).
- ✓ Add equal volume of SRBC and Alsever's solution (10 ml of SRBC + 10 ml of Alsever's solution) and mix gently. Then, label and incubate 4°C refrigerator for 72 hours. The use of Alsever's solution is to preserve SRBC.
- ✓ Then, the supernatant was discarded or washed 3 times with veronal buffer diluents until clear supernatant observed.
- ✓ The packed cells volume was measured after the last washing (before discarding the supernatant).
- ✓ The volume of the packed cells was read by placing an identical tube in to the reader
- ✓ Finally, a 3% suspension of SRBC was prepared.

2. Amboceptor titration /hemolytic system

It is prepared by mixing amboceptor working solution of 0.5 ml with 3% SRBC

- ✓ Two rows of 5 test tubes were arranged on a rack
- ✓ In other test tube, 1: 700 (1 ml of hemolytic serum + 699 ml veronal solution were pre-diluted.
- ✓ 1ml of 1: 700 pre-diluted amboceptor was transferred to the test tube of rows
- ✓ 0.5ml VBD was added to each of the rest of tubes of both rows
- ✓ Amboceptor was then diluted serially in 0.5 ml amount in all rows.
- ✓ Then, to each tube of the rows, 1ml of VBD was added
- ✓ Then, 0.5 ml of 3 % SRBC was added to each test tubes and were shaken well
- ✓ The tube were left on the table for 10 minutes
- ✓ 1 ml of complement at working dilution was added and incubated at 37 °C for 30 minutes.
- ✓ The tube showing complete hemolysis, minimum hemolytic dose (MHD) was read.

3. Evaluation of the complement

- ✓ Freeze dried complement was reconstituted
- ✓ 1: 100 complement dilution was prepared
- ✓ Complement was added into 9 wells increasing by 5µl every time, starting with 10µl
- ✓ Diluent was added into the 9 wells in decreasing amounts by 5 µl , starting with 40 µl
- ✓ 25µl of a diluent was added into the wells with Cornwall syringe
- ✓ The plate was placed in water bath at 37°C for 1 hour
- ✓ 25µl 3 % SRBC was added to all wells

- ✓ 25µl amboceptor at working dilution 1:1000 was added to wells
- ✓ Components were mixed by shaking and incubated again in water bath at 58 °C for 30 minutes.

The test was read by recording the minimum hemolytic dose of complement (MHD) the first well showing complete hemolysis. The next well contains the full hemolytic dose (FHD). The working dilution of complement was then computed: complement dilution= 2FHD/ initial dilution of complement.

4. Antigen titration

Micro titer plate I:

- ✓ First, 25µl of VBD was placed to every wells of a micro titer plate
- ✓ 25µl of a pre diluted antigen was added to all wells of row A
- ✓ By serial doubling (two fold) dilution 25µl of antigen was transferred from row A to B and from row B to C until row G by multi-channel pipette, then 25µl mixture was discarded from row G

Micro titer plate II

- ✓ 50µl VBD was added in all wells
- ✓ 50µl of pre-diluted inactivated positive control serum was added to all wells of column 1
- ✓ 50µl was serially transferred by two fold dilution, from column 1 to column 2, and from column 2 to column 3, until column 11 from where 25 µl was discarded (column 12 had only VBD)

Mixing plate I and II

- ✓ 25µl was transferred from plate II to plate I
- ✓ 25µl of complement in at working dilution (1:40) was added to all wells of plat I
- ✓ Plate I was incubated at 37 °C for 30 minutes (sealed)(warm fixation)
- ✓ Then, 25µl of equal volumes of pre-mixed 3% SRBC and amboceptor (working dilution) were added to all wells.
- ✓ The plates were covered with sealing tape and shacked placed in an incubator (37° C) for 30 minutes (warm fixation)

Interpretation:

The last wells with 50% sedimentation was read and recorded which was regarded as the right corner value. In this case, the corner value was 1: 25 dilution and was used throughout the test. The 50% sedimentation was taken as one unit and the working dilution of the antigen was two units.

