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SEROEPIDEMIOLOGY AND MOLECULAR IDENTIFICATION OF *BRUCELLA* SPECIES IN LIVESTOCK AND HUMANS IN SOUTH OMO ZONE, SOUTH ETHIOPIA REGIONAL STATE, ETHIOPIA

PhD Dissertation

By:

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Addis Ababa University, College of Veterinary Medicine and Agriculture, Department of Veterinary Epidemiology and Public Health

PhD program in Veterinary Public Health

May, 2025

Bishoftu, Ethiopia

SEROEPIDEMIOLOGY AND MOLECULAR IDENTIFICATION OF *BRUCELLA* SPECIES IN LIVESTOCK AND HUMANS IN SOUTH OMO ZONE, SOUTH ETHIOPIA REGIONAL STATE, ETHIOPIA

A dissertation submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Veterinary Public Health

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Title: **Seroepidemiology and Molecular Identification of *Brucella* Species in Livestock and Humans in South Omo Zone, South Ethiopia Regional State, Ethiopia**

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As the members of the Examining Board of the final PhD open defense, we certify that we have read and evaluated the Dissertation prepared by Mekonnen Sorsa Berecha titled: **Seroepidemiology and Molecular Identification of *Brucella* Species in Livestock and Humans in South Omo Zone, South Ethiopia Regional State, Ethiopia**, and recommend that it is accepted as fulfilling the dissertation requirement for the Degree of Philosophy in Veterinary Public Health.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS

AAU	Addis Ababa University
ALIPB	Aklilu Lemma Institute of Pathobiology
AMOS	*Abortus, Melitensis, Ovis, Suis*
C-ELISA	Competitive Enzyme-Linked immunosorbent assay
CFT	Complement Fixation Test
CFU	Colony Forming Units
CSA	Central Statistical Agency
DALYs	Disability-Adjusted Life Years
DNA	Deoxyribonucleic Acid
FPA	Fluorescence Polarization Assay
LPS	Lipopolysaccharide
mRBPT	Modified Rose Bengal Plate test
MRT	Milk Ring Test
NVI	National Veterinary Institute
OD	Optical Densities
OIE	World Organization for Animal Health
Omp	Outer Membrane Protein
OPD	O-Phenylenediamine Dihydrochloride
OR	Odds Ratio
PA	Pastoral Associations
RFM	Retained Fetal Membrane
RT-PCR	Real-time Polymerase Chain Reaction
SAT	Slow Agglutination Test
SDA	Serum-Dextrose Agar
S-LPS	Smooth Lipopolysaccharide
SRBC	Sheep Red Blood Cells
SSA	Sub-Saharan Africa
TMP-SMZ	Trimethoprim-Sulfamethoxazole
TSA	Tryptose Soy Agar

STATEMENT OF AUTHOR

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Addis Ababa University

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DEDICATION

This dissertation is dedicated to my mother **Dunge Chokorsa Wakjira**, who never went to school herself but she paid a unique sacrifice for me beyond the natural mothership in the journey of my academic life.

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Mekonnen Sorsa Berecha

PhD Dissertation

Addis Ababa University

ABSTRACT

Brucellosis, a bacterial infection caused by the genus Brucella affecting animals, poses economic and public health risks, particularly for communities reliant on livestock. Limited data exists on the epidemiology of brucellosis and Brucella species affecting livestock and humans in Ethiopia, particularly in the South Omo Zone of the South Ethiopia Regional State. A cross-sectional study was conducted in South Omo Zone, Ethiopia, from January 2017 to June 2020 to determine the prevalence, isolate and identify Brucella species in cattle, sheep, goats, and humans, and pinpoint risk factors associated with the disease across five districts: Bena-Tsemay, Dassenech, Hamer, Gnangatom, and Malie. A multistage sampling approach was used to select study subjects, encompassing 1,920 cattle, 1,536 sheep, 1,536 goats, and 768 humans for serological examiantion using combined serial tests RBPT, competitive ELISA and CFT. These sample sizes for animals, and humans were distributed to each district in proportion to their livestock,and human populations, respectively. Additionally, a structured questionnaire survey was conducted to explore risk factors associated with brucellosis in both livestock and humans. Furthermore, a total of 340 clinical specimens (n=53 milk from cattle, n=39 milk from goats, n=28 milk from sheep, n=34 vaginal swab from cattle, n=48 vaginal swab from goats, n=16 vaginal swab from sheep) having recent history of abortion and whole blood samples from pastoralists with febrile illness visiting health centers (n=122) were collected for Brucella species isolation and molecular identification using real-time polymerase chain reaction. Data were analyzed with STATA version 14.0, applying logistic regression to assess risk factors associations.

The findings revealed that in cattle, overall seroprevalence of 5.26% (95% CI:5.05-8.04) and 36.43% (95% CI:33.18-43.76) at individual and herd level, respectively. Age, sex, herd size,

abortion history, retained fetal membrane, and parity were statistically significant with seropositivity for brucellosis. Moreover, the findings established that pastoralists had low level of awareness about brucellosis and carryout risky practices that could expose them to brucellosis. In sheep, the study indicated 5.40% (95% CI: 3.34-7.47) and 39.74% (95% CI: 36.50-48.80) seroprevalence at individual and flock level, respectively. Age groups, sex, flock size, district, history of abortion, and body condition were stastistically significantly associated risk factors with Brucella seropositivity (P<0.05). In goats, individual animal level and flock level seroprevalences were recorded as 6.90% (95% CI:5.18-10.90) and 34.31% (95% CI:30.52-42.41), respectively. Statistically significant associations were found between seroprevalence and factors such as sex, age, flock size, body condition, parity number, production system, history of abortion, retained fetal membrane, and stillbirth (P< 0.05). In humans, the overall seroprevalence of brucellosis recorded was 14.58% (95% CI: 12.07-18.24). The multivariable logistic regression analysis indicated statistically significant association (P<0.05) between Brucella seropositivity and factors such as gender, age, education level, occupation, consumption of raw animal products such as milk, meat and blood, direct animal contact, assisting animals during parturition with bare hand, contact with aborted materials without protective gloves, and lack of knowledge of zoonosis and brucellosis. The isolation and molecular detection revealed that 15 Brucella isolates were obtained (4.41%): 8 B. melitensis (53.33%) from (3 goats' milk sample, 3 goats' vaginal swab samples, and 2 human blood); 5 B. abortus (33.33%) from (2 cattle milk, 1 cattle vaginal swabs, and 2 human blood); and 2 B. ovis (13.33%) both from vaginal swab of sheep based on bacteriological and biochemical tests. The isolation and molecular identification of these Brucella species from multiple hosts including humans demonstrate potential cross-species transmission.

In conclusion, brucellosis is endemic in both livestock and humans in South Omo Zone, driven by multiple risk factors and exacerbated by low community awareness. The findings underscore a serious public health concern requiring urgent need for integrated control measures, including public education, improved veterinary services, safe handling of animal products, and further research on disease transmission dynamics in wildlife-livestock-human interfaces.

Key words: *Brucella species, Ethiopia, Isolation and molecular identification, Livestock, Public Health. Seroepidemiology, South Omo zone.*

1. INTRODUCTION

1.1. Background of the study

Brucellosis is a significant zoonotic disease caused by a gram-negative, facultative intracellular bacterium belonging to the genus *Brucella*, which can infect a wide variety of animal species as well as humans (Fichi, 2003; Benkirane, 2006). Although *Brucella* species are not host specific, they do exhibit host preferences. For instance, *Brucella abortus* (*B. abortus)* is primarily associated with cattle, *Brucella melitensis* (*B. melitensis)* mainly affects small ruminants, *Brucella ovis (B. ovis*) affects sheep (Poester *et al.,* 2013; Olsen and Gallagher, 2017), and *Brucella suis* (*B. suis)* is commonly found in swine (Abdelbaset *et al*., 2018). Of the several *Brucella* species identified in both humans and animals, *B. abortus, B. melitensis, B. suis,* and *Brucella canis* (*B. canis)* are recognized as the primary causes of zoonotic infections (Benkirane, 2006; Ducrotoy *et al.,* 2017). It also affects a wide range of wild mammals (OIE, 2021).

The disease is characterized by reproductive dysfunction in livestock, leading to significant economic losses in animal production systems due to abortion, reduced fertility, and decreased milk yield (Olsen and Gallagher, 2017). In humans, brucellosis manifests as flu-like symptoms, including undulating fever, malaise, sweating, arthritis and in some chronic cases, can lead to serious complications affecting the bone and reproductive system leading to sterility (Kiros *et al*., 2016; Rahdar *et al.,* 2019; Gowin *et al*., 2020).

Brucellosis endemic areas are primarily located in the low- and middle-income countries of the world. It is endemic in regions such as the Mediterranean basin, the Arabian Peninsula, the Middle East, Africa, and Central America and South America and Asia, with major regional differences (Ducrotoy *et al.,* 2017). The highest prevalence in livestock was observed in countries of the Middle East and sub-Saharan Africa, China, India, Peru, and Mexico (Ducorotoy *et al.,* 2017; Franc *et al.,* 2018). Only a few countries in the world are free from brucellosis and are mainly in developed regions in Western and Northern Europe, Canada, Japan, Australia, and New Zealand (OIE, 2021). Recent estimates indicate an annual global prevalence of approximately 2.1 million human brucellosis cases, significantly surpassing earlier figures (Laine *et al*., 2023). Africa and Asia exhibit the highest incidence rates.

The epidemiology of *Brucella* species in livestock is influenced by factors such as husbandry practices, environmental conditions, and animal movement. In the context of Ethiopia, pastoral and agro-pastoral systems dominate rural livelihoods, where livestock is often raised in close communal settings and consumption of raw animal products is common, facilitating the transmission of infectious diseases (Hussein *et al*., 2020).

Studies have shown that high seroprevalence rates of brucellosis exist in various regions across Ethiopia, with reports indicating seroprevalence rates of ranging 0.4 % (Ferede *et al.*, 2011) from Bahir Dar to 13.7% (Wedajo *et al.*, 2015) from Afar region in sheep; 1.5% (Bekele *et al*., 2011) from pastoral area to 17.36% (Teshome *et al*., 2022) from Borena in goats;. and 0.6% reported by Temesgen *et al*. (2021) from central high land to 9.7% reported by Chaka *et al.* (2018) from Southern Ethiopia Agropastoral areas in cattle. Moreover, the absence of effective control programs and limited veterinary services further worsen the situation.

Brucellosis is recognized among the top five priority zoonotic diseases (E.G. Pieracci *et al.* 2016) in Ethiopia, where a significant proportion of the population relies on livestock for their livelihoods. Human infection commonly occurs through the consumption of unpasteurized dairy products and direct contact with infected animals (Dagnachew *et al*., 2020). Airborne transmission through inhalation of infected aerosolized particles can also occur (Zhang *et al.*, 2020). Activities like assisting during birthing or calving, illiteracy, and lack of awareness of zoonosis and or brucellosis expose humans to brucellosis (Khoshnood *et al*., 2022; Narimisa *et al*., 2024).

The prevalence of human brucellosis in Ethiopia varies significantly across different regions and populations. For instance, a low prevalence rate of 1.3% was reported by Tsegaye *et al.* (2021) among abattoir workers in Bishoftu and Mojo. In contrast, Tschopp *et al.* (2021) reported much higher rates among pastoralist communities, with 34.9% in the Somali Regional State and 48.3% in the Afar Regional State. Notably, the highest seroprevalence, 48.8%, was documented by Abdulkadir (2019) in the Afar Regional State.

The South Omo Zone, located in the South Ethiopia Regional State, is home to a diverse population comprising various ethnic groups, each with distinct cultural and traditional ways of life (Solomon *et al*., 2021). The zone is predominantly inhabited by pastoralist communities who primarily depend on livestock for their livelihoods, with minimal engagement in agro-pastoral practices. This heavy reliance on livestock may increase the community's vulnerability to zoonotic diseases, such as brucellosis.

1.2. Statement of the problem and rationale of the study

Ethiopia is one of the fastest growing countries in Africa where agricultural sector plays the leading economic role in its development. Livestock production in particular has been contributing considerable to the country's economy in providing draught animal power for crop cultivation, as a source of animal proteins to the society and also provide export commodities, such as live animals, hides, and skins to earn foreign exchanges to the country. For instance, recently in 2023, agriculture accounted for 35.79% of the country's Gross Domestic Product (GDP). Livestock, a significant component of agriculture, contributed approximately 19% to the national GDP and up to 45% to the agricultural GDP (World Bank, 2024). These figures underscore the crucial role of both agriculture and livestock in Ethiopia's economic landscape.

Ethiopia is known by its livestock populations ranked the first in Africa and tenth in the world. According to a CSA (2021) livestock sample survey, the country possesses approximately 70.29 million cattle, 42.9 million sheep, 52.46 million goats, 8.14 million camels, 57 million poultry, 2.14 million horses, 0.38 million mules, and 10.79 million donkeys

In spite of these huge livestock resources in Ethiopia and favorable future prospect of international livestock trading scheme, the contribution of the livestock sector to country's economy has been constrained by the widespread prevailing diseases of livestock having both economic and public health impacts coupled with other factors. Among these diseases brucellosis is the most important zoonotic bacterial diseases affecting various species of animals cause high morbidity resulting in significant economic losses and restriction of live animal and/or animal product export to the international livestock market. In addition to this, the widespread existence of zoonotic infectious diseases such as brucellosis, and other diseases in

livestock of the country coupled with absence of effective diseases control strategies have posed serious public health hazard to human population of Ethiopia (Megersa *et al.,* 2011).

Pastoralism is a social and economic system where people primarily rely on herding domesticated animals, mainly cattle, sheep, goats, and/or camels for their livelihood, often in dry grassland or rangeland environments. Pastoral areas are often neglected when it comes to disease surveillance and health services, due to the remoteness of the areas, challenging logistics, harsh environment, lack of infrastructure and sometimes security issues. The South Omo Zone however, is an important pastoral area in Ethiopia in terms of livestock herds, livestock economics and human and animal cross-border movements as the area is boardered by Kenya and South Sudan. This zone thus, needs special attention and epidemiology of *Brucella* species and its public health significances must be investigated.

The South Ethiopia Regional State of Ethiopia possesses the country's 20% cattle and 15% small ruminant populations (CSA, 2021). The South Omo Zone's contribution to the total livestock population of the regional state is very significant. The South-Omo pastoralist are known for rearing different species of animals including cattle, goat and sheep in large numbers with huge livestock composed of different species of livestock, of which 1, 230, 399 (goat), 489, 449 (sheep), 1, 134, 120 (cattle), 495 (camel), 424, 538 (poultry) and 132, 500 (bee colonies) (South Omo Zone Agricultural office, unpublished, 2016).

In Ethiopia, there are empirical evidences that pastorl communities consume predominantly raw animal products such as raw milk, and children directly consume milk from the udder of animals as reported by Kebede *et al*. (2019) from Borana area. Similarly, the pastoral communities of the South Omo zone, have the practice and habit of consumption of raw animals' products like milk, meat, and raw animals' blood (Solomon *et al*., 2021). These conditions are potential risk factors for transmission of zoonotic diseases such as brucellosis, as the source of brucellosis for human is almost exclusively infected livestock (Robinson *et al*., 2003).

In Ethiopia, there was a limited report regarding isolation, identification and molecular characterization of *Brucella* species both in animals and human (Gumi *et al., 2013*; Yohannes *et*

al., 2013) except recent report of isolation of *B. melitensis* by Sintayehu *et al*. (2015) and *B. abortus* by Minda *et al.* (2016), *B. abortus* by Edao *et al*. (2020), *B. melitensis* by Tekle *et al*. (2019), and *B.melitensis* by Wakjira *et al.* (2022). There are few seroprevalence reports on human brucellosis (Regassa *et al.,* 2009; Tsegaye *et al.,* 2017; Tschopp *et al.,* 2021; Getahun *et al*., 2022) in Ethiopia. In South Omo zone of South Ethiopia Regional State there are only few reports on seroprevalence of brucellosis in human.

This highlights a potential gap in monitoring and public health surveillance concerning this zoonotic disease. Moreover, there is information gap and no research has been conducted on transmission dynamics of brucellosis at the interface between livestock species and human populations especially in pastoral community of South Omo Zone where the traditional habits of consumption of raw milk, milk products, and raw blood of livestock, handling of aborted materials, manipulation of reproductive excretions with bare hands while assisting parturition and herding of a large number of animals mixed with other animals, frequent contact among different species of livestock at communal grazing land and water sources are widely practiced (Tigist *et al.,* 2011). This lifestyle and strong dependence of the pastoral communities on their livestock would highly favor the transmission and persistence of zoonotic diseases like brucellosis. Therefore, information on epidemiology, transmission dynamic, potential transmission risk factors and public health significance of brucellosis in livestock and pastoralist community of South-Omo zone should be generated.

Furthermore, different literatures showed that there is epidemiological variation between *B.melitensis* (primary cause of brucellosis in goats) and *B. ovis* (primary cause of brucellosis in sheep) in terms of virulence, clinical pictures, *in-vitro* growth requirements (Perez-Etayo *et al*., 2018). Generally, goats are more susceptible to *Brucella* infection than sheep, and this could be partly due to the fact that sheep excrete the organism for shorter periods than goats.This may reduce the potential for spread of the disease within and between sheep flocks (Radostits *et al.,* 2007). In Ethiopia, most prevalence studies on brucellosis in small ruminants have treated sheep and goats as a collective category, neglecting the distinct epidemiological dynamics between these two species. Given the differing characteristics of brucellosis in sheep and goats, it is

crucial to conduct separate investigations into the seroprevalence and associated risk factors in each population.

Understanding the epidemiology of *Brucella* species in livestock and humans in South Omo Zone is crucial for several reasons. Firstly, determining seroprevalence levels and identification of the causative *Brucella* spp will inform the veterinary and public health authorities about the burden of brucellosis and the species of circulating *Brucella spp.* in livestock and the community, enabling targeted interventions. Secondly, identifying risk factors associated with transmission will aid in developing effective control strategies. Furthermore, given the potential implications for food security and economic stability in pastoral communities, addressing brucellosis is vital for improving the overall health status of both animals and humans. It will also generate information about brucellosis in both livestock and humans in the study area that can be used as input for the national brucellosis prevention and control program. Hence, to enhance understanding and address the challenges posed by brucellosis in livestock and humans, focused research is warranted.

1.3. Research questions

Based on the statement of the problem explained this PhD dissertation work was initiated to address the following basic research questions:

1. What is/are/ the magnitudes of brucellosis in cattle, sheep, goats and human populations in South Omo zone, South Ethiopia Regional State, Ethiopia?
2. What are the major putative risk factors associated to the occurrence and transmissions of brucellosis in cattle, sheep, goats and human in the study site?
3. Which *Brucella* species is/are circulating and responsible to cause brucellosis in cattle, sheep, goats and humans in the study area?

1.4. Research hypotheses

To guide the study design the following hypotheses are tailored for a study on the seroepidemiology, isolation, and molecular identification of *Brucella* species in livestock and humans in South Omo Zone of South Ethiopia Regional State:

- **H1:** Brucellosis is highly prevalent in cattle, sheep, goats, and humans in South Omo

zone, South Ethiopia Regional State.

- **H2:** The prevalence of livestock brucellosis is significantly higher in pastoral settings compared to agro-poastoralist in the South Omo Zone.
- **H3**:The presence of reproductive health issues (such as abortions, stillbirths, and retained fetal membranes), herd or flock sizes, and the age of livestock are significantly associated with the prevalence of brucellosis in livestock in the study area.
- **H4**: *Brucella abortus* and *Brucella melitensis* are the major *Brucella* species circulating among livestock and humans populations in South Omo zone of SouthEthiopia Regional State.
- **H5**: There is a difference in prevelance of brucellosis between goat and sheep in the study area.
- **H6**: The prevalence of human brucellosis is significantly associated with factors such as education level, occupation, consumption habits of raw milk, raw blood, and meat, knowledge of zoonosis and brucellosis, assisting animal birthing, contact with animals and aborted materials, as well as demographic factors.
- H7**:** There is a significant correlation between the prevalence of livestock brucellosis and the incidence of human brucellosis, indicating a zoonotic transmission pathway.

1.5. Objectives of the study

1.5.1. General objective

The general objective of this PhD research is to study the seroepidemiology of brucellosis in livestock and humans, isolate and molecularly identify the causative agent in both species, and identify the risk factors for its transmission in livestock and zoonotic transmission to humans in the South Omo pastoral area of Ethiopia.

1.5.2. Specific objectives

The objectives of the study were:

- To estimate the seroprevalence and identify potential risk factors for the occurrence and transmission of brucellosis in cattle in the South Omo Zone of South Ethiopia Regional State,

- To assess the seroprevalence and related risk factors of ovine brucellosis in the South Omo Zone of South Ethiopia Regional State,
- To determine the seroprevalence and putative risk factors of caprine brucellosis in the South Omo Zone of South Ethiopia Regional State,
- To estimate the seroprevalence and determine the potential risk factors for the occurrence and transmission of human brucellosis in the South Omo Zone of South Ethiopia Regional State, and
- To isolate and molecularly identify *Brucella* species circulating in livestock and human populations within the South Omo Zone of South Ethiopia Regional State.

2. LITERATURE REVIEW

2.1. Introduction

Brucellosis is a disease that may date back to the time of the Fifth Plague of Egypt, around 1600 BC. Archaeological studies of ancient Egyptian skeletal remains from around 750 BC have shown evidence of sacroiliitis and various osteoarticular conditions, which are recognized complications of brucellosis (Pappas and Papadimitriou, 2007). The bacterium responsible for the illness, originally named *Micrococcus melitensis,* was first isolated by David Bruce in 1887 from the spleen of a British soldier who died from a febrile illness prevalent among military personnel in Malta, referred to as Malta fever. For nearly twenty years after the identification of *M. melitensis*, the origins of Malta fever remained unclear, with the disease initially thought to be transmitted by vectors. However, in 1905, Themistocles Zammit made a groundbreaking discovery by isolating *B. melitensis* from goat milk, thereby demonstrating the zoonotic transmission of the disease (Wyatt, 2005). It was previously believed that goats could not spread the infection since they exhibited no signs of illness when subjected to *Brucella* cultures. This pivotal realization that healthy goats could harbor the bacteria marked a significant advancement in the field of epidemiology (Sriranganathan *et al*., 2009).

In 1897, Danish veterinarian Bernard L.F. Bang discovered Bang's bacillus (*B. abortus*), the causative agent of Bang's disease, also known as the bacillus responsible for cattle abortion (Colville, 2007). American researcher Alice Evans performed essential studies on pathogenic bacteria found in dairy products, linking Bang's disease with Malta fever. She later renamed the genus *Brucella* in tribute to David Bruce (Madkour, 2001). Evans's work was instrumental in the promotion of pasteurization as a vital method to reduce the risk of human brucellosis in the United States. Furthermore, the identification of *Brucella* species in marine mammals in the early 1990s broadened the understanding of brucellosis, showing that it is not limited to land animals (Sriranganathan *et al*., 2009).

Brucellosis primarily affects livestock, including cattle, pigs, sheep, goats, camels, and dogs, but it can also impact other ruminants and marine animals. The disease is referred to by various names, such as Malta fever, Mediterranean fever, Cyprus fever, Gibraltar fever, undulant fever,

rock fever (Mantur and Amarnath, 2007), as well as Bang's disease or enzootic abortion in humans and animals, respectively (Radostits *et al*., 2007).

2.2. Etiology

2.2.1. Taxonomic Classification

The genus *Brucella* is classified within the family *Brucellaceae*, order *Rhizobiales*, class *Alphaproteobacteria*, and phylum *Proteobacteria*. The phylum *Proteobacteria* constitutes the largest group of bacteria, encompassing numerous genera of pathogens, including *Escherichia*, *Salmonella*, *Vibrio*, and *Helicobacter*, among others. Members of the *Proteobacteria* phylum are characterized as Gram-negative bacteria, possessing an outer membrane primarily composed of lipopolysaccharides (Garrtty and Krieg, 2010).

Historically, the genus *Brucella* has been recognized as containing six 'classical' species, which were categorized based on their antigenic differences and the primary host species from which they were isolated. These classical species include *B. abortus, B. melitensis, B. suis, B. ovis, B. canis*, and *B. neotomae* (Adams and Schutta, 2010). Additionally, several species affecting marine mammals have been identified and classified, such as *B. ceti* and *B. pinnipedialis*, as well as a *Brucella* species found in the common vole (*B. microti*) (Foster *et al*., 2007; Holger *et al*., 2008). More recently, *Brucella inopinata* was isolated from a breast implant infection in a woman showing clinical signs of brucellosis (Scholz *et al*., 2010). In total, there are 12 recognized species of *Brucella* (Hull and Schumaker, 2018) (Table 1).

Table 1: *Brucella* species classified by host and their zoonotic potential

Species	Natural Host	Zoonotic Potential	References
1.B. melitensis	Sheep, goats, and camels	Yes - High	Hallin *et al*. (2005)
2. B. abortus	Cattle, elk, and bison	Yes - High	Bruce (1887)
3. B. suis	Pigs, hares, reindeer/caribou	Yes - High	Hallin *et al*. (2005)
4.B. canis	Domestic and wild dogs	Yes - Moderate	Scholz *et al*. (2016)
5.B. ovis	Sheep	Yes-Low	Hofer *et al. (* 2012)
6.B. neotomae	Desert wood rats	Infections reported	Suarez-esquivel *et al*. (2017)
7. B. ceti	Cetaceans	Yes - Low	Whatmore *et al.* (2014)
8.B. pinnipedialis	Pinnipeds	Yes - Low	Hallin *et al*. (2005)
9. B. microti	Red foxes, common voles, frogs	No reported infections	Scholz *et al*. (2016)
10. B. inopinata	Unknown	Yes - High	Hofer *et al. (*2012)
11. B. papionis	Non-human primates	No reported infections	Whatmore *et al.* (2014)
12. B. vulpis	Red fox	No reported infections	Scholz *et al*. (2016)

2.2.2. Characteristics of Brucella

Brucella species are facultative intracellular, Gram-negative, non-spore-forming, non-capsulated coccobacilli that exhibit partial acid-fastness, and can be found as endospores or native plasmids. They can endure freezing and thawing cycles; however, they are susceptible to most disinfectants effective against Gram-negative bacteria. Pasteurization is an effective method for eliminating *Brucella* in milk (Scholz *et al*., 2010). These bacteria generally measure between 0.5 to 0.7 μm in diameter and 0.6 to 1.5 μm in length. Classic *Brucella* species test positive for oxidase, catalase, and urease. Although these species are considered non-motile, they possess all necessary genes, except for the chemotaxis genes, to develop a functional flagellum (Radostits *et al*., 2007).

The genomes of *Brucella* species are comparable in size and gene composition (Halling *et al*., 2005). Each species within this genus has a typical genome size of around 3.29 Megabases (Mb), which consists of two circular chromosomes. Chromosome I average about 2.11 Mb, while Chromosome II averages approximately 1.18 Mb. The G+C content is 57.2% for Chromosome I and 57.3% for Chromosome II across all *Brucella* genomes (Halling *et al*., 2005). Notably, *Brucella* does not possess classic virulence genes associated with capsules, plasmids, pili, or

exotoxins. Compared to other bacterial pathogens, limited information is available regarding the mechanisms that allow *Brucella* to persist in hosts and multiply within phagocytes. Additionally, many elements of the interaction between *Brucella* and its host remain poorly understood (Seleem *et al*., 2008).

In cattle, bison, and buffalo, brucellosis is primarily attributed to *Brucella abortus*. Up to nine biovars of *B. abortus* (1-9) have been identified, though some of these biovars show minimal differences, and the classification status of certain varieties is uncertain. Other species of *Brucella* that have been occasionally linked to diseases in cattle include *B. melitensis* and *B. suis* (Radostits *et al*., 2007; Seleem *et al*., 2010).

2.3. Pathogenesis

The genus *Brucella's* capability to replicate and maintain itself within host cells is closely linked to its ability to induce chronic illness and evade both innate and adaptive immune responses (FAO, 2003). While the precise mechanisms by which *Brucella* species invade host cells remain somewhat obscure, it is evident that the internalization process necessitates alterations in the cytoskeleton, even though specific receptors for *Brucella* on host cells have yet to be identified (Radostits *et al*., 2007). Interestingly, invasion via the gastrointestinal tract does not provoke a notable inflammatory response from the host. Consequently, *Brucella* species can enter the host undetected by the innate immune system. *Brucella* has evolved mechanisms that inhibit the activation of the host's innate immune response (Poester *et al*., 2013). Notably, a protein containing a Toll/interleukin-1 receptor (TIR) domain from *Brucella* obstructs Toll-like receptor (TLR) 2 signaling by disrupting MyD88 and hinders the maturation of dendritic cells (DCs), including their cytokine release and antigen presentation capabilities (Cirl *et al*., 2008).

Brucella abortus also initiates the suppression of pro-inflammatory mediator transcription in trophoblastic cells soon after infection begins. Trophoblasts, the placental cells targeted during infections in pregnant cows, initially exhibit reduced pro-inflammatory transcripts, but *B. abortus* subsequently induces the expression of pro-inflammatory chemokines in cultured trophoblastic cells. This change aligns with the expression profile observed in the placentas of naturally infected cows (Poester *et al*., 2013).

Interestingly, *Brucella* species do not possess traditional bacterial virulence factors such as exotoxins, cytolysins, capsules, fimbriae, flagella, plasmids, lysogenic phages, endotoxic lipopolysaccharides (LPS), or pro-apoptotic agents. Nonetheless, certain types of LPS are critical for *Brucella's* virulence as they prevent complement-mediated bacterial destruction and confer resistance to antimicrobial peptides like defensins and lactoferrin (Seleem *et al*., 2008). Another key virulence factor in *Brucella* is the BvrR/BvrS two-component regulatory system, essential for altering the host cell cytoskeleton during *Brucella* invasion and modulating the expression of outer membrane proteins needed for full virulence (Poester *et al*., 2013). Cyclic β-1, 2-glucans, also part of the outer membrane, is necessary for the intracellular survival of *Brucella*. The species expresses a type IV secretion system (T4SS), encoded by the virB operon, which is vital for its intracellular survival and overall virulence in vivo (Radostits *et al*., 2007; Cirl *et al*., 2008).

Once *Brucella* invades, it typically localizes within the lymph nodes that drain the infection site, leading to hyperplasia of lymphoid and reticuloendothelial tissues and the infiltration of inflammatory cells. The bacteria can persist within macrophages by surviving the phagolysosome, which facilitates localized infection and allows *Brucella* to escape the lymph nodes into the bloodstream. During the bacteremia phase, organs such as bones, joints, eyes, and the brain may become infected, although the bacteria are most frequently isolated from the supramammary lymph nodes, milk, iliac lymph nodes, spleen, and uterus (Radostits *et al*., 2007). In bulls, the reproductive organs and associated lymph nodes show a pronounced susceptibility to infection. During the acute phase, a significant number of *Brucella* cells can be found in semen; however, as the infection becomes chronic, the excretion of *Brucella* cells decreases, although they may still be intermittently excreted for years (Nielsen *et al*., 2016).

If the infected animal is pregnant, *B. abortus* extensively colonizes and proliferates in the chorionic trophoblasts of the developing fetus. Erythritol, a substance produced by the fetus that stimulates *B. abortus* growth, is found in high concentrations within placental and fetal fluids, directing the infection to these tissues. Invasion of the pregnant uterus leads to severe ulcerative endometritis in the intercotyledonary areas. The allantochorion, fetal fluids, and placental cotyledons are infiltrated, resulting in the destruction of villi (Poester *et al*., 2013). The organism

has a strong preference for the ruminant placenta. In acute infections of pregnant cows, up to 85% of the bacteria can be found in the cotyledons, placental membranes, and allantoic fluid, causing tissue necrosis of the fetal membranes that facilitates bacterial transmission to the fetus. Ultimately, this colonization of chorionic and fetal tissues can lead to abortion during the third trimester of pregnancy (Radostits *et al*., 2007).

Sexually immature and non-pregnant cattle can also become infected, but they typically lose their humoral antibodies to *Brucella* much more rapidly than infected pregnant cattle. In adult non-pregnant cows, *Brucella* colonizes the udder, and if these cows become pregnant later, the uterus may become infected due to periodic phases of bacteremia originating from the udder (Radostits *et al*., 2007). While infected udders appear clinically normal, they represent a significant source of reinfection for the uterus, as well as potential infection routes for calves or humans consuming the milk, and form the basis for agglutination tests using milk and whey (Cirl *et al*., 2008).

Brucella spp. is a facultative intracellular bacterium that primarily infects mammals, including humans, causing brucellosis. A critical factor in the pathogenesis of *Brucella* infections is the presence of erythritol, a sugar alcohol found abundantly in reproductive tissues and certain organs, which acts as a growth factor for *Brucella* (Pappas *et al*., 2006). This explains the predilection of *Brucella* for infecting specific sites such as the liver, spleen, joints, and reproductive organs, including the placenta and mammary glands (Corbel, 2006). These organs are crucial to the disease's chronic nature, as the bacteria can persist in these sites, leading to long-term infection and complications. The liver and spleen are central to *Brucella's* survival, as the bacteria can reside within macrophages, making these reticuloendothelial organs critical in the disease's progression (Godfroid *et al*., 2011). The consumption of raw liver from infected animals is a significant risk factor, as *Brucella* is often present in these tissues, increasing the likelihood of zoonotic transmission through direct contact or consumption (Pappas *et al.*, 2006). In pastoral communities like pastoralists of South Omo zone, where raw or undercooked animal products are commonly consumed, this risk is heightened, contributing to the higher incidence of human brucellosis in such areas (OIE, 2018). Thus, both the consumption of raw animal products

and the anatomical affinity of *Brucella* for certain organs are critical factors in the transmission and pathogenesis of brucellosis

2.4. Clinical Signs and Post-Mortem Findings

In cattle, *B. abortus* leads to abortions and stillbirths, typically occurring in the latter half of pregnancy. Some calves may be born alive but weak and could perish shortly after birth. Retained placenta is possible, which may lead to secondary metritis. There can also be a reduction in milk production (Seleem *et al*., 2010). Following an initial abortion, further pregnancies are usually normal; however, cows may excrete the bacteria in their milk and uterine discharges.

In bulls, conditions like epididymitis, seminal vesiculitis, orchitis, or testicular abscesses can sometimes occur. Infertility may occasionally be observed in both genders due to conditions such as metritis or orchitis/epididymitis (Radostits *et al*., 2007). Hygromas, particularly around leg joints, are frequently reported in certain tropical regions. Long-term infections can also lead to arthritis. Systemic symptoms are typically absent in uncomplicated cases, with fatalities being rare, save for in foetuses or neonates. Non-pregnant females usually show no noticeable symptoms (Nielsen *et al*., 2016).

Similar clinical signs are observed in camels, bison, water buffalo, bighorn sheep, and other ruminants (Musa *et al*., 2008; Bechtol *et al*., 2011). Some carnivore species have exhibited symptomatic infections as well, including abortions, epididymitis, polyarthritis, and various symptoms in *B. abortus*-infected dogs (Xavier *et al*., 2010). In horses, *B. abortus* can inflame the supraspinous or supra-atlantal bursa, known respectively as fistulous withers and poll evil (Radostits *et al*., 2007).

During necropsy, granulomatous inflammatory lesions may be found in the reproductive organs, udder, supramammary lymph nodes, other lymphoid tissues, and occasionally in the joints and synovial membranes. Abortion may result in mild to severe endometritis. The placenta is often thickened, edematous, and could have exudate on its surface (Poester *et al*., 2013). Regional lymph nodes may be enlarged, and lesions may be present in the mammary gland. Some aborted

foetuses may appear normal, while others show signs of autolysis or varying degrees of subcutaneous edema and bloody fluid within body cavities. The liver may present with enlargement and discoloration, and the lungs might display fibrous pleuritis and pneumonia (Buzgan *et al*., 2010). In bulls, one or both scrotal sacs may be swollen due to orchitis, epididymitis, or abscesses. Hygromas can be identified at slaughter in both sexes on the knees, stifles, hocks, where the haunch meets the body, and in areas between the nuchal ligament and primary thoracic vertebrae (Radostits *et al*., 2007).

In cattle that are naive and unvaccinated, *B. abortus* can spread rapidly, leading to abortion storms, where the prevalence of abortion can range from 30% to 80%. In herds where this organism has become endemic, symptoms typically appear sporadically, and cows may face abortion during their first pregnancies (Buzgan *et al*., 2010). Death in adult animals of most species is infrequent; however, *B. abortus* can be fatal in experimentally infected moose and may also affect bighorn sheep (Radostits *et al*., 2007).

In humans, *Brucella* induces systemic infections that can present as acute, subacute, or chronic relapsing illness. The clinical presentation of human brucellosis is nonspecific and varies widely. Patients often experience a range of symptoms, including undulant fever, headache, chills, myalgia, and arthralgia. The disease is also linked to complications such as abortion, orchitis, acute renal failure, endocarditis, splenic abscess, spondylitis, arthritis, and encephalitis (Buzgan *et al*., 2010).

2.5. Transmission in livestock and human

Different *Brucella* species exhibit varying levels of adaptation to their hosts. In cattle, the primary cause of infection is commonly *B. abortus*, although *B. melitensis* and, less frequently, *B. suis* can also infect cattle, with transmission methods being generally comparable to those associated with *B. abortus*. These infections pose significant risks to humans due to the high virulence found in many strains of *B. melitensis* and *B. suis*, as well as the substantial quantity of bacteria excreted by infected animals (Seleem *et al*., 2010).

In cattle and other members of the *Bovidae* family, *Brucella* is typically transmitted through close contact among animals following abortion events. Pastures or animal housing can become contaminated, and ingestion is likely the most common route of acquisition. Other potential transmission methods include inhalation, inoculation through the conjunctiva, skin exposure, and contamination from infected milking equipment (Radostits *et al*., 2007). Using pooled colostrum to feed newborn calves may also facilitate the spread of infection. While sexual transmission has a minor impact on the spread of bovine brucellosis, artificial insemination can lead to disease transmission, making it crucial to collect semen only from animals confirmed to be free of the infection (Garrtty and Noel, 2010).

In sheep and goats, brucellosis is highly contagious due to the pathogenic nature of *B. melitensis* and the close proximity created by densely crowded flocks, interactions between different owners' animals, and significant exposure in confined spaces. Animal-to-animal transmission occurs largely because of the massive quantities of bacteria released into the environment (Buzgan *et al*., 2010).

Camels can also be susceptible to brucellosis caused by *B. melitensis* and *B. abortus* (Abbasa, 2002; Gwida *et al.,* 2012), particularly when they graze alongside infected sheep, goats, and cattle. This suggests that factors such as large herd sizes, shared watering points with ruminants, and poor hygiene practices in pastoral management contribute to the spread of camel brucellosis, especially during abortions or deliveries involving infected females (Abbasa, 2002).

For humans, the primary sources of infection are animals that carry the disease, particularly significant food-producing species such as cattle, sheep, goats, and pigs. The risk and severity of disease often depend on the specific type of *Brucella* involved, which can be influenced by the host animal responsible for transmitting the infection (Pappas *et al*., 2006).