Test Procedures

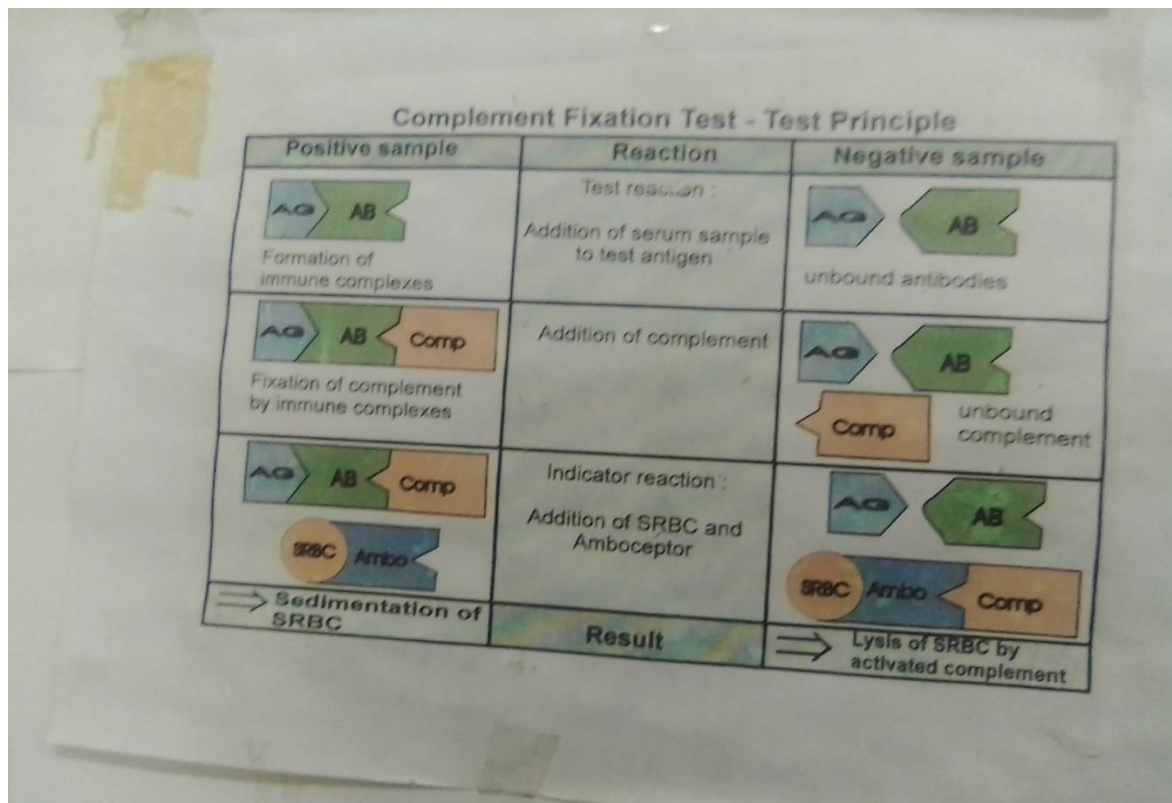
- Sera were pre diluted at 1:2.5 and incubated at 58° C in a water bath for 30 minutes in order to inactivate the native complement (to de-complement)
- 25µl of diluted test sera was placed in wells of first and second rows of U-bottom plate, and 25µl of veronal buffer was added to all wells except those of the first row
- Serial doubling dilution were then made by transferring 25µl volumes of serum from 2nd row on wards continuing for at least four dilution
- 25µl of antigen diluted to working dilution excluding those of anti-complementary controls, which received 25µl VBD was added to all wells
- 25µl of complement (1: 40 working dilution) in working dilution was added to all wells except control wells

- **Control wells containing:** the serum control has serum + complement + diluent, and the antigen control has antigen + complement + diluent. The complement control has complement + diluent, and the hemolytic system has diluent set up to contain 75µl total volume in each case before hemolytic system was added
- The plates were incubated for 30 minutes at 37⁰ C with agitations (warm fixation)
- 25µl of 3 % SRBC and amboceptor (hemolytic system) mixture was added into all the wells
- Plates were sealed with sealing tape and placed on a shaker and incubator (37⁰ C) for 30 minutes
- Before reading the result, the plates were left in the refrigerator at +4⁰C for one hour in order to allow non- lysed cells to settle
- Plates were taken out from refrigerator and results were read after being left on the table for 10 minutes at room temperature
- Positive reactions were indicated by the absence of hemolysis, sedimentation of SRBC while negative reactions was by the hemolysis of SRBC

Interpretation:

Sera with at least 50% fixation of the complement at a dilution of 1:10 were taken as positive. A hemolytic reaction of 50% or less at a dilution of 1:5 was considered as the minimum sero-positive threshold (Dohoo *et al.*, 1985).

General Principle of CFT



Photos taken while screening the samples with CFT



Annex 6: RBPT and CFT Test Results



AHI



TEST RESULT REPORT FORM

Customer name: Gashaw Adane

Reference No. SR/RE/79/2022

Contact: phone : 0914067704, email : gashawdane@gmail.com

Test Report No. 79/2022

Sampling Place: Region: Amhara, District: Basona warena & Debre Birhan, Kebele: 01, 02, 06, 10, saria & Angolela

Sampling type: Purposive

Date reported: 06-04-2022

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed	Owner
6722-6944	SERUM	Bovine	14/01/2022	04/02/2022	30/03/2022	Gashaw Adane
					30/03/2022	