In general, humans contract brucellosis through consumption of contaminated or unpasteurized milk and dairy products or through direct contact with infected animals or their carcasses. Abortion materials, uterine secretions, and colostrum are particularly infectious. Additionally, contact between mucous membranes or skin abrasions and fluids or tissues from aborted fetuses

or infected animals represent a notable risk for *Brucella* transmission (Earhart *et al.,* 2009). Key infection routes in human include penetration of the oral or gastric mucosa from eating unpasteurized or contaminated dairy products, inhalation or penetration of the ocular mucosa, and direct introduction into the bloodstream via skin abrasions or during vaccinations (Falagas and Bliziotis, 2006). Furthermore, consumption of raw bloods from infected animals could be also potential risk for humans.

Human-to-human transmission of brucellosis is extremely rare but has been documented in specific cases, such as corneal transplants and sexual transmission (Pappas *et al.,* 2006). *Brucella* spp., primarily *B. melitensis*, is the most virulent species causing severe systemic disease in humans, leading to complications like osteoarticular infections, endocarditis, and neurological issues (Godfroid *et al*., 2011). *B. melitensis* is the most significant cause of zoonotic brucellosis, particularly in Ethiopia, where it contributes significantly to human infections, especially in regions with high livestock exposure (Mekonnen *et al.,* 2015). Its ability to infect both animals and humans makes it a major public health concern.

2.6. Risk Factors Associated with *Brucella* Infection in livestock

The factors that affect the onset, transmission, persistence, and/or management of bovine brucellosis are linked to the genetic makeup of the animal host population, agricultural practices, and the biological characteristics of the pathogens (Radostits *et al*., 2007). For human brucellosis, risk factors involve interacting with infected animals, consuming contaminated animal products such as unpasteurized milk and dairy items (including milk from cows, goats, and camels), and eating meat, having a travel history to regions where the disease is common, and working with *Brucella* species cultures in laboratory settings (Buzgan *et al*., 2010).

2.6.1. Host Related Risk Factors

The vulnerability of livestock to *Brucella* infections is affected by various factors, including the age, sex, and reproductive condition of the animal. Younger animals tend to be less vulnerable to *Brucella* compared to their older counterparts, while animals that are sexually mature and pregnant exhibit a higher susceptibility to the infection than those that are not yet sexually mature. During the late stages of pregnancy, placental trophoblasts secrete increasing levels of

erythritol, coinciding with the higher susceptibility of pregnant cattle to *B. abortus* infection. Pathogenic strains of *Brucella* exhibit a preference for using erythritol over glucose for their growth. Erythritol is known to stimulate the proliferation of certain *Brucella* strains; however, *Brucella* has also been detected in the reproductive systems of animals that do not have measurable erythritol levels. This raises questions about the significance of erythritol in the organism's virulence. *B. abortus* Strain 19, a naturally occurring attenuated mutant, is commonly employed for vaccinating cattle and is unique among *B. abortus* strains in that it is inhibited by erythritol.

2.6.2. Management Related Risk Factors

The factors that increase the risk of disease transmission within a herd include the presence of unvaccinated animals in infected herds, the size of the herd, population density, housing methods, and the use of maternity pens. Larger herds are often sustained by acquiring replacement cattle, which may carry infections. Managing extensive herds can be challenging, potentially leading to managerial oversights that facilitate disease spread (Díaz Aparicio, 2013). There is a correlation between population density defined as the number of cattle relative to land area and the prevalence of disease, primarily due to increased interactions between susceptible and infected animals. Implementing maternity pens during calving is linked to a reduction in infection rates, likely because it minimizes the exposure of susceptible animals to those that are infected (Mcdermott and Arimi, 2002).

The transmission of disease between herds and across regions typically results from the relocation of an infected animal from a contaminated herd to a susceptible, uninfected herd. The uncontrolled movement of cattle from infected herds or regions to areas free of brucellosis presents a significant challenge to eradication efforts. Once a herd becomes infected, the duration necessary to achieve brucellosis-free status is prolonged by factors such as herd size, ongoing abortions, and inadequate housing conditions (Radostits *et al*., 2007).

2.6.3. Pathogen Related Risk Factors

The bacterium features a unique non endotoxic lipopolysaccharide that enhances its resistance to antimicrobial defenses and influences the immune response of the host. These characteristics

establish lipopolysaccharide as a critical virulence factor for the survival and replication of *Brucella* within its host (Bricker, 2002). This organism can persist on grass for varying durations dictated by environmental factors. In temperate regions, its infectivity can last up to 100 days during winter and around 30 days in summer. While it is vulnerable to heat, sunlight, and conventional disinfectants, freezing allows it to remain viable for nearly indefinite periods (Radostits *et al*., 2007).

2.7. Public Health Significance

Brucellosis is recognized as the most common zoonotic disease worldwide, yet it is classified among the seven most overlooked diseases by the World Health Organization (WHO) (Hull and Schumaker, 2018). Its vague clinical symptoms in humans can lead to chronic and debilitating conditions, resulting in significant economic consequences due to the disruption of daily activities (Buzgan *et al*., 2010).

Out of the twelve identified *Brucella* species, seven are capable of infecting humans (Table 1). The species deemed most harmful and invasive to humans include *B. melitensis, B. suis,* and *B. abortus,* which are listed as select agents by the Centers for Disease Control and Prevention in the USA (FAO, 2003). This classification arises from the highly contagious characteristics of these three species, as they can be spread through aerosols. Additionally, identifying an outbreak of brucellosis can be challenging since the early symptoms often resemble those of influenza (Buzgan *et al*., 2010). There are around 500,000 documented cases of human brucellosis each year; however, it is estimated that the actual number of cases ranges from 5,000,000 to 12,500,000 annually (Godfroid *et al.,* 2013). The countries most significantly impacted include Syria, which has the highest reported incidence (1,603.4 cases per 1,000,000 people), followed by Mongolia (3, 910), Iraq (268.8), Tajikistan (211.9), Saudi Arabia (149.5), and Iran (141.6) according to WHO statistics (Pappas *et al.,* 2006; Zhang *et al.,* 2010; Hull and Schumaker, 2018). Turkey and Kyrgyzstan experienced incidences exceeding 200 in the past decade but have recently declined to 49.5 and 88.0, respectively (Dean *et al*., 2012; Hull and Schumaker, 2018). The risk populations in these endemic regions mainly consist of workers in animal husbandry, shepherds, slaughterhouse employees, and rural communities closely interacting with animals and animal products (Yumuk *et al*., 2012; Tsend *et al*., 2014; Aloufi *et al*., 2016). It is important

to recognize that many nations known to have endemic human brucellosis lack reliable data, primarily due to insufficient surveillance and reporting to the WHO, as well as a dearth of peer-reviewed studies detailing the incidence of the disease (Pappas *et al*., 2003; Hull and Schumaker, 2018).

In sub-Saharan Africa, human brucellosis is endemic, yet serological evidence is sporadic (Mcdermott and Arimi, 2002; Moreno, 2014). Furthermore, clinicians often find diagnosing human brucellosis in this region challenging due to the wide range of clinical presentations and the unavailability of reliable tests. This situation frequently leads to misdiagnoses with other febrile illnesses, resulting in substantial underreporting of brucellosis cases (Mcdermott and Arimi, 2002). Despite the disease's significant prevalence in many low-income countries, it tends to receive inadequate attention from health systems (Buzgan *et al*., 2010).

In Ethiopia, data on human brucellosis is limited, but a few studies involving high-risk occupational groups like slaughterhouse and farm workers have yielded an average prevalence of 2.7% (Kassahun *et al*., 2006; Tibeso *et al.*, 2014). However, high seroprevalence (48.3% from Afar Region and 34.9% from Somali Region) of brucellosis was reported recently by Tschopp *et al.* (2021). Moreover, the highest seroprevalence of 48.8% was also reported from the Afar Regional state by Abdulkadir (2019).

The prevalence of infection in animal reservoirs is pivotal to understanding human occurrences Consequently, human infections result from direct contact with infected animals or their byproducts, as well as through the consumption of contaminated food products like milk, meat, or carcasses (Makita *et al.*, 2008). Thus, brucellosis poses an occupational risk for veterinarians, slaughterhouse employees, farmers, animal caretakers, and laboratory staff (Memish and Mah, 2001; Pappas *et al*., 2005). It is also recognized as the most prevalent laboratory-acquired infection globally (Weinstein and Singh, 2009) primarily due to the low infectious dose, estimated to be between 10–100 bacterial cells via aerosol or subcutaneous exposure (Mense *et al.*, 2004). Limited cases of human-to-human transmission have been documented (Godfroid *et al.*, 2005).

Asymptomatic infections may occur in humans. Clinically, the disease presents with highly variable and non-specific symptoms. Typically, brucellosis begins as an acute febrile illness, with non-specific flu-like symptoms such as fever, headache, fatigue, back pain, myalgia, and general discomfort (Seleem *et al*., 2010). Patients can experience severe night sweats, and potential complications include arthritis, spondylitis, chronic fatigue, and epididymo-orchitis. Neurological manifestations, including meningitis, uveitis, optic neuritis, and personality changes, may also arise, in addition to conditions such as anemia, internal abscesses, nephritis, endocarditis, and dermatitis. Other internal organs and tissues can be affected, leading to a wide array of symptoms (Radostits *et al*., 2007; OIE, 2016).

The economic burden of brucellosis on individuals stems from hospital treatment costs, medication expenses; out-of-pocket costs incurred by patients, and lost wages due to illness. Research by Felix *et al*. (2003) found that implementing mass vaccination for animals prevented a total of 49,027 disability-adjusted life years (DALYs) through achieving a 52% reduction in brucellosis transmission. However, the overall burden of human DALYs due to brucellosis on a global scale remains to be thoroughly assessed. The underreporting and underestimation of human brucellosis cases compared to animal brucellosis highlight its significance. It often presents as an acute febrile illness, frequently confused with malaria or typhoid fever (WHO, 2009).

The potential risk factors for human exposure to brucellosis include consumption of unpasteurized dairy products, direct contact with infected animals, placentas, or aborted fetuses, occupational exposure such as in pastoral communities, professionals having exposure to infected animals with brucellosis such as veterinarians, abattoire workers, and laboratory technologists exposued to *Brucella* while processing biological samples containing the pathogen, farmtakers or attendants, assisting during birthing or calving, illiteracy, and level of awareness of zoonosis and or brucellosis (Khoshnood *et al*., 2022; Narimisa *et al*., 2024).

2.8. Diagnosis

Accurate diagnosis of *Brucella* species infections is crucial for managing the disease in animals, which in turn affects humans. Clinical diagnosis of brucellosis often lacks reliability and is

primarily based on the history of reproductive issues in livestock; however, this remains a presumptive diagnosis that must be validated through laboratory methods (Fernando *et al*., 2010). Thus, laboratory testing plays a vital role in detecting and confirming the disease in animals, as well as accurately identifying it in humans. A definitive diagnosis is typically achieved by isolating and identifying the causative agent, which can determine the species and biovars of *Brucella* in biological samples ((Fernando *et al*., 2010). This definitive isolation process is time-intensive and requires highly trained personnel due to the associated hazards. Consequently, serological tests are generally preferred. Over the past twenty years, there has been significant progress in brucellosis serology, with the emergence of highly sensitive and specific new tests (Seleem *et al*., 2010). Additionally, modern genetic characterization methods for *Brucella* using molecular DNA technology have been developed. A variety of PCR-based assays have been introduced, enabling rapid genus identification and the differential detection of various species and strains (Seleem *et al*., 2010; Xavier *et al.,* 2010).

2.8.1. Direct Methods for Diagnosing Brucellosis

2.8.1.1. Bacteriological Diagnosis

The isolation of the *Brucella* organism is recognized as the gold standard for diagnosing brucellosis due to its specificity and capacity to enable the biotyping of isolates, which is significant from an epidemiological perspective (Bricker, 2002). Despite its high specificity, culturing *Brucella* species is not straightforward. These bacteria are fastidious and necessitate rich media for initial cultures. Additionally, successful isolation calls for a substantial quantity of viable bacteria in clinical specimens, appropriate incubation conditions, and prompt transportation to the diagnostic facility (Seleem *et al*., 2010; OIE, 2016). The handling of live cultures also carries the risk of laboratory exposure and potential infection. Consequently, molecular typing techniques have been adopted as alternatives to biochemical methods for the swift identification and characterization of *Brucella* species (Gopaul *et al*., 2007; Lopez-Goni *et al*., 2008; Matero *et al*., 2011). Several studies have highlighted the lower sensitivity of microbiological culture in comparison to PCR (Cortez *et al.,* 2001; Leary *et al.,* 2008; OIE, 2016).

In cultural diagnosis for brucellosis, the organism can be retrieved from various materials, typically depending on the clinical presentation. In animals, the placenta poses the highest risk of infection, containing the largest concentration of bacteria, followed by lymph nodes and milk, with blood being the primary source in humans (OIE, 2016). Other materials that may harbor the pathogen include stomach contents, spleen and lung tissues from aborted fetuses, vaginal swabs, semen, and joint or hygroma fluids from mature animals (Bricker, 2002). Vaginal secretions should be collected after abortion or parturition, ideally using a swab with a transport medium to facilitate sampling and isolation of the bacteria for up to six weeks post-delivery or abortion (OIE, 2016). Milk samples should be pooled from all four mammary glands, and non-pasteurized dairy products can also be used for *Brucella* isolation (Fernando *et al*., 2010; OIE, 2016).

For cultures taken from animal carcasses, the preferred tissues include the mammary gland, supramammary, medial and internal iliac, retropharyngeal, parotid, and prescapular lymph nodes, along with the spleen. During sampling from animal tissues, each specimen must be individually packaged, cooled, and shipped immediately in leak-proof containers to the laboratory (Bricker, 2002; Poester *et al.,* 2006). In humans, blood cultures are the primary material of choice, but specimens should be collected early in the disease process. Samples need to be maintained in a cold chain or frozen until they are ready for culture (OIE, 2016).

Typically, *Brucella* cultures are carried out on solid media. This approach is generally the most effective as it allows for the clear isolation and recognition of developing colonies while restricting the growth of non-smooth mutants and excessive contaminants. However, in cases of larger samples or when primary culture enrichment is required, liquid media might be recommended (OIE, 2016). Various commercial dehydrated basal media are available, such as *Brucella* medium base or tryptose soy agar (TSA).

To cultivate strains like *B. abortus* bv.2, the addition of 2–5% bovine or equine serum is essential, and many laboratories consistently add serum to basal media like blood agar or Columbia agar to achieve optimal results. Other viable media options include serum-dextrose agar (SDA) or glycerol-dextrose agar, with SDA often favored for observing colonial morphology. A non-selective, biphasic medium called Castañeda’s medium is suggested for the

isolation of *Brucella* from blood, body fluids, or milk, especially when enrichment culture is recommended due to the tendency of *Brucellae* to dissociate in broth media, which complicates traditional biotyping methods (OIE, 2018).

Field samples are frequently contaminated with other bacteria; hence, selective media are necessary to prevent overgrowth by faster-growing organisms. All previously mentioned basal media can be modified for selective purposes, with appropriate antibiotics added to inhibit the growth of non-*Brucella* organisms. The most commonly utilized selective medium is the modified Farrell's medium (FM) (Stack *et al*., 2002), which includes the following components per liter of agar: polymyxin B sulfate (5000 units = 5mg), bacitracin (25,000 units = 25mg), natamycin (50mg), nalidixic acid (5mg), nystatin (100,000 units), and vancomycin (20mg). A corresponding freeze-dried antibiotic supplement is commercially available. However, at the concentrations found in FM, nalidixic acid and bacitracin can inhibit certain strains of *B. abortus, B. melitensis,* and *B. suis* (OIE, 2016). Therefore, using FM along with the less selective Thayer–Martin's modified (mTM) culture media has been established as an effective strategy for the primary isolation of *Brucella* from field veterinary samples. Nevertheless, the mTM is not suitable for direct observations of colonial morphology because of the opaque nature caused by the hemoglobin in its basal component, which is critical for the presumptive identification of *Brucella* (Miguel *et al*., 2011).

A recently developed selective and translucent culture medium, termed CITA, has been formulated. CITA is created using blood agar base as a basal component, supplemented with 5% sterile calf serum and containing vancomycin (20mg/litre), colistin methanesulfonate (7.5mg/litre), nitrofurantoin (10mg/litre), nystatin (100,000 International Units (IU)/litre), and amphotericin B (4mg/litre) (Miguel *et al*., 2011). This new medium inhibits most contaminant microorganisms while allowing for the growth of all *Brucella* species. It has proven to be more sensitive than both mTM and Farrell's media in isolating all smooth *Brucella* species from environmental samples, establishing it as the selective medium of choice for comprehensive *Brucella* isolation. Optimal diagnostic sensitivity can be achieved by utilizing both FM and CITA in tandem (OIE, 2018).

After 48-72h of incubation at 37°C, *Brucella* colonies are usually 0.5 to 1.0 mm in diameter with a convex and circular outline. Smooth strains are transparent and pale yellow, resembling droplets of honey with a shiny surface when observed in transmitted light. Rough colonies are opaquer with a granular surface. The 'smooth' or 'rough' colony morphology is exhibited depending on LPS structure (Whatmore, 2009). The 'smooth' phenotype is due to the presence of a complete LPS which is composed of lipid A, a core oligosaccharide and an O-side chains. Most *Brucella* species are considered 'smooth' although 'rough' mutants can occur especially following repeated laboratory subculture. *B. ovis* and *B. canis* are naturally occurring 'rough' species (Whatmore, 2009).

The dissociation of *Brucella* can be identified by emulsifying a colony in a 0.1% w/v solution of aqueous acriflavine. Smooth colonies generate a homogeneous yellow suspension, while rough colonies result in granular agglutinations. Additional variations in colonies can be observed by inspecting the plates under angled lighting after staining the colonies with crystal violet (Whatmore, 2009). Smooth colonies exhibit a translucent and pale yellow appearance, whereas rough colonies are stained red, purple, or blue, displaying an opaque and granular texture. The identification of a culture as belonging to the genus Brucella is based on characteristics such as colonial morphology, staining, slide agglutination using anti-*Brucella* serum (for both smooth and rough types), as well as urease, catalase, and oxidase tests (OIE, 2016). Once the culture is confirmed to be *Brucella*, it is crucial to classify the specific species and biovars, which should ideally be performed in specialized or reference laboratories. This further classification involves complex tests including CO_2 requirements, hydrogen sulfide (H_2S) production, sensitivity to dyes (like thionin and basic fuchsin), phage lysis, and agglutination with specific A, M, or R antisera. In some instances, the oxidative metabolic method might also be required (OIE, 2016).

2.8.2. Molecular Diagnosis

Molecular methods serve as crucial instruments for both diagnostic purposes and epidemiological research, offering valuable insights for the identification of species and biotypes within the *Brucella* genus. These techniques enable the distinction between virulent strains and those used in vaccines (Xavier *et al.,* 2010). The molecular identification of *Brucella* species can be performed directly on clinical specimens without the need for prior isolation of the bacteria.

Furthermore, these approaches can augment the findings derived from phenotypic assessments (Xavier *et al.*, 2010).

The Polymerase Chain Reaction (PCR) has emerged as the preferred diagnostic method for detecting *Brucella* DNA at various levels, including genus, species, and biovar. A wide range of PCR techniques has been developed, with applications spanning from disease diagnosis to the characterization of field isolates for epidemiological studies, including taxonomic research (Fernando *et al*., 2010). PCR, along with its variations that amplify specific genomic sequences of *Brucella* species, has become the most commonly utilized molecular technique for diagnosing brucellosis (Bricker, 2002; OIE, 2016).

Standard PCR assays typically utilize a pair of primers to amplify the target genomic sequences found in *Brucella* spp. Commonly employed primer pairs target sequences that encode 16S rRNA (Xavier *et al.,* 2010), outer membrane proteins (such as omp2a, omp2b, and omp31) (Xavier *et al.,* 2010), the immunogenic 31 kDa protein from *B. abortus* (BCSP 31 B4/B5) (Cloeckaert *et al*., 2000), the interspace region of 16S-23S ribosomal DNA (ITS66/ITS279) (Keid *et al*., 2007), and the insertion sequence IS711 (Cloeckaert *et al*., 2000; Elfaki *et al.,* 2005)

The initial species-specific multiplex PCR known as the AMOS-PCR assay is designed to identify and differentiate between *B. abortus* biovars 1, 2, and 4, as well as *B. melitensis*, *B. ovis*, and *B. suis* biovar 1. This differentiation is based on the polymorphisms that arise from the species-specific localization of the insertion sequence IS711 within the *Brucella* chromosome (Fernando *et al*., 2010). Enhancements to this method were made by adding extra strain-specific primers to the primer mix, allowing for the identification of the vaccine strains *Brucella abortus S19* and RB51. A subsequent refinement of this protocol, termed BaSS-PCR (*Brucella abortus* Strain Specific PCR assay), was created to identify and differentiate field strains of *B. abortus* biovars 1, 2, and 4 from vaccine strains and other *Brucella* species found in cattle (Fernando *et al*., 2010). Additionally, a new primer was developed, which, when used alongside the IS711 AMOS primer, facilitated the PCR identification of isolates from *B. abortus* biovars 3, 5, 6, and 9 (Fernando *et al*., 2010; OIE, 2016)

In addition to the widely utilized PCR assays, a novel Multiplex-PCR assay has been created that specifically detects *B. neotomae, B. pinnipedialis, B. ceti,* and *B. microti*. It further distinguishes between *B. abortus* biovars 1, 2, and 4 from biovars 3, 5, and 6, as well as differentiating *B. suis* biovar 1 from biovars 2, 3, 4, and 5 (David *et al*., 2006). A Bruce-ladder multiplex PCR assay was also introduced for the identification and differentiation of classical *Brucella* species, including those associated with marine mammals and vaccine strains, all within a single assay (David *et al*., 2006). However, subsequent assessments of this assay indicated its inability to differentiate certain *B. canis* strains from *B. suis* (Lopez-Goni *et al*., 2008). Ongoing improvements to the Bruceladder Multiplex PCR assay have enabled the identification of the nine recognized *Brucella* species, including *B. microti, B. ceti, B. inopinata*, and *B. pinnipedialis* (Mayer-Scholl *et al*., 2009). Furthermore, the previously ambiguous identification of some *B. canis* strains as *B. suis* has been rectified by substituting the primers. BMEI1436f/BMEI1435r in the initial primer mix with the updated primers BMEI1426/1427 (Lopez-Goni *et al*., 2011).

Real-time PCR offers a quicker and more sensitive alternative to traditional PCR methods. It eliminates the need for post-amplification handling of PCR products, which minimizes the chances of laboratory contamination and reduces the likelihood of false-positive results. Recently, real-time PCR assays have been reported for the detection of *Brucella* cultured cells (Redkar *et al*., 2001) as well as for the identification of *Brucella* in urine (Queipo-Ortuño *et al.,* 2005), blood, and paraffin-embedded tissues (Kattar *et al.,* 2007).

To specifically detect seven biovars of *B. abortus*, three biovars of *B. melitensis*, and biovar one of *B. suis*, three distinct real-time PCR assays were created utilizing fluorescence resonance energy transfer. The forward primers for these real-time PCR assays were sourced from the insertion element IS711, while the reverse primers and FRET probes were chosen from unique chromosomal locations specific to each species or biovar. The *B. abortus*-specific assay demonstrated a sensitivity as low as 0.25 pg of DNA, which equates to 16-25 genome copies, with comparable detection limits also noted for the *B. melitensis* and *B. suis* specific assays (Redkar *et al*., 2001).

The superiority of real-time PCR regarding its sensitivity and specificity has been extensively documented across various studies. For instance, Queipo-Ortuno *et al*. (2005) conducted real-time PCR using SYBR Light Cycler Green I on blood cultures, serum samples, and whole blood from brucellosis patients, employing primers B4 and B5 that target the bcsp31 gene. Their findings were compared with those obtained from PCR-enzyme linked immunosorbent assay (Kattar *et al.,* 2007), revealing superior sensitivity of real-time PCR in serum samples. Surucuoglu *et al.* (2009) utilized the TaqMan real-time PCR method, focusing on the IS711, bcsp31, and per genes in patients exhibiting different clinical manifestations of brucellosis; they also contrasted their results with various conventional methods based on serum samples. Their assay targeting IS711 proved to be the most sensitive, specific, efficient, and reproducible for detecting *Brucella* species. Numerous studies have further confirmed the rapid results, specificity, and sensitivity of various real-time PCR assays (Navarro-Martínez *et al*., 2008; Colmenero *et al.*, 2011).

Single nucleotide polymorphism (SNP) analysis serves as a powerful tool for accurately portraying the phylogenetic relationships within a species, especially within a genetically stable group like *Brucella*. This methodology relies on a series of discrimination assays examining SNPs that are distinctive to specific *Brucella* species. Scott *et al.* (2007) demonstrated the application of SNPs to create a multiplex SNP detection assay using primer extension technology, enabling rapid and clear identification of an isolate as belonging to one of the six common *Brucella* species or the newly identified marine mammal group (Foster *et al*., 2008). An alternative method was also introduced for the rapid, straightforward, and unequivocal identification of *Brucella* at the species level, as well as distinguishing vaccine strains through minor groove binding protein (MGB) probes implemented on a real-time PCR platform (Foster *et al*., 2008; Gopaul *et al*., 2013).

Multilocus Sequence Typing (MLST) and Multiple Loci Variable Number of Tandem Repeats Analysis (MLVA) have been found to be highly discriminatory, effectively clustering strains according to currently acknowledged *Brucella* species and biovars. These techniques have further been utilized to pinpoint subtypes within each species or biovar based on geographic origin or host specificity (Vergnaud *et al*., 2018). Several studies have indicated that MLVA

typing is particularly beneficial in outbreak and epidemiological investigations (Kiliç *et al.,* 2011; Garofolo *et al*., 2013). However, MLVA and MLST have been reported to be inadequate in detecting new mutations arising from novel clades (Jeffrey *et al*., 2009). Whole genome sequencing has been recognized as a robust and unbiased method for elucidating relationships within closely related species, such as those in the *Brucella* genus (Alice *et al*., 2012; Wattam *et al.,* 2014).

2.8.3. Indirect Diagnostic Methods for Brucellosis

2.8.3.1. Serological Testing

Serological tests play an essential role in the laboratory diagnosis of brucellosis, as they form the backbone of many control and eradication initiatives. These tests utilize inactivated whole bacteria or purified components, such as lipopolysaccharides or membrane proteins, to serve as antigens for identifying antibodies produced by the host in response to infection. Antibodies directed against smooth *Brucella* species, including *B. abortus, B. melitensis*, and *B. suis*, exhibit cross-reactivity with antigens derived from *B. abortus.* In contrast, antibodies targeting rough *Brucella* species, such as *B. ovis and B. canis,* only cross-react among themselves (OIE, 2016).

There are several serological assays that focus on either whole-cell antigens or sLPS (Nielsen *et al.,* 2010). Generally, serological tests are regarded as the primary method for initial screening. However, a notable limitation is the potential for cross-reactivity with organisms sharing similar sLPS, which include *Yersinia enterocolitica*, *Vibrio cholerae*, *Ochrobactrum anthropi, Salmonella enterica serotype Urbana, Francisella tularensis,* and *Escherichia coli* O157:H7. This overlap can lead to false positive serological reactions (Godfroid and Käsbohrer, 2002) and various factors can cause false negative results, such as delayed seroconversion, blocking antibodies, and the prozone phenomenon (Pappas *et al*., 2006).

To effectively validate serological tests, results should be interpreted in light of the true infectious status of the animal. Detecting anti-*Brucella* antibodies indicates exposure to *Brucella* spp. but does not clarify which specific species triggered the antibody response. Additionally, seropositivity does not necessarily imply that the animal is currently infected at the time of testing (Godfroid, 2002). Research into both experimental and natural infections has revealed

that nearly all animal species susceptible to *Brucella* can lose their antibody titers over time, suggesting that the actual prevalence of brucellosis could surpass what is identified through antibody screenings. Therefore, the "gold standard" for diagnosing brucellosis remains the isolation of *Brucella* spp. through culture. In suspected cases of brucellosis in livestock or wildlife indicated by positive serological results, isolating the pathogen is mandatory and should always be pursued (Godfroid *et al.,* 2010).

Nonetheless, it has been found that while culture from human clinical samples demonstrates 100% specificity, its sensitivity is often low, particularly in chronic cases (Lulu *et al*., 1989). Cultures from individuals with acute brucellosis can show sensitivity levels ranging from 50% to 80%, whereas chronic cases yield positive cultures in less than 5% of instances (Rhyan *et al*., 2009; Hull and Schumaker, 2018). A study conducted in the United States revealed that only 30-50% of seropositive animals are culturable, raising questions about the fate of the remaining 50-70% of seropositive animals that are negative in cultures (Hull and Schumaker, 2018). The presence of antibodies against *Brucella* also varies with the infection stage, where specific IgM antibodies are prevalent during the acute phase, while IgG antibodies appear in the serum during later stages and in relapsing patients (Smits *et al.,* 2003). ELISA testing has been noted for its ability to differentiate between IgM and IgG antibodies, providing a general assessment of the illness's stage (Mohan *et al*., 2016).

Although numerous serological tests are currently in use, they can be categorized as screening tests (e.g., Rose Bengal Plate Test - RBT), monitoring or epidemiological surveillance tests (e.g., milk ring test), and complementary or confirmatory tests (e.g., 2-mercaptoethanol, complement fixation tests, ELISAs, and fluorescence polarization assays). The selection of a specific test should consider the species involved and adhere to local regulations (Nielsen *et al.*, 2010).

Slow Agglutination Test (SAT/SAW): The Slow Agglutination Test, or Slow Agglutination of Wright, is designed primarily to identify agglutinin antibodies, particularly of the IgM type, that target *Brucella* species under optimal conditions of antigen and antibody concentration. This test forms substantial antigen-antibody complexes that settle at the bottom of the test tube; the process is protracted as it requires overnight incubation at 37°C, unlike quicker agglutination

tests. A micro-method, known as the micro agglutination test, can also be employed, utilizing a reaction volume of 100 µL without sacrificing performance. The addition of a dye to stain the cells aids in reading the results (Poester *et al.,* 2010). The specificity of this test can be enhanced by treating serum with a chelating agent, such as EDTA, which mitigates cross-reactivity due to IgM. Although it is no longer endorsed by the OIE for diagnosing bovine brucellosis, it is still frequently utilized in diagnosing human brucellosis (Godfroid *et al.,* 2010; OIE, 2016).

Buffered *Brucella* Antigen Tests: The Rose Bengal Plate Test (RBPT) and Buffered Plate Agglutination (BA) tests are well-established buffered *Brucella* antigen tests characterized by rapid agglutination within just 4 minutes on a glass surface, facilitated by an acidic-buffered antigen (pH 3.65 ± 0.05). These tests have been adopted widely in many countries as a standard screening method due to their simplicity and perceived greater sensitivity compared to the SAT. According to the OIE, these tests are classified as "prescribed tests for trade" (Bricker, 2002; OIE, 2018).

The Rose Bengal Plate Test (RBPT) is a widely used diagnostic method for the detection of brucellosis, a zoonotic disease caused by the *Brucella* species. It is a serological test that detects antibodies against *Brucella* antigens in the blood, particularly immunoglobulin M (IgM) and immunoglobulin G (IgG), which are produced in response, to infection (Godfroid *et al*., 2011). This test is favored for its simplicity, rapid results, and cost-effectiveness, making it ideal for use in both field settings and laboratory diagnostics. The RBPT is based on the agglutination reaction, where the serum sample containing *Brucella* antibodies is mixed with a suspension of *Brucella* antigens (usually *B. abortus* or *B. melitensis*), which are sensitized with the Rose Bengal stain. If the serum contains specific antibodies against *Brucella*, these antibodies will bind to the antigens, forming visible agglutination. A positive result is characterized by the formation of clumps, while a negative result is indicated by a clear suspension without clumping (OIE, 2018).

The RBPT is with a number of advantages. Firstly, its simplicity and speed are the primary attribute. It requires minimal equipment, with results available in less than 30 minutes. This makes it particularly useful in field conditions or remote areas where laboratory resources may

be limited (Corbel, 2006). Secondly, its cost-effectiveness, the test is inexpensive compared to other diagnostic methods like culture or PCR. This cost-effectiveness makes it an attractive option for large-scale screening in areas with limited financial resources (Ferguson, 2013). Thirdly, its sensitivity to detect antibody produced against *Brucella*. The RBPT has shown high sensitivity and good specificity when used in the detection of brucellosis in cattle, sheep, goats, and humans (Godfroid *et al*., 2011). While it may not differentiate between active and past infections, it is effective in identifying exposed individuals (Fergusen, 2013). For field use, it is a valuable tool, especially when combined with other diagnostic methods, such as the complement fixation test (CFT) or the enzyme-linked immunosorbent assay (ELISA). Furthermore, RBPT has good adaptability. The test can be adapted for use in various species, including livestock and humans. This makes it useful for zoonotic surveillance and control programs in countries where brucellosis is endemic (Godfroid *et al*., 2011).

However, the RBPT is not without limitations. Its main limitations include: cross-reactivity leading to false positive, false negative result, and lack of differentiation. One of the limitations of the RBPT is the cross-reactivity with other pathogens that can cause similar immune responses. For example, *Yersinia enterocolitica* and *Francisella tularensis* may induce false-positive reactions due to the similarities in their antigens (Alton *et al.,* 1988). This is particularly problematic in areas where co-infection with other bacteria is common. The other limitation of RBPT is false-negative results. In chronic or early-stage infections, the RBPT may produce false-negative results due to insufficient antibody levels in the serum. This can occur if the immune response is not yet fully developed or if the animal or human is in the incubation period of infection (Ferguson, 2013). In addition, lack of differentiation is the potential limitation of the RBPT. The RBPT is not capable of distinguishing between active infection and past exposure, and it cannot provide information about disease severity. For example, an individual who has been vaccinated against brucellosis may produce antibodies detectable by the RBPT, leading to a false positive if they have not been actively infected (Corbel, 2006; Godfroid *et al.,* 2011).

The RBPT has wider area of application for screening of brucellosis in livestock, human surveillance, epidemiological studies, and zoonotic surveillance. The RBPT is often used in livestock populations for large-scale screening, especially in areas where brucellosis is endemic.

The test is frequently employed in disease surveillance programs to monitor infection rates in animals and to help control the spread of brucellosis in cattle, sheep, and goats. In human cases, the RBPT is used to identify individuals who have been exposed to *Brucella*, particularly in high-risk populations, such as farmers, slaughterhouse workers, and veterinarians. This test is especially useful in developing countries, where access to more advanced diagnostic tools is often limited (Godfroid *et al*., 2011). The RBPT has been widely employed in epidemiological surveys in countries where brucellosis is a public health concern. It can provide a rapid assessment of disease prevalence, helping guide decisions on vaccination programs and other public health interventions. Due to the zoonotic nature of brucellosis, the RBPT is also used to monitor transmission between animals and humans, aiding in the identification of hotspots for brucellosis transmission and the development of targeted control measures (Ferguson, 2013).

Complement Fixation Test: The Complement Fixation Test (CFT) detects anti-*Brucella* antibodies capable of activating complement. The relevant immunoglobulins in cattle that can engage bovine complement are IgG and IgM. Some literature suggests that while this test may not exhibit high sensitivity, it possesses excellent specificity. However, due to challenges in standardization, it is increasingly being supplanted by ELISA tests (Godfroid *et al.,* 2010). The OIE also classifies this test as a "prescribed test for trade" (OIE, 2016).The Complement Fixation Test (CFT) is a serological assay extensively used for diagnosing brucellosis, a zoonotic disease caused by *Brucella* species. The CFT is highly reliable and specific, detecting antibodies in the serum of infected individuals, typically in both humans and animals. This diagnostic tool is based on the ability of antibodies in a serum sample to fix complement in the presence of *Brucella* antigens, allowing for accurate detection of infection.

The test works by detecting antibodies that bind to specific *Brucella* antigens. The process involves several key steps: antigen preparation, serum sample addition, complement addition, and finally, the addition of indicator red blood cells. Initially, a suspension of *Brucella* antigens, usually from *B. abortus* or *B. melitensis*, is prepared. A serum sample is then added to the antigen suspension, and if specific antibodies (IgM and IgG) are present, they will bind to the *Brucella* antigens. Following this, complement proteins, which aid in the immune response, are introduced to the reaction. If antibodies are present, they will fix the complement. The

complement-bound immune complexes then cause the lysis (destruction) of red blood cells that have been previously coated with an indicator substance. The degree of hemolysis is measured, with no hemolysis indicating a positive result and partial hemolysis suggesting a negative or weakly positive result (OIE, 2018).

One of the key strengths of the CFT is its high specificity, which allows it to accurately detect brucellosis without cross-reacting with other similar zoonotic diseases, such as *Yersinia enterocolitica* or *Francisella tularensis*. These pathogens can interfere with less specific tests, like the Rose Bengal Plate Test (RBPT) (Corbel, 2006). Due to its accuracy, the CFT is often regarded as the gold standard in brucellosis serology, especially for detecting active infections and high antibody titers, which are common in chronic brucellosis or cases of disease exacerbation. It is particularly effective in detecting IgG antibodies, which are indicative of long-standing infections (Alton *et al*., 1988; Godfroid *et al.,* 2011). Additionally, the CFT provides quantitative results, offering a more detailed picture of the infection's progression and severity. It can determine antibody titers, which is useful for monitoring disease dynamics, assessing treatment efficacy, and evaluating vaccination programs (Pappas *et al*., 2006). The test is often used to confirm suspected cases of brucellosis following initial screening with simpler tests, such as the RBPT or enzyme-linked immunosorbent assay (ELISA), providing a definitive diagnosis.

The Complement Fixation Test (CFT) has several limitations. First, it is labor-intensive and time-consuming, which makes it less suitable for rapid field diagnosis or for use in settings with limited laboratory infrastructure (Ferguson, 2013). Additionally, the test requires skilled technicians to accurately perform and interpret the results (Corbel, 2006). While CFT is more specific than some other serological tests, it can still cross-react with other infections that cause similar immune responses, particularly in cases of co-infection or chronic infection, making it difficult to differentiate brucellosis from other diseases (Godfroid *et al*., 2011). Furthermore, the CFT is often not sensitive in the early stages of infection (Ferguson, 2013). Finally, it must be conducted under standardized conditions to ensure accurate results, as variations in the laboratory environment, such as temperature, can affect the complement fixation reaction and lead to inconsistent results (Pappas *et al*., 2006)

Enzyme Linked Immunosorbent Asay Tests (ELISA): ELISA tests are categorized into two types: indirect ELISA (I-ELISA) and competitive ELISA (c-ELISA). The majority of iELISA utilize purified smooth lipopolysaccharides (LPS) as antigens, though there is considerable variation in the anti-bovine immunoglobulin conjugates applied (Godfroid *et al.,* 2010). Most I-ELISA primarily target IgGs or IgG subclasses. Their standout feature is their high sensitivity; however, they are also subject to non-specific reactions, particularly those associated with *Yersinia enterocolitica O:9* infections. These cross-reactivity issues observed in iELISA have led to the development of c-ELISA. The O-chain of the smooth LPS from *Brucella* has specific epitopes not found in the LPS of *Yersinia enterocolitica* O:9. Consequently, by employing monoclonal antibodies that are specific to certain epitopes within the LPS of *Brucella*, more precise c-ELISA methods have been developed. Although these tests are more specific, they are less sensitive compared to I-ELISA (Poester *et al.,* 2010). The OIE classifies these tests as "prescribed tests for trade" (OIE, 2018).

The use of cELISA in addition to, or as a replacement for, conventional tests for brucellosis offers many benefits. The cELISA procedures for detection of porcine antibody to *Brucella* spp. are identical to those used for bovine antibody to *B. abortus* and *B. melitensis*, making the test valid for multiple animal species. This assay is capable of eliminating some reactions due possibly to *Y. enterocolitica serotype 0.9* or other cross-reacting antibodies, such as IgM, which have lower affinity for *Brucella* epitopes than does the mAb used in the assay (Riber and Jungesten, 2007). Competitive ELISA is a prescribed test for international trade but none of the conventional serological tests has been shown to be entirely reliable for routine diagnosis in individual pigs (OIE, 2008). Competitive ELISA is a rapid assay, it is faster than CFT, and it can be automated and therefore the results can be measured objectively. Competitive ELISA can be used on a variety of animal species, and an added advantage is its suitability for use on poor-quality samples such as those affected by hemolysis.

I-ELISA is based on the binding of antibodies (usually IgG) present in the test serum to *Brucella* antigens coated on a microplate. After binding, an enzyme-conjugated secondary antibody is added, followed by a substrate that produces a color change proportional to the antibody concentration. The test's high specificity and sensitivity make it invaluable for detecting chronic

and active brucellosis infections (OIE, 2016; Godfroid *et al*., 2011). For both animal and human cases, I-ELISA offers several advantages, including the ability to detect low antibody titers, particularly in chronic infections where other serological tests might fail. It also allows for the detection of antibodies specific to various *Brucella* species, making it more effective than tests like Rose Bengal Plate Test (RBPT), which cannot distinguish between species (Pappas *et al*., 2006; Bricker, 2002).

The I-ELISA is highly reliable in cattle and small ruminants, which are the primary reservoirs for *Brucella* species. While it demonstrates high sensitivity (often exceeding 90%) and specificity (ranging from 95% to 98%), its effectiveness can be influenced by factors such as the stage of infection, antigen preparation, and sample type (Alton *et al*., 1988). Recent studies suggest that combining I-ELISA with other diagnostic methods (e.g., PCR or *Brucella*-specific antigen assays) can enhance diagnostic accuracy, particularly in endemic areas.

Despite its advantages, I-ELISA has some limitations. It may show reduced sensitivity in detecting early-stage infections and is not useful for differentiating between current infection and vaccination. Cross-reactivity with other zoonotic infections, such as *Yersinia enterocolitica*, can also complicate the interpretation of results (Pappas *et al*., 2006; Nielsen & Gall, 2001). Moreover, its use in milk testing shows reduced sensitivity compared to serum samples. However, studies indicate that using pooled milk samples can help mitigate this issue (OIE, 2016).

Recent advancements in I-ELISA technology include the development of recombinant antigens and more standardized assays, which are expected to improve the sensitivity and specificity of the test. The introduction of lateral-flow I-ELISA methods, offering quicker turnaround times and field applications, is a promising direction for brucellosis diagnosis (Abd El-Ghany *et al.*, 2021). Additionally, the growing adoption of high-throughput screening techniques, including multiplex I-ELISA, is increasing the ability to test large numbers of animals, which is particularly beneficial in epidemiological surveys and mass vaccination programs.

The Fluorescence Polarization Assay (FPA) detects *Brucella* antibodies by measuring light depolarization, with larger molecules causing less depolarization. It is fast and automatable, offering rapid results (Nielsen & Gall, 2001; Godfroid *et al*., 2010). The Milk Ring Test detects *Brucella* antibodies in milk by forming a purple ring when complexes form, though its sensitivity is low (Nielsen & Gall, 2001; OIE, 2018). ELISA and FPA are also used for milk, with reduced sensitivity compared to serum (Nielsen & Gall, 2001). The Skin Test assesses immune response to *Brucella*, suitable for herd assessments but not individual certification (Godfroid *et al*., 2010; OIE, 2018). The Skin Test detects *Brucella* infection by inducing a localized inflammatory response through the injection of brucellergen. It is specific for identifying true brucellosis cases but has low sensitivity, limiting its use for individual certification. It's more suitable for herd assessments and cannot distinguish between infection and vaccination (Godfroid *et al.,* 2010; OIE, 2018).

2.9. Epidemiology

The global landscape of brucellosis is in a state of flux, with new or re-emerging outbreaks being reported. The epidemiological patterns of human brucellosis have shifted significantly over time due to a variety of health, economic, and political factors, as well as an increase in international travel. Recently, fresh instances of human brucellosis have been recorded, particularly in Central Asia (Seleem *et al.,* 2010). In animals, the disease is prevalent across the globe, with the exception of nations that have successfully eradicated it, defined as having no reported cases for a minimum of five years. These nations include Australia, Canada, Cyprus, Denmark, Finland, the Netherlands, New Zealand, Norway, Sweden, and the United Kingdom. Regions such as Mediterranean, Europe, northern and eastern Africa, countries in the Near East, India, Central Asia, as well as Central and South America still grapple with the disease. Although *B. melitensis* has never been identified in certain countries, there is a lack of conclusive evidence that it has been completely eliminated from small ruminant populations in any nation aside from those listed above (Robinson, 2003).

In sub-Saharan Africa (SSA), the introduction of exotic animal breeds with improved productivity is somewhat limited by infectious diseases, one of which is bovine brucellosis (Mcdermott and Arimi, 2002). There is a considerable amount of serological evidence indicating

the prevalence of brucellosis across SSA. Reports have documented the occurrence and impact of brucellosis in cattle from various countries, including Ethiopia, Egypt, Kenya, Uganda, Zambia, and Zimbabwe (Holt *et al.,* 2011; Megersa *et al.,* 2011; Muendo *et al*., 2012). However, this information tends to be inconsistent both temporally and geographically, and high-quality data suitable for rigorous epidemiological analysis are relatively lacking (Mcdermott and Arimi, 2002; Ducrotoy *et al.,* 2014). More than a decade ago, (McDermott and Arimi, 2002) cautioned that the reported figures should be interpreted carefully due to uncertainties around test implementation and validation. Despite these diagnostic challenges, several studies in Africa suggest that the individual seroprevalence of brucellosis may be associated with the incidence of abortions (Mcdermott and Arimi, 2002; Megersa *et al.,* 2011).

The debate continues over whether brucellosis seroprevalence is greater under extensive or intensive breeding systems (Ducrotoy *et al.,* 2014). In their review, McDermott and Arimi (Mcdermott and Arimi, 2002) noted that individual seroprevalence figures for cattle vary significantly across sub-Saharan African pastoralists, ranging from 7.5% to 40% in arid and semi-arid environments, 0.3% to 25.4% in cash/subsistence crop-livestock systems in sub-humid areas, and 1.5% to 16.2% in crop-livestock systems situated in tropical highland regions. This broad spectrum of values implies that, based on early findings, it is challenging to draw general conclusions about the significance of these production systems concerning individual prevalence, a point already highlighted by Mangen *et al.* (2011).

Information regarding brucellosis in small ruminants in SSA is limited. It is widely believed that brucellosis in these species is primarily caused by *B. melitensis*, but due to the scarcity of bacteriological studies, various epidemiological scenarios remain possible (Mcdermott and Arimi, 2002; Ducrotoy *et al.,* 2014). The few recent investigations typically indicate a low individual prevalence, which may signify the presence of chronically infected flocks and herds. However, there has been a notable lack of reports on flock or herd prevalences, which are crucial for understanding the broader implications of the disease and assessing potential risks associated with changing breeding practices and epidemiological conditions (Muma *et al*., 2012).

In arid and semi-arid regions of the East Africa, camels frequently react positively in serological tests for brucellosis, usually indicating a low individual seroprevalence (Mcdermott and Arimi, 2002; Megersa *et al.*, 2011; Wesinew *et al*., 2013; Ducrotoy *et al.*, 2014; Ducrotoy *et al.*, 2017). Nevertheless, herd seroprevalence in camels has been reported to be relatively high, with figures of 15% in Borana, Southern Ethiopia (Megersa *et al.*, 2012), and 24% in Afar, Eastern Ethiopia (Adugna *et al*., 2013). However, it is important to note that the tests utilized in these surveys have not been validated for camels (Sprague *et al*., 2012).

2.9.1. Bovine Brucellosis in Ethiopia

Brucellosis has been documented in cattle across various regions of Ethiopia, affecting both intensive and extensive farming practices. Research indicates a seroprevalence rate of 38% for bovine brucellosis in western Ethiopia (Rashid, 1993), although the majority of studies have reported a lower seroprevalence of less than 5% in cattle, as outlined in (Table 2).

Table 2: Seroprevalence of Bovine brucellosis across various regions of Ethiopia

Study Area	Number Tested	Prevalence (%)	95% Confidence Interval	Type of Test	Production System	Author(s)
Central Oromia	1,238	2.9	2.0-4.0	RBT, CFT	Extensive & Intensive	Jergefa *et al.* (2009)
Tigray Region	816	3.19	2.0-4.6	RBT, CFT	Extensive	Berhe *et al*. (2007)
Central High Land of Ethiopia	352	0.6	0.0016–0.0209	CFT	Extensive &Intensive	Temesgen *et al*. (2021)
Jimma Zone	1,595	3.1	2.2-4.0	RBT, CFT	Extensive & Intensive	Ibrahim *et al.* (2010)
Jigjiga Zone	435	1.38	0.5-3.0	RBT, CFT	Agro-pastoral	Degefu *et al.* (2011)
West Tigray	1,354	4.9	3.7-6.2	RBT, CFT	Extensive & Intensive	Haileselassie *et al.* (2011)
Southern & Eastern Ethiopia	1,623	3.5	2.6-4.4	RBT, CFT	Extensive and Pastoral	Megersa *et al*. (2011)
Addis Ababa	1,202	1.5	0.8-2.4	RBT, CFT	Intensive	Tesfaye *et al*. (2011)
Borana	575	8.0	5.9-10.5	RBT, CFT	Pastoral	Megersa *et al.* (2012)
East Wollega	406	1.97	0.8-3.8	RBT, CFT	Extensive	Yohannes *et al*. (2012)
Western Ethiopia	1,152	1.0	0.5-1.8	RBT, CFT	Extensive	Adugna *et al.* (2013)
Northern Ethiopia	441	0.4	0.05-1.6	RBT, CFT	Intensive	Asmare *et al.* (2013)
Southern Ethiopia	719	3.2	2.0-4.7	RBT, CFT	Intensive	Asmare *et al.* (2013)
Central Ethiopia	567	1.6	0.7-2.9	RBT, CFT	Intensive	Asmare *et al.* (2013)
Somali & Borena	862	1.4	0.7-2.4	RBT, ELISA	Pastoral	Gumi *et al*. (2013)
Arsi	417	1.7	0.6-3.4	RBT, i-ELISA	Intensive	Rea *et* al. (2013)
East Shewa	300	2.0	0.7-4.3	RBT, CFT	Intensive	Alemu *et al*. (2014)
Alage	804	2.4	1.4-3.6	RBT, c-ELISA	Extensive and Intensive	Asgedom *et al*. (2016)
Asella & Bishoftu	570	1.4	0.6-2.7	RBT, CFT	Intensive	Minda *et al*. (2016)
Harar & Dire Dawa	967	1.3	0.7-2.2	RBT, CFT	Intensive	Terefe *et al*. (2016)
Adami Tullu	450	4.5	0.03-1.05	RBT,CFT	Intensive	Gebawo *et al.* (2014)
Nechsar Park	268	9.7	7.0-14.7	RBT/i-ELISA, CFT	Extensive	Chaka *et al*. (2018)
Sidama Zone	560	3.1	0.04-5.30	RBT, CFT	Extensive	Nuradis *et al.* (2009)
Addis Ababa	1,550	0.06	0.002-0.3	RBT, C-ELISA	Intensive	Edao *et al*. (2018)
Ambo	764	0.2	0.02-2.00	RBT	Extensive	Bashitu *et al.* (2015)

Study Area	Number Tested	Prevalence (%)	95% Confidence Interval	Type of Test	Production System	Author(s)
DebreBerhan	970	0.70	7.05-12.60	RBT,CFT	Intensive	Bashitu *et al.* (2015)
North West Gonder	960	4.90	0.070-3.01	CFT	Extensive	Alehegn *et al*. (2016)
North Shewa	384	0.78	0.23-9.23	RBT	Extensive	Pal *et al*. (2016)
Gomogofa zone	564	1.04	6.02-14.0	RBT	Extensive	Meles and Kibeb (2018)
Gambella Zone	384	3.1	3,20-9.45	CFT	Extensive	Tamirat *et al*. (2023)
In and around Adama	384	1.04	0.26, 18.78	CFT	Intensive	Bulcha *et al*. (2020)
Jimma	424	3.3	1.82-5.48	C-ELISA	Intensive/Extensive	Monenus *et al*. (2022)
Sendafa, Central Ethiopia	503	0.40	3.44–7.01	CFT	Intensive/Extensive	Bifo *et al*. (2020)
Jimma Zone	423	4.3	2.15–5.89	CFT	Extensive	Dereje and Benti (2020)
Bench Maji Zone	772	1.94	0.97-0.2. 92	CFT	Extensive	T Kenea, B Megersa (2021)
Borana	788	7.6	2.12-6.55	ELISA	Pastoral	Tilahun *et al*. (2022)
Afar Regional Stae	488	7.2	5.0-10.2	ELISA	Pastoral	Tschopp *et al*. (2021)
Somali Regional State	116	6.9	3.5-13.2	ELISA	Pastoral	Tschopp *et al*. (2021)

As can be seen on Table 2, above relatively higher seroprevalce of bovine brucellosis was recorded in pastol settings (8% from Borana as reported by Megersa *et al*., 2012; 7.2% from Afara Regional State reported by Techopp *et al*., 2021; 6.9% fro Somali Regional State reported by Techopp *et al.*, 2021) as compared to other farming system.

2. 9.2. Small Ruminant Brucellosis in Ethiopia

Various studies in Ethiopia have documented the presence of brucellosis in small ruminants. The majority of research on this subject has focused on pastoral livestock production systems, where sheep and goats are typically raised together. The reported prevalence of brucellosis in small ruminants varies, ranging from 0.4% in the mixed crop-livestock production system of Bahir Dar (Ferede *et al.*, 2011) to 13.7% in the pastoral region of Afar (Wedajo *et al.*, 2015).

Recent studies have highlighted notable epidemiological differences between two significant strains of the *Brucella* bacteria: *Brucella* melitensis, the primary agent behind brucellosis in goats, and *Brucella ovis,* which primarily affects sheep. Research conducted by Perez-Etayo *et al.* (2018) indicates variations in virulence, clinical manifestations, and growth requirements in vitro for these two species. Research findings suggest that goats exhibit a higher susceptibility to *Brucella* infections compared to sheep. This increased vulnerability may be partly attributed to the fact that sheep tend to excrete the bacteria for shorter durations than goats. As a result, the potential for disease transmission within and between sheep populations is likely lower, as noted by Radostits *et al.* (2007). The (Table 3) below summarizes the seroprevalence of brucellosis in sheep in Ethiopia.

Table 3: Seroprevalence of Ovine brucellosis in various regions of Ethiopia

Study Area	Sample Size Tested	Prevalence (%)	95% Confidence Interval	Testing Method	Production Type	Authors
Afar and Somali	928	5.6	8.4 - 11.1	i-ELISA	Extensive	Teshale *et al.*, 2006
Afar Region	563	3.2	3.8 - 6.0	CFT	Extensive	Ashenafi *et al.*, 2007
South Omo	384	4.2	2.4 - 6.6	RBT, CFT	Extensive	Ashagrie *et al.*, 2011
Bahir Dar	500	0.4	0.04 - 1.4	RBT, CFT	Extensive	Ferede *et al.*, 2011
South Omo, Jigjiga	421	1.2	0.7 - 2.6	CFT	Extensive	Bekele *et al.*, 2011
Dire Dawa	171	8.77	6.4 - 12.4	CFT	Extensive	Negash *et al.*, 2012
Yabello, Oromia	384	1.56	0.5 - 3.3	RBT, CFT	Extensive	Golo and MA, 2013
Southern Tigray	985	3.5	2.4 - 4.7	CFT	Extensive	Teklue *et al.*, 2013
Afar Region	1050	13.0	11.0 - 15.2	RBT, CFT	Extensive	Adugna *et al.*, 2013
Southern & Central	3315	1.9	1.4 - 2.4	RBT, CFT	Extensive	Asmare *et al.*, 2013
Somali and Oromia	420	3.6	2.0 - 5.8	RBT, CFT	Extensive	Tsehay *et al.*, 2014
Afar Region	414	13.7	10.5 - 17.4	RBT, CFT	Extensive	Wedajo *et al.*, 2015
Mojo Abattoir	853	1.76	1.0 - 2.8	RBT, CFT	Extensive	Tsegay *et al.*, 2015

Study Area	Sample Size Tested	Prevalence (%)	95% Confidence Interval	Testing Method	Production Type	Authors
Arsi and East Shewa	840	4.6	3.3 - 6.3	RBT, i-ELISA	Extensive	Abiot *et al.*, 2015
Afar Region	1190	12.4	10.5 - 14.4	RBR, CFT	Extensive	Tegegn & Feleke, 2016
Somali Region	291	1.37	0.4 - 3.4	RBT, CFT	Extensive	Mohammed *et al.*, 2017
Afar Region	825	1.8	1.1–3.1	CFT	Extensive	Sintayehu *et al.*, 2015
Borana	135	2.2	0.6–6.9	CFT	Extensive	Sintayehu *et al*., 2015
Afar Region	613	8.9	6.7–11.9	ELISA	Pastoral	Tschpp *et al*., 2021
Somali Region	243	6.6	4.0–10.	ELISA	Pastoral	Tschpp *et al*., 2021
West Hararghe	153	5.9	0.6-3.0	ELISA	Extensive	A.M. Wubaye *et al*. 2024

This noticeable wider range (0.4% to 13.7%) seroprevalence of ovine brucellosis presented in Table 3 above in Ethiopia, could be related to differences in production system, flock siz, sample size or number of tested animals, the type of tests used due to sensitivity and specificity variation.

Table 4.Seroprevalce of Caprine brucellosis from different areas of Ethiopia

Study area	Sample Size Tested	Prevalence (%)	95% Confidence Interval	Testing Method	Production Type	Authors
Oromia, Somali &Dire Dawa	510	7.8%	12.03-19.90	I-ELISA	Pastoral	Gumi *et al*., 2013
Dire Dawa	213	9.39%	0.45-0.87	CFT	Extensive	Negash *et al*., 2012
South Omo	384	4.2%	2.56-7.91	CFT	Pastoral	Ashagrie *et al*., 2011
Jigjiga, Hamer, Dasenech	309	1.5%	17.02-19.34	CFT	Pastoral	Bekele *et al*., 2011
Afar	1,005	5.8%	5.04-7.72	CFT	Pastoral	Ashenafi *et al.*, 2007
Afar & Somali	1,072	13.2%	0.23-3.40	I-ELISA	Pastoral	Teshale *et al*., 2006
Borana	213	2.8%	14.15-16.06	CFT	Pastoral	Dabassa *et al*., 2013
	3,694	3.1%	2.69–3.57	CFT	Pastoral	
Afar	2,294	3.9%	3.20–4.80	CFT	Pastoral	Sintayehu *et al*., 2015
Borana	474	4.4%	2.80–6.80	CFT	Pastola	Sintayehu *et al*., 2015
Somali Region	191	1.57%	0.32-4.50	CFT	Pastolal	Mohammed *et al*., 2017
Borana	667	3.7%	0.8-9.2	C-ELISA	Pastoral	Edao *et al*., 2020
Afar Region	1486	9.7%	8.3–11.7	ELISA	Pastoral	Tschpp *et al.,* 2021
Somali Region	980	9.5%	7.3–12.3	ELISA	Pastoral	Tschpp *et al.,* 2021
West Hararghe	291	6.9%	0.5–2.7	ELISA	Extensive	A.M. Wubaye *et al.* 2024
Borana	789	17.36%	4.78, 20.19	c-ELISA	Pastolal	Teshome *et al.*, 2022

The seroprevalence of caprine brucellosis in Ethiopia widely distributed in different parts across the regions, and production system (Table 4) ranging between the lower 1.5% (Bekele *et al*., 2011) and higher 17.36% (Teshome *et al*., 2022).

2.9.3. Human Brucellosis in Ethiopia

Research on human brucellosis in Ethiopia is limited when compared to studies on animal brucellosis, with even less available data regarding the risk factors associated with human infections. A review of the literature reveals that investigations into human brucellosis primarily took place in hospitals or health clinics and often involved patients presenting with febrile illnesses. Nevertheless, there is a scarcity of research focused on groups with occupational exposure. The prevalence and incidence rates of human brucellosis across different geographic areas in Ethiopia are summarized in (Table 5).

Table 5: Seroprevalence of human brucellosis across various regions of Ethiopia.

Study Area	Number Tested	Prevalence/ Incidence (%)	95% Confidence Interval	Type of Test	Sampled Population	Authors
West Gojjam	653	2.6	1.8-4.70	SAT	Febrile patients	Animut *et al.*, 2009
Yabello	88	34	24.3-45.0	IgM/IgG lateral flow assay	Febrile patients	Genene *et al.*, 2009
Matama	100	3	0.6-8.50	IgM/IgG lateral flow assay	Febrile patients	Genene *et al.*, 2009
Jimma	56	3.6	0.4-12.30	RBT/CFT	Febrile patients	Tolosa *et al*., 2007
Afar Region	200	15	10.3-20.7	RBT/CFT	Febrile patients	Zewolda & Wereta, 2012
Addis Ababa	336	4.8	2.7-7.60	2-Mercapto Ethanol Test	Occupationally linked	Kassahun *et al.*, 2006
Western Tigray	246	2.1	0.3-3.50	RBT/CFT	Occupationally linked	Haileselassie *et al*., 2011
Adami Tulu	93	2.1	0.3-7.50	RBT/CFT	Occupationally linked	Tibeso *et al.*, 2014
Bishoftu & Modjo	149	1.3	0.2-4.70	RBT/CFT	Abattoir workers	Tsegay *et al.*, 2017
Arba Minch	254	10.6	7.1-15.0	Standard Tube titration	Blood donors	Workalemahu *et al*., 2017
Sidama zone	360	5.8	3.45-9.0	CFT	Febrile patients	Asmare *et al.*, 2007
Borana	341	2.6	1.20-4.90	C-ELISA	Pastoralists	Edao *et al*., 2020

Study Area	Number Tested	Prevalence/ Incidence (%)	95% Confidence Interval	Type of Test	Sampled Population	Authors
Afar Region	384	15.8	12.7-19.7	CFT	Febrile patients	Ahmed *et al.*, 2008
Afar Region	594	48.3	43.9-52.7	ELISA	Pastoralists	Tschopp *et al*., 2021
Somali Region	216	34.9	28.1-42.4	ELISA	Pastoralists	Tschopp *et al*., 2021
Afar Region	376	31.5	9.12-16.05	ELISA	Patients	Mehari *et al*., 2021
Moyale,Southern Ethiopia	293	13	9.5-16.5	ELISA	Febrile Patients	Sileshi *et al*., 2024
Afar Region	630	4.4	0.65-1.78	CFT	Febrile Patients	Zerfu *et al*., 2018
Central Oromia	166	1.2	0.32–4.27	CFT	Owners & Farm workers	Temesgen *et al*., 2022
Somali Region	19	2.8	0.9–6.4.	ELISA		Ibrahim *et al.*, 2021
Afar Region	120	3.33	1.63-6.2	RBPT/CFT	Pastoralists	Wegi *et al*., 2021
Afar Region	172	48.8	41.20-56.3	I-ELISA	Pastoralists	Abdulkadir Mohammed, 2019

CFT=Complement fixation test. ELISA=Enzyme linked immunesorbant assay, RBPT=Rose Bengal plate test, I-ELISA=Indirect enzyme immunesorbant assay

2.9.4. Camel Brucellosis in Ethiopia

In Ethiopia, camels comprise a significant portion of the country's livestock, with an estimated population of 1.1 million. These animals are crucial for the pastoral and agropastoral communities, as they are exceptionally suited to thrive in the hot and arid climates that are challenging for other domestic animals (Tilahun *et al*., 2013). Traditionally, these communities raise camels primarily for their milk (Demeke, 1997). Despite the substantial number of camels found in the pastoral regions, the issue of camel brucellosis remains largely under-researched in Ethiopia. Initial studies on camel brucellosis (Domenech, 1977) conducted in the Sidamo, Harar, and Tigray provinces revealed an individual seroprevalence rate of 4.4% (n=977). Furthermore, Teshome *et al.* (Teshome *et al*., 2003) found a seroprevalence of 4.2% after testing 1,442 camels in the arid and semi-arid regions of Afar, Somali, and Borena, which are known for camel rearing. In the Borena lowland area, Megersa *et al*. (2005) and Megersa *et al*. (2011) reported the presence of anti-*Brucella* antibodies in 1.8% (58/3,218) and 2.2% (17/756) of the camels tested, respectively. In the southeast lowland regions of the Somali Area, Tilahun *et al*. (2013) documented individual and herd seroprevalence rates of 2.43% (n=822) and 10.3% (n=185), respectively. Wegari *et al*. (2021) have reported seroprevalence of 3.2% (95% CI: 1.63-6.2) by combined RBPT and CFT in camels (n=250) in pastoral area of Amibara distric of Afar Reginal State, Ethiopia.

2.10. *Bruella* species causing brucellosis in livestock and human

In different African countries *Brucella* species infecting both animals and human were reported. Efrem *et al. (*2024) have isolated and characterized *B.abortus* and *B.melitensis* from animals with *B.melitensis* most dominant from Eritrea. From the same area, Eritrea *B.melitensis* was reported by Bereket *et al.* (2021) from goats.

In Ethiopia, there was a limited report regarding isolation and molecular characterization of *Brucella* species both in animals and human except recent report of isolation of *B. melitensis* by Sintayehu *et al.* (2015) and *B. abortus* by Minda *et al.* (2016), *B. abortus* by Edao *et al.* (2020) and *B. melitensis* by Tekle *et al.* (2019). There is no confirmed *Brucella* species report yet from human in Ethiopia except some seroprevalence records. In Ethiopia, the limited seroprevalence reports on human brucellosis include studies by Regassa *et al*. (2009), Tsegaye *et al.* (2017), Tschopp *et al.* (2021), and Getahun *et al.*

(2022). This could be evident that these species of *Brucella* isolated in livestock are circulating in human population as a source of brucellosis is invariably infected animals.

2.11. Treatment of Brucellosi in Human

Brucellosis is classified as a neglected zoonotic disease. In humans, the intracellular positioning of *Brucella* and its ability to adapt to various environmental conditions it encounters during replication, particularly within macrophages, contribute to high rates of treatment failure and relapse. These outcomes are influenced by the choice of drug combinations and adherence to the treatment regimen (Seleem *et al.*, 2008). There is substantial evidence indicating that tetracyclines, particularly doxycycline and minocycline, are the most effective treatments for brucellosis (Hall, 1990). The selection of the treatment regimen and the duration of antimicrobial therapy should take into consideration the presence of focal disease and any underlying conditions that may contraindicate certain antibiotics, such as cases involving pregnant women or young children (Solera *et al.*, 1997).

Most patients with acute brucellosis respond favorably to a combination therapy of doxycycline along with either aminoglycosides or rifampicin for duration of six weeks. Alternatives include monotherapy with doxycycline or minocycline, or a combination of doxycycline with trimethoprim-sulfamethoxazole (TMP-SMZ), as well as quinolones and rifampicin. Patients suffering from focal infections, such as spondylitis or endocarditis, may require prolonged antibiotic courses based on their clinical progression (Corbel, 2006). For those experiencing persistent symptoms after an extended course of antibiotics, and where focal disease or relapse has been excluded, managing these cases can be challenging. This debilitating condition, often referred to as chronic brucellosis, resembles chronic fatigue syndrome and necessitates symptomatic treatment (Solera, 2013).

2.12. Prevention and Control of brucellosis in livestock

For over forty years, veterinary services in developed nations have executed control and eradication initiatives for brucellosis in livestock. Initially, these programs focus on controlling the infection through mandatory vaccination; this is followed by a gradual reduction and eventual cessation of vaccination, transitioning to a “test-and-slaughter” strategy aimed at eradicating the disease once incidence levels are sufficiently low to facilitate this process. Typically, completing a brucellosis eradication program utilizing a “test-and-slaughter” approach takes more than ten years, with a crucial element for success being the availability of adequate financial compensation for farmers whose livestock are culled

(European Commission, 2009). In the European Union (EU), these national initiatives receive co-funding from both the EU and Member States (MSs). This strategy has been effectively applied to control bovine, ovine, and caprine brucellosis in Northern MSs, although some Southern European MSs have yet to finish their eradication programs, especially regarding ovine and caprine brucellosis (Godfroid *et al.,* 2013).

In low-resource countries, establishing an effective eradication strategy poses significant challenges, necessitating the development of innovative solutions that consider both financial constraints and community perceptions where human brucellosis is recognized as a public health issue (Marcotty *et al.*, 2009). One such innovative approach explored in Mongolia assessed the economic benefits, cost-effectiveness, and health improvements associated with controlling brucellosis through mass vaccination of livestock (Zinsstag *et al*., 2005). In Tajikistan, the use of biannual conjunctival vaccination of small ruminants with Rev 1 led to 80% decrease in seroprevalence over five years, while the percentage of households with infected animals declined from 25.1 to 7.5% (Ward *et al*., 2012).

In many Southeast European (SEE) and Mediterranean nations, the resurgence of *B. melitensis* infection in sheep and goats has impacted public health adversely over the past decade. Brucellosis has shifted from a low-prevalence issue to a significant endemic disease, particularly in the Balkans, where high rates are observed in sheep, goats, and humans (FAO, 2010, Kirandjiski *et al*., 2010). This trend can partly be attributed to political upheavals, conflicts, and wars that arose in the early 1990s following the dissolution of Yugoslavia (Donev *et al.,* 2010). Additionally, funding shortages have diminished vigilance and halted the execution of recommended control measures. Coupled with increased international travel, cross-border livestock movement, and poorly regulated trade in livestock and agricultural goods, these factors have contributed to the resurgence and intensification of brucellosis prevalence in SEE nations (Donev, 2010).

In Sub-Saharan Africa, vaccination against brucellosis is reportedly infrequent outside of southern Africa, and where it does occur, it is often on an irregular basis rather than as part of an organized campaign. In several countries within this region, the disease is classified as notifiable, and measures such as surveillance, movement control, and culling or vaccination efforts have been implemented in Botswana, Namibia, Lesotho, and South Africa (Mcdermott and Arimi, 2002). In Zimbabwe, control strategies focused on specific cattle production systems, while a partial test-and-slaughter program for

goats in KwaZulu-Natal showed some success in reducing prevalence but failed to completely eliminate the disease. Challenges in implementing this policy mirrored those encountered elsewhere, including time and financial limitations and farmers' dissatisfaction with compensation (Emslie, 2002).

Despite significant investment in controlling animal brucellosis, outcomes have not always aligned with expectations. This discrepancy may stem from several factors, including those intrinsic to the disease regardless of the causative agent or host as well as the limited sensitivity of certain diagnostic tests in specific epidemiological contexts, alongside factors related to the causative agent and the host (FAO, 2003; Corbel, 2006). Nonetheless, three primary strategies have proven effective in controlling brucellosis in domestic animals when utilized in combination: rigorous biosecurity measures at the farm level, test-and-slaughter initiatives, and vaccination of at-risk populations (Mcdermott and Arimi, 2002).

Employing any one of these strategies alone is significantly less effective; optimal outcomes are achieved when at least two are used together. The best approach will vary based on the epidemiological context, available resources, and additional considerations. Furthermore, beyond these conventional strategies, complementary measures such as animal identification, movement control, and financial compensation should be factored into the program to enhance its chances of success (Nicoletti, 2010).

2.12.1. Management and Biosecurity

Efforts to manage and implement hygienic practices against *Brucella* infections should concentrate on reducing the likelihood of contact with viable *Brucella* organisms, which include both infected animals and contaminated environments. The primary means by which *Brucella* can enter an unaffected farm include the acquisition of infected animals that may release the bacteria into the surroundings, thereby putting susceptible individuals at risk, as well as through exposure to contaminated materials and pastures (Rodriguez and Crespo, 2010).

The application of effective biosecurity measures is essential for preventing the introduction of the disease into a susceptible population. Key strategies involve enforcing quarantine protocols prior to bringing new animals onto the premises, isolating animals of uncertain health status, regulating animal movements, appropriately managing replacements, isolating pregnant females prior to giving birth, and maintaining stringent quality control over semen (Nicoletti, 2010). Additionally, it is vital to reduce or

eliminate interactions between bulls designated for artificial insemination and wildlife in areas where wild animals have been identified as potential sources of infection (Mcdermott and Arimi, 2002).

In environments where *Brucella* infections are present, alongside the aforementioned biosecurity protocols, it is crucial to implement hygienic measures to limit and manage the bacterial load within the environment. This practice should be systematically enforced to mitigate the risk of contact with viable *Brucella* species. Examples of such measures include the disposal of aborted products, thorough cleaning and disinfection of facilities, removal of infected manure, and incineration of contaminated materials (Nicoletti, 2010). For humans, the most sensible strategy to prevent brucellosis is through the control and elimination of the infection within animal reservoirs. Furthermore, it is necessary to educate farmers about the careful handling and disposal of aborted fetuses, fetal membranes, and associated discharges. Informing the wider public about the risks of consuming unpasteurized milk and dairy products, as well as training abattoir workers about the transmission of the infection, particularly through skin abrasions, is of significant importance (Sriranganathan *et al.*, 2009; Corbel, 2006; Pappas *et al*., 2006).

2.12.2. Test and Slaughter Programs

The primary objective of this strategy is to swiftly identify and eliminate potential sources of infection, thereby preventing the spread of *Brucella*. Although the diagnostic methods employed are effective, there remains a risk of infection from asymptomatic carriers who can harbor the pathogen within the herd. Should the herd's immunity decline, this could trigger a surge in abortions (Corbel, 2006). This approach is particularly beneficial in areas with low prevalence of infection, where financial resources and veterinary expertise are sufficient to support these measures (Díaz Aparicio, 2013). Test-and-slaughter programs can also play a critical role in managing outbreaks, especially when the number of animals involved makes traditional stamping-out methods impractical. In certain scenarios, the only effective means of completely eradicating the bacteria from a herd necessitates culling, followed by exhaustive cleaning and disinfection of the facility, along with the introduction of *Brucella*-free livestock (FAO, 2003; Corbel, 2006).

2.12.3. Vaccination

Vaccination efforts are typically concentrated on the most vulnerable individuals to promote a protective immune response against the targeted pathogen. However, in the context of animal brucellosis, vaccination initiatives focus on populations that are less susceptible, deliberately avoiding the vaccination of the highly susceptible category due to potential adverse effects on pregnant animals (Nicoletti, 2010).

The characteristics of the "ideal vaccine against brucellosis" would include (Olsen, 2013): (i) the ability to provide robust and long-lasting immunity against various *Brucella* species without requiring re-vaccination, (ii) safety regardless of the animal's reproductive status, (iii) minimal or negligible residual virulence for humans, coupled with susceptibility to standard antibiotics used in treating human brucellosis, (iv) the prevention of cross-reacting antibodies in traditional serological assays used for control and eradication programs, (v) affordability, and (vi) stability under varying environmental temperatures.

While an ideal vaccine is yet to be developed, the use of available vaccine strains remains the most effective strategy to prevent and manage brucellosis in cattle, being a vital aspect of global brucellosis control and eradication initiatives (Dorneles *et al.*, 2015). Numerous countries have implemented control measures against bovine brucellosis to lower prevalence rates or completely eradicate the disease in livestock, with the intention of preventing transmission to humans and reducing economic losses (Ignacio *et al*., 2004). The vaccination of female calves is a central component of any brucellosis control plan, having proven effective in reducing disease prevalence (Olsen, 2013). Recognizing that vaccination alone is insufficient to control and eradicate the disease, it should be combined with the ongoing removal of infected animals, which are a source of new infections. Therefore, in addition to vaccination, many bovine brucellosis eradication programs also incorporate test-and-slaughter methods, monitoring, and hygiene practices (Ignacio *et al*., 2004).

A few vaccines have been widely administered for cattle immunization against *B. abortus*, including S19, RB51, 45/20, and SR82, with S19 and RB51 being the most prevalent (OIE, 2018). However, various candidates for *B. abortus* vaccines have been developed, such as DNA, subunit, recombinant *B. abortus*, and recombinant vector vaccines. Most of these candidates have primarily undergone

evaluation in experimental mouse models and, with few exceptions, have not been tested on cattle or proved ineffective against the target species (Dorneles *et al*., 2015).

The smooth S19 strain is the most commonly used vaccine for managing bovine brucellosis. Although it is currently regarded as the reference strain, it shares similar limitations with the *B. melitensis* Rev.1 strain, despite its proven effectiveness (OIE, 2016). Consequently, the rough strain RB51 is increasingly preferred in certain regions as an alternative for bovine brucellosis vaccination, as it does not trigger cross-reacting antibody production, although its efficacy in specific epidemiological contexts is still debated (Ignacio *et al*., 2004).

Reports indicate that administering full doses (1-3.4x10^{10} colony-forming units (CFU) of *B. abortus* strain RB51 intravenously can lead to significant placentitis and placental infection in most vaccinated cattle, resulting in vaccine strain excretion in milk for a notable number of vaccinated animals. Field experiences suggest that it may also cause abortion and increased perinatal mortality when given to pregnant cattle (OIE, 2018). Reducing the vaccine dose (1×10^9 CFU) has not been associated with abortions or placentitis lesions; however, many vaccinated animals may still shed the vaccine strain. Despite this lower dose, it fails to protect against *B. abortus* when used in calves and provides only moderate protection as an adult vaccine (Ignacio *et al*., 2004).

Brucella melitensis Rev.1 is the predominant vaccine employed for the prevention of brucellosis in sheep and goats. Despite its limitations, it is regarded as the benchmark vaccine against which all other vaccines are evaluated. In contrast, the rough *B. abortus* RB51 vaccine does not provide protection against *B. melitensis* infections in sheep (Ignacio *et al*., 2004). The Rev.1 vaccine is prepared as a freeze-dried suspension of the live *B. melitensis* Rev.1 strain and is administered to sheep and goats. It is recommended for lambs and kids aged three to five months, delivered as a single subcutaneous or conjunctival injection, with five months being the upper age limit to minimize the antibody response, allowing compatibility with subsequent serological testing. Regardless of the method of administration, the standard dose must contain between 0.5×10^9 and 2.0×10^9 viable organisms (OIE, 2016). Lower doses provide significantly less protection than the standard ones and are not advisable for vaccinating sheep and goats. Subcutaneous vaccination leads to long-lasting serological responses, which can substantially interfere with serological tests and is not advised in combined eradication strategies. However, administering the vaccine conjunctivally at the standard dose yields comparable protection

without causing a persistent antibody response, thus supporting the implementation of vaccination alongside eradication programs (OIE, 2018).