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	223	2	221	0	Brucella: Ab detection	Rose Bengal Plate Test (RBPT)	AHI-TM-SRL-006

*Accredited Test (AHI is accredited for tests marked *)

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed	Owner
6740-6774	SERUM	Bovine	14/01/2022	04/02/2022	30/03/2022	Gashaw Adane
					30/03/2022	

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	2	1	1	0	Brucella: Ab detection	Complement Fixation Test (CFT)	AHI-TM-SRL-006

*Accredited Test (AHI is accredited for tests marked *)

Opinions and Interpretations

out of 223 sera sample tested for brucella antibody by RBPT 2 samples were positive and the 2 positive sample were retested by CFT and from 2 samples 1 samples was positive for CFT test

Verified by:
Getachew Tuli

Approved by:

Dr. Getnet Abie Mekonnen
Animal Disease Diagnostic &
Study Core Process
Coordinator

Page 1 / 7

NOTE: the results shown on this test report only refers the number of samples tested and the respective customer not for other purpose. NAHDIC is responsible for all the information provided on this report, except for the information provided by the customer.

Effective Date: 02/10/2019	Document Type: FRM	Approved by: Abera Kabede
PO BOX 04: Sebeta Town, Ethiopia	+251113380894/95/96/97	Document Number: NAHDIC-FRM-GEN-025-7A
		Fax: +251113380220 Website: http://www.nahdic.gov.et/



AHI



TEST RESULT REPORT FORM

Customer name: Gashaw Adane

Reference No. SR/RE/117/2022

Contact: phone : , email :

Test Report No. 117/2022

Sampling Place: Region: Amhara, District: Angolelana Tera, Kebele: Chaki

Date reported: 06-04-2022

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed	Owner
8239-8353	SERUM	Bovine	24/02/2022	07/03/2022	22/03/2022	Gashaw Adane
					22/03/2022	

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	115	2	113	0	Brucella: Ab detection	Rose Bengal Plate Test (RBPT)	AHI-TM-SRL-006

*Accredited Test (AHI is accredited for tests marked *)

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed	Owner
8251-8254	SERUM	Bovine	24/02/2022	07/03/2022	22/03/2022	Gashaw Adane
					22/03/2022	

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	2	0	2	0	Brucella: Ab detection	Complement Fixation Test (CFT)	AHI-TM-SRL-006

*Accredited Test (AHI is accredited for tests marked *)

Opinions and Interpretations

Out of 115 sera sample tested for brucella antibody by RBT 2 samples were positive and the 2 positive sample were retested by CFT while the 2 samples were negative for CFT

Verified by:

Getachew Tuli

Approved by:

Dr. Getnet Abie Mekonnen
Animal Disease Diagnostic &
Study Core Process
Coordinator

NAHDIC Original Document



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Effective Date: 02/10/2019	Document Type: FRM	Approved by: Abera Kabebe
PO BOX 04: Sebeta Town, Ethiopia	+251113380894/95/96/97	Document Number: NAHDIC-FRM-GEN-025-7A
	Fax: +251113380220	Website: http://www.nahdic.gov.et/



AHI



TEST RESULT REPORT FORM

Customer name: Gashaw Adane

Reference No. SR/RE/151/2022

Contact: phone : , email :

Test Report No. 151/2022

Sampling Place: Region: Amhara, District: Angolelana Tera, Kebele: Chaki

Sampling type: Purposive

Date reported: 06-04-2022

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed	Owner
11003-11090	SERUM	Bovine	15/03/2022	22/03/2022	30/03/2022	Gashaw Adane
					30/03/2022	

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	88	2	86	0	Brucella: Ab detection	Rose Bengal Plate Test (RBPT)	AHI-TM-SRL-006

*Accredited Test (AHI is accredited for tests marked *)

Lab Code	Sample Type	Species	Sampling Date	Date Received	Date Analyzed	Owner
11072-11075	SERUM	Bovine	15/03/2022	22/03/2022	30/03/2022	Gashaw Adane
					30/03/2022	

Testing Lab	No.	Positive	Negative	Doubtful	Test	Method	SOP
AHI	2	1	1	0	Brucella: Ab detection	Complement Fixation Test (CFT)	AHI-TM-SRL-006

*Accredited Test (AHI is accredited for tests marked *)

Opinions and Interpretations

Out of 88 sera sample tested for brucella antibody 2 samples were positive by RBPT and 1 sample from 2 tested samples was positive for CFT.

Verified by:
Getachew Tuli

Approved by:

NAHDIC Original Document



Dr. Getnet Abie Mekonnen
Animal Disease Diagnostic &
Study Core Process
Coordinator

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NOTE: the results shown on this test report only refers the number of samples tested and the respective customer not for other purpose. NAHDIC is responsible for all the information provided on this report, except for the information provided by the customer.

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