Care should be exercised when utilizing the *B. melitensis* Rev.1 vaccine to prevent possible environmental contamination or human infection. In numerous developing nations and areas where the disease is endemic, vaccinating the entire population may be the most effective strategy for disease control (BLASCO, 1997). However, it is important to note that the Rev.1 vaccine can frequently lead to abortion and milk excretion if animals are vaccinated during pregnancy, regardless of whether a full or reduced dose is used (BLASCO, 1997). These adverse effects are significantly diminished when adult animals are vaccinated conjunctivally (full dose) during lambing/kidding, lactation, or prior to mating. Consequently, when mass vaccination is the sole method available for disease control, it is advisable to conduct a vaccination campaign using the standard dose of Rev.1 via the conjunctival route, targeting animals that are not pregnant or during late lambing/kidding and pre-breeding periods (BLASCO, 1997).

In general, one of the significant drawbacks of brucellosis vaccination in animals is the prohibition against vaccinating pregnant animals, which complicates the rapid increase of the resistant portion of the population by reducing transmission among animals through mass vaccination (Olsen, 2013). The effectiveness of live vaccines for brucellosis immunization in animals relies on achieving a balance between adequate colonization of the host to elicit robust protection against other *Brucella* field strains, while also limiting replication to reduce the residual virulence of these vaccine strains (Ignacio *et al*., 2004). Although the immunological mechanisms triggered by live vaccines are not entirely understood, they are believed to stimulate innate immunity, activate CD8+ and CD4+ cells, and generate a sufficient population of memory cells, among other mechanisms, to foster strong protection (Olsen, 2013).

Most issues associated with using live vaccines could potentially be addressed by employing killed bacteria or subunit vaccines. Nevertheless, the capacity of these inactivated vaccines to produce a robust and enduring immune response against *Brucella* has traditionally been deemed inferior to that elicited by live attenuated vaccines. Ongoing research is exploring new strategies to overcome the primary limitations of inactivated vaccines, aiming to induce protective immunity in domestic livestock (Ignacio *et al*., 2004; Dorneles *et al*., 2015).

Ethiopia has already started implementation of the national brucellosis prevention and control strategy (2023-2032). In Ethiopia, brucellosis has been recognized for over 50 years, with numerous studies reporting its prevalence in both livestock and humans. The seroprevalence in cattle ranges from 0.06% to 50%, and herd-level seroprevalence can vary from 0.8% to 51.7%. *Brucella abortus* and *Brucella melitensis* are commonly isolated from livestock, and brucellosis is increasingly recognized at the wildlife-livestock interface. The disease imposes an estimated annual loss of USD 377.93 million from cattle alone, and a significant public health impact, with an estimated 1,324,828.809 Disability-Adjusted Life Years (DALYs) lost in humans annually.

In response to this ongoing challenge, Ethiopia has developed a strategic plan aimed at controlling and eventually eliminating brucellosis in both humans and animals by 2032. This plan follows the Staged Tool for the Elimination of Brucellosis (STEB), a stepwise approach that guides countries from an endemic state to brucellosis-free status. The strategy focuses on governance, enhancing laboratory capacity, improving surveillance systems, disease prevention, and communication. The program will begin in high-burden regions with a phased approach, eventually scaling up to cover the entire country.

This strategic plan underscores the importance of a multi-sectorial, risk-based approach to brucellosis control, emphasizing the need for collaboration between public health, agriculture, and veterinary sectors, alongside community education and capacity building to ensure the success of brucellosis elimination efforts in Ethiopia (January 2023, Addis Ababa Ethiopia, National Brucellosis Preventionand Control Program, 2023-2032).

3. MATERIALS AND METHODS

3.1. Description of study area

The present PhD study was conducted in five pastoral districts (Benatsemay, Malie, Hamer, Dassenech and Gnangatom) of South Omo zone. South Omo zone is located in the South Ethiopia Regional State of Ethiopia (Fig. 1). The study area is located approximatelt at 800 km south of Addis Ababa.

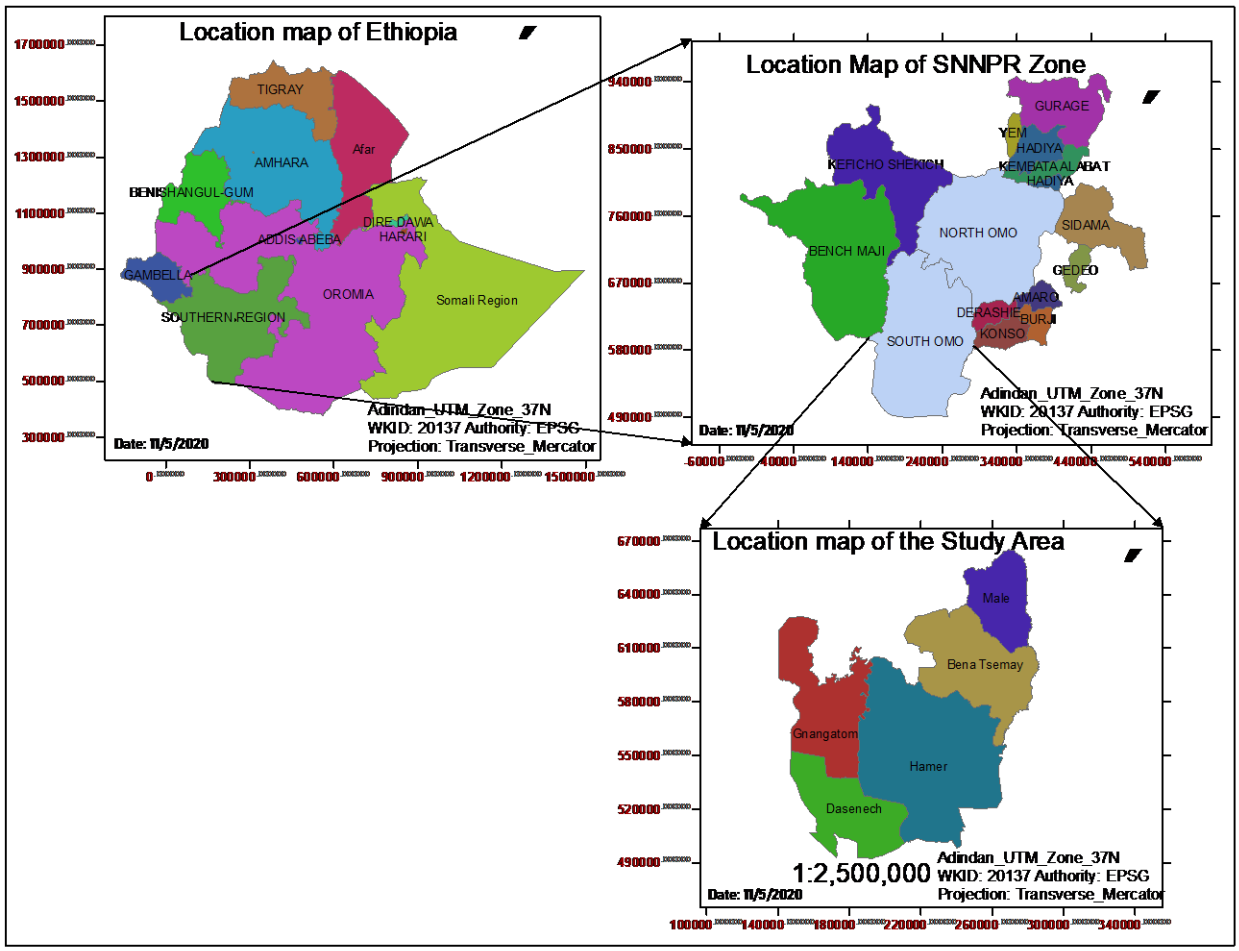

Figure 1. Map showing the study area (South Omo zone)

The altitude of the zone ranges between 500 meters to 1, 500 meters above sea level. The average annual temperature ranges between 18 to 32°C and the average annual rainfall is about 390 mm. In the study area, rain is erratic and usually bimodal occurring from September to November and from March to May. The weather condition is characterized by semi-arid and arid climate. The major livestock production system in the Zone is pastoral and comprises the higher livestock population of the region (CSA, 2021).

South Omo Zone is in South Ethiopia Regional State, bordered to the south by Kenya, to the west by West Omo Zone and South Sudan, to the northwest by Keffa Zone, to the north by Ari Zone and Gofa Zone, to the northeast by Gardula, Ale Zone and Konso and to the east by the Oromia Region. The administrative center of South Omo is Dimeka (https://en.wikipedia.org/wiki/, 2024).

This zone is named for the Omo River, a river that flows into Lake Turkana on the western side. Mago National Park and Tama Wildlife Reserve are located at the eastern bank of Omo River. There is Lake Chew Bahir surrounded by Stephanie Wildlife Sanctuary located at the eastern border of this zone. South Omo is the most sparsely populated part of Ethiopia, inhabited by nomadic and semi-nomadic ethnic groups (CSA, 2021).

Livestock rearing is central to the livelihoods of South Omo's communities. Households typically manage mixed herds of cattle, goats, and sheep, with herd compositions varying among ethnic groups. For instance, the Benna communities own more cattle and sheep per household compared to the Hamer and Tsemay pastoralists, owing to relatively favorable climatic conditions and lower incidence of diseases like trypanosomiasis in their area. Conversely, the Hamer pastoralists have more goats per household due to the suitability of their area for goat rearing. According to the zone Agriculture and Pastoral Community Development office (2020) the average herd or flock composition in the area approximately 25 cattle, 15 goats, and 10 sheep per household with varying levels and some pastoralists posses upto 100 animals. Cattle are not only economic assets but also hold significant cultural value. They are integral to social practices such as bride price negotiations and community rituals. Milk and its products are staple foods, often consumed raw, and in some communities, raw blood from cattle is also part of the diet (Solomon *et al*., 2021).

The South Omo Zone is a mosaic of ethnic groups, each with distinct languages, traditions, and social structures and lifestyles. Among the major ethinic groups, the Hamer are known for their pastoral lifestyle and the "bull-jumping" ceremony, marking a young man's passage to adulthood, while women wear red ochre-plaited hairstyles and symbolic jewelry. The Bena focus on cattle and sheep herding, benefiting from favorable climate conditions. The Gnangatom, or Bume, are agro-pastoralists practicing flood-retreat agriculture and detailed leatherwork. The Bodi celebrate the "Ka'el" ceremony, where men undergo a high-calorie diet from milk and fresh blood (Solomon *et al*., 2021) to gain weight,

symbolizing beauty and prestige. Collectively, these communities reflect the zone's deep interconnection between culture, environment, and livestock, forming a vibrant mosaic of traditions shaped by both social structure and ecological adaptation.

3.2. Study population

3.2.1. Animal study

The study animals were indigenous cattle, sheep and goats kept under mainly pastoral farming system. Livestock production system is generally predominated by extensive pastoral and few agro-pastoral system in which indigenous animals are allowed to forage freely during day time and kept in barn during the night time. All cattle, sheep and goats in the study area with the age of 6 months and above and both sexes were considered as the study animals for seroprevalce study of brucellosis. For isolation of the *Brucella* species, female animals (cattle, sheep, and goats) with recent history of abortion up to 1 month were considered. The total population of livestock in the study districts include 1, 134, 120 cattle, 1, 230, 399 goats, and 471, 449 sheep (South Omo Agricultural office, unpublished, 2016).

3.2.2. Human study

The study also includes human or pastroalists who are livestock owners and those attending the nearby health centers of each selected districts due to febrile illness. Owing livestock for febrile patients visiting the health centers was considered as inclusion criteria for participation in the current study and for sample collection. Furthermore, to evaluate the zoonotic transmission dynamics febrile patients whose animals (cattle, or sheep, and/or goats) sampled for the same study were identified making interview by the health professionals collecting samples and included as study participants as both livestock and human sample collections were undertaken parallelly for each districts. Both males and females, all age groups of people visiting the health centers due to febrile condition were included in the study but permission of participation for <18 years old individuals was obtained from the parents with them via both oral and written consent signature.The most recent population estimate for the South Omo Zone is around 918,440 residents, based on 2023 estimates from the South Omo Zone plan commission. This includes 459,586 males and 458,854 females (Lidetu *et al*., 2023).

3.3. Study design

A cross-sectional study design was employed to determine the seroprevalence and associated risk factors of brucellosis in cattle, sheep, goats and humans from January 2017 to June 2020.

A multi-stage sampling technique was implemented, with zone as highest and village or pastoral association as lowest regarding the sites selection. On the other hand regarding animal structure herd/flock was taken as as highest and individual animals as lowest sampling stages. Selection of the study unit at each stage was based on a mixed design of convenience (zone and district selection) and random samplings (villages, herds/flocks, and individual animal selection). The zone was purposely selected as the study was intended to be carried out in pastoralist area with no or few similar studies conducted so far. Similarly, only predominant pastoral farming stystem districts were selected purposely for the study. Out of the eight districts (Selamago, Kuraz, Bako Gazer, Benatsemay, Malie, Hamer, Dassenech, and Gnangatom) in the zone, five pastoral districts namely Benatsemay, Malie, Hamer, Dassenech, and Gnangatom were selected for the study. Villages were randomly (using lottery method) selected following obtaining lists of villages in each district. Simple randomly sampling technique was applied to select individual animal to be sampled from the herds or flocks.

For the isolation of *Brucella* species from livestock and humans from biological samples (milk and vaginal discharge) obtained from livestock with a recent history of abortion and whole blood samples from humans exhibiting febrile symptoms who visited health centers in five selected districts of the South Omo Zone during the study period. Animals specifically cattle, goats, and sheep with a history of recent abortion (15-30 days) were purposely sampled for bacteriological culture. For human samples, 5ml of blood was drawn from each febrile patient; 2ml was promptly placed in a sterile heparinized-vacutainer tube, labeled with the same identification code, and reserved for bacteriological culture, while the remaining 3ml was used for serological testing. The 2ml reserved whole blood from each individual underwent bacteriological culturing after serological screening using the Rose Bengal Plate Test (RBPT), with only those samples that tested positive being considered for isolation.

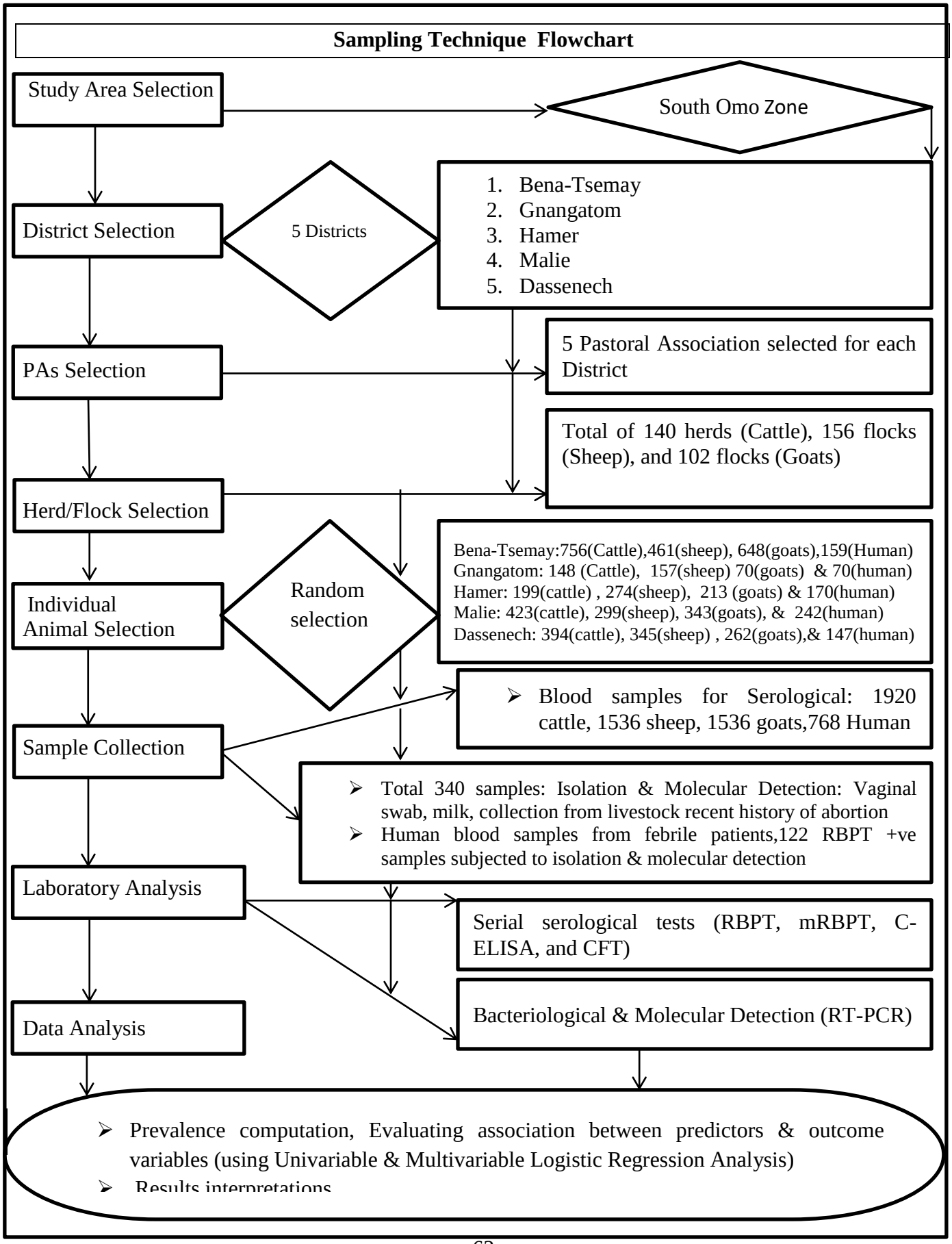

Figure 5. Sampling technique flow chart

3.4. Sample size determination

Sample size was determined according to Thrusfield (2007) using 95% confidence level, 5% precision. The 50% expected prevalence of brucellosis was used since there was no previous report on prevalences of brucellosis in different species of livestock and human in the study area.

The formula used for sample size determination was:

$$n = \frac{(1.96)^2 * Pexp * (1- Pexp)}{d^2}$$

Where: n = required sample size, Pexp= expected prevalence and d= desired absolute precision (5%)

Using the above formula, the minimum sample size calculated for the study for each species (cattle, sheep, and goats) was 384. However, to increase the level of precision, and considering the study covers wider area (five districts) and availability of resources for the study, the sample sizes were increased to a total of 1, 920(cattle), 1, 536(goats), 1,536(sheep), and 768 for humans. For isolation of the *Brucella* species from biological samples from both livestock and humans a total of 340 samples were collected based on the inclusion criteria (recent history of abortion in livestock and presence of febrile symptom in human). The determined total sample size was distributed to each district based livestock and humans population using population proportion. For the questionnaire survey data were obtained from each sampled animal and human.

Table 6: Sampling distribution for cattle

District	**Cattle population**	**Population proportion**	**Sample size of each district**	**Sample size**	**Herds Number**
Bena Tsemay	420,764	0.394	0.394*1, 920	756	45
Malie	235,474	0.22	0.22*1, 920	423	30
Hamer	110,696	0.10	0.10*1, 920	199	20
Dassenech	219,380	0.21	0.21*1, 920	394	30
Gnangatom	81,806	0.08	0.08*1, 920	148	15
Total	**1, 068, 120**	**1.00**	**1*1, 920**	**1, 920**	**140**

Since almost there is no significant difference in the number of villages or pastoral association among the districts, five villages were purposely selected from each district. Based on sample size distributed for each district number of herds to be considered in the study districts were distributed purposely 15

herds for district with small sample size calculated and distributed, 45 herds for district with the highest sample size calculated, and 20 and 30 herds for the remaining districts within between the highest and lowest sample size distributed. Accordingly, number of herds assigned to each district 45 herds (Benatsemay), 30 herds (Malie), 20 herds (Hamer), 30 herds (Dassenech), and 15 herds (Gnangatom) (Table 6).

Table 7: Sampling distribution to districts for sheep

District	**Sheep population**	**Population proportion**	**Sample size of each district**	**sample size**	**Flocks Number**
Bena Tsemay	141,350	0.299	0.299*1, 536	461	47
Malie	91,748	0.195	0.195*1, 536	299	30
Hamer	84,186	0.179	0.179*1, 536	274	28
Dassenech	105,905	0.2254	0.225*1, 536	345	35
Gnangatom	48,260	0.1021	0.102*1, 536	157	16
Total	**471,449**	**1.00**	**0.999*1536**	**1, 536**	**156**

The required sample size (1, 536) determined was distributed to each district proportionally based on their sheep population (Table 7) (Kothari, 2014). Accordingly, Bena Tsemay (n=461), Male (n=299), Hamer (n=274), Dassenech (n=345), and Gnangatom (n=157), sheep were sampled. The number of sheep flocks sampled in each district were determined by dividing the total sample size by the number of sheep sampled within each flock.

According to Animal production and Veterinary Service office of each district on average each flock comprised of ten sheep. Therefore, the flocks were selected from each district by dividing the distributed sample size by ten. In those flocks which had only ten or less animals, all sheep older than six months were sampled whereas for those flocks that had more than ten sheep the required number of samples were selected randomly from the flock. In this regard, 47 flocks from Bena Tsemay, 30 flocks from Malie, 28 flocks from Hamer, 35 flocks from Dassenech, and 16 flocks from Gnangatom districts were sampled.

Table 8: Sampling distribution to districts for goats

District	Goat population	Population proportion	Sample size fo each district	Sample size	Flocks sampled
1.Hamer	167, 670	0.1395	0.139*1, 536	213	14
2.Dassenech	206, 185	0.170	0.170*1, 536	262	17
3.Gnangatom	55, 100	0.046	0.046*1, 536	70	5
4.BenaTsemay	511, 197	0.422	0.422*1, 536	648	43
5. Malie	270, 247	0.223	0.223*1, 536	343	23
Total	**1, 210, 399**	**1.00**	**1*1536**	**1, 536**	**102**

The determined sample size of 1, 536 were allocated to districts based on their respective goats' populations (Table 8) proportionally. Specifically, Bena Tsemay received 648 samples, Malie 343, Hamer 213, Dassenech 262, and Gnangatom 70. The sampling within each district involved dividing the total sample size by the average size of fifteen goats per flock, as provided by the Animal Production and Veterinary Service offices. In flocks consisting of exactly fifteen goats, all older than six months were sampled, while larger flocks had samples chosen randomly, resulting in 14, 17, 5, 43, and 23 flocks being sampled from the respective districts of Hammer, Dassenech, Gnangatom, Bena-Tsemay, and Malie, respectively.

For human study, the sample size determined was distributed to the selected districts proportionally based on each district population (Table 9).

Table 9: Sampling distribution to the districts for human

Districts	Human Population	Population proportion	Sample size Each district	Sample size
Malie	84,657	0.318	0.318*768	242
Gnangatom	17, 632	0.066	0.066*768	50
Hamer	59,160	0.220	0.220*768	170
Bena-Tsemay	55, 590	0.207	0.207*768	159
Dasenech	51,559	0.192	0.192*768	147
Total	**268,598**	**1.00**		**768**

Based on the inclusion criteria described above for isolation of *Brucella species*, a total of 340 biological specimens were collected from livestock (cattle, sheep, and goats) and human as indicated in (Table 10).

Table 10: Biological samples collected for isolation of *Brucella* sp. from animals and humans

Species/Human	Sample Type	Districts					
		Bena-Tsemay	Dassenech	Hamer	Gnangatom	Malie	Total
1. Bovine	1.Milk	7	15	13	7	11	53
	2.Vaginal swab	9	10	6	4	5	34
2. Caprine	1.Milk	6	13	11	4	7	39
	2.Vaginal Swab	19	6	10	6	7	48
3. Ovine	1.Milk	5	7	6	6	4	28
	2.Vaginal Swab	2	4	4	3	3	16
4. Humans	1.Whole blood	24	32	21	12	33	122
Total		**72**	**87**	**71**	**42**	**70**	**340**

3.5. Questionnaire Survey

3.5.1. Questionnaire survey for livestock

For cattle, during collection of serum samples, a pretested semi-structured questionnaire survey was administered to respondents or owner/herder of the cattle included in the study through interview by local language using local veterinary experts. The questionnaire was focused on age category, sex, herd size, abortion history, and history of retained fetal membrane, stage and frequency of abortion, body condition scores and parity number for each individual animal. Moreover, structured questionnaire survey was also carried out to assess understanding, awareness and practices by pastoralists on brucellosis. Accordingly, a total of 140 herds' owners or herders were included in this study. The structured questionnaire interviewed with the respondents emphasized on awareness about brucellosis, knowledge of zoonotic disease transmitted from animals to humans through consumption of milk and other animal products, knowledge of pathogenic causes of abortion in animal, knowledge and understanding about disease transmitted during handling of infected animal and its product, knowledge about diseases transmitted during delivery assistance, ways of disposal of aborted materials, fate of frequently aborted cows in the herd, risk of assisting parturition with bare hands, and habit of consumption of raw animals' blood and milk were assessed at the study area.

For sheep and goats, during the collection of blood samples, a structured questionnaire was developed and filled separately for each animal and flock by interviewing owners or herders using local language with the help of veterinary experts working in the district veterinary clinic to assess potential risk factors

for ovine brucellosis. The questionnaire was focused on age category (Young ≤1 year and Adult ≥ 1 year), sex (Male and Female), flock size (Small ≤ 25 sheep/goats, medium: 26 to 49 sheep/goats, and Large ≥50 sheep/goats), abortion history (present and absent), stillbirth in the flock (Yes and No), history of the retained fetal membrane in the sampled animal (Yes and No), stage of abortion (Early and Late), body condition scores (good, medium and poor), production system (Pastoral, Agro- pastoral) and parity number for each animal (1, and ≥1).

3.5. 2. Questionnaire survey for humans

During the collection of blood samples, a structured questionnaire was developed and filled for each individual by interviewing the participant using local language with the help of medical personnel (Clinical nurse) who recruited to collect blood sample to assess potential risk factors for human brucellosis. The questionnaire was focused on district (Bena-Tsemay, Hamer, Dassenech, Gnangatom, and Malie), gender (Male, Female), age category (<18 years, 18-30 years, 31-40 years, 41-50 years, 51-60 years and >60 years), marital status (single, married), education level (illiterate, elementary school, high school, diploma, bachelor degree or obove), occupation or production (agro-pastoralist, pastoralist), raw milk consumption (yes, no), raw meat consumption (yes, no), animals' raw blood consumption (yes, no), close contact with animals (yes, no), sharing water points with animals (yes, no), knowledge of zoonosis (yes, no), assisting animals during parturition (yes, no), ways of assisting parturition of animals (bare hand, protected), contact with aborted material (yes, no), knowledge of brucellosis (yes, no), and disposal of aborted material (burn, burry, throw away).

3.6. Sample collection

3.6.1. Blood sample collection from livestock for serological test

Following proper physical restraining from each animal, approximately 10ml of whole blood was aseptically collected from the jugular vein using sterile plain vacutainer tubes and needles (BD Vacutainer Systems, Plymouth, UK). The collected blood samples were labeled with individual animal identification code and the blood samples were kept at a slant position and allowed to clot at room temperature for 12 hours. Sera were then collected into sterile cryo-vials for each sample, labeled and transported on ice in the icebox to the Aklilu Lemma Institute of Pathobiology brucellosis laboratory and stored at -20°C until tested using serological tests (Schwarz *et al*., 2017).

For sheep and goats, approximately 5-7 ml of whole blood samples were collected by venipuncture from the jugular vein using sterile disposable plain vacutainer tubes and needles (BD Vacutainer Systems, Plymouth, UK). The blood samples were kept at a slant position and allowed to clot at room temperature for 12 hours. The sera were transferred into into sterile cryo-vials and transported on ice in the icebox to the Aklilu Lemma Institute of Pathobiology brucellosis laboratory and stored at -20°C until serologically tested.

3.6.2. Blood sample collection from Humans for serological test

After obtaining a both oral and signed written consents form from consented study participants with the assistance of experienced clinical nurses, approximately 5 ml of peripheral blood was drawn from the median cubital vein of voluntary febrile patients visiting health centers in the selected districts. The collected whole blood samples were labeled with a specific code, date of collection, and location, and then transported under cold chain conditions to the Aklilu Lemma Institute of Pathobiology (ALIPB) *Brucella* laboratory for analysis. From the collected blood sample, 2ml of whole blood was stored at +2°C temperature for immediate bacteriological culture processing while the remaining blood samples were left to clot at room temperature; the serum was then separated, transferred into sterile cryovials labeled with the same code as the blood samples, and stored at -20°C for further laboratory examination (OIE, 2018). Finally, after screening test of the serum sample whole blood samples (n=122) were subjected for isolation of the pathogen.

3.6.3. Sample collection for Brucella isolation and molecular detection

For bacterial isolation a total of 340 (Table 10) clinical samples were collected which comprises of 98 vaginal swab (34 from cows and 64 from small ruminants), 120 milk samples (53 from cows and 67 from small ruminants), and 122 whole blood from humans. The milk and vaginal discharge swab samples were collected from animals with recent history of abortion within the last 15-30 days during sample collection. The vaginal or uterine discharges were collected aseptically with sterile swab in screw caped tube and transported in cold-chain using Stuart transport medium (Himedia, HiMedia Laboratories Private Limited, Maharashtra, India). Approximately 15ml of pooled milk samples from all teats were collected from each animal following the standard protocol (OIE, 2018) for the recovery of *Brucella* species. Briefly, the milk samples were collected after washing and drying the whole udder and disinfecting the teats using 70% alcohol. The first streams were discarded and the required (15ml)

sample milked directly into a sterile screw caped container. The collected samples were transported to ALIPB *Brucella* laboratory under the cold chain and preserved at -20^{0}c until bacteriologically processed.

3.7. Laboratory Methods

3.7.1. Serological tests

The collected serum from cattle, sheep, goats and humans were serologically analyzed for the presence of antibody against *Brucella* using Rose Bengal plate test (RBPT) as screening test and serially combined RBPT and Competitive Enzyme Linked Immunosorbent Aassay (C-ELISA) and combine RBPT and Complement Fixation Tests (CFT) as confirmatory tests. RBPT was used as screening test as it is the most sensitive test up to 100% (Zhang *et al*., 2018; Abinet *et al.*, 2023) to detect antibiodies against *Brucella* followed by ELISA but CFT is the least sensitive (up to 72%). Complement fixation test is the most specific (100% speficity) to detect antibodies against *Brucella* and recommended as confirmatory test commonly combined with RBPT (OIE, 2021). Use of serial serological tests in diagnostic algorithms improves diagnostic accuracy (Loubet *et al*., 2024).

3.7.1.1. Rose Bengal Plate Test (RBPT)

All sera samples collected were initially screened by RBPT using RBPT antigen (Animal and Plant Health Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) according to OIE (2018) procedures for the presence of *Brucell*a antibodies. The test was carried out in brucellosis laboratory at Aklilu Lemma Institute of Pathobiology Addis Ababa University. Sera and antigen were removed from refrigerator and kept at room temperature for 30 minutes before the test to bring to the room temperature. Briefly, for bovine and human serum samples,30µl of serum was added onto a clean plastic plate and then equal amount of RBT antigen (30µl) was added after thoroughly homogenizing near to the serum (Appendix 4). However, for sheep and goats serum samples, a modified method (mRBPT) was implemented by mixing three volumes (75 µl) of serum and one volume of antigen (25 µl) instead of an equal volume of each according to the recommendation of OIE (2018) (Appendix 5). The antigen and test serum were thoroughly mixed using plastic rod, agitated for 4 minutes using laboratory rotary shaker, and immediately read for agglutination. The results were read by examining the degree of agglutination in good light. Any visible reaction or agglutination observed by the naked eye was considered to be a positive and sample with no agglutination was recorded as negative.

3.7.1. 2. Competitive ELISA test

All RBPT positive serum samples for both livestock and human were further tested using the COMPELISA 160 and 400, as per the manufacturer's protocol (Annex 11) (Animal and Plant Health Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) in brucellosis laboratory at Aklilu Lemma Institute of Pathobiology Addis Ababa University (ALIPB, AAU). The test was carried out as per the manufacturer's instructions. The test was conducted in 96-well polystyrene plates that are pre-coated with *Brucella* species lipopolysaccharide antigen. Twenty micro liter of each test serum was added to each well followed by 100µl of prepared conjugate solution. The plates were then shaken vigorously for two minutes and incubated at room temperature for 30 minutes on rotary shaker, at 160 revolutions per minute. Plates were washed 5 times and dried by tapping on soft paper. Hydrogen peroxidase substrate and chromogen solution was prepared for10 minutes. One hundred µl of O-Phenylenediamine dihydrochloride (OPD) solution was added to all wells and the plates were incubated at room temperature for 20 minutes. Micro plate reader was switched on and the units allowed stabilizing for 10minutes. The reaction was then being stopped using stopping solution. Optical densities (OD) were read at 450nm using micro plate reader. The lack of color development indicated that the sample tested was positive. A positive or negative cut-off was calculated as 60% of the mean of the OD values of the four conjugate control wells. Any test sample giving an OD value equal to or below this value was regarded as being positive (Appendix 11).

3.7.1.3. Complement fixation test (CFT)

Further, all positive samples with RBPT- were subjected to complement fixation test as a confirmatory test at the National Veterinary Institute (NVI) for livestock serum samples, Bishoftu, Ethiopia. While CFT test for human serum samples were carried out at ALIPB, Addis Ababa, Ethiopia. The *Brucella* antigen and control sera (positive and negative) used for the test were produced by Veterinary Laboratory Agency, UK, batch 16. The standardization of the antigen was made at 1:20 working dilution. The *Brucella* antigen, complement and 3% sensitized sheep red blood cells were added after the test sera were serially diluted (1:5, 1:10, 1:20, and 1:40) in microtitre plates. Then the plates were incubated at 37°C for 30minutes. The microplates were centrifuged at 2500 rpm for 4 minutes using a sigma centrifuge and read for the result. The test was considered positive when the reading was as partial fixation (50% haemolysis) or complete fixation (no haemolysis) with clear water supernatant at 1:10 dilution. Complete lack of fixation (complete haemolysis) was recorded as negative. The validity of the test was considered when there was complete hemolysis in negative control serum and the positive

control shows inhibition of hemolysis. Subsequently, interpreted as a serum with strong reaction, more than 75% fixation of complement at a dilution of 1: 5 and at least with 50% fixation of complement at a dilution of 1:10 and dilution of 1:20 were classified as positive (OIE, 2018) (Appendix 6).

3.7.2. Sample processing and inoculation for isolation of Brucella species

3.7.2.1. Culture media preparation

The isolation process begins with the preparation and use of selective growth media, specifically designed to favor the growth of *Brucella* while inhibiting other microorganisms. The primary medium employed includes *Brucella* media agar sourced from Oxoid (Oxoid, Basingstoke, United Kingdom). The medium was supplemented with antimicrobial agents to enhance selectivity as supplement. This agent comprises a precise combination of antimicrobial compounds: 2,500 IU of polymyxin B, 12,500 IU of bacitracin, 50 mg of cycloheximide, 2.5 mg of nalidixic acid, 50,000 IU of nystatin, and 10 mg of vancomycin. In addition to the selective agents, the culture medium was enriched with heat-inactivated horse serum at a concentration of 5%. The serum treated by heating at 56 °C for 30 minutes, which serves to eliminate any potential microbial contaminants while retaining growth factors beneficial for *Brucella*. To further optimize the culture conditions, 0.5% dextrose and 0.5% methanol were also included.

Briefly, *Brucella* selective media was prepared by suspending the required amount of *Brucella* medium base in sterile 5% V/V inactivated horse serum (ie, horse serum held at 56°C for 30 minutes). Rehydrated contents of *Brucella* selective supplement were aseptically added to the sterilized *Brucella* basal medium and homogenized before plating and then 15 to 20 mL of the medium was poured into the Petri dish and allowed to solidify. The plates were incubated at 37°C for 48 hours for sterility check and no bacterial colony growths were considered as sterile and used for culture (Appendix 12).

3.7.2.2. Sample preparation and inoculation

Bacteriological samples were performed in a Biosafety level III (BSL3) laboratory at ALIPB with high personal protection. All samples and media prepared were taken out from refrigerator and thawed for 30 minutes at room temperature before inoculation. Milk samples underwent centrifugation at 3,000 rpm for 10 minutes at a temperature of 20°C, which resulted in the formation of a sediment pellet at the bottom and a layer of supernatant cream on top. After carefully aspirating and discarding the middle layer, the

fatty layer and the sediment pellet were combined with a sterile swab. The same swab was then used to inoculate *Brucella* agar plates prepared. Then the samples were streaked on *Brucella* selective basal media in duplicate and incubated both at 37°C in aerobic conditions and anaerobic with 5% CO_2 using CO_2 incubator and daily followed to assess the growth for 7-14 days (OIE, 2018).

3.7.2.3. Phenotypic and biochemical tests

In the identification of *Brucella* colonies, the distinctive characteristics, which include a round shape, a shiny surface, and a translucent appearance with smooth edges, exhibiting a pinpoint size and a resemblance to honey droplets, consistent with standard identification methods were used. To further confirm the presence of *Brucella*, these suspected colonies underwent a series of tests. The tests began with Gram staining and the Modified Ziehl-Neelsen staining technique, followed by observations of hemolytic activity on blood agar. Key biochemical assessments including urease and oxidase tests, along with evaluations of the colonies' growth in the presence of certain dyes, specifically basic fuchsine (20 µg/ml) and thionin at concentrations of 20 and 40 µg/ml were employed. In addition, the colonies' requirements for CO_2, their ability to produce hydrogen sulfide properties were assessed, all in accordance with established protocols (OIE, 2018).

3.7.3 Molecular identification of the isolates

3.7.3.1. Genomic DNA extraction

Genomic DNA was isolated from colonies grown on solid media using boiling technique, as previously detailed (Ali *et al.,* 2009). A small number of colonies (3-5) were taken and suspended in 500 µL of sterile double-distilled water within a 1.5 mL microcentrifuge tube, then heated in a boiling water bath for 10 minutes. After centrifuging at 12,000 rpm for 3 minutes, 5 µL of the supernatant was used as DNA template for Real-time PCR analysis.

3.7.3.2. Real- time PCR Analysis

The species-specific assay was conducted according to the protocols described by Gopaul *et al.* (2010) and kit manufacturer's instruction. A reaction mixture without template DNA was used as a negative control. Sequences of the primers and probes used for the individual species defining assays are illustrated below.

After thorough bacteriological, primary, and secondary biochemical tests were conducted the next process was applying Real-time polymerase chain reaction (RT-PCR) to further screen the isolates to the genus level using universal primers specific to *Brucella* (IS711) and finally characterizing the isolates to the species level using species specific primers and probes. The forward primer sequence used was 5'-GCT-TGA-AGC-TTG-CGG-ACA-GT-3', and the reverse primer sequence was 3'-GGC-CTA-CCG-CTG-GGA-AT-5'. Additionally, a FAM-labeled probe (FAM-AAG-CCA-ACA-CCC-GGC-CAT-TAT-GGT-BHQ-1) was incorporated alongside an internal positive control, utilizing the IPC 10x Exo IPC Mix and 50x Exo IPC DNA *(*Matero *et al*., 2011).

For the specific identification of *Brucella abortus*, particular primers were employed: the forward primer sequence was 5'-GCA-CAC-TCA-CCT-TCC-ACA-ACAA-3', and the reverse primer was 3'-CCC-CGT-TCT-GCA-CCA-GACT-5'. The corresponding probe used for this detection was FAM-TGG-AAC-GAC-CTT-TGC-AGG-CGA-GAT-C-BHQ-1. In the case of *Brucella melitensis,* the forward primer sequence was 5'-TCG-CAT-CGG-CAG-TTT-CAA-3', paired with a reverse primer sequence of 3'-CCA-GCT-TTT-GGC-CTT-TTCC-5'. The probe designed for *B. melitensis* detection was FAM-CCT-CGG-CAT-GGC-CCG-CAA-BHQ-1 *(*Gopaul *et al*., 2010).

Extracted DNA samples were amplified with an applied Biosystems 7500 PCR system. The master mix components are made for universal primers (IS711) for the genus *Brucella*. The reaction mixture (IS711) Real-time TaqMan® PCR was set up in a final volume of 25 µl and run for 45 cycles with 3. 9 µl of RNAse free water, 12.5 µl of Taq man universal PCR master mix (2x), each primer forward, reverse and TaqMan® probe at concentrations of 0.2 µM, 10x Exo IPC 2.5µl,50x Exo IPC DNA 0.5µl and 5 µl of DNA template. For *B. melitensis* and *B. abortus*, the master mix components were made separately in a test tube (0.20µM primer F, 0.20µM primer R, 0.20µM probe, Taq man universal PCR master mix (2x) 12.5µ, RNAse free water 6.9µ and DNA template 5µl for each *(*Navarro *et al*., 2015). For double-strand DNA denaturation a temperature of 95°C for10 minutes, amplification/extension at 95 ^{0}c for 15 seconds, annealing at 45^{0}c for 30 seconds, and 60^{0}c for 1 min for a final extension were used. Each experimental run included positive controls and negative controls, allowing for proper validation of results. Samples were considered positive if their cycle threshold (CT) values were ≤45. This specific real-time PCR approach was tailored to identify *Brucella* at both the genus level and for the species *B. abortus* and *B. melitensis,* utilizing both genus-specific and species-specific probes targeted for the *Brucella* species that predominantly affecting livestock and human (Appendix 14).

3.8. Data management and analysis

All data collected during the study period were checked, coded, and entered in to Microsoft Excel spreadsheet and analyzed using STATA version 14.0 for Windows (Stata Corp. College Station, TX, USA). The total prevalence for individual animal level was calculated on the basis of RBPT, Combined RBPT and C-ELISA and combined RBPT and CFT positivity, dividing the number of *Brucella* positive reactors by total number of tested animals multiplied by 100. A herd is said to be positive when it has one individual positive animal in it. Herd level prevalence for seropositivity was calculated by dividing the number of herds with at least one positive for brucellosis by the number of all herds included in the study multiplied by 100. Similarly for small ruminants flock level prevalence was determined by dividing the number of flocks with at least one positive for brucellosis by the number of total flocks testedmultiplied by 100 .

Descriptive analysis was utilized to present the data as frequencies and percentages. An explanatory research design was also implemented to clarify the connections between the explanatory variables and the dependent variable (brucellosis positivity). Univariable logistic regression analyses were performed to assess the relationship between potential risk factors and seropositivity for brucellosis, with odds ratios calculated at 95% confidence intervals (CIs). All risk factors that were significant in univariate analysis were considered as candidate for further analysis and included in the multivariable logistic regression analysis to explore the relationship between risk factors and the incidence of livestock and human brucellosis, also at a 95% CI. A P-value below 0.05 was deemed statistically significant. Odds ratio (OR) was used to measure the degree of association between risk factors and seroprevalence of brucellosis.

The choice of relevant variables in multivariable linear regression analysis often relies on univariable analysis, which evaluates the individual relationships between each predictor and the outcome. A typical approach involves using a cutoff P-value, commonly set at < 0.05, to identify candidate variables for inclusion in the multivariable linear regression. However, this threshold is frequently criticized for being insufficient. A variable may appear statistically insignificant when analyzed alone, yet prove to be significant when considered in conjunction with other variables in a multivariable framework, or vice versa. To mitigate these issues, higher P-value cutoffs of 0.2 or 0.25 are suggested (Malhotra R K., 2020). Therefore, in constructing the final multivariable logistic regression model, only those variables with a P-value less than 0.25 from the univariable analyses were included for further analysis. The

Hosmer–Lemeshow test was used to assess how well a logistic regression model fits the observed data and goodness-of-fit of the model or to evaluates how closely the predicted probabilities from a logistic regression model match the actual outcomes. P-value>0.05 showed good model fitness.

3.9. Ethical Consideration

This PhD study was partly supported by the research funded by the National Institute of Health with grant number U01HG007472-01, and the research obtained ethical approval from College of Veterinary Medicine and Agriculture certificate Ref. No: VM/ERC/10/03/12/2020 and Aklilu Lemma Institute of Pathobiology of Addis Ababa University, Ethiopia by Ref. No.: ALIPBIRB/54/2011/19 and the National Research Ethics Committee (ref no. 3.10/785/07) under the project both for animal and human study. Prior to the initiation of the research, transparency was prioritized; the animal owners were provided with comprehensive information regarding the study's objectives. This approach ensured that the participants were fully aware of the nature of the research and its implications. Importantly, verbal consent was secured from the animal owners, fostering an environment of cooperation and respect for animal welfare. This ethical framework underlines the commitment to responsible research practices, ensuring that animals are treated with care and dignity while facilitating valuable scientific inquiry. Such adherence to ethical standards is essential for maintaining public trust in veterinary research and advancing knowledge in the field.

For humans, individuals admitted to health centers with febrile condition were clearly communicated about the study and samples were collected only up on their willingness and with oral and written signed consent. Ethical considerations were prioritized through the acquisition of both oral and informed written signed consents from research participants (Appendix 15). This process ensured that individuals were fully aware of the study's purpose, procedures, and potential risks involved. For participants under the age of 18, consent was duly obtained from their families, recognizing the need for parental or guardian approval in accordance with legal and ethical standards. This approach not only safeguarded the rights of the minors involved in the research but also upheld the integrity of the study by ensuring that all participants provided consent in a responsible manner. Samples from human participants were collected by the help of professional nurse.

4. RESULTS

4.1. Seroprevalence and Risk factors

4.1.1. Seroprevalence and risk factors of bovine brucellosis

Out of the total 1920 serum samples collected from cattle, 121 (6.30%) (95% CI:4.17-10.12) were positive to RBPT. The 121 RBPT positive sera were retested with C-ELISA and the result showed 103 (5.36%) (95% CI: 4.17-8.38)were found to be positive for antibodies against natural infection by *Brucella*. Similarly, out of 121 RBPT positive sera 101 (5.26%) (95% CI: 5.45.8.04) were found to be positive with the combined RBPT and CFT tests. The overall seroprevalence of bovine brucellosis in south Omo Zone is thus 5.26% (101/1920) with combined RBPT and CFT tests. The individual animal seroprevalence of bovine brucellosis in the five district of South Omo Zone ranged from 3.31% to 9.46% (Table 11). There was statistically significant differences in individual animal seroprevalence of brucellosis among the five districts ($p<0.05$). Comparatively, the highest seroprevalence of brucellosis was recorded in Gnangatom district (9.46%) (95% CI: 1.35-14.25, P=0.00) while the lowest seroprevalence was observed in Malie district (3.31%) (95% CI: 0.90-6.20, P=0.05).

Table 11: Sero-prevalence of bovine brucellosis in five districts of South Omo Zone

District	**Number tested**	**RBPT Positive**	**95%CI**	**Combined RBPT & C-ELISA Positive**	**95% CI**	**Combined RBPT& CFT Positive**	**95% CI**	**P-Value**
Benatsemay	756	39 (5.03%)	1.25-6.45	35 (4.63%)	1.70-5.50	35 (4.63%)	0.60-6.40	0.067
Malie	423	14 (3.31%)	2.05-4.50	14 (3.31%)	1.65-5.05	14 (3.31%)	0.90-6.20	0.051
Hamer	199	20(10.0%)	7.50-12.55	15 (7.54%)	5.50-9.25	14 (7.04%)	1.40-9.55	0.002
Dassenech	394	29(6.35%)	3.30-9.50	25(6.35%)	3.34-8.50	24 (6.09%)	1.20-7.80	0.001
Gnangatom	148	19 (10.14%)	6.75-17.60	14 (9.46%)	4.5-13.60	14(9.46%)	1.35-14.25	0.000
Total	**1920**	**121(6.30%)**	**4.17-10.12**	**103(5.36%)**	**4.17-8.38**	**101(5.26%)**	**5.05-8.04**	

RBPT=Rose Bengal Plate Test, C-ELISA=Competitive Enzyme Linked Immune Sorbant Assay, CFT=Complement Fixation Test, CI=Confidence Interval

Out of the total 140 herds tested for brucellosis in the study area overall 51 (36.43%) (95% CI: 33.18-43.76) herds were found to be positive with the combined RBPT and CFT test. The highest herd level prevalence was recorded in Dassenech district with 56.67% (95% CI: 51.50-64.30). Village level seroprevalence ranged from 40% to 100%. The overall village level bovine brucellosis seroprevalence of

64% (95% CI: 59.63-71.69) was recorded in the study area. The highest village level seroprevalence 100% (95% CI: 101-102.45) was recorded for Dasesenech, whereas the lowest village level seroprevalences of 40% (95% CI: 32.05-49.75 and 33.70-47.40) was for Benatsemay and Malie districts of the study area (Table 12).

Table 12: Herd and village level prevalence with combined RBPT-CFT test

Districts	**Herd Level prevalence**			**Village Level Prevalence**		
	No. of Herds tested	**No. of Herds positive (%)**	**95% CI**	**No. of villages tested**	**No.of villages positive (%)**	**95% CI**
Benatsemay	45	14(31.11%)	27.45-42.20	5	2(40%)	32.05-49.75
Malie	30	7 (23.33%)	20.75-26.35	5	2(40%)	33.70-47.40
Hamer	20	5(25%)	17.20-27.25	5	4(80%)	73.80-89.50
Dassenech	30	17(56.67%)	51.50-64.30	5	5(100%)	101-102.45
Gnangatom	15	8 (53.33%)	49.00-58.70	5	3(60%)	57.60-69.33
Total	**140**	**51(36.43%)**	**33.18-43.76**	**25**	**16 (64%)**	**59.63-71.69**

RBPT=Rose Bengal Plate Test, CFT=Complement Fixation Test, CI=Confidence Interval

The Hosmer–Lemeshow test used to assess how well a logistic regression model fits the observed data and goodness-of-fit of the model result showed p-value of 0.69. This implies that the final logistic regression model had goodness of fit to the data (P-value>0.05).

Regarding the result of risk factors associated to seroprevalence of brucellosis at individual animal level, the current study revealed statistically significant associations (P<0.05) between variables (age, sex, herd size, abortion history, history of retained fetal membrane, body condition, and parity) and seropositivity for brucellosis except body condition score and frequency of abortion (Table 13).

There was a relative low seroprevalence (3.29%) of bovine brucellosis in animals with age range of 0.5 to 2 years old compared to animals with age range of 2 to 4, and above 4 years old with 8.77%, and 3.56%, respectively. In this study, from the total of 101 seopositive cattle 86(5.87%) seroprevalence was observed in older age category (>2 years of age) while only 15 (3.29%) seroprevalence was observed in younger age category 6 months to 2years old.

In the present study, a statistically significant association between sex and seroprevalence of brucellosis was observed (P<0.05). Almost all 93.07 % of the seropositive animals were females. Seroprevalence in

females was 7.22 % while 1.13% in males. In multivariable logistic regeression analysis the female animals were 2.10 times at higher risk of being seropositive to brucellosis than males for brucellosis (OR=2.10, 95% CI: 1.58-6.34).The multivariable logistic regression analysis revealed that herd size was significantly correlated (P< 0.05) with seropositivity of bovine brucellosis. Animals tested for brucellosis were categorized within herd size of <25animals, 25-50 animals, and >50 animals and the result illustrated seroprevalence of 2.43%, 5.19% and 7.91%, respectively. Those animals in large herd size were found to be at higher risk of seropositive than those in small herd size with the odds of 7.08 (95% CI: 5.00-8.05).

The results of multivariable logistic regression showed that the occurrence of abortion is positively and significantly associated with the seroprevalence of brucellosis (P<0.05). The seroprevalence was 37.26% and 1.38% in cows with and without history of previous abortion, respectively (Table 13). Similarly, history of occurrence of retained fetal membranes was statistically significantly associated with seropositivity for brucellosis (P<0.05). The seroprevalence was 23.06% and 1.15% in cows with and without history of retained fetal membrane, respectively. However, frequency of occurrence of abortion was not statistically significantly (P>0.05) associated with seroprevalence of brucellosis. Body condition scores in the current study had no statistical significant association between bovine brucellosis seropositivity (P>0.05) though higher prevalence of 3.85%, 4.50%, and 7.10% were recorded in increasing order in good, medium and poor body condition animals, respectively. Poor body condition scored animals have higher odds of risk of infection by *Brucella* (OR=4.50, 95% CI: 0.45-7.66) than medium and good body condition (OR=1.02, 95%CI: 1.01-3.04).

The study also showed that there is risk of *Brucella* infection as parity number increases. Statistically significant differences had been observed between seropositivity and among the three parity groups (P < 0.05). The study revealed that seroprevalence of 2.43%, 7.64%, and 10.81% were recorded in cows with parity number of 1 calf, 2 calves, and more than 3 calves, respectively. Cows with higher parity number have found to demonstrate higher risk of being seropositive to *Brucella* insfection (OR=10.02, 95% CI: 9.06-17.80) than those cows with low parity number (Table 13).

Table 13: Multivariable logistic regression analysis of risk factors of *bovine brucellosis*

Factors	**Level**	**Number Tested**	**Test Positive RBPT+CFT**	**OR**	**95% CI**	**P-Value**
Age	0.5-2 years [a]	456	15 (3.29%)	-	-	
	2-4 years	650	57 (8.77%)	5.75	4.30-7.45	
	Above 4 years	814	29 (3.56%)	3.05	2.20-5.50	0.02
Sex	Male [a]	619	7(1.13%)	-	-	
	Female	1301	94(7.22%)	2.10	1.58-6.34	0.00
Herd size	<25 animals [a]	575	14(2.43%)	-	-	
	25-50 animals	713	37(5.19%)	1.02	1.01-3.04	
	>50 animals	632	50(7.91%)	7.08	5.00-8.05	0.01
Abortion	Yes [a]	212	79(37.26%)	-	-	
History	No	1089	15 (1.38%)	9.01	5.07-9.09	0.00
Stage of	First trimester [a]	62	13(20.97%)	-	-	
Abortion	Second trimester	111	68(61.26%)	14.05	12.0-16.06	
	Third trimester	39	13(33.33%)	2.80	1.03-8.09	0.00
History of	Yes [a]	345	83(23.06%)	-	-	-
RFM	No	956	11(1.15%)	2.14	1.40-11.2	0.00
Frequency of	Only Once					
Abortion	More than one	148	70(47.30%)	7.08	0.90-15.45	0.07
		64	9(14.06%)			
Body	Good [a]	572	22(3.85%)	-	-	
condition	Medium	644	29(4.50%)	1.02	0.70-1.83	
	Poor	704	50(7.10%)	4.50	0.45-7.66	0.32
Parity	1calf [a]	329	8(2.43%)	-	-	
	2 calves	602	46(7.64%)	5.05	4.04-7.00	
	≥3calves	370	40(10.81%)	10.02	9.06-17.80	0.04

[a] *Others were computed in reference to this category, OR=Odds ratio, CI=Confidence interval*

This study also attempts to assess level of knowledge, awareness of brucellosis and practices posing risk to pastoralists in the study area (Table 14). Accordingly, majority of participants or pastoralists, 97.86% were not aware of bovine brucellosis. About 79% of the participants were not aware of zoonotic diseases transmitted through milk consumption, 65% of the participants also don't know pathogenic causes of

abortion in their cattle and 86% of them lack knowledge about diseases that can be transmitted during assisting parturition.

Concerning practices undertaken by the pastoralists in the study area that could be potential risk factors in transmission of brucellosis, 45% disposed dead fetus to open dump in the environment, and 55 % of participants fed aborted materials to dogs. All of the participants, 100% replied that the fate of frequently aborted cows was retained in the herd. All participants 100% practiced assisting parturition without any protective materials with their bare hands. All participants 100% in the study area confirmed that they have habit of consuming raw milk, and 85% of them have the habit of consuming raw animals' blood (Table 14).

Table 14: Knowledge about brucellosis, and Practices posing risk to pastoralists

Variables	Alternatives	Total No. of herders	Response
Awareness about brucellosis	Yes	140	3 (2.14%)
	No		137(97.86%)
Knowledge of zoonotic disease transmitted by milk consumption	Yes	140	29 (20.71%)
	No		111(79.29%)
Knowledge of infectious diseases causing abortion in animal	Yes	140	48(34.29%)
	No		92(65.71%)
Knowledge of diseases transmitted during handling of infected animal	Yes	140	37(26.43%)
	No		103(73.57%)
Knowledge of diseases transmitted during delivery assistance	Yes	140	19(13.57%)
	No		121(86.43%)
Ways of disposal of aborted material	Burning	140	0(-)
	Burying		0(-)
	Open dumping		63(45%)
	Feeding dogs		77(55%)
Fate of frequently aborted cows	Culling	140	0(-)
	Retaining		140(100%)
Assist parturition with bare hands	Yes	140	140(100%)
	No		0(-)
Habit of Consumption of raw animals' blood	Yes	140	119(85%)
	No		21(15%)
Habit of Consumption of raw milk	Yes	140	140(100%)
	No		0(-)

4.1.2. Seroprevalence and risk factors of ovine brucellosis

Out of 1536 sheep sera samples tested, 6.84% (105/1536; 95% CI: 3.06-9.07), 5.98% (92/1536; 95% CI: 5.10-8.27), and 5.40% (83/1536; 95% CI: 3.34-7.47) were found to be positive for brucellosis by mRBPT, combined mRBPT and C-ELISA, and combined mRBPT and CFT tests, respectively at individual animal level (Table 15).

Table 15: Individual animal level seroprevalence of ovine brucellosis

Districts	Number Tested	mRBPT positive	95%CI	mRBPT+ C-ELISA positive	95%CI	mRBPT+CFT positive	95% CI
1.Bena Tsemay	461	19 (4.12%)	0.45-4.68	17 (3.69%)	0.50-5.55	17(3.69%)	0.52-4.00
2.Malie	299	12 (4.01%)	0.38- 5.29	10 (3.34%)	0.80-4.20	10 (3.34%)	1.75-4.85
3.Hamer	274	20 (7.30 %)	2.16-8.04	18 (6.57%)	1.05-9.45	13 (4.74%)	1.78-6.90
4.Dassenech	345	40 (11.59%)	4.24-15.13	35 (10.14%)	7.50-12.35	34 (9.85%)	8.12-14.40
5.Gnangatom	157	14 (8.91%)	8.06- 12.20	12 (7.64%)	5.40-9.80	9 (5.73%)	4.50-7.20
Total	**1536**	**105 (6.84%)**	**3.06-9.07**	**92 (5.98%)**	**5.10-8.27**	**83 (5.40%)**	**3.34-7.47**

mRBPT=Modified Rose Bengal Plate Test, CFT=Complement Fixation Test, CI=Confidence Interval

Concerning flock level seroprevalence, out of 156 flocks tested 62 were found positive (39.74%) 95% CI: 36.50-48.8) by combined mRBPT and CFT test (Table 16). Regarding distribution of ovine seroprevalence among the districts, the highest seroprevalence was recorded in Gnangatom district 100% (16/16; 95% CI:97.58-103.30) followed by Dassenech 62.86% (22/35; 95% CI:53.06-65.45) and Hamer 32.14% (9/28; 95% CI:30.43-34.42), respectively in descending order (Table 16) at flock level.

Table 16: Flock level seroprevalence of ovine brucellosis

Districts	Number of Flocks Tested	mRBPT+CFT positive	95% CI
1. Bena Tsemay	47	13 (27.66%)	22.50-29.05
2. Malie	30	2 (6.67%)	5.65-13.70
3. Hamer	28	9 (32.14%)	30.43-34.42
4. Dassenech	35	22 (62.86%)	53.06-65.45
5. Gnangatom	16	16 (100%)	97.58-103.30
Total	**156**	**62 (39.74%)**	**36.50-48.8**

mRBPT=Modified Rose Bengal Plate Test, CFT=Complement Fixation Test, CI=Confidence Interval

In the present study ovine brucellosis at PAs (the smallest administrative units) showed 3 of 5 (60%) PAs from Bena Tsemay, 2 of 5 (40%) PAs from Malie, 4 of 5 (80%) PAs from Hamer, 5 of 5 (100%) PAs from Dassenech, and 5 of 5 (100%) PAs from Gnangatom districts contain at least one seropositive sheep for brucellosis.

The results of univariable and multivariate logistic regression analysis of individual animal level showed that district, sex, age, body condition, history of abortion, history of retained fetal membrane, parity, flock size, and production system had a statistically significant association with *Brucella* seropositivity ($P<0.05$).

Table 17: Univariable and multivariable logistic regression analysis of ovine brucellosis

			Univariate Logistic Regression Analysis			Multivariate Logistic Regression Analysis	
Variables	Alternatives	Number of sheep tested	mRBPT +CFT positive	OR (95% CI)	P-Value	OR (95% CI)	P-Value
Districts	Bena Tsemay	461	17(3.69%)	Reference			
	Malie	299	10 (3.34%)	0.1 (0.05-2.12)	0.12		
	Hamer	274	13 (4.74%)	0.6 (0.50-3.10)	0.06		
	Dassenech	345	34 (9.85%)	3.2 (1.98-6.58)	0.00	2 (1.56-7.50)	0.03
	Gnangatom	157	9 (5.73%)	2.6 (1.48-5.32)	0.00		
Sex	Male	549	5 (0.91%)	Reference			
	Female	987	78 (7.90%)	3.5 (2.45-7.50)	0.00	3 (1.55- 6.78)	0.00
Age	Young (<1 Year)	390	10 (2.56%)	Reference			
	Adult (>1 Year)	1146	73 (6.37%)	2 (1.80-9.50)	0.00	1.7 (1.40-2.50)	0.01
Flock Size	Small (≤25 sheep)	730	24 (3.28%)	Reference			
	Medium (26 to 49 sheep)	579	25 (4.32%)	1.1 (0.78-4.34)	0.04		
	Large (≥ 50 sheep)	227	34 (14.98%)	2.50 (1.55-6.90)	0.00	1.8 (1.05-4.05)	0.00
Production system	Agropastoral	275	11 (4.0%)	Reference			
	Pastoral	1261	72 (5.71%)	3 (1.95- 3.57)	0.01	3 (1.75-7.00)	0.03
History of Abortion	No	410	8 (1.95%)	Reference			
	Yes	577	70 (12.13%)	4 (2.65- 7.90)	0.00	4 (2.00-7.90)	0.00
Stage of Abortion	Early	185	16 (8.65%)	Reference			
	Late	392	54 (13.77%)	1.9 (1.78-4.92)	0.01	1.2 (1.08-2.69)	0.02
History of RFM	No	432	13 (3.00%)	Reference			
	Yes	555	65 (11.71%)	0.1 (0.05- 1.10)	0.07	1.05 (0.5-7.60)	0.07
Still birth	No	333	30 (9.0%)	Reference			
	Yes	654	71 (10.86%)	1.3 (0.78-3.80)	0.09	0.86 (0.07-3.55)	0.08
Body Condition Score	Good	490	11 (2.24%)	Reference			
	Medium	540	9 (1.67%)	0.12 (0.05-2.57)	0.04		0.03
	Poor	506	63 (12.45%)	3.1 (1.25-9.85)	0.01	1.7(1.05- 2.40)	0.01
Parity Number	One	372	22 (5.82%)	Reference			
	More than one	615	56 (9.10%)	0.5 (0.1-3.95)	0.13	0.45 (0.10-9.50)	0.09

CI=Confidence Interval, OR=Odds Ratio, RFM=Retained Fetal Membrane

Multivariable logistic regression analysis (Table 17) showed that ewes were 3.0 times more likely to be seropositive to brucellosis as compared to rams (OR=3.0, 95% CI: 1.55- 6.78, P=0.00). Adult sheep were

found to be 1.7 times more likely to be seropositive to brucellosis than young ones (OR=1.7, 95% CI: 1.40-2.50, P=0.01). Sheep in large flocks were 1.8 times more likely to be seropositive to brucellosis as compared to medium and small flock size (OR=1.8, 95% CI: 1.05-4.05, P=0.00). Ewes with a history of abortion were 4 times more likely to contract brucellosis than ewes without a history of abortion (OR=4, 95% CI: 2.00-7.90, P=0.00). Furthermore, sheep with poor body condition score were found to be 1.7 times more likely to be seropositive to brucellosis as compared to those with good body condition (OR=1.7, 95% CI: 1.050-11.32, P=0.01)

4.1.3. Seroprevalence and risk factors of caprine brucellosis

4.1.3.1. Overall seroprevalence

In this study out of the total 1, 536 goats' sera samples tested for seroprevalence of antibodies against *Brucella* natural infection using a series of serological tests, 185 (12.04%) (95% CI: 5.05-15.0), 108 (7.03%) (95% CI: 5.2-11.4), and 106 (6.90%) (95% CI: 5.18-10.90) prevalences were recorded using mRBPT, combined mRBPT and C-ELISA, and combined mRBPT and CFT tests, respectively (Table 18) at individual animal level.

Table 18: Individual animal level seroprevalence of caprine brucellosis

Districts	**No.of goats tested**	**mRBPT Positive**	**95% CI**	**mRBPT+ CELISA Positive**	**95% CI**	**mRBPT+CFT Positive**	**95% CI**
1. Hamer	213	30 (14.08%)	(1.70-12.50)	18 (8.45%)	(6.5-9.50)	17 (7.98%)	(5.90-11.60)
2. Dassenech	262	22 (8.40%)	(5.35-14.56)	19 (7.63%)	(3.55-11)	18 (6.87%)	(3.75-9.66)
3. Gnangatom	70	11 (15.71%)	(11.7-17.4)	9 (12.86%)	(8.80-16)	9 (12.86%)	(9.70-15.20)
4. Bena-Tsemay	648	63 (9.72%)	(1.35-11.50)	38 (5.86%)	(2.50-12.2)	38 (5.86%)	(2.20-9.70)
5. Malie	343	59 (17.20%)	(12.57-20.5)	24 (6.99%)	(4.65-8.3)	24 (6.99%)	(4.57-8.40)
Total	**1, 536**	**185 (12.04%)**	**(5.05-15.0)**	**108 (7.03%)**	**(5.2-11.4)**	**106 (6.90%)**	**(5.18-10.90)**

mRBPT = modified Rose Bengal Plate Test, C-ELISA = Competitive Enzyme Linked Immunosorbent Assay, CFT = Complement Fixation Test

Regarding the magnitude of seroprevalence of caprine brucellosis among the flocks and districts, out of the total of 102 flocks tested, 35 (34.31%) (95% CI: 30.52-42.41) were positive for caprine brucellosis (Table 19). The highest flock level seroprevalence was recorded in Gnangatom (60%) (95%CI:52.40-

62.90), followed by Malie (39.13%) (95% CI: 35.05-44.75), and Dassenech (35.29%) (95% CI: 31.55-38.20) districts.

Table 19: Flock level seroprevaalence of caprine brucellosis

District	Number of Flocks Sampled	Number of Positive Flocks (mRBPT+CFT result)	95% CI
1. Hamer	14	4 (28.57%)	26.85 -34.05
2. Dassenech	17	6 (35.29%)	31.55 -38.20
3. Gnangatom	5	3 (60%)	52.40 -62.90
4. Bena-Tsemay	43	13 (30.23%)	26.75 -32.15
5. Malie	23	9 (39.13%)	35.05 -44.75
Total	**102**	**35 (34.31%)**	**30.52-42.411**

4.1.3.2. Associated risk factors of caprine brucellosis

An analysis of risk factors with univariable logistic regression revealed significant associations with *Brucella* seropositivity at the individual animal level. Factors such as sex, age, body condition, history of abortion, history of retained fetal membrane, parity number, flock size, and production system were statistically significant ($P<0.05$) while there was no statistically significant difference among the districts ($P>0.05$) (Table 20).Female goats were found to be nearly 1.5 times more seropositive to brucellosis than males (OR=1.46, 95% CI: 1.96-2.23 and P=0.04). Adult goat faced a twofold increased risk compared to younger ones (OR=2.35, 95% CI: 1.75-2.40, and P=0.02). Furthermore, goats in larger flocks had 1.85 times the likelihood of being seropositive to brucellosis compared to the small flock size (OR=1.85, 95% CI: 1.14-3.02 and P=0.01). Goats in pastoral settings had higher (3 times) odds of contracting brucellosis as compared to those in agro-pastoral setting of the study area (OR=3.26, 95% CI: 2.50-7.06 and P=0.00). Goats with a history of abortion showed 9.64 times higher risk of being seropositive to brucellosis as compared to does without history of abortion (OR=9.64, 95% CI: 7.20-14.57 and P=0.00). Furthermore, goats with late stage of abortion had very higher odds of 23.64 times being seropositive to brucellosis - as compared to those abortion history of early stage (OR=23..64, 95% CI: 19.24-28.13 and P=0.00). In addition goats with history of retained fetal membrane had higher odds of being exposed to brucellosis as compared to those without the history of retained fetal membrane (OR=11.46, 95% CI: 9.55-12.12, and P=0.00). The study also showed that does with still birth had higher odds of risk as compared to those without still birth experience (OR=8.05, 95% CI: 5.32-12.19,

and P=0.00). Finally, goats with more than one parity number had recorded higher odds of exposure to *Brucella* seropositivity with odds of 4.61 as compared to those goats with only one parity number (OR=4.61, 95%CI: 3.06-6.96, and P=0.00).

Table 20: Univariable logistic regression analysis of risk factors of *Brucella* seropositivity in goats

Variables	**Alternatives**	**Number Tested**	**Prevalence mRBPT+CFT**	**OR (95%CI)**	**P-Value**
Districts	Hamer	213	17 (7.98 %)	Reference	
	Dassenech	262	18 (6.87%)	0.85 (0.43-1.69)	0.65
	Gnangatom	70	9 (12.88%)	1.70 (0.72-4.01)	0.23
	Bena-Tsemay	648	38 (5.86%)	0.72 (0.39-1.30)	0.28
	Malie	343	24 (7.00%)	0.87(0.46- 2.66)	0.67
Sex	Male	618	34 (5.50%)	Reference	
	Female	918	72 (7.84%)	1.46 (1.96-2.23)	0.04
Age	Young	257	5 (1.95%)	Reference	
	Adult	1279	101 (7.90%)	2.35 (1.75-2.40)	0.02
Flock size	Small	553	27 (4.88%)	Reference	0.04
	Medium	440	32 (7.27%)	1.53 (1.90-3.59)	0.01
	Large	542	47 (8.67%)	1.85 (1.14- 3.02)	0.01
Production	Agro-pastoralist	520	20 (3.85 %)	Reference	
system	Pastoralist	1016	86 (8.46%)	3.26 (2.50-7.06)	0.00
History of	No	814	9 (1.10%)	Reference	
Abortion	Yes	104	63 (60.58%)	9.64 (7.20-14.57)	0.00
Stage of	Early	23	6 (26.08%)	Reference	
abortion	Late	81	57 (70.37%)	23.64 (19.24-28.13)	0.00
History of	No	847	54 (6.38%)	Reference	
RFM	Yes	71	52 (73.24%)	11.46 (9.55-12.12)	0.00
Still birth	No	707	50 (7.07%)	Reference	
	Yes	211	56 (26.54%)	8.05 (5.32- 12.19)	0.00
Body	Poor	521	70 (13.44%)	Reference	
Condition	Medium	539	28 (5.19%)	0.35 (0.22-0.56)	0.00
	Good	476	8 (1.68 %)	0.11 (0.05- 0.23)	0.00
Parity	One	463	33 (7.13%)	Reference	
Number	More than one	455	39 (8.57%)	4.61 (3.06-6.96)	0.00

Following the univariable regression analysis, the choice of relevant candidate variables for multivariable linear regression analysis variables that recorded P-value cutoffs of 0.2 or 0.25 are suggested (Malhotra R K., 2020).

Table 21: Multivarible logistic regression analysis of factors associated to brucellosis in goats

Variables	**Alternatives**	**Number Tested**	**Seroprevalence mRBPT+CFT**	**OR (95% CI)**	**P-Value**
Sex	Male	618	34 (5.50%)	Reference	
	Female	918	72 (7.84%)	2.06 (1.02-4.19)	0.00
Age	Young	257	5 (1.95%)	Reference	
	Adult	1279	101 (7.90%)	3.72 (1.33-6.56)	0.00
Flock size	Small	553	27 (4.88%)	Reference	
	Medium	440	32 (7.27%)	0.94 (0.90-2.44)	0.02
	Large	542	47 (8.67%)	1.82 (1.5-3.69)	
Production system	Agro-pastoralist	520	20 (3.85 %)	Reference	0.00
	Pastoralist	1016	86 (8.46%)	3.26 (2.82-5.94)	
History of Abortion	No	814	9 (1.10%)	Reference	
	Yes	104	63 (60.58%)	9.34 (7.90-15.87)	0.01
Stage of abortion	Early	23	6 (26.08%)	Reference	
	Late	81	57 (70.37%)	14.35 (13.55-27.99)	0.00
History of RFM	No	847	54 (6.38%)	Reference	
	Yes	71	52 (73.24%)	8.13 (6.85-13.22)	0.00
Still birth	No	707	50 (7.07%)	Reference	
	Yes	211	56 (26.54%)	1.59 (1.44-5.75)	0.04
Body Condition	Poor	521	70 (13.44%)	Reference	
	Medium	539	28 (5.19%)	0.49(0.26-0.92)	
	Good	476	8 (1.68 %)	0.16 (0. 06-0 .42)	0.00
Parity Number	One	463	33 (7.13%)	Reference	
	More than one	455	39 (8.57%)	3.81 (1.29-4.50)	0.02

OR = Odds ratio, CI = Confidence interval

The result of multivariable logistic regression analysis shows that female goats were 2 times more likely to be seropositive for brucellosis than males (OR=2.06, 95% CI: 1.02-4.19, and P=0.00). Adult goats were found tobe 3.72 times more likely to be seropositive to brucellosis as compared to the young goats (OR=3.72, 95%CI: 1.33-6.56, and P=0.00). Furthermore, goats in large flock size were 1.82 times more likely to be seropositive as compared to those in small flock size (OR=1.82, 95%CI:1.5-3.69, and P=0.02). Goats in pastoral production system were 3.26 times more likely to be seropositive to brucellosis as compared to those in the agr-pastoral production system (OR=3.26, 95%CI: 2.82-5.94, and P=0.00). Those goats with history of abortion were 9.34 times more likely to be brucellosis seropositive than those without history of abortion (OR=9.34, 95% CI:7.90-15.87, and P=0.01). Goats with history of abortion occurrence in the late stage of pregnancy were found to be 14.35 time more likely to be seropositive to brucellosis than those at early stage of abortion (OR=14.35, 95%CI:13.55-27.99,and P=0.01). Goats with history of retained fetal memebrane were about 8.13 times more likely to be brucellosis seropositive as compared to those without retained fetal memebrane (OR=8.13, 95%CI=6.85-13.22, and P=0.00). Goats experiencing stillbirth had 1.59 times more likely to be seropositive to brucellosis as compared to those without stillbirth (OR=1.59, 95% CI: 1.44-5.75, and P=0.04), and finally goats with parity number greater than one were 3.81 times more likely to be brucellosis seropositive than those with one parity number (OR=3.81, 95%CI: 1.29-4.50, and P=0.02).

4.1.4. Seroprevalence and risk factors of human brucellosis

4.1.4.1. Overall seroprevalce of human brucellosis

In the current study that intended to examine antibody detection for human brucellosis, a total of 768 individuals were sampled. The findings indicated seroprevalence rates of 15.88% (n=122) (95% CI: 14.04-20.02), 14.97% (n=115) (95% CI: 12.83-18.09), and 14.58% (n=112) (95% CI: 12.07-18.24) when assessed using RBPT, combined RBPT and C-ELISA, and combined RBPT and CFT tests, respectively. The overall seroprevalence was calculated to be 14.58% (95% CI: 12.07-18.24). The investigation into human brucellosis seroprevalence in the South Omo Zone revealed significant differences among the five study districts. Notably, the Gnangatom district displayed a particularly elevated seroprevalence rate of 20% followed by 17.06% in Hamer (Table 22). This noticeable seroprevalence could be related to the higher prevalence of brucellosis in livestock. Very interestingly, out of the five districts higher seroprevalence of bovine brucellosis was recorded by the study which is part of this PhD. dissertation (Sorsa *et al.,* 2021). In these areas it is observed that a large number of

pastoralists settled with their animals temporarily during dry season around Mago Park to get access to the grazing land and water point for the animals. Most importantly, the animals have contact with wild life in the park and brucellosis may transmit between the livestock and wild life, which needs further investigation.

Table 22: Seroprevalence of human brucellosis in south Omo Zone across the study Districts

District	**Number Sampled**	**RBPT Result**	**95% CI**	**RBPT&C-ELISA Combined Result**	**95%CI**	**RBPT&CFT Combined Result**	**95% CI**
Bena-Tsemay	159	24(15.09%)	13.30-18.60	20 (12.58%)	10.44-14.55	20 (12.58%)	10.45- 14.38
Hamer	170	32(18.82%)	15.50-23.75	30(17.65%)	15.05-19.55	29(17.06%)	12.55- 18.20
Dasenech	147	21(14.28%)	11.40-16.77	21(14.28%)	11.00-17.50	21(14.28%)	10.95-19.67
Gnangatom	50	12(24%)	20.20-26.50	11(22%)	17.78-24.05	10(20%)	17.25-23.05
Malie	242	33(13.64%)	9.78-14.50	33(13.64%)	9.90-14.80	32(13.22%)	9.15-15.89
Over all	**768**	**122(15.88%)**	**14.04-20.02**	**115(14.97%)**	**12.83-18.09**	**112 (14.58%)**	**12.07-18.24**

RBPT=Rose Bengal Plate Test, CI=Confidence interval, C-ELISA=Competitive Enzyme linked Immunosorbent Assay, CFT=Complement Fixaion Test

4.1.4.2. Risk factors for human brucellosis seropositivity

Table (23) presents the prevalence and univariable logistic regression analysis regarding the associations of various risk factors linked to *Brucella* seropositivity in humans. The key exposure variables identified as predictors of the outcome variable include district, gender, age, educational level, occupation or production system, consumption of raw milk, intake of raw meat, consumption of raw animal blood, direct contact with potentially infected animals, sharing water sources with animals, knowledge of zoonotic diseases, involvement in parturition assistance, methods of providing support during parturition, handling aborted materials, and the disposal practices of aborted materials. The findings indicate that most of the recorded variables exhibited statistically strong association with seropositivity to *Brucella* infection ($P<0.05$).

Table 23: Univariable logistic regression analysis of risk factors of human brucellosis

Risk Factors	Level	sample Tested	Number of Positive (%)	OR (95%CI)	P-Value
District	Bena-Tsemay	159	20 (12.58%)		
	Hamer	170	29(17.06%)	1.16(0.77- 2.65)	0.26
	Dasenech	147	21(14.28%)	1.74(0.60-2.24)	0.66
	Gnangatom	50	10(20.00%	1.06(0.75-4.01)	0.19
	Malie	242	32(13.22%)	1.43(0.58-1.93)	0.85
Gender	Male	328	15(4.57%)		
	Female	440	97(24.05%)	5.9 (3.35-10.38)	0.00
Age	<18 Years	99	23(23.23%)		
	18-30 Years	135	21(15.55%)	0.61(0.32-1.18)	0.14
	31-40 Years	204	11(5.39%)	0.19(0.09-0.40)	0.00
	41-50 Years	129	10(7.75%)	0.28(0.13-0.62)	0.00
	51-60 Years	101	14(13.86%)	0.54(0.256-1.11)	0.09
	>60 Years Old	100	33(33.00%)	1.63(0.87-3.04)	0.13
Marital Status	Single	337	55(16.32%)		
	Married	431	57(13.23%)	0.79(0.52-1.17)	0.23
Level of education	Illiterate	539	101(18.73%)		
	Elementary School	183	10(5.46%)	0.25(0.13-0.49)	0.00
	High school	31	1(3.23%)	0.15(0.02-1.18)	0.06
	Diploma	10	0	0.000(0-5.30)	0.99
	Bachelor Degree	5	0	0.000(0-7.72)	0.99
Production/Occupation	Agro-pastoralist	303	23(7.59%)		
	Pastoralist	465	89(19.14%)	2.88 (1.78-4.68)	0.00
Raw milk consumption	No	255	4(1.57%)		
	Yes	513	108(21.05%)	16.73 (13.09-19.96)	0.00
Raw meat consumption	No	245	6(2.45%)		
	Yes	523	106(20.27%)	10.13 (4.38-23.40)	0.00
Animals' Blood consumption	No	405	30(7.41%)		
	Yes	363	82(22.59%)	3.65 (2.34-5.69)	0.00
Sharing Water points with animals	No	44	8(18.18%)		
	Yes	724	104(14.36%)	0.76 (0.34-1.67)	0.49
Knowledge of Zoonosis	No	540	100(18.52%)		
	Yes	228	12(5.26%)	0.44 (0.13-0.46)	0.00
Close contact with animals	No	181	10(5.52%)		
	Yes	587	102(17.38%)	3.60 (1.84-7.05)	0.00
Assisting parturition	No	376	29(7.71%)		
	Yes	392	83(21.17%)	3.20 (2.05-5.04)	0.00
Contact with aborted material	No	395	38(9.62%)		
	Yes	373	74(19.84%)	2.33 (1.53-3.54)	0.00
Mechanism of assisting parturition	Bare hand	495	86(17.37%)		
	Protected	273	26(9.52%)	0.50 (0.31-0.79)	0.00
Knowledge of brucellosis	No	495	91(18.38%)		
	Yes	273	21(7.69%)	0.37 (0.22-0.61)	0.00
Disposal of aborted materials	Burn	21	0		0.04
	Burry	94	3(3.19%)	1.57 (0.35-9.45)	0.99
	Throw away	653	109(16.69%)	6.53 (0.11-9.16)	0.99

OR=Odds Ration, CI=Confidence Interval

The multivariable logistic regression analysis revealed that all predictor variables except mechanism of assisting parturition were found to be statistically significantly associated with increased risk of *Brucella* seropositivity in humans (P<0.05) (Table 24). Briefly, the current study revealed that seroprevalence recorded in females (24.05%) is higher than males (4.57%) with (OR= 6.9, 95% CI: 3.37-13.98 and P=0.00); regarding age of humans sampled and tested, relatively higher seroprevalence of brucellosis was recorded in young <18 years old, and >60 years old, 23.23%, and 33%, respectively as compared to other age categories. Older individuals were 2.5 times more likely to be seropositive for *Brucella* infection than other age groups in the study area and it is statistically significant (OR=2.5, 95%CI: 1.92-6.52, P=0.00); there is statistically significant difference between seropositivity and level of education of the sampled humans (*P*<0.05). Higher seroprevalence was recorded in illiterate individuals (18.73%) than other higher education level; seropositivity for natural *Brucella* infection in the pastoralist demographic stands at 19.14%, significantly higher than the 7.59% prevalence observed among agro-pastoralists; individuals who consumed raw milk had 12.4 times higher odds of *Brucella* seropositivity than those who had not (OR= 12.40, 95% CI: 4.05-18.22 and P=0.00), and statistically significant; the present study revealed that seroprevalence of human brucellosis was found to be higher in those individuals who consumed raw meat (20.27%) than those who don't consume raw meat (2.45%) and the difference is statistically significant (OR=13.9, 95% CI: 5.07-17.96, and P=0.00). Patients who had raw animals blood consumption experience were 3.2 times more likely to be brucellosis seropositive as compared to those without consumption of raw animals' blood (OR=3.2, 95% CI:1.72-5.99, and P=0.00). Furthermore, those patients assisting parturition of animals were 2.5 times more likely to be seropositive to brucellosis as compared to those not assisting parturition (OR=2.5, 95% CI: 1.32-4.54, aand P=0.00). Similarly, patients who had contact with aborted materials were 2.2 times more likely to be seropositive to brucellosis than those who had not (OR=2.2, 95% CI: 1.19-4.09, P=0.01).

Table 24: Multivariable logistic regression analysis of factors associated to brucellosis in human

Risk factors	Categories	Tested No	RBPT+CFT Positive (%)	OR (95% CI)	P-Value
Gender	Male	328	15(4.57%)	Reference	
	Female	440	97(24.05%)	6.9(3.37-13.98)	0.00
Age Categories	<18 Years old	99	23(23.23%)	Reference	
	18-30 Years	135	21(15.55%)	0.7(0.25-1.79)	
	31-40 Years	204	11(5.39%)	0.3(0.09- 0.76)	0.00
	41-50 Years	129	10(7.75%)	0.4(0.12- 1.08)	
	51-60 Years	101	14(13.86%)	0.6(0.22-1.67)	
	>60 Years	100	33(33.00%)	2.5(1.92- 6.52)	
Educational Level	Illiterate	539	101(18.73%)	Reference	
	Elementary School	183	10(5.46%)	0.3(0.12- 0.75)	0.04
	High school	31	1(3.23%)	0.1(0.01-0.13)	
Occupation/production	Agro-pastoralist	303	23(7.59%)	Reference	
	Pastoralist	465	89(19.14%)	3.3(1.63- 6.78)	0.00
Consumption of raw milk	No	255	4(1.57%)	Reference	
	Yes	513	108(21.05%)	12.4(4.05-18.22)	0.00
Consumption of Raw Meat	No	245	6(2.45%)	Reference	
	Yes	523	106(20.27%)	13.9(5.07- 17.96)	0.00
Close contact with animals	No	181	10(5.52%)	Reference	
	Yes	587	102(17.38%)	3.4(1.44- 8.15)	0.01
Consumption of raw Animal Blood	No	405	30(7.41%)	Reference	
	Yes	363	82(22.59%)	3.2(1.72-5.99)	0.00
Assisting Parturition	No	376	29(7.71%)	Reference	
	Yes	392	83(21.17%)	2.5(1.32-4.54)	0.01
Mechanism of Assisting Parturition	Bare hands	495	86(17.37%)	Reference	
	Protected	273	26(9.52%)	0.5(0.26-1.04)	0.07
Contact with abortion material	No	395	38(9.62%)	Reference	
	Yes	373	74(19.84%)	2.2(1.19-4.09)	0.01
Knowledge of Zoonosis	No	540	100(18.52%)	Reference	
	Yes	228	12(5.26%)	0.4(0.17- 0.91)	0.03
Knowledge of Brucellosis	No	495	91(18.38%)	Reference	
	Yes	273	21(7.69%)	0.4(0.22-0.87)	0.02

OR=Odds Ration, CI=Confidence Interval

Regarding knowledge of zoonosis of the research participants, in the present study out of 768 participants 540 (70.31%) had no knowledge of zoonosis. The multivariable logistic regression analysis findings indicated that individuals with low or no knowledge regarding zoonosis recorded higher rates of seropositivity 18.52% for brucellosis (OR=0.4, 95% CI:0.17-0.91, and *P*=0.03) while low rate of seropositivity of 5.26% for brucellosis was recorded in individuals having knowledge regarding zoonosis. The analysis also revealed a significant disparity in seroprevalence rates, where participants

without awareness of brucellosis showed a higher seroprevalence of 18.38% (OR= 0.4, 95% CI: 0.22-0.87, and *P*=0.02), compared to relatively lower seroprevalence of 7.69% recorded among those who had awareness about the disease.

4.2. Isolation and molecular identification of *Brucella* species in livestock and human

4.2. 1. Bacteriological and biochemical analysis results

In the current study out of 340 biological samples (n=53 milk sample and n=34 vaginal swab from bovine, n=39 milk and n=48 vaginal swab from goats, n=28 milk and n=16 vaginal swab from sheep, and n=122 whole blood samples from human) inoculated on *Brucella*-selective supplemented media, 15 samples (4.41%), revealed the growth of typical *Brucella* colonies. Specifically, six (40%) of these isolates originate from vaginal swabs (1 from bovine, 3 from caprine, and 2 from ovine) evidencing high levels of bacteria are found in the birth fluids of an infected animal, five (33.33%) from milk samples (2 from bovine and 3 from caprine) of animals, and the remaining four (26.67%) from whole blood of human samples. The recovered isolates included three from bovine, six from caprine, two from ovine, and four from humans. All colonies exhibited distinctive *Brucella* colony features.

Geographically, the distribution of the isolates indicated the highest prevalence in the Hamer district with six isolates (40%), followed by Gnangatom with four (26.67%), and two each from Desenech and Bena-Tsemay (13.33% each), and one from Malie (6.67%).

Notably, the isolates from the humans were one each from the Bena-Tsemay and Gnangatom, and two from Hamer district. This finding highlights the occurrence of *Brucella* in multiple hosts including humans and distributed within all studied districts of South Omo Zone (Table 25).

Table 25: Phonotypical characteristics of *Brucella* isolates from livestock and human

Sample source	Samples Type and size		Growth positive	Colony	Urease Test	Oxidase	H_2S Production	CO_2 Requirement	Growth On Basic fuchsine Dye	Growth on Thionin dye
Bovine	Vaginal Swab	34	1	S	(+)	(+)	(+)	(+)	(+)	(-)
	Milk	53	2	S	(+)	(+)	(+)	(+)	(+)	(-)
Caprine	Vaginal Swab	48	3	S	(+)	(+)	(-)	(-)	(+)	(+)
	Milk	39	3	S	(+)	(+)	(-)	(-)	(+)	(+)
Ovine	Vaginal Swab	16	2	R	(-)	(-)	(-)	(+)	(-)	(+)
	Milk	28	-	-	-	-	-	-	-	-
Human	Whole Blood	122	2	S	(+)	(+)	(-)	(-)	(+)	(+)
			2	S	(+)	(+)	(+)	(+)	(+)	(-)

S=smooth colonies, R=Rough colonies, (+) =Positive, (-) =Negative

The colony morphology showed that out of the total isolates all have smooth colonies except two of the isolates which demonstrated rough colonies. Isolates with observed rough colonies were recovered from vaginal swabs taken from ovine. All isolates with smooth colonies were found to be positive for oxidase and urease tests while the two isolates with rough colonies have reacted negative for both oxidase and urease tests. The two isolates from ovine grow in 5-10%CO_2 and thionine dye but fail to grow in the presence of basic fuchsine dye and also no production of H_2S.

Out of the total 15 isolates, 8 (53.33%) of them have exhibited similar phenotypic features all with smooth colonies, positive for urease, oxidase tests and able to grow in the presence of basic fuchsine and thionine dyes but negative for H_2S and CO_2. While 5(33.33%) exhibited similar features such as smooth colony, positive for urease, oxidase, H_2S production and grow in CO_2, basic fuchsine dye but fail to grow in media containing thionine dye. Finally, the remaining 2 had shown rough colonies, negative for urease, oxidase, H_2S, and unable to grow in basic fuchsin (20 μg/mL) dye but able to grow in high concentration of CO_2 and in media containing thionin dye. Based on these features eight isolates demonstrated similar phenotypic character can be *B. melitensis* suspected, five isolates having similar features are suspected to be *B. abortus,* and the two isolates that had same characteristics were *B. ovis* suspected species. Finally, after the series of primary and secondary biochemical tests and identifying

the isolates to the suspected species, all the isolates were subjected to Real time PCR analysis for further confirmation of the isolates to the genera and species level.

4.2.2. Real-Time PCR Analysis result

All the isolates (15) identified based on phonotypical and biochemical tests were further analyzed by Real-Time PCR to characterize to the genus and species levels, using universal primer (IS711) gene and species specific primers and probes. Accordingly, all the isolates 15(100%) were confirmed as genus *Brucella* (Figure 2) as all the isolated have amplified by the universal *Brucella* genus specific primer and probes. Out of the screened genus of *Brucella*, 3(20%) were from samples obtained from bovine, 6(40%) were from samples collected from caprine, and the remaining 4(26.67%) were in samples from humans.

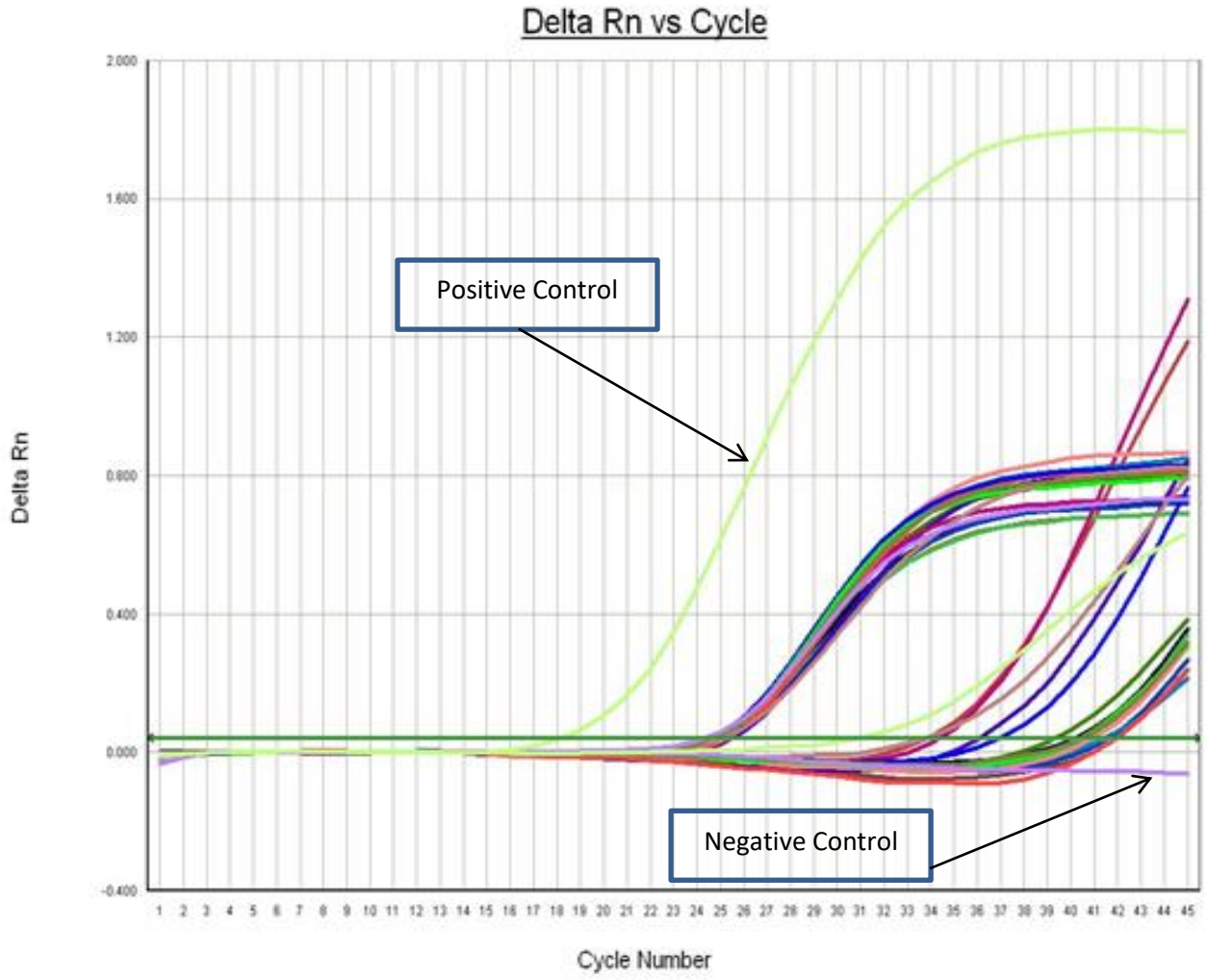

Figure 2. Real Time PCR amplification result of genus *Brucella* screening test using the IS711

Legend:

- All 15 isolates subjected to RT-PCR were amplified using universal primer with some containg high DNA concentration amplified at early cyle before 45 cycles while those with low DNA concentration amplified later with different colored curves due to florescent empregnated probes.

Following confirmation of the isolates to the genus *Brucella* level, the isolates were further analyzed to identify to the species level. All the 15 isolates amplified DNA by the universal primer and probe were analyzed using *B.melitensis* specific primer and probe. Consequently, out of 15 isolates, 8 were identified as *B. melitensis, a*mong which 6 were derived from caprine sources and 2 from human samples, as indicated by analysis with species-specific primers and probes via Real-Time PCR (Figure 3).

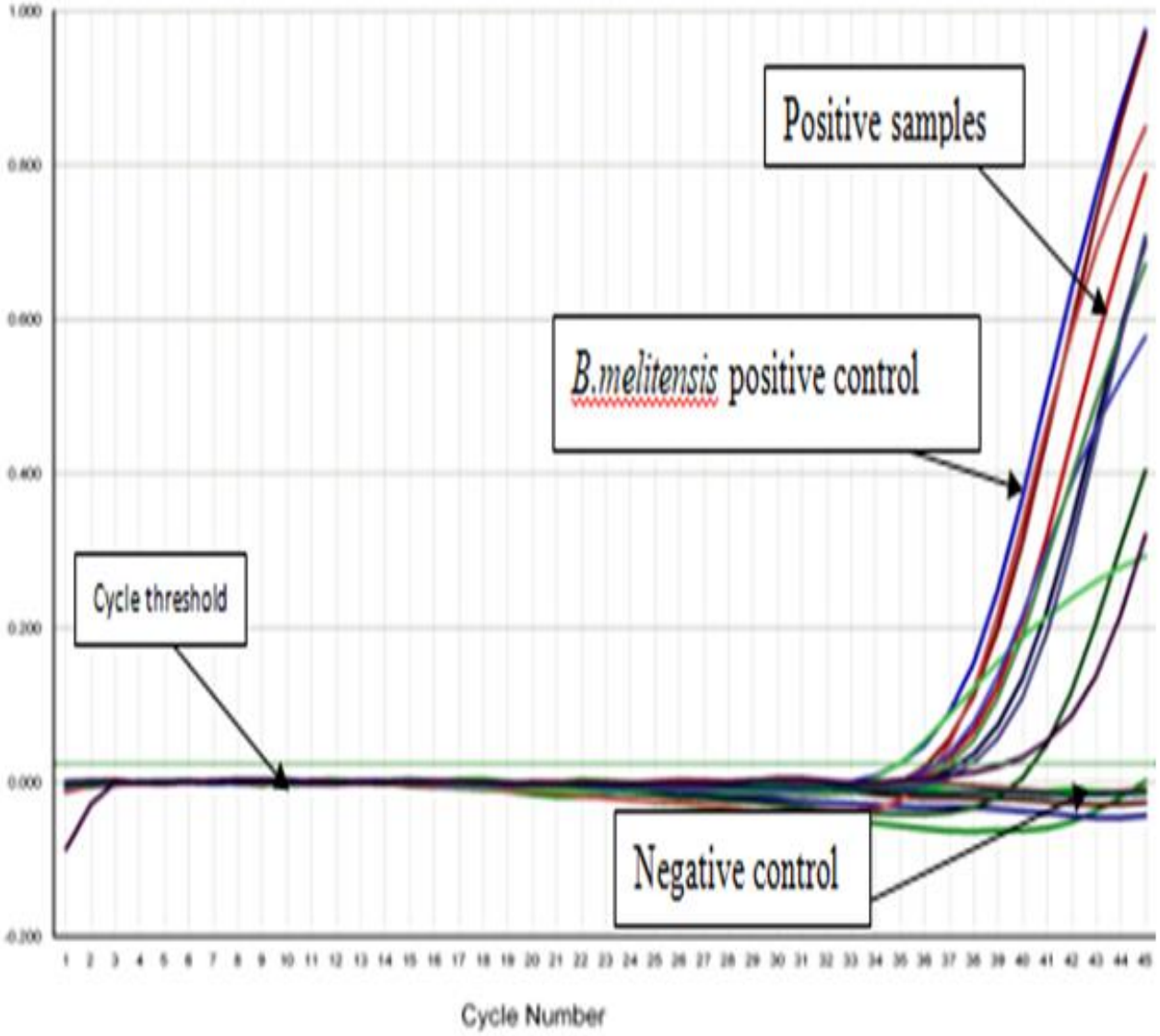

Figure 3. Real time PCR amplification result of *B.melitensis* species specific primer use

After running RT-PCR with *B.melitensis* specific primer and probes and identifying the 8 isolates as *B.melitensis*, all the isolates were also tested using *B. abortus* specific primer and probe. Accordingly, out of 15 isolates, 5(33.33%) were proven as *B. abortus*, 3 from bovine samples and 2 from human samples (Figure 4). While the remaining 2 isolates, accounting for 13.33%, were amplified with a universal primer but failed to react with the species-specific primers and probes used (*B. melitensis* and *B. abortus*). Due to lack of specific primers and probes for other species of *Brucella*, we couldn't confirm the specific species of the isolates recovered from sheep in this study. However, based on phenotypic and biochemical characterization the two isolates were suggested to be *B. ovis*. The two

isolates displayed rough colony morphology, demonstrated a reliance on CO_2 for growth, and showed no hydrogen sulfide (H_2S) production. Moreover, they tested negative for both oxidase and urease and were capable of growth in the presence of thionin dye but not in basic fuchsin dye. This suggests distinct biochemical properties that differentiate them from *B. melitensis* and other species.

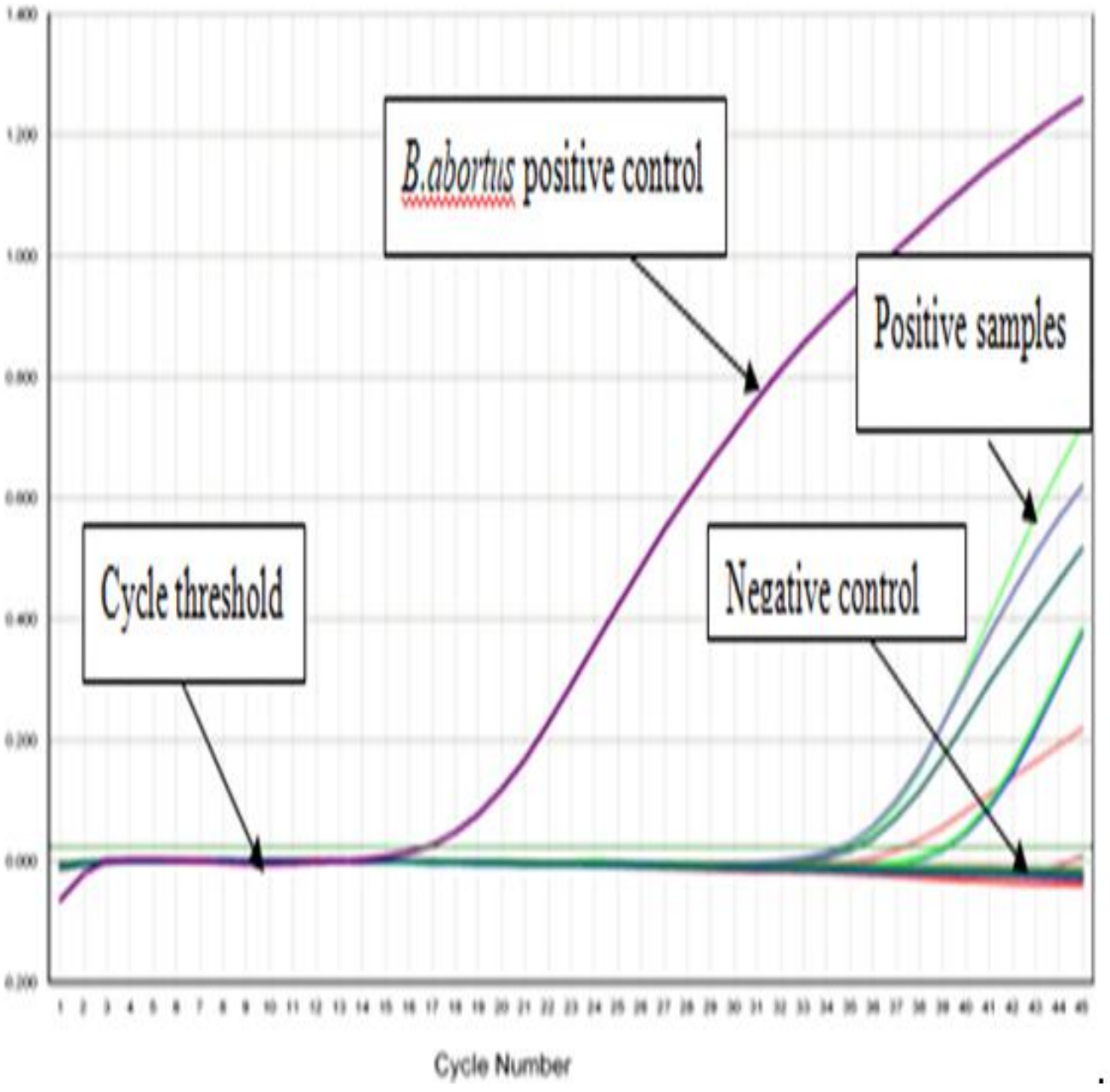

Figure 4. Real-Time PCR amplification result of *B. abortus species specific* primer

5. DISCUSSIONS

This PhD study is the most intensive work in terms of large sample size, wider geographical coverage in South Omo zone of South Ethiopia Regional State and inclusion of diverse species of livestock (bovine, ovine, and caprine), and human from the same site with the aim to determine the seroepidemiology and isolate and molecularly identify the causative agents with their potential zoonotic transmison pattern of brucellosis to the pastoralist in the study area. The study involves 1920 cattle, 1536 sheep, 1536 goats, and 768 human samples for estimation of seroprevalence and determines associated risk factors of brucellosis. In addition, the study collected a total of 340 clinical specimens from the target population of the livestock and humans, isolated and molecularly characterized *Brucella* species circulating in animals and human populations in the study area.

The current study used serially RBPT as screening and combined RBPT and C-ELISA and combined RBPT and CFT as confirmatory tests. The use of serial tests is recommended to maximize the precision of test results. RBPT is highly sensitive test and C-ELISA and CFT are highly specific and used as assenting tests (Radostits *et al.*, 2007; Godfroid *et al.*, 2010). Combining multiple serological tests in diagnostic algorithms improves diagnostic accuracy (Loubet *et al*., 2024).

Sensitivity and specificity of serological tests for brucellosis detection varies with speficic serological test used and species of animals tested. For example Abinet *et al*. (2023) found sensitivity level of RBPT to be 100% for shoats, and 74% cattle serum for brucellosis testing. However, the same study revealed specificity of RBPT to be 98.69%, 99.28%, 100% in sheep, goats, and cattle, respectively. Specificity on CFT was 100% for sheep, goats, and cattle. Moreover, Zhang *et al*. (2018) reported CFT as the most specific for brucellosis diagnosis followed by C-ELISA and RBPT as the least specific. Interms of sensitivity, RBPT was found to be the most sensitive followed by C-ELISA but CFT as least sensitive.

5.1. Seroprevalence and risk factors of bovine brucellosis

The overall individual level seroprevalence in five districts of south Omo Zone was 5.26% (95% CI:5.05-8.04) (Table 11). This prevalence is in agreement with previous findings reported by Wubishet *et al*. (2019), 5.3% from Borana.This prevalence was comparable with previous studies reported from different parts of Ethiopia, such as Hunduma and Regassa (2009), who reported 4.70% from Borana; Shewit *et al.* (2008), who reported 4.90% prevalence from Western Tigray; Gebawo *et al*. (2014)

documented a 4.50% in Adami Tulu; and Alehegn *et al.* (2016), who reported 4.90% in and around Gonder. The finding of the current study is also in line with reports from other African countries, such as Tanzania 5.30% prevalence by Swai *et al.* (2009), Egypt 5.40% by Samaha *et al.* (2008), and Eritrea 5.60% by Omer *et al.* (2000).

Contrary to the current study, lower seroprevalences were documented in previous studies. These lower seroprevalences range between 0.14% Yayeh (2003) to 3.40% Megersa *et al*. (2011), Hunduma and Regassa (2009) reported 3.0% from Jigjiga, Megersa *et al.* (2011) reported 3.40% from South Omo Zone, Bashitu *et al.* (2015) 0.2% from Ambo, Bashitu *et al*. (2015) 0.7% from Debre-Birhan, Minda *et al.* (2016) 0.78% from Bishoftu, Gelaye *et al*. (2010) 1.70% from Sidama Zone, Nuradis *et al*. (2009) 3.10% from Jima Zone, Berhe *et al.* (2007) 3.19% from Tigray, Yilma (2016) 1.04% in and around Chench, Gamo Goffa, Southern Ethiopia, Tolosa *et al.* (2008) 2.40% from Jimma Zone, Jergefa *et al.* (2009) 2.90% from Central Oromia, *Adugna et al.* (2013) 1.0% from Western Ethiopia, and Fikadu *et al*. (2014) reported 2% from East Shewa Zone. Moreover, Bisrat (2007) reported a 1.9% prevalence from Debre Zeit, Asmare *et al.* (2007) documented a 2.46% prevalence in the Sidama Zone of southern Ethiopia, Hailu *et al.* (2011) reported a seroprevalence of 1.38% in the Jigjiga zone of the Somali Regional State, and Gebreyohans (2004) reported 1.5% seroprevalence in Addis Ababa dairy farms. These lower seroprevalences recorded in the previous studies could be associated with production systems, breed differences, reproduction status, farm hygiene, and sample size. Majority of this lower seroprevalence reports were from intensive production system from urban and peri-urban areas where relatively good farm hygiene is practiced.

Relatively the higher level seroprevalence 5.26% of the current work in the study area might be related to poor hygienic practices, pastoralists' lack of awareness and knowledge on brucellosis, frequent contact among herds at grazing and watering points exposing the animals, pastoralists have high tendency of maintaining cows though abortion occurs in the herd.

On the other hand, the current study finding was lower than previous study reports from Ethiopia. These higher seroprevalences are 15.50% in East Shewa Zone (Hunduma & Regassa 2009), 10.60% in Borana (Megersa *et al.,* 2011), 11.0% in Wuchale-Jida district (Kebede *et al.,* 2008), 10% in different private dairy farms of Addis Ababa (Jiksa, 2002), and 7.7% in Tigray region (Mekonnen *et al.,* 2010).

Herd level prevalence in the current study was found to be 36.43% (95% CI: 33.18-43.76). Out of 140 herds tested in five districts of the South Omo Zone, 51 of them had at least one confirmed antibody against the natural infection of brucellosis (Table 12). The serological prevalence of 36.43% encountered at the herd level in the present study was consistent with previous findings, which ranged between 34% and 37.84% (Alehegn *et al.*, 2016; Asgedom *et al.*, 2016). Alehegn *et al.* (2016) reported that from a total of 47 herds, 16 (34%) herds were found with at least one animal tested positive. Herd level seroprevalence of 14 (37.84%) was recorded by Asgedom *et al*. (2016). However, lower herd level seroprevalences were recorded, as reported by Megersa *et al*. (2011), with a herd level prevalence of 26.1% (35/134), and from a total of 227 herds included in the study, there were 34 (15.0%) herds with at least one animal tested positive (Nuraddis *et al*., 2009).

In contrary to the present study, higher herd level prevalences were reported by Megersa *et al*. (2012) 51.70% and Mekonnen *et al*. (2010) 63.60% from Ethiopia. Similarly, 84.9% by Mai *et al.* (2012) from Nigeria, 77.50% by Hesterberg *et al*. (2008) from South Africa, 100% by Magona *et al.* (2009) from Uganda, 78.60% by Bernard *et al*. (2005) from Uganda, 46.40% by Bernard *et al*. (2005) from Uganda, and 63% (Muma *et al*., 2007a; Muma *et al*., 2007b) from Zambia. These higher differences are mainly due to differences in types of tests (Rose Bengal Plate test had been used in this higher prevalence) and production systems.

Differences in the seroprevalence rates observed at herd level in this study, as compared to those recorded by previous researchers, may be due to several factors such as differences in herd size, management systems, and sample size. In addition the higher herd level seroprevalence observed in the current study could be attributed to the nature of pastoral production system in which high herd mobility, multiple livestock species herding and increased number of animals per holdings increase animal exposure to brucellosis.

The present study attempted to look into the existence of association between seropositivity for brucellosis and age, sex, herd size, body condition, history of abortion and retained fetal membrane, parity, stage of abortion, and frequency of abortion (Table 13). Accordingly, the current study revealed that statistically significant association was observed between seropositivity and most of the variables ($P<0.05$) while there was no statistical significant association between seropositivity and few of the variables ($P>0.05$).

Among the potential risk factors considered in the present study, the age of cattle was shown to have a significant effect on the serological prevalence rate of bovine brucellosis ($P < 0.05$). There was a significantly low seroprevalence (3.29%) of bovine brucellosis in animals with age range of 0.5 to 2 years old compared to animals with age range of 2 to 4, and above 4 years old with 8.77%, and 3.56%, respectively (P=0.001). In this study, from the total 101 seropositive cattle 86(85.15%) seroprevalnce was observed, in older age category (>2 years of age) while only 15(14.85%) seroprevalence was observed in younger age category (6 months to 2years). This observation is in agreement with the report of Asmare *et al.* (2007), in which the majorities (97.87%) of sero-reactors were detected in the animals older than 2 years. The logistic regression result also showed that older animals with an age range between 2 and 4 years are 5.75 times at higher risk than younger animals. The association of age with seropositivity to *Brucella* infection in the current study is consistent with the findings of previous studies (Berhe *et al*., 2007; Hailemelekot *et al*., 2007; Mussie *et al.*, 2007; Kebede, 2008; Mekonnen *et al*., 2010; Asmare *et al.*, 2010; Ibrahim *et al*., 2010; Megersa *et al*., 2011) in Ethiopia. Age is one of the inherent factors that influence the susceptibility to *Brucella* infection. Brucellosis appears to be more associated with sexual maturity. Young and sexually immature animals tend to be more resistant to infection and frequently clear infection, although latent infections do occur (Radostits *et al.*, 2000), and higher seroprevalence is commonly reported in sexually matured animals. This may be due to the fact that sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms, tend to increase in concentration with age and sexual maturity (Al Hassan *et al*., 2014). Besides, the higher prevalence of brucellosis in older cattle can be attributed to the constant exposure of the cattle over time to the agent.

Another important risk factor considered in the current study was sex of animals. In the present study, a statistically significant association between sex and seroprevalence of brucellosis was observed. About 93.07% of the seropositive animals were females. Seropositivity in females (7.22%) was significantly higher ($P = 0.006$) than that of males (1.13%). The females were 2.10 times at higher risk of being seropositive for brucellosis (OR=2.10, 95% CI:1.58-6.34, P=0.00) than males (Table 13). Female animals are maintained in herds over extended time period thus, have ample time for exposure to the pathogen and being source of infection for other animals. Moreover, female animals are more prone to exposure to infectious agents like *Brucella* due to physiological stress causing factors such as pregnancy. In the current study, there was no positive reactor among male animals in two districts namely Bena-tsemay and Hamer. The absence of seroreactors in males in these districts in the current study is in

agreement with the previous reports by Yayeh (2003) in North Gondar Zone, Tolosa *et al*. (2008) in Jimma Zone, Bashitu *et al.* (2015) in Debrebirhan and Ambo towns, who reported only female positive reactors. This could be due to limited number of male animals involved in samples as farmers hold limited number of males and relatively large number of females for production purpose.

In this study, herd size was found to be statistically significantly correlated with seropositivity for brucellosis (P<0.05). Animals tested for brucellosis were categorized within herd sizes of <25 animals, 25-50 animals, and >50 animals, and the results showed seroprevalence of 2.43%, 5.19%, and 7.91%, respectively. Those animals in large herd sizes were found to be at higher risk than those in small herd sizes (OR=7.08, 95% CI: 5.00-8.05, and P=0.01). This result is in agreement with reports of (Berhe *et al.,* 2007; Nuraddis *et al*., 2009; Ahmad *et al*., 2009; Mekonnen *et al.,* 2010; Megersa *et al.,* 2011; Bulcha *et al*., 2020; Edae *et al*., 2020) from Ethiopia, Samui *et al.* (2007) from Zambia, and Patel *et al*. (2014) from India. An increase in herd size is usually accompanied by an increase in stocking density, one of the determinants for exposure to *Brucella* infection, especially following abortion or calving (Radostits *et al*., 2000).

In contrary to our finding, Kebede *et al*. (2008) and Jergefa *et al.* (2009) reported higher seroprevalence in small herd sizes than in large herd sizes. This could be associated with the large sample size taken relative to large herd size.

In the current study, out of the total cows positive for brucellosis, 84.04% had a history of abortion. Similarly, of the total cows positive for antibody against the natural infection of brucellosis, 88.30% had a history of retained fetal membrane. Cows with history of abortion were 9 times morelikely to be seropositive to brucellosis (OR=9.01, 95% CI: 5.07-9.09, P=0.00).

This could be explained by the fact that abortion or stillbirth and retained fetal membrane are typical outcomes of brucellosis. This statistically significant association between history of abortion and retained fetal memebrane and seropositivity for brucellosis was supported by many authors (Berhe *et al*., 2007; Asmare *et al.,* 2010; Adugna *et al.,* 2013; Alehegn *et al*., 2016; Wubishet *et al.,* 2019). The report of Bulcha *et al.* (2020) is also in line with the current work findings that the presence of abortion history and retained fetal membrane were significantly associated with the animal level seropositivity of bovine brucellosis (p<0.05). This could be explained by the fact that erythritol sugar in the placenta and fetal

fluid is elevated during the gestation period. This stimulates the growth and multiplication of the bacteria in the reproductive organs.

The result of the current study showed that there was no statistically significant association between bovine brucellosis seropositivity and body condition score of the animals (P>0.05). However, prevalence of 3.85%, 4.50%, and 7.10% were recorded in good, medium and poor body condition animals, respectively. Poor body condition scored animals have higher odds of risk of infection by *Brucella* (OR=4.50, 95%CI: 0.45-7.66, P=0.32) than medium and good body condition (OR=1.02, 95%CI: 0.70-1.83).

This study also revealed that there is statistical significant association between parity and seropositivity of bovine brucellosis ($p < 0.05$), and hence, parity was one of the potential risk factors in the study animals. The study revealed that seroprevalence of 2.43%, 7.64%, and 10.81% were recorded in cows with increased parity numbers of 1 calved, 2 calved, and more than 3 calved, respectively. Cows with a higher parity number have been found to demonstrate a higher risk of being seropositive to brucellosis (OR = 10.02, 95%CI: 9.06-17.80) than those cows with a low parity number (Table 13). This is probably due to increased contact with fetal materials and vaginal discharge from infected cows there, increasing the chance of being infected by *Brucella.* This association was in agreement with the findings of other investigators (Kebede *et al*., 2008; Hunduma & Regassa, 2009; Degefu *et al*., 2011; Alehegn *et al*., 2016).

The present study also attempted to assess the level of knowledge and awareness of brucellosis and practices posing a risk to pastoralists in the study area (Table 14). In view of that, the majority of participants, or pastoralists, 97.86%, were not aware of bovine brucellosis. About 79% of the participants were not even aware of zoonotic diseases transmitted through milk consumption; 65% of the participants also didn’t know the pathogenic causes of abortion in their cattle; and 86% of them lacked knowledge about diseases transmitted during assisting parturition. This finding is closely in agreement with reports of Bulcha *et al*. (2020), who recorded that 83.3% of the respondents had never heard of brucellosis from Ethiopia, Buhari (2015) from Nigeria, and Addo *et al*. (2011) from Ghana, which found very low awareness of the zoonotic nature of brucellosis. However, the finding was in contravention of Minda *et al*. (2016), who reported high awareness (77%) of brucellosis among participants in Bishoftu

and Asella, Ethiopia. This difference implied that the level of awareness of brucellosis among the pastoral community is very low as compared to the non-pastoral community.

Concerning practices undertaken by the pastoralists in the study area that could be potential risk factors in transmission of brucellosis, 27% disposed dead fetus to open dump in the environment, 55% of participants fed aborted materials to dogs, and 17% of the participants indicated that aborted fetus/still birth can be consumed. All of the participants, 100%, replied that the fate of frequently aborting cows was retained in the herd. All participants 100% practiced assisting parturition without any protective materials with their bare hands. All participants 100% in the study area confirmed that they have the habit of consuming raw milk, and 85% of them have the habit of consuming raw animals' blood (Table 14).

The finding implied that the communities in the current study area are engaged in risky practices that could expose them to infection with brucellosis. All respondents consumed raw milk, assisted animals during abortions or parturition, and handled aborted materials/fetal membranes without protective clothing. Such risky practices have been shown to be important risk factors for brucellosis transmission to humans.

5.2. Seroprevalence and risk factors of ovine brucellosis

In our results out of 105 (6.84%, 95% CI: 3.06-9.07) sera samples positive with RBPT 12.38% and 20.95% were seronegative with combined mRBPT and C-ELISA and combined mRBPT andCFT, respectively. This implies that RBPT suffers from false-positive reactions. Out of 1, 536 sheep sera samples tested for brucellosis, 6.84% (105/1536), 5.98% (92/1536), and 5.40% (83/1536) were positive for brucellosis with mRBPT, combined tests with mRBPT and C-ELISA and combined tests with mRBPT and CFT tests, respectively (Table 15).

This work is the first extensive study of its kind on the prevalence of ovine brucellosis in the study area in terms of the sample size and coverage area. The overall seroprevalence of 5.40% (95% CI: 3.34-7.47) was recorded at the individual animal level. This shows that ovine brucellosis is endemic in the study area.

The seroprevalence result of the present study (5.40%) is nearly similar to the findings of Benkirane *et al*. (2015) who reported 5.6% from Afar and Somali regional states, Tsehay *et al.* (2014) observed 5.42% from Oromia and Somali regional states, Shimeles and Andualem (2018) detected 5.87% from Amhara regional state, Ethiopia. The finding of the current study was also in close agreement with previous reports from other countries: 5.2% in Nigeria by (Shehu *et al*., 1999; Lone *et al*., 2013). However, the current work prevalence was higher than the previous reports in Borana by Teshale *et al.*, (2006)1.09%, in a similar place by Mihreteab *et al.* (2011) 1.56%.

In contrary to our findings, higher ovine seroprevalences of brucellosis were recorded 6.70% by Nigatu *et al.* (2014) in Ethiopia, 24.44% (165/675, 95% CI 21.4-27.8) by Yesuf *et al*. (2010) elsewhere. This variation may be due to differences in age, sex, production system, sample size, test methods used for diagnosis, flock composition, and agro-climate.

Of the 1, 536 sheep sera sampled for this study, 30.01% (461/1536) were from the Bena-Tsemay, 19.47% (299/1536) were from the Male, 17.84% (274/1536) were from Hamer, 22.46% (345/1536) were from Dassenech, and 10.22% (157/1536) were from Gnangatom districts. Sheep from Dassenech and Gnangatom districts had the highest seroprevalence of 9.85% (95% CI: 8.12-14.40) and 5.73% (95% CI: 4.50-7.20), respectively with statistically significant difference ($P<0.05$) between the districts. Univariable logistic regression analysis showed that the odds of acquiring brucellosis in the Dassenech district was about 3.2 times higher as compared to sheep in Bena Tsemay districts (OR=3.2, 95%CI: 1.98-6.58, and P=0.03) and a significant difference was also evident in the final model (Table 17).

This significant difference in Dassenech and Gnangatom districts as compared to others could be attributed to higher flocks aggregation on the Omo river basin in search of water and grass which increases contact among flocks that may be infected by brucellosis. In addition, the relative higher seroprevalence observed in the Gnangatom district could also be related to contact or interface between sheep flocks and wildlife at Omo National park even though the status of brucellosis in wildlife in the area is unknown. Furthermore, the lack of adequate veterinary infrastructure and services in the area as the aforementioned districts are the remotest area in the Zone as well in the country might have contributed to the relative higher seroprevalence and the widespread of the disease in the districts.

Males comprised 35.75% (549/1536) of the total sheep sampled and females comprised 64.25% (987/1536) of sheep sampled for this study. A higher rate of seroprevalence of 7.90% (95% CI: 2.45-7.50) was recorded in females as compared to males at rate of 0.91%. The difference was statistically significant (P<0.001). Univariable logistic regression analysis demonstrated that the odds of acquiring brucellosis in female sheep is about 3.5 times higher as compared to the male sheep (OR=3.5, 95% CI: 2.45-7.50, and P=0.00). Similarly, a significant difference was also evident in the final model. The result of the present study is in line with the previous report of (Shimeles & Alemu, 2012) in smallholder production system, significantly higher (P < 0.001) seroprevalence was observed in females (8.21%, 95% CI: 5.2-11.8) than in male sheep (3.01%, 95% CI: 2.41-4.34). Likewise, statistically significant difference (P<0.05) was also observed among male and female sheep (Yesuf *et al*., 2010).This implied that it is a fact that male animals are less susceptible to *Brucella* infection, due to the less concentaration of erythritol, and the absence of physiological stress-causing factors like pregnancy in males. In contrary to our finding, previous reports from Ethiopia verified that higher seropositivity was observed in males over females though no statistically significant (P>0.05) difference was found between males and females sheep (Teshale *et al*., 2006; Ashenafi *et al*., 2007).

The age of the sheep was grouped into two categories: young (<1 Year) (n=390), and adult (>1 Year) (n=1146). Among the age categories, the higher seroprevalence of 6.37% was observed for those sheep above one year old, and 2.56% seroprevalence was recorded for those below 1-year-old. Both univariable and multivariable logistic regression analysis showed a statistical significant association between ovine seropositivity and age categories (P=0.00). Furthermore, the odds of brucellosis exposure in aged sheep (adult) were about 2 times higher than that of young sheep (OR=2, 95% CI: 1.80-9.50, and p=0.00).

Similar to the present finding, significantly higher seroprevalence was reported in sexually matured (adult) than in young sheep from Ethiopia (Ashenafi *et al.,* 2007; Yosef &Nardos, 2010; Negash *et al*., 2012; Adugna *et al*., 2013). This phenomenon could be due to two important conditions. The first reason is that younger animals are more resistant to infection and frequently clear an established infection. The second reason could be related to the fact that sex hormones and erythritol which stimulate the growth and multiplication of *Brucella* organisms tend to increase in concentration with age and sexual maturity.

The present study covered a total of 156 flocks of sheep from five districts of the South Omo zone. The number of flocks sampled was distributed to each district based on the sheep population. Accordingly, 30.13% (47/156) flocks were from Bena Tsemay, 19.23% (30/156) flocks were from Malie, 17.95% (28/156) flocks were from Hamer, 22.44% (35/156) flocks were from Dassenech, and 10.25% (16/156) flocks of sheep were from Gnangatom districts. The overall flock level seroprevalence of ovine brucellosis was found to be 39.74% (95% CI: 36.50-48.8). The highest flock level seroprevalence of 100% (16/16) (95% CI: 97.58-103.30) was recorded in Gnangatom district followed by 62.86% (22/35) (95% CI: 53.06-65.45) in Dassenech district, and 32.14% (9/28) (95% CI: 30.43-34.42) in Hamer district. The least flock level seroprevalence of 6.67% of ovine brucellosis was detected in the Malie district. The possible explanation for very high flock level seroprevalence in Gnangatom and Dassenech districts as compared to others could be related to the larger flock density observed in the two districts which might increase the chance of spread of the disease among the livestock population. On the other hand, the least flock level prevalence observed in the Malie district might be correlated with smaller flock density, limited movement of flocks in search for feed and water since the production system in the district is partly agro-pastoral.

In the current study, we classified flock size into three groups as small flock size (for a flock comprising ≤25 sheep), medium flock size (for a flock comprising 26 to 49 sheep), and large flock size (for a flock comprising ≥ 50 sheep). The current study indicated a strong statistically significant difference between seropositivity of ovine brucellosis and flock size. Higher seroprevalence was recorded in large flock size with a rate of 14.98% compared to the medium and small flock size with a rate of 4.32% and 3.28%, respectively. The univariable logistic regression test verified that the odds of acquiring brucellosis for sheep in large flock size was 2.5 times higher (OR=2.5, 95% CI: 1.55-6.90, and P=0.00) than those in small flock size. A similar result was obtained by multivariable logistic regression analysis.

The present study was in line with the previous investigations of (Shimeles & Alemu, 2012; Gebremedhin, 2015) who reported that seroprevalence of ovine brucellosis was significantly different among the flock size categories (small, medium and large) and seroprevalence increases with flock size in Ethiopia. Another previous study was also evidenced that lower seroprevalence in small flocks compared to medium-sized and large-sized flocks (Chavez *et al.*, 2013) from other countries elsewhere.
Out of 987 female sheep sampled, 75.38% (577/987) had a history of abortion. Of 577 sheep with a history of abortion, 12.13% with odds ratio of 4 (OR=4, 95% CI: 2.65- 7.90) were found to be positive

for antibody against natural infection by *Brucella*. Out of 410 sheep without a history of abortion, 1.95% (8/410) were detected seropositive. The seroprevalence of brucellosis in female sheep was statistically significant with a history of abortion ($P < 0.05$). Regarding the association between seropositivity and history of abortion, the result of the current study is consistent with other reports (Samadi *et al*., 2010; Tegegn *et al*., 2016). Previous studies from Ethiopia reported that there was a significant association ($P<0.05$) between seropositivity to brucellosis and history of previous abortion in sheep (Gumaa *et al*., 2014).

Abortion is the most predominant symptom of brucellosis in naturally infected sheep. The animals commonly abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies. Some infected animals carry the pregnancy to term and shed the organism (Lone *et al.,* 2013; Samadi *et al.,* 2010). It can be concluded that abortion of infected animals is important for public health (Benkirane *et al.,* 2015).

This study revealed a higher seroprevalence of 11.71% (65/555) in sheep with a history of the retained fetal membrane than those without a history of retained fetal membrane 3.0% (13/432) even though there was no statistically significant difference ($P>0.05$) observed. This result is consistent with other reports (Tegegn *et al.,* 2016). This indicates that abortions or stillbirths and retained placenta are typical outcomes of brucellosis (Radostits *et al*., 2007).

Higher prevalence of 10.86%, (95% CI: 0.78-3.80) was recorded in sheep with stillbirth while the prevalence of 9% was observed in sheep without stillbirth though no statistically significant differences. Various authors reported that stillbirth, abortion, infertility, and the birth of weak offspring are recorded as the common clinical signs of brucellosis in natural hosts (Samadi *et al.,* 2010; Sonawane *et al*., 2011).

The body condition of the sheep was one of the risk factors considered in the present study. Accordingly, the highest prevalence of 12.45%, was recorded in sheep with poor body condition score while the lowest prevalence of 1.67%, was observed in sheep with medium body condition score. Sheep with poor body condtion were 1.7 time more likely to be seropositive to brucellosis compared to those with good body condition according to the result of final logistic regression model (OR=1.7, 95% CI: 1.05-2.40, and P=0.01)

In the present study higher seroprevalence of brucellosis (9.10%) was recorded in ewes with more than one parities than those with only one parity (5.82%). However, there was no statistical significance difference between different numbers of parties. This result is closely consistent with other reports from Ethiopia (Tegegn *et al*., 2016; Edao *et al*., 2020). The increase in seroprevalence recorded with the increasing number of parity numbers could be related to the increased age of the animals that increases the chance of exposure to brucellosis.

This study discovers the prevalence rate of brucellosis in sheep in South Omo Zone in one of the pastoralist areas of Ethiopia known to supply large number of small ruminants for the domestic and export market. This epidemiological information could help in controlling of the the disease. Most of the studies conducted so far in the country focus on goat brucellosis than sheep eventhough epidemiological variations are there. This study was the first intensive work on ovine bruceloosis which recruited large sample size in pastoral areas of the country. This piece of work has also identified potential risk factors associated to the occurrence of ovine brucellosis and its public health significances. *Brucella ovis* predominantly affects sheep and is often less virulent than *Brucella melitensis*, which can infect goats (caprine) and is associated with higher rates of human disease (Radostits *et al*., 2007). In ovine brucellosis, the organism is primarily localized in reproductive organs and is mainly secreted in the semen and reproductive fluids, especially during breeding seasons. Infected rams can shed *Brucella ovis* in their semen, infecting females during mating.In goats, *Brucella melitensis* is known to be excreted in milk and can persistently shed, even in lactating animals asymptomatically. This poses a higher risk for transmission to humans (Perez-Etayo *et al*., 2018).

5.3. Seroprevalence and risk factors of caprine brucellosis

In the current study, a total of 1, 536 goats were tested across five districts of South Omo Zone of Southern Ethiopia. The key objectives of study were to investigate caprine brucellosis seroprevalence and associated risk factors. To estimate the seroprevalence three different testing methods were employed to determine the presence of antibodies against natural infection of *Brucella*, specifically the mRBPT as screening test, the combined mRBPT and C-ELISA, and combined mRBPT and CFT as confirmatory tests. To determine the associations of the putative risk factors with seropositivity of the diseases both univariable and multivariable logistic regression analysis were used.

The overall individual animal level seroprevalence of 12.04% by mRBPT, with corresponding rates of 7.03% and 6.90% by combined tests of mRBPT and C-ELISA and combined tests of mRBPT and CFT, respectively were recorded (Table 18). The present study finding was comparable with seroprevalence reports of 7.8% by Gumi *et al.* (2013) from Oromia, Somali & Dire Dawa, 9.39% by Negash *et al.* (2012) from Dire Dawa, 7.52% by Tekle (2016) from Afar regional state of Ethiopia.

Contrast to the our study result, in different pastoral areas of Ethiopia relatively low level of seroprevalence of caprine brucellosis was reported. For example, a prevalence of 1.7 % was observed in Somali pastoral region of Ethiopia (Lakew *et al.*, 2019) whereas a prevalence of 4.8 % was reported in goats in Afar pastoral region (Ashenafi *et al*., 2007), prevalence of 1.7 % reported by Teshale *et al*. (2006), a prevalence of 3.7% was reported in Borana by Edao *et al*. (2020).

In addition to individual animal-level analysis, the survey also considered the flock level seroprevalence and out of 102 flocks, 35 (34.31%) (95% CI: 30.52-42.41) tested positive for caprine brucellosis. Notably, the highest flock seroprevalence was found in Gnangatom, in which 60% (95%CI:52.40-62.90) of the flocks were positive, followed by Malie at 39.13% (95%CI:35.05-44.75) and Dassenech at 35.29% (95%CI:31.55-38.20). This finding is comparable with previous report by Edao *et al*. (2020) from Borana, Ethiopia that indicated overall flock level prevalence of 22.7% (95% CI=13.8-33.8).

Furthermore, district level seroprevalence of caprine brucellosis revealed that in Hamer, out of 213 goats tested, 30(14.08%) were positive using the mRBPT, 18(8.45%) and 17(7.98%) were positive by combined mRBPT and C-ELISA and combined mRBPT and CFT tests, respectively. In Dassenech district out of 262 goats tested, 22(8.40%) were positive with mRBPT, 19(7.63%) were positive with combined test C-ELISA and 18(6.87%) seroprevalence was recorded with CFT test. In Gnangatom district the highest individual seroprevalence was recorded in which out of 70 goats tested, 11(15.71%) were positive by mRBPT, while both C-ELISA and CFT indicated a positivity of 9 (12.86%). In Bena-Tsemay, 648 goats were tested and seroprevalence of 63(9.72%), 38(5.86%), and 38 (5.86%) were recorded with mRBPT, C-ELISA and CFT, respectively. Finally, in Malie district a notable rate of 17.20% (59 positives out of 343) by mRBPT, but 24(6.99%) seroprevalence was recorded by C-ELISA and CFT tests. However, there was no statistical significance difference in the seroprevalence among the five districts ($p>0.05$).

The study analyzed different factors such as sex, age, flock size, production system, history of abortion, stage of abortion, history of retained fetal membrane, still birth, body condition of the animals, and parity number for their association with caprine brucellosis seropositivity using logistic regtression analysis (Tables 20 and 21).

Accordingly, the multivariable logistic regression analysis concerning the association of sex of the goats with brucellosis seropositivity, revealed that there is a sex-based difference in seroprevalence rates (Table 21). Among males (n=618), only 34 (5.5%) were found to show positive seroprevalence, while females (n=918) exhibited a higher rate of 72 (7.84%)(Table 21). Females goats had an odds ratio (OR) of 2.062 with a (95% CI: 1.02-4.19, and P=0.00), which implies that female goats are approximately twice as likely to test seropositive for brucellosis compared to male goats. This suggests that female goats are more susceptible to brucellosis compared to their male counterparts. This higher susceptibility of female goats could be related to reproductive and physiological factors such as survival ability of the *Brucella* targeting reproductive tissue (placenta or uterus) of female animals due to presence of erythritol (sugar alcohol) which supports *Brucella* replication, and physiological status of the animal like pregnancy related stress could increase female goats susceptibility to infection. Brucellosis is commonly transmitted through contact with infected birth fluids, placentas, and milk, which are more likely to be encountered by females, especially those that have been exposed to contaminated birthing environments.

The seroprevalence of goats prevalence recorded related to sex of the animal was in line with previous report of Teshome *et al*. (2022) who indicated female goats were more frequently infected with *Brucella* species (20.19 %) than their male counterparts (11.42 %). The odds of infection were nearly 7 times higher in female goats than in males ($P < 0.001$).

However, other previous reports found no statistical significant difference between males and females (Ashenafi *et al*., 2007; Asmare *et al*., 2013). This could be due to the small sample size of males and males are also kept in the flock for shorter period which decrease their exposure to the disease.

The seroprevalence of brucellosis among the tested goats was notably different between the two age groups. In young goats, only 5 out of 257 tested positive, resulting in a prevalence rate of 1.95%. Conversely, adult goats exhibited a significantly higher prevalence, with 101 out of 1, 279 tested positive, leading to a prevalence rate of 7.90%. The odds ratio for adult goats was 3.72 compared to the

reference category of young goats (OR=3.72, 95%CI:1.33-6.56, and P=0.00). This indicates that adult goats were 3.72 times more likely to test positive for brucellosis compared to young goats.. This indicates a strong correlation between age and the presence of brucellosis, suggesting that the observed difference in prevalence rates is unlikely due to random chance. The higher prevalence of brucellosis in adult goats compared to young ones could be related to reproductive maturity, increased and cumulative exposure over time, the potential for chronic carrier states among adult animals, specific management practices, and the protective effects of maternal antibodies in young goats.

This finding is comparable with previous report from Ethiopia by Teshome *et al*. (2022) who indicated the prevalence was higher in adult goats (26.17%) than in younger ones (8.07 %). Adult goats were thus 12 times more likely to be seropositive for brucellosis than their younger counterparts ($P < 0.001$). *Brucella* infection can affect animals of any age, but it is most prevalent among sexually mature individuals, particularly those that are pregnant (Radostits *et al*. 2007). These animals are at a higher risk of infection compared to their younger, sexually immature counterparts. The primary exposure to *Brucella* occurs during the calving process of infected females. Research indicates that seroprevalence of brucellosis rises with age and sexual maturity, being notably lower in young stock than in adults (Negash *et al*., 2012). This lower prevalence among younger animals may be attributed to the capability of harboring the bacteria without manifesting detectable antibodies until they experience their first birth or abortion. The bacteria can reside in regional lymph nodes without triggering an immune response until pregnancy occurs, when substances like erythritol, which fosters *Brucella* growth, become available (McDermott *et al.,* 2002). Sex hormones and erythritol stimulate the bacteria's replication, contributing to increased prevalence with age (Megersa *et al*., 2012). Infected animals that abort typically develop immunity but can remain carriers, shedding *Brucella* in fetal fluids (Langoni *et al*., 2000).

In present PhD. Dissertation study, the association between flock size and brucellosis seroprevalence in goats was explored. The tested goats were categorized into three distinct flock sizes: small, medium, and large based on number of goats at house hold level in the study area. The key variables of interest were the seroprevalence rates across these categories, the odds ratios (OR) reflecting the likelihood of brucellosis infection in relation to flock size, and the corresponding p-values indicating the statistical significance of these findings. The findings of the study revealed that in small flock size out of 553 goats tested, 27(4.88%) were found to be seropositive for brucellosis while in medium flock sized of 440 goats, 32(7.27%) and among large flocks of 542 goats, 47(8.67%) seroprevalence was recorded with OR

of 1.82 (95% CI: 1.5- 3.69), and overall p-value of 0.02 indicating that goats in large flocks were nearly 1.82 times more likely to test positive for brucellosis compared to those in small flocks. The possible reasons for goats in larger flocks are more likely to be seropositive for brucellosis could be due to higher animal density facilitates disease spread through direct contact or contaminated materials; increased interactions among goats in larger groups also elevate the risk of transmission; shared resources like water and feeding areas contribute to cross-contamination; increased flock size also increases the chances of birthing, abortion events, and excretion of *Brucella*-contaminated fluids which in turn increases environmental contamination and the stress of overcrowding can weaken the goats' immune systems, making them more vulnerable to *Brucella* infections.

Similar to the present study result, Teshome *et al*. (2022) reported higher prevalence observed in goats sampled from larger herd size compared to those goats tested from medium and small size herds. Teshome *et al.* (2022), reported serprevalence of 13.71%, 12.36%, and 23.05% in small, medium, and large flock size, respectively. Furthermore, the association of flock size with the prevalence of anti-*Brucella* antibody has also been previously reported (Teklue e*t al*., 2013; Asmare *et al*., 2013).

The other key risk factor analyzed in the current study was the association of production system and caprine brucellosis seropositivity. The research involved testing goats from two primary production settings: agro-pastoral and pastoral systems. The findings related to this revealed that in agro-pastoral system out of 520 goats tested, 20(3.85%) were seropositive while in pastoral setting out of the total 1, 1016 goats tested for the presence of antibody against natural infection of *Brucella*, large number 86(8.46%) were tested positive with the odds ratio of 3.26 (95% CI: 2.82-11.94) and overall p-value of 0.00. The calculated odds ratio of 3.26 indicates that goats raised in pastoral systems werer over 3 times more likely to be seropositive for brucellosis compared to those in agro-pastoral systems. Furthermore, the statistical significance (p-value<0.05) associated with the differences in seroprevalence rates signifies a strong association between the type of production system and the likelihood of brucellosis infection in goats.

This increased prevalence could be linked to various factors inherent in pastoral farming, including higher animal density, low veterinary service access, and limited disease management strategies. Pastoral systems involve moving flocks over vast distances to find grazing and water sources, which increases brucellosis transmission due to mingling with other animals. Shared resources like grazing

land and water sources can become contaminated with *Brucella* from infected animals. The mobility of these pastoralists, who often lack veterinary services, complicates the isolation of infected animals, raising overall infection rates as sick animals remain with healthy ones. Congestion at water and grazing sites further contaminates the environment, while larger flock sizes heighten animal density, facilitating the spread of brucellosis and escalating the disease's prevalence. Contrary to the pastoral system, the low incidence of brucellosis in agro-pastoral production system may be attributed to the mixed farming practices that often include crop cultivation alongside livestock rearing. This diversity can potentially mitigate disease spread through improved management practices This finding highlights the urgent need for targeted interventions in the pastoral production systems to mitigate risk factors contributing to the disease's spread.

The study examined goats with recorded histories of abortion in relation to their seroprevalence for brucellosis. Understanding the relationship between a history of abortion and the seroprevalence of brucellosis is critical for managing animal health and controlling outbreaks. A total of 918 female goats were tested for seroprevalence of brucellosis out of which 814 were having no history of abortion and 104 hadabortion history. The results of a multivariate logistic regression analysis revealed that the prevalence of brucellosis in goats with a history of abortion was found to be high at 60.58%, compared to 1.10% prevalence rate in those without abortion history with the odds ratio for goats with a history of abortion (OR=9.34, 95% CI: 7.90-15.87, P-value of 0.01), indicates that those goats with history of abortion were over 9 times more likely to test positive for brucellosis compared to those without an abortion history. This significant association suggests that a history of abortion could be a critical risk factor for the disease. The result suggests that there is statistically significant association between history of abortion and goats' seropositivity.This higher seroprevalence of caprine brucellosis in goats with the history of abortion could be attributed to the presence and replication of the *Brucella* organism in the target reproductive tissue particularly in the placenta of gravid uterus and results in infection leading to placentitis and subsequent abortion.

The result of the present study was in agreement with other reports elsewhere that caprine brucellosis is correlated with history of reproductive problems. For instance, Teshome *et al*. (2022) reported higher prevalence of 28.09%in goats with history of abortion while lower prevalence of 10.8% was recorded in goats without history of abortion.

Regarding the assessment of correlation between retained fetal membrane (RFM) and seropositivity of goats for brucellosis in the current study, the result revealed that out of total 918 female goats sampled, 847 had no history of RFM while the remaining 71 had recorded history of RFM. Out of 847 goats without history of RFM tested, relatively low serprevalence of 54(6.38%) was recorded. In contrast to this, goats with a history of RFM exhibited a dramatically higher prevalence rate of 52 (73.24 %) with OR of 8.13 and 95% CI of 6.85- 13.22 and p-value of 0.04. The results of this study underline a substantial association between a history of RFM and an increased risk of brucellosis in goats. The odds ratio of 8.13 suggests that goats with RFM are over 8 times more likely to test positive for brucellosis compared to those without this history. It is hypothesized that the disrupted reproductive health linked to RFM may stem from underlying infections, including brucellosis. Additionally, the inflammatory process associated with retained membranes could create an environment conducive to further infection. Goats with a history of RFM should be closely monitored for signs of brucellosis, and public health initiatives might benefit from emphasizing the importance of regular screening in at-risk populations. This study elucidates a compelling connection between retained fetal membrane and brucellosis seroprevalence in goats.

Brucellosis can have a serious effect on the reproductive health of animals and may be more common in goats experiencing retained fetal membranes (RFM) compared to those without this condition, as it specifically targets the reproductive organs and tissues, causing inflammation and potentially resulting in complications such as RFM. Goats affected by brucellosis tend to have compromised reproductive health, which can heighten the risk of RFM due to their diminished ability to combat infections and maintain normal reproductive functions. Additionally, *Brucella* has a strong affinity for reproductive organs, including the uterus, and infected reproductive tissues are more susceptible to damage and inflammation. This vulnerability can lead to complications during the birthing process and additional postpartum issues like RFM. Since *Brucella* can inhabit and persist within reproductive tissues, the infection disrupts the natural detachment of the placenta from the uterine wall, causing it to become retained. Moreover, *Brucella* produces endotoxins that can interfere with the placental attachment process, exacerbating issues during delivery that hinder the natural expulsion of the placenta. This imbalance can lead to abnormal uterine contractions, further increasing the risk of RFM. Moreover, RFM can create conditions that are more conducive to infection, as retained tissues may become necrotic and promote bacterial growth. In situations where brucellosis is already present, RFM aggravates the

scenario, increasing bacterial loads and intensifying the infection in affected goats. This ongoing cycle can contribute to a higher prevalence of brucellosis in goats suffering from RFM.

In this study, goats were tested for brucellosis, and the presence or absence of stillbirth was recorded. The study sample consisted of a total of 918 goats, among which 707 experienced no stillbirth, while the remaining 211 goats exhibited stillbirths. The prevalence of brucellosis seropositivity was calculated and further examined using multivariate logistic regression to determine the odds ratio (OR) for the association between stillbirth and brucellosis seroprevalence. The result indicates that out of 707 goats with no history of still birth, 50(7.07%) goats tested positive for brucellosis. In contrast to this, significantly large number of goats that experienced still birth tested positive for brucellosis with prevalence rate of 56(26.54%). The odds ratio calculated from the regression analysis for stillbirth among the brucellosis-positive group was found to be 1**.**59 (95% CI: 1.44-5.75, P=0.04). The calculated odds ratio of 1.59 signifies that goats with brucellosis have 1.6 times higher odds of experiencing stillbirth compared to those without the disease. The 95% confidence interval does not cross one, indicating a statistically significant association between brucellosis and stillbirth. This result provides statistical evidence supporting the association between brucellosis seroprevalence and the incidence of stillbirth.

The finding of this study was in line with previous reports that indicate that abortion; stillbirths and retained placenta are typical outcomes of brucellosis (Radostits *et al*., 2007; Samadi *et al*., 2010; Sonawane *et al.*, 2011; Tegegn *et al.*, 2016).

Goats classified in medium body condition had a notably lower seroprevalence of brucellosis of 28(5.19%). The odds ratio of 0.49 (95% CI: 0.26-0.92) suggests that these goats have around 51% lower odds of being seropositive for brucellosis compared to their poor body condition counterparts. The confidence interval indicates that this result is statistically significant, reinforcing the protective effect of better body condition against the disease. Goats in good body condition exhibited even more pronounced reduction in odds, with OR = 0.16 (95% CI: 0.06- 0.42). This suggests that goats in good body condition are 84% less likely to be seropositive for brucellosis compared to those in poor condition, and the P-value demonstrated strong statistical significance difference. This association is underscored by a low confidence interval, highlights a critical link between optimal nutrition and health outcomes in goats. These findings imply that the analysis provide compelling evidence that body condition is significantly

associated with brucellosis seroprevalence in goats. Poor body condition is a notable risk factor, while good body condition offers a protective advantage.The result of the current study is in agreement with previous reports from Ethiopia and elsewhere (Yeshwas Ferede *et al.*, 2011; Mohammed *et al*., 2017). They pointed out that higher seroprevalences were recorded in goats with poor body condition compared to those with good nutritional status.

This study finally explored the relationship between the number of parities (the number of times a goat has given birth) and seropositivity to brucellosis in goats in the study area. Understanding the factors that influence the prevalence of brucellosis is essential for effective disease management and control strategies. In this regard, two groups of goats were examined in relation to their parity number: those that had given birth once (parity one) and those with more than one birth (multiple parity). The objective was to ascertain if there was a statistically significant difference in seropositivity rates for brucellosis between these groups. The result of the analysis revealed that out of the total of 918 goats tested for brucellosis, 463 belong to the parity one group and 455 in the multiple parity groups. The analysis indicates that 33 (7.13%) goats from the parity one group tested positive for brucellosis. In contrast, 39 (8.57%) goats from the more than one parity group were found to be seropositive. The odds of seropositivity for goats with more than one parities compared to those with only one parity were significant, with an odds ratio (OR) of 3.81 (95% CI:1.29-11.26). This indicates that goats with multiple parities are over 3 times more likely to test positive for brucellosis compared to their counterparts with a single parity. The P-value of 0.02 underscores the statistical significance of these findings, suggesting a strong association between higher parity numbers and increased seropositivity for brucellosis.

The findings from this analysis indicate that goats with multiple parities show a markedly higher rate of seropositivity to brucellosis compared to those with a single parity. This can be attributed to several factors like increased exposure, infection cycle, immunity and age of the animals. Goats that have given birth multiple times may have had increased exposure to the bacteria, especially if they are exposed to environments where brucellosis is endemic. The reproductive cycle may play a role in the transmission of the disease, as brucellosis can be spread during breeding and birthing processes.

The findings of the current study was found to be in agreement with previous report of Teshome *et al*. (2022) whose study revealed parity number was significantly associated with prevalence of *Brucella* infection (OR=1.43; 95% CI: 1.17-1.73, $P < 0.001$). Furthermore, other reports by different authors also

revealed positive correlation of number of parities and brucellosis seropositivity (Tegegn *et al.*, 2016; Edao *et al*., 2020). These results have important implications for the management and control of brucellosis in goat populations.

5.4. Seroprevalence and risk factors of human brucellosis

In this study, a total human seropositivity for *Brucella* was found to be 14.58% (95% CI: 12.07-18.24) as shown in (Table 22). This result aligns closely with earlier findings from the Afar region, which reported seroprevalences of 15% by Zewolda and Wereta (2012) and 16.5% by Ahmed *et al*. (2008). The overall seroprevalence rate of brucellosis was 15.8% (95% CI; 12.70-19.70%) from Afar region as reported by Mehari *et al.* (2021). This similarity may be linked to the resemblance in the study population's context, as both belong to a pastoralist community. Similarly, nearly closely 16.5%, finding was reported from among pastoral community visiting clinics due to febrile illness in Kenya by Muturi *et al*. (2018). Furthermore, the prevalence of human brucellosis in this study is in agreement with the findings from febrile individuals of different Sub-Saharan African countries like Tanzania (15.4%) reported by Chipwaza *et al.* (2015), Northern Uganda (18.7%) reported by Muloki *et al*. (2018), and Northeastern Kenya (13.7%) reported by Mukhtar (2010).

However, a significantly high seroprevalence rate of 34% was observed in Yabello, as indicated by the findings of Genene *et al*. (2009),48.3% in Afar and 34.9% in Smali region as repoted by Tschpp *et al*., (2021), and 48.8% prevalence of human brucellosis was reported by Abdulkadir (2019) from Afar Regional State, Ethiopia.

In contrary to the findings of the current study, previous reports have indicated lower prevalence rates of human brucellosis in Ethiopia, including 2.6% by Animut *et al*. (2009) from West Gojam, and 2.6% by Edao *et al.* (2020) from Borana Pastoral region, 3.6% by Tolosa *et al.* (2007) from Jimma, 1.34% by Tsegay *et al*. (2017) from Debre Zeit and Mojo export abattoirs, and 4.8% by Kassahun *et al*. (2006) from Addis Ababa. Additionally, Haileselassie *et al*. (2011) and Tibeso *et al*. (2014) reported prevalence rates of 2.1% from Western Tigray and Adami Tulu, respectively, while Workalemahu *et al.* (2017) reported 10.6% from Arba Minch. The variations in seroprevalence may be linked to factors such as the sample size used in the research, the types of confirmatory tests employed, the specific production systems or settings in the study area, type of samples (patient or apparently healthy), and the presence

and level of animal brucellosis in the study area, since animals are a source of human brucellosis. The variations observed in different studies could be associated with prevalence of brucellosis in the livestock population, duration of exposure, sample size epidemiological settings of the study population and variability related to diagnostic test and method applied.

In this study, several potential risk factors for human brucellosis were examined, including gender, age, education level, type of occupation or production system, consumption of raw milk and meat, consumption of animal blood, direct interaction with animals, sharing water points with animals, practice of involvement in assisting with animal births (either with bare hands or using protection), exposure to aborted materials, methods for disposing of aborted fetus and fetal remains, and the level of awareness about brucellosis and zoonotic diseases (Tables 23 and 24). Among these explanatory variables those which exhibited a P-value less than 0.25 in univariable logistic regression analysis (Table 23) were subjected to further analysis by multivariable logistic regression (Table 24). Accordingly, on multivariable logistic regression analysis, all predictor variables except mechanism of assisting parturition were significantly associated with increased risk of *Brucella* seropositivity in humans ($p<0.05$) (Table 24).

The current study revealed that seroprevalence recorded in females (24.05%) was found to be higher than males (4.57%) with (OR: 6.9, 95% CI: 3.37-13.98 and P=0.00). The odds of human seropositivity were 6.9 times higher in females as compared to males. This could be linked to custom that females have responsibility of milking animals and care for animals in the barn in pastoralist community while males are taking animals to grazing areas and such practice might increase the chance of contracting brucellosis (Kinati *et al,* 2018). In contrary to the current study Mehari *et al.* (2021) reported higher seroprevalence of 23.3% (95% CI: 17.70-30.10) in males than in females from Afar regional state of Ethiopia. This could be correlated to gender specific roles and responsibilities that are mainly associated with cultural practices predispose certain genders to higher risk for human brucellosis. In pastoral settings of South Omo Zone, females have limited access to information on disease prevention, especially regarding zoonosis, when compared to men; females are primarily engaged in milking and processing animal products that could expose them to zoonotic disease. According to Alemu *et al.* (2023), study conducted in Hamer aand Dasenech, south Omo zone, women are vulnerable to brucellosis because they are in charge of all household activities, surroundings and treatment procedures of animals.

Regarding age of humans sampled and tested, relatively higher seroprevalence of brucellosis was recorded in young <18 years old, and >60 years old, with rate of 23.23%, and 33%, respectively as compared to other age categories. Older individuals were 2. 5 times more likely to be seropositive for *Brucella* infection than other age groups in the study area and it is statistically significant (OR=2.5, 95%CI: 1.92-6.52, and P=0.00). The possible reasons of higher seroprevalence of brucellosis recorded in <18 years old and elder people >60 years old could be these age categories consume raw milk and declined immunity against diseases as age increases. Similar to the current study, according to Mehari *et al.* (2021), a study conducted in Afar Region, Ethiopia indicated relatively higher 26.5% seroprevalence (95% CI: 14.20-43.90) in older people of >45 years and in young individuals between 2-14 years old with 12.1.% seroprevalence (95% CI: 5.8-23.4) as compared to other age range groups.

There is statistically significant difference between seropositivity and level of education of the sampled humans (*P*=0.042). Higher seroprevalence was recorded in illiterate individuals (18.73%) than other higher education level. This implies that illiterate community may not have awareness about the diseases transmitted from animals to humans and the likely exposure to such diseases could increase. This finding is consistent with the report of Edao *et al.* (2020) from Borana pastoralists who indicated higher seroprevalence of 2.4% in illiterate participants than others. Mehari *et al.* (2021) also reported higher seroprevalence of 20.4% human brucellosis (95% CI: 15.40-26.60) in illiterate and compared to relatively lower seroprevalence of 11.9% (95% CI: 8.40-16.70) in individuals having primary school and above education in Afar Regional State of Ethiopia. Moreover, according to the report of Omballa *et al.* (2016) and Osoro *et al*. (2015) having at least basic education is protective from brucellosis. Furthermore, Alhoshani *et al.* (2016) reported that the least educated individuals in Saudi Arabia had a higher prevalence rate than individuals with higher education.

The multivariable logistic regression analysis revealed that seropositivity for natural *Brucella* infection in the pastoralist was 19.14%, significantly higher than the 7.59% observed among agro-pastoralists. The statistical odds ratio suggests that pastoralists were 2.88 times more likely to test positive for *Brucella* infection compared to agro-pastoralists (OR=2.88 95% CI: 1.78-4.68, and P=0.00). The observed higher seropositivity in pastoralists can be linked to their increased exposure to livestock through daily interactions. Unlike agro-pastoralists, who may have a more diversified livelihood strategy, pastoralists' dependence on their herds places them at greater risk for brucellosis. This study highlights a significant disparity in the prevalence of *Brucella* infection between pastoralist and agro-pastoralist communities,

suggesting a pressing need for public health interventions aimed at the pastoral population. Future research should explore targeted strategies to mitigate the risks associated with this infection in high-seroprevalence groups. Mehari *et al.* (2021) noted higher seroprevalence of 20% at (95% CI: 15.2-25.9) in pastoralists as compared to others which recorded 11.8% seroprevalence at (95% CI: 8.2-16.7).

In the current study, higher seroprevalence of 21.05% was recorded in febrile patients who experienced consumption of raw milk , the finding indicated that individuals who consumed raw milk had 16.73 times higher odds of *Brucella* seropositivity than those who had not (OR=16.73, 95% CI: 6.09-22.96, and P=0.00). This finding was in line with the previous report by Mehari *et al.* (2021) seroprevalence of 23.2% in individuals consuming raw milk with odds of 28.65 and 95% CI of 3.86-212.42, *P*=0.001. Edao *et al*. (2020) also reported higher 4.0% seroprevalence of human brucellosis from individuals consuming raw milk in Borana pastoralists of Ethiopia (OR= 6.0, 95%CI: 0.7-50.4, and P=0.098) and zero seroprevalence from those who don't consume raw milk. This finding is in agreement with other studies in Kenya (Namanda *et al*., 2009; Muturi *et al*., 2018), and Central Uganda (Tumwine *et al*., 2015). Furthermore, the result of the present investigation is in line with previous reports that consumption of raw milk has been designated as a risk factor for brucellosis transmission from animals to humans (Kozukeev *et al*., 2006; Earhart *et al*., 2009; Arif *et al*., 2017). The occurrence of human brucellosis is highly correlated with *Brucella* sero-prevalence in livestock, where infected animals are constantly shedding bacteria in milk and at parturition increasing the likelihood of infection among human. In the current study area we studied the seroprevalence of bovine, ovine, and caprine simultaneously in the same villages where human samples were taken and analyzed for presence of antibody against natural infection of *Brucella* and we confirmed that *Brucella* infection is prevalent and circulating in bovine and ovines' population (Sorsa *et al*., 2021; Sorsa *et al*., 2022). Added to that in the study area there is misconception by most pastoralists that boiling milk reduce the nutritional quality of milk and this could also be among factors that increase the risk of transmission of brucellosis.

The present study revealed that seroprevalence of human brucellosis was found to be higher in those individuals who consumed raw meat (20.27%) than those who don't consume raw meat (2.45%). The difference is statistically significant (*P*=0.00) with OR=10.13, 95%CI: 4.38-23.80). This finding is consistent with previous report indicating that *Brucella* seropositive individuals were reported among abattoirs workers in Ethiopia (Tsegay *et al*., 2017). Similar result was recorded in the study of Edao *et al.* (2020), indicating 3.1% seroprevalence of brucellosis in individuals practicing raw meat consumption

(OR=1.4, 95% CI: 0.4-5.0, and P= 0.61) and relatively low seroprevalence of 2.2% in those that don't consume raw meat in the study area. The finding of this study is also consistent with other reports that showed the incidence of brucellosis in human is directly related to the prevalence of the disease in animals, eating habits, poor hygiene and practices that expose humans to infected animals or their products (Swai *et al.*, 2009; John *et al*., 2010). For instance, in Nigeria eating raw or undercooked contaminated meat has been incriminated in the spread of human brucellosis (Adamu *et al*., 2015). From this it can be deduced that consumption of raw and undercooked meat could be among the potential risk factors for human brucellosis. It is suggested that awareness creation should be aggressively undertaken in the study area.

The current study showed that 363 (47.27%) of the participants (febrile patients) had practice of consuming raw animals' blood while the remaining 405 (52.73%) were not. The study identified this practice as one of the potential risk factors for the occurrence of human brucellosis in the study area. The multivariable logistic regression analysis has revealed significant association between consumption of raw animals' blood and seropositivity (OR=3.65, 95% CI: 2.34-5.69, and P=0.00). Higher seroprevalence was recorded in individuals with this practice, the consumption of raw animals' blood at rate of 22.59% than those who don't consume raw animals' blood with prevalence of 7.41%. The odds of human seropositivity were 3.2 times higher in individuals consuming raw animals' blood than those who don't consume raw animals' blood. This finding is in agreement with study from Ethiopia (Edao *et al.*, 2020), from Kenya (Nanyende, 2010; Osoro *et al*., 2015).

Assisting during parturition was also significantly associated with seropositivity of human brucellosis in this study. The odds of human seropositivity were 3.2 times higher in individuals assisting animals during parturition than those who don't assisting (OR=3.2, 95%CI: 2.05-5.04, and P=0.00). Similarly, a study by Edao *et al.* (2020), in Borana pastoralists from Ethiopia showed that assisting during parturition was found to be potential risk factors of seropositivity and for transmission of brucellosis to humans (OR=9.9, 95% CI:1.4-72). Furthermore, the association between assisting animals with delivery and increased risk of infection has been reported in other studies carried out in similar settings in East Africa, in Tanzania (John *et al*., 2010) and in Kenya (Osoro *et al*., 2015: Muturi *et al*., 2018). The *Brucella* organism is known to preferentially inhabit the reproductive organs, especially the placenta and aborted fetus. Therefore, assisting animals that may be infected during the birthing process could elevate the risk of contracting the infection.

The current study revealed that higher seroprevalence of 17.38% in febrile patients having close and frequent contact with animals compared to those with less frequent contact with animals which accounts for the prevalence of 5.52%. Individuals having close contact with animals had 3.6 times more likely to be seropositive for *Brucella* infection than those with less frequent contact with animals (OR=3.6, 95% CI: 1.84-7.05, and P=0.00). This implies that the ultimate sources of infection for human brucellosis are infected animals. Similar studies from Ethiopia have shown the association of occurrence of human brucellosis and the direct contact with infected animals, such as assisting during parturition, milking, feeding etc and indirect contact such as consumption of raw milk and milk products from infected animals, are risk factors for zoonotic transmission of brucellosis from infected animals to humans (Edao *et al.,* 2020).This finding was also in agreement with previous studies from East African countries such as Southern Uganda (Asiimwe *et al.,* 2015), Central Uganda (Tumwine *et al*., 2015), Kenya (Osoro *et al.,* 2015), and Nigeria (Alhaji *et al*., 2016).

Another potential risk factor of human brucellosis identified in this study was contact with aborted materials such as fetus, fetal membrane and discharges. The multivariable logistic regression analysis showed that the odds of humans having contact with aborted materials were 2.33 times more likely to be seropositive for *Brucella* infection as compared to those without contact with aborted materials (OR=2.33, 95% CI: 1.53- 3.54, P=0.00). This finding is consistent with previous report from Afar Regional State, Ethiopia by Mehari *et al. (*2021), who indicated 35.8% human brucellosis (OR=2.82, 95% CI: 1.16-6.86, P=0.022) in those individuals having contact with aborted materials. Furthermore, other report from Ethiopia in Boranal pastoralists by Edao *et al*. (2020) has confirmed the positive and significant association between human seropositivity for brucellosis and contact with aborted materials (OR=3.4, 95% CI: 0.7-19.1 and P=0.17). Other studies conducted in different parts of Kenya in Marsabit (Osoro *et al*., 2015) and Turkana (Nanyende, 2010) have reported positive association between seropositivity for brucellosis and contact with aborted materials and helping animals during birth.

Regarding knowledge of zoonosis of the research participants, in the present study out of 768 participants 540 (70.31%) had no knowledge of zoonosis. The multivariable logistic regression analysis findings indicated that individuals with low or no knowledge regarding zoonosis recorded higher rates of seropositivity 18.52% for brucellosis (OR=0.44, 95% CI:0.13- 0.46, and P=0.00) while low rate of seropositivity of 5.26% for brucellosis was recorded in individuals having knowledge regarding

zoonosis. A statistically significant association ($p < 0.05$) was observed between knowledge levels and the presence of antibodies against natural infection of *Brucella*, suggesting that enhancing public knowledge may effectively reduce the risk of brucellosis infection.

A questionnaire-based survey was administered to 768 participants to assess their knowledge of brucellosis and their engagement in practices that could potentially expose them to infection. The responses were statistically analyzed using multivariable logistic regression to determine the relationship between knowledge of brucellosis and seroprevalence rates. The findings indicate that a substantial portion of the surveyed population, totaling 495 individuals (64.45%), lacked knowledge about brucellosis. As a result this led them to practice risky activities, including assisting animals during parturition without protective equipment, consuming raw milk and animals' raw blood, and handling aborted materials without gloves. The analysis revealed a significant disparity in seroprevalence rates, where participants without awareness of brucellosis showed a higher seroprevalence of 18.38% (OR=0.37, 95% CI: 0.22-0.61, and P=0.00), compared to relatively lower seroprevalence of 7.69% recorded among those who had awareness about the disease. However, contrary to the current study findings regarding the association between seropositivity of human brucellosis and knowledge of the diseases, Tumwine *et al*. (2015) have reported low disparity of seroprevalence of 17.5% in participants without knowledge of brucellosis, and 16.3% seropositivity in those having knowledge about brucellosis from Central Uganda. This high seropositivity could be linked to fact that good knowledge of brucellosis among pastoralists and herders has a crucial effect in preventing and controlling of the disease both in animal and human populations. Therefore, there is a clear need to promote health education about transmission, prevention and risk factors for brucellosis in the study area to reduce the risk of acquiring brucellosis.Concerning disposal mechanism of aborted materials, patients who responded that they throw away had higher seroprevalence of 16.69% with odds ratio of 6.53 (95% CI: 0.11-10.16, and P=0.99).

5.5. Isolation and molecular identification of *Brucella* species

The current study utilized the gold standard method, isolation to recover *Brucella* from specimens suspected to contain *Brucella* such as vaginal swabs (uterine discharge), milk samples from animals experiencing recent abortion, and whole blood samples from human with fever visiting health centers in the study districts. For the human, for the economical usage of scarce culture media resources only those positive reactors samples were subjected to isolation after screening serologically.

In the present study, out of 340 clinical specimens collected and inoculated on to culture media for isolation, 15 (4.41%) were found to be positive for culture. Different scholars have indicated that isolation of *Brucella* from the infected hosts is considered as the gold standard for diagnosis of brucellosis (Radostits *et al*., 2011; Rahman *et al*., 2012).

It is known that in Ethiopia, there is extremely limited works on isolation and characterization of *Brucella* species though serological studies undertaken so far confirmed brucellosis is endemic in the country. Only few recently published studies are available so far on isolation and identification of *Brucella* species from livestock and no report from human and ovine from Ethiopia. The first isolation attempt was by Sintayehu *et al*. (2015) who reported *B.melitensis* isolates from slaughtered seropositive goats with rate of 14.29% (2/14). The second published work was report of isolation of *B. abortus* at isolation rate of 6.5% (3/46) from samples collected from abortion materials from cows in Asela town, central Ethiopia (Minda *et al.,* 2016). The third published report on isolation and molecular detection of *Brucella* species was by Tekle *et al*. (2019) who reported *B. melitensis* from samples collected from abortion cases of goats from the Amibara district of Afar region at the isolation rate of 12.5% (8/64). The fourth report on isolation and molecular characterization of *Brucella* species was by Edao *et al*. (2020). Edao *et al*. (2020) reported 22.7% (15/66) culture positive out of 66 postmortem samples collected from culled dairy cattle as a measure to control brucellosis following serological testing from Adami Tulu Agricultural Center. Nine isolates were recovered from mammary gland lymph nodes, three from uterine tissues and three from vaginal swabs. Edao *et al*. (2020) confirmed all the isolates were *B.abortus* by the use of different advanced molecular tools and finally characterized to the lineage level. Very recent study on *Brucella* species was isolated from 6/13 (46.15%) vaginal swab samples cultured on *Brucella* selective agar, and shown to be *B. melitensis* using Real-Time PCR reported by Wakjira *et al.* (2022). The result revealed that out of six isolates, two were from vaginal swabs of cattle and the remaining four were from vaginal swabs of small ruminants. All the isolates were detected as *B.melitensis* using real-time polymerase chain reaction.

Regarding species of animals and human as sources of the isolates, in the present study the *Brucella* was isolated at the rate of 3.4% (3/87) from cattle, 6.90% (6/87) from goats, 4.44% (2/44) from sheep, and 3.28% (4/122) from human samples with overall isolation rate of 4.41% (15/340) from all livestock and

human. The highest rate of isolation was observed in goats as compared to other species of animals, implying that goats are more susceptible to *Brucella* infection.

This isolation rate of the current study was comparable with previous reports (Minda *et al*., 2016) eventough there is variation in the study settings. However, relatively less compared to other reports (Sintayehu *et al*., 2015; Tekle *et al*., 2019; Edao *et al*. 2020). This variations could be attributed to types of specimen like cases of postmortem sample collection enhances chance of isolation like the case of Edao *et al*.(2020), recentness of abortion history of animals sampled, storage condition of the samples and duration of culture, type and enrichment of culture media used, and sample size like the case of Wakjira *et al*. (2022) where only 13 vaginal swabs were cultured and 6 of them showed growth.

Regarding the clinical specimen types from which the isolates are identified, the isolation rate was 40% (6 out of 15), 33.33% (5 out of 15), and 26.67% (4 out of 15), from vaginal swab, milk, and whole blood samples respectively (Table 24). After isolation of the pathogen on *Bucella* selective media with supplement, further colony characterization and biochemical tests were conducted. Following phenotypic characteristics of the isolates Real-time PCR was applied to identify the isolates to the genera and specific species level using universal primer and species specific primers and probes.

More importantly, the four *Brucella* isolates recovered from human samples were from patients having risky practices such as assisting animals giving birth without protective materilas, consumption of raw animals' products such as raw milk, and blood in addition to other risk factors. The implication of this finding is conclusive that traditional practices involved by people and feeding habit expose them to brucellosis and other zoonotic diseases which calls for practical interventions in creating awareness in this regard.

This isolation of *Brucella* in human from whole blood in the present study is consistent woth previous report from India by Barua *et al.* (2016) who isolated at rate of 11.76% (18/102) from blood samples collected from febrile patients suspected to have brucellosis. Similar to the present study on application of biochemical tests for phenotypical characterization of the isolates, Barua *et al.* (2016) also employed growth on *Brucella* specific medium, biochemical reactions, CO_2 requirement, H_2S production, dye sensitivity to basic fuchsin and thionin.

Districtwise, the distribution of the isolates indicated the highest prevalence in the Hamer district with six isolates (40%), followed by Gnangatom with four (26.67%), and two each from Desenech and Bena-Tsemay (13.33% each), and one from Malie (6.67%). Notably, the isolates from the humans were one each from the Bena-Tsemay and Gnangatom, and two from Hamer district. This finding underlines the occurrence of *Brucella* in multiple hosts including humans and distributed within all studied districts of South Omo Zone. The seroprevalences rates determined in livestock and human in the current study was also supportive of this situation.

The isolates showed phenotypic characteristics typical for the genus *Brucella*. Out of the fifteen (15) isolates, two (2) isolates had characterized by rough colonies, negative for urease, oxidase and H_2S production, and inability to grow on basic fuchsine dye while they require 5-10% CO_2 for growth and abled to grow in the presence of thionin dye can rule out other species of *Brucella*. This result of CO_2-dependence for the growth was in line with different literatures indicating this is a trait of *B. ovis* and most strains of *B. abortus* biovars 1–4 (OIE, 2018). Gram's and Modified Zeihl Nielsen stain techniques confirmed gram negative bacteria. Based on these phenotypic features these two isolates were presumed to be *B. ovis.* This result is in line with previous reports (OIE, 2018).

Furthermore, out of the fifteen (15) isolates, eight (8) of them had colonies with pinpoint small size, smooth surface, honey drop colored, shiny and translucent. The Gram's stain revealed that the organisms were gram negative, single coccobacilli. The isolates were oxidase positive, produced urease within 24 hrs of incubation grew in the presence of both basic fuchsin and thionin dyes but didn't require CO_2 for growth and no production of H_2S. Based on the typical phenotypic demonstration of the isolates these isolates were presumed to be *B. melitensis.* This result is in alignment with previous reports (OIE, 2018). These isolates were further confirmed as *B. melitensis* species based molecular characterization by RT-PCR.

Based on the phonotypical analysis the remaining five (5) isolates demonstrated smooth, pinpoint, honey drop colonies, positive for oxidase, urease, H_2S tests and required CO_2 for growth, and able to grow on media incorporated with basic fuchsin dye but failed to grow in the presence of thionin dye. Gram's stain and Modified Zeihl Neelsen stain techniques also showed gram negative coccobaccili cellular morphology. Based on these series of biochemical tests the five isolates were presumed *B. abortus.* This phonotypical based result of the current study aligns with the previous reports (OIE, 2018). Out of the

five isolates demonstrating such character three were from specimens collected from bovine while the remaining two were from human sample.

After phonotypical and biochemical based classification of the isolates further molecular detection was conducted using Real-Time PCR. The results of Real-Time PCR revealed that all the isolates 15 (100%) were amplified by the universal primer (IS711) gene verifying that all the fifteen asolates were *Brucella*. Furthermore, the Real-Time PCR confirmed that specific *Brucella* species circulating in the study area.

In this regard, eight isolates were identified as *B. melitensis* species (3 from vaginal swab of goats, 3 from milk samples of goats, and 2 from human whole blood samples). This finding indiacted that *B. melitensis* has host prefence primarly infecting caprine and also humans. This result is in agreement with a literature (OIE, 2022) that indicates *B. melitensis* is the main causative agent of infection with *Brucella* in small ruminants, particulary goats. This result agreed with previous reports of Hegazy *et al*. (2011) that pointed out that *B. melitensis* is the most virulent of the *Brucella* species affecting both humans and animals, followed by *B. abortus* and *B. suis* (Mahmoud and Hamdy, 2018).

In brucellosis endemic areas, transmission of *Brucella* to humans can occur through consumption of unpasteurized milk, particularly in areas where milk ring tests are not routinely practiced for screening *Brucella* specific antibodies in milk (Islam *et al*., 2018). The identification of *B. melitensis* in goats' milk in the current study constitutes a public health hazard as people in pastoralist settings of Ethiopia in general and in the study area in particular consumed unpasteurized or raw milk. The isolation of *B. melitensis* from goat vaginal or uterine discharge could suggest that the discharge can contaminate the grazing area and facilitate the transmission of brucellosis in the study area.

In this study, five (33.33%) isolates were detected and confirmed as *B. abortus* species using *B.abortus* specific primer. Out of these five *B.abortus*, 3(60%) were isolated from bovine vaginal swabs and the remaining 2(40%) isolated from humans blood samples. This finding highlights *B. abortus* as the primary agent responsible for brucellosis in cattle and also affecting humans. The results of bacteriological methods and a classical biotyping schemes used in this study for characterization of *Brucella* species and subspecies levels were presumed these five isolates as *B. abortus.* This result was in line with reports of Whatmore *et al*. (2016) which indicate that *Brucella* can be identified based on routine bacteriological and biotyping schemes and can be further identified to the species level and

below that using polymerase chain reaction. The result of the current study was in accordance with various previous reports that *Brucella* is known to be shed in the aborted materials of cattle such as: uterine discharge, vaginal swab, placenta and fetus (Islam *et al*., 2019). In the current study, two *B. abortus* were recovered from milk obtained from cows and two of them from human blood samples suggesting that humans acquire the disease likely through consumption of raw milk and other occupation risk factors.

Similar to the current study, other previous studies from Ethiopia have reported *B.abortus* from cattle. For instance, Minda *et al*. (2016), isolated and identified *B.abortus* from dairy cattle from Asella Ethiopia based on biochemical tests at the rate of 3 (6.52%), out of 46 clinical samples collected from dairy cattle with history of abortion. One isolate was recovered from placental cotyledon and the remaing two were isolated from vaginal swab. Furthermore, Edao *et al*. (2020) reported 15 (22.7%) culture positive out of 66 postmortem samples collected from culled animals as a measure to control brucellosis following serological testing from Adami Tulu Agricultural Center in which nine isolates were recovered from mammary gland lymph nodes, three from uterine tissues and three from vaginal swabs.

The isolation and molecular detection of *B.abortus* from Human in the current study is the first report in Ethiopia. Out of the total 768 blood samples collected from febrile patients visiting the nearby health centers across the five districts of South Omo zone all the positive reactors samples for RBPT 122 (15.88%) were subjected to isolation on *Brucella* selective supplemented media. Of the total 122 whole blood streaked, four samples (3.28%) were found positive for culture. Based on a series of biochemical, cellular, and molecular tests undertaken these four recovered *Brucella* from human were confirmed two of them as *B.abortus* and the remaining two as *B. melitensis*. This first time successful isolation and molecular detection of *Brucella* species in humans in Ethiopia marks a significant milestone in public health research on brucellosis and shows transmission pattern that humans acquire brucellosis from infected livestock. This achievement enhances understanding of zoonotic diseases in the region, enabling better diagnostics and prevention strategies. It also underscores the urgent need for focused health interventions to mitigate *Brucella*-related infections in vulnerable populations.

5.6. Limitations of the study

The main strengthes of this PhD dissertation include all the objectives proposed both in livestock and human were properly addressed. Firstly, the study covered a wider area including five districts, involving multiple animals species (cattle, sheep and goats) and human. Secondly, the study isolated and detected *Brucella* species from cattle, sheep, goats and human from the same study sites which is a potential indicator of the transmission of the pathogen among the livestock and human populations in the area. Thirdly, the study revealed the first report on isolation and molecular detection of *Brucella species* from the human and ovine hosts in the country.

However, this work is not without limitations. Attempts to collect blood sample from humans parallel to animals sample collection were not successful due unwillingness of the pastoral community to give their blood. As the target study participants are mainly pastoral communities, and due to high mobility of livestock and pastorlists in search for grazing land and water sources, it was infeasible to trace positive animals after screening and collecting samples from the human (owners/herders/any family members) to generate concrete evidence for the pattern of transmission among livestock and human. However, to assess the public health significance of the disease and transmission dynamics at interface between animals and humans, we collected samples from febrile patients visiting the health centers in the study area with strict inclusion critieria such as patient febrile illness, patient ownership of the livestock (to assure contact with animals) and their livestock (cattle, sheep, and/or goats) must be sampled for the same study done on livestock. Therefore, seroprevalence in human is limited to febrile patients which can't be inferred to the apparently healthy human populations.

The sampling design and size has limitation that we used large sample size in livestock, infact considering the wider area coverage of the study. The isolates recovered from sheep were not confirmed by molecular methods except presumption of the isolates to the genus level using universal primer and probe in Real-Time PCR, and primary and secondary biochemical tests due to lack of *B.ovis* specific primer and probe in the market. Furthermore, due to the lack of advanced molecular techniques facility, the isolates identified from livestock and human were not characterized to the biovar and lineage level.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

South Omo zone is predominantly inhabited by pastorlaists of different tribes that are accustomed to traditional pastoalist living styles like consumption of raw animls' products such as milk, meat, and blood and interaction and dependency on livestock for food and income, high mobility of the pastoralists with their livestocks. All the inhabitants rear a diversified livestock species and the area consists of various wild animals specially those districts boardering Mago National Park where there is huge settlements of the pastoralists along with their livestock in search for grazing land and water. The pastoralists have close contact with their livestock and can be exposed to direct contact with contaminated uterine discharge during partuartion. These situations might have influenced the epidemiological picture of *Brucella* infection and the circulating *Brucella* species in livestock and human population in the area.

This study was conducted to investigate the seroepidemiology of brucellosis in livestock (cattle, sheep, and goats) and humans, isolate and molecularly identify the causative agent, and identify the risk factors for its transmission in livestock and zoonotic transmission to humans in the South Omo Zone of the South Ethiopia Regional State.

The findings revealed that brucellosis remains a significant public health and veterinary concern in the area, with varying degrees of seroprevalence observed among different livestock species (cattle, sheep, and goats) and humans. Several risk factors contributing to the occurrence and transmission of the disease were identified, including management practices, close contact between livestock, herd and flock size, production system, history of reproductive problems and other host related factors in livestock. In humans, the potential risk factors contributing to the occurrence and transmission of brucellosis identified include, level of education, occupation, consumption of raw animals' products such as milk, meat, and blood, assisting parturition without proctective material, and limited awareness of zoonotic diseases including brucellosis. Furthermore, the study successfully isolated and molecularly identified *B.melitensis, B. abortus*, and *B.ovis* circulating in animals and human populations from multiple hosts (cattle, sheep, and goats) including human. The isolation and molecular identification of *Brucella*

species from human and sheep in the current study is the first report in the study area as well as in Ethiopia. The seroprevalence results and isolation and molecular identification of *Brucella* species from humans having close contact with livestock, consuming raw animals' products mainly milk and blood, is a clear evidence for the zoonotic transmission of brucellosis from animals to humans in the study area.

6.2. Recommendations

Based on the findings of the study and concluding remarks drawn, the following recommendations are forwarded:

- The finding of the present PhD study designated correlation between seroprevalence rate and isolation of *Brucella* species and involvement of people in risky practices and consumption habits (raw animals milk, meat, and blood) of the people; hence, awareness creation to the pastoralists must be done to mitigate the impact of brucellosis on public health and livestock productivity,

- The significant seroprevalence of brucellosis both in the studied animals and humans in the districts adjacent to Mago National Park calls for future study to explore the transmission dynamics between livestock, wildlife, as well as humans to develop effective control strategies,

- To control the transmission of brucellosis from animals to human population in the study area vaccine production and vaccination campaign in livestock is recommended,

- Appropriate antibiotic treatment alongside patient education on transmission, prevention methods is recommended for infected human at the health center in the study area,

- *Brucella* species isolates of the current study need further detail molecular characterization at biovariant level to know the lineage of the isolates for the better understanding of regional and international Epidemiology of *Brucella*,

- Economic impacts of brucellosis in livestock and human should be investigated as the disease is endemic in the area,

- Humans are at significant risk of infection; therefore, effective control of zoonotic transmission is essential to reduce public health impacts, and

- Overall, implementation of One Health approach framework to attain optimal health for people and domestic animals in the area is recommended to safeguard the health of society

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8. APPENDICES

Appendix 1: Human sample/data collection Recording sheet/

No	District	Sex (Male, Female)	Age	Marital status (Single, Married)	Educational level (Illitrate, primary,	Occupation(Pasto, Agro-past)	Sharing water point with animals	Close contact with animals (Yes,	Know ledge of brucellosis (Yes,No)	Knowledge of Zoonosis (Yes, No)	Consumption behaviour(Yes, No)			Assist during Parturition (Yes, No)	Mechanism of assisting parturition (Bare hand, Protected)	Dispose of aborted material (Burn, Burry, Throw away)	Contact with aborted material (Yes,	Milking cows and goat (Yes, No)
											Raw milk (Yes, No)	Raw meat (Yes, No)	Raw blood (Yes, No)					
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Appendix 2: Individual animal's data recording sheet for *Bovine*

No	District	Sex (Male, Female)	Age (<25, 26-50, >50 animals)	Herd size (Small, Medium, Large)	Abortion History (IYes, No)	Stage of Abortion (Firsttrimester Second trimester,Third trimester	History of RFM (Yes,No)	Frequency of Abortion (Once, more than one)	Body condition (poor, Medium, Good)	Parity (One, two, three)	Owners/Herders/ Knowledge and understanding about brucellosis, and Practices posing risk to pastoralists									
											Awareness about brucellosis (Yes,, No)	Knowledge of zoonotic transmitted by milk	Knowledge of Causes of abortion in animal (Y,N)	handling of infected animal and its product (Yes, No)	diseases transmitted during delivery (Yes	Ways of disposal of aborted (Burning, Burying,, Open	Fate of frequently aborted cows (Culling, Retaining)	Assist parturition with bare hands (Yes, No)	Habit of Consumption of raw animals' blood (Yes, No)	Habit of Consumption of raw milk (Yes, No)
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Appendix 3: Individual animal's data recording sheet for small ruminants

No	District (Bena-Tsemay, Hamer, Gnangatom, Dassenech, Malie)	Sex (Male, Female)	Age (Young,, Adult)	Flock size (Small, Medium, Large)	Abortion History (IYes, No)	Stage of Abortion (Early, Late)	History of RFM (Yes,No)	Production system (Pastoral, Agro-Pastoral)	Body condition (poor, Medium, Good)	Still birth (Yes, No)	Parity (One, More than one)	Remarks
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Appendix 4: Rose Bengal Plate Test Procedure (OIE, 2018) for cattle and human samples

1. Sera (control and test sera) and antigen for use are left at room temperature for half an hour before testing; since active materials straight from the refrigerator react improperly.
2. 30µl serum is mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
3. The antigen and serum are mixed thoroughly using an applicator stick
4. Rock plate by hand/rotary shaker/ for about 4 minutes
5. Examine for agglutination in a good light
6. Use magnifying glass when micro agglutination suspected

Interpretation:

0 = no agglutination

+ = barely perceptible

++ = fine agglutination,

Some clearing +++ = coarse clumping, definite clearing

Those samples identified with no agglutination are recorded as negative those with +, ++, +++, ++++ are recorded as positive.

Appendi 5: Rose Bengal Plate Test Procedure (OIE, 2018) for small rumninants samples

1. Sera (control and test sera) and antigen for use are left at room temperature for half an hour before testing; since active materials straight from the refrigerator react improperly.
2. To enhance sensitivity, a modified RBPT (mRBPT) applied, utilizing three volumes (75 µL) of the serum with one volume (25 µL) of antigen mixed on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
3. The antigen and serum are mixed thoroughly using an applicator stick
4. Rock plate by hand/rotary shaker/ for about 4 minutes
8. Examine for agglutination in a good light
9. Use magnifying glass when micro agglutination suspected

Interpretation:

0 = no agglutination

+ = barely perceptible

++ = fine agglutination,

Some clearing +++ = coarse clumping, definite clearing

Those samples identified with no agglutination are recorded as negative those with +, ++, +++, ++++ are recorded as positive.

Appendix 6: Complement Fixation Test Procedure

1. Test sera and appropriate working standards are diluted with an equal volume of veronal buffered saline in small tubes and incubated at 58°C for 50 minutes in order to inactivate the native complement.

2. Using standard 96-well U-bottom microtitre plates, 25µl volumes of diluted test serum are placed in the wells of the first and second rows, and 25µl volumes of veronal buffered saline are added to all wells except those of the first row.

3. Serial doubling dilutions are then made by transferring 25µl volumes of serum from the second row onwards continuing for at least four dilutions.

4. Repeat steps 2 and 3 above for each serum to act as anticomplementary serum controls (see below).

5. Volumes (25µl) of complement at 1.25 MHD, are added to each well and 25µl of antigen, diluted to working strength, are added to all wells excluding those of the anticomplementary controls. These latter wells receive 25µl of veronal buffered saline instead.

6. Control wells containing: diluent only, negative serum + complement + diluent, antigen + complement + diluent, and complement + diluent, are set up to contain 75µl total volume in each case.

7. The plates are incubated at 37°C for 30 minutes with agitation at least for the initial 10 minutes, or at 4°C for 14-18 hours.

8. Volumes (25µl) of sensitised SRBC suspension are added to each well, and the plates are reincubated at 37°C for 30 minutes with agitation at least for the first 10 minutes.

9. The results are read after the plates have been left to stand at 4°C for up to 1 hour to allow unlysed cells to settle.

Interpretation: Sera with strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of complement (2+) at a dilution of 1:10 and above are classified as positive and lack of fixation/complete hemolysis is considered as negative

Appendix 7: The Gram's Stain Procedure (Quinn *et al*., 2011)

Take a loop full of bacteria emulsify it in a small drop of water or saline on the slide. This should be a thin, not milky, suspension or it will not stain properly. Air dries the slide. This is done automatically in the virtual module. To begin:

1. Heat fixes the slide: click on the Bunsen burner; pass the slide gently two or three times (1-2 seconds) through the flame. Do not overheat - this will cause distortion of the cells.
2. Flood the slide with crystal violet for 1 minute
3. Rinse with running tap water
4. Flood the slide with iodine for 1 minute
5. Rinse with water
6. Decolorize with alcohol for 5-10 seconds
7. Rinse with water
8. Flood the slide with safranin for 1 minute
9. Rinse with water
10. Dry the slide in air and observe the slide under the microscope

Interpretation: Gram positive bacteria appear blue, Gram negative bacteria stained red

Appendix 8: Modified Zeihl Neelsen Stain (*Brucella* Staining) Procedure (Quinn *et al*., 2011)

1. Fix a smear by heat
2. Overlay the slide completely with dilute carbolfuchsin for 15 minutes
3. Differentiate the smear for 15 seconds in 0.05% sulphuric acid/0.5%acetic acid and wash it with tap water
4. Counter stain with 3% malachite green solution or methylene blue for 2minutes, wash again with water and dry it.

Interpretation: *Brucella* species appeared red. But other bacteria and the background appear green

Appendix 9: Oxidase Test Procedure (Quinn *et al*., 2011)

The oxidase test is based on the bacterial production of an oxidase enzyme. The oxidase reaction is due to the presence of a cytochrome oxidase system, which activates the oxidation of reduced cytochrome by molecular oxygen. This in turn as an electron acceptor in terminal stage of electron transfers system. The cytochrome is an iron containing hemo proteins that acts as the last link in the chain of anaerobic respiration by trasfering electron (hydrogen) to oxygen with the formation of the water or H_2O_2.

Oxidase reagents: Tetramethyl-p-phenylenediamine dihydrochloride (1%water solution) – kovac's reagent Dimethyl-p-phenylenediamine dihydrochloride (1% water solution) – Gordon and Mcleod's reagent.

Procedure: Direct plate procedure

Add 2-3 drops of oxidase reagent directly to a few suspected colonies growing on plate medium. Do not flood the entire plate.

Do not invert the plate

Observe color change after 15-30 seconds (kovac's reagent) Indirect paper procedure Place a 6cm2 pieces of whatman no. 1 filter paper in petri dish Add 2-3 drops of Kovac's reagent to acenter of the paper Smear loop full of a suspected colony on to the reagent impregnated paper in aline 3-6 cm long A positive color reaction occur within 5-10 seconds

Interpretation: Oxidase positive: the colonies form dark blue color after few seconds where as oxidase negative shows no color formation

Appendix 10: Urease Test Procedure (Quinn *et al*., 2011)

It is to determine the ability of the organisms to split urea, forming two molecules of ammonia by the action of the enzyme urease.

Procedure:

Urea agar/broth inoculated with a loop full of pure culture of the test organisms and incubates at the 35^{o}c for 18-24 hrs.

Interpretation:

Organisms that hydrolyze urea rapidly may produce positive reaction within 1-2 hrs less active spp may require 3 or more days.

Rapid urea splitters – Red (pink) colour throughout the medium

Slow urea splitters – Red (pink) initially in slant only gradually converting the entire tube.

No urea hydrolysis – Medium used original yellow colour.

Appendix 11: C-ELISA Procedure

APHA SCIENTIFIC

INSTRUCTIONS FOR USE

(for in-vitro and animal use only)

COMPELISA 160 & 400

A competitive ELISA kit for the detection of antibodies against Brucella in serum samples. The COMPELISA kit has been standardised for use in diagnosing brucellosis infection in cows, sheep and goats.

Kit contents

Plates	Plates pre-coated with *B. melitensis* LPS antigen
Conjugate	As supplied (store at -20°C)
Control	Positive serum Negative serum
Chromogen **Substrate**	OPD tablets (Warning: Toxic!) Urea Hydrogen Peroxide tablets (Warning: Irritant!)
Diluting buffer	Tablets of phosphate buffered saline PBS) Phenol red indicator Tween 20
Stopping solution	Citric Acid (Warning: Irritant!)
Wash solution	Na_2HPO_4 Di-sodium hydrogen orthophosphate

Equipment Required

Microtitre plate reader with 450nm filter

Single and multichannel variable volume pipettes Disposable tips for the above

Reagent troughs for multichannel pipetting 10 litre container for wash fluid

4°C ± 3°C refrigerator

Rotary shaker, capable 160Revs/Min Microtitre plate shaker

Sterile distilled or deionised water

Bottles, tubes and beakers for storage of sera and reagents Absorbent paper towels

Freezer for storage of conjugate Water bath (optional)

Incubator (optional)

Reagent Preparation

- Reagents provided are sensitive to changes in temperature and light. They must be prepared and stored as per instructions if they are to be effective in the test.
- Very clean glassware, sterile distilled water or good quality drinking water are vital for the preparation and storage of reagents.
- **Please read and comprehend instructions fully before attempting to use the kit.**

Diluting Buffer

Prepare diluting buffer by adding 5 tablets of PBS, 500µl phenol red indicator and 250µl of Tween 20 to 500ml distilled water. The pH must be between 7.2 and 7.6, phenol red will turn yellow below pH 7.2 and violet above pH 7.6. If the pH of the diluting buffer is not within target range it should be discarded. Store at 4°C ± 3°C. Discard after one month of preparation.

Wash Solution

Prepare the wash solution by adding the contents of the ampoule of Na_2HPO_4 and 1ml of Tween 20 to 10 litres of distilled water. This can be stored at room temperature (21°C ± 6°C). Discard after one month of preparation.

Conjugate

Prepare the conjugate for use according to instructions on the ampoule. Once the conjugate has been prepared it must be used immediately. (See Method – Step 1)

Stopping Solution

Prepare the stopping solution by diluting the contents of the ampoule of citric acid with 38ml of distilled water. Store at 4°C ± 3°C. Discard after one month of preparation.

Controls

Reconstitute the positive and negative control samples each with 1ml sterile distilled water. Allow tostand until fully reconstituted, ensure the entire contents are completely re-suspended before use. Store at 4°C ± 3°C. If the control samples are to be kept for more than one week, store at -20°C ± 5°C in aliquots.

OPD

If a magnetic stirrer is not available, the urea hydrogen peroxide tablets can be dissolved in distilled water up to 20 minutes prior to use although the OPD tablets must only be added and dissolved immediately before use. (See Method – Step 9).

Method

1. Warm the diluting buffer to room temperature, it is recommended that the diluting buffer is warmed in a water bath at 23ºC (± 3ºC). Mix the conjugate concentrate (BM40) thoroughly and dilute to working strength in the warmed diluting buffer according to the instructions on the conjugate ampoule. This solution cannot be stored.

NB When removing the conjugate from -20°C it is advisable to hand warm the ampoules before adding to the warmed diluting buffer. When emptying the conjugate vial, a small volume of conjugate can remain in the vial. Use a fine tip pipette to remove the remainder out; failure to do this may affect the outcome of the test.

2. Add 20µl of each test serum per well. Leave columns 11 and 12 for controls (See microtitre plate layout page 7).
3. Add 20µl of the positive control to wells F11, F12, G11, G12, H11 and H12.
4. Add 20µl of the negative control to wells A11, A12, B11, B12, C11 and C12.
5. The remaining wells in columns 11 and 12 have no serum added and act as the conjugate control. D11, D12, E11, E12.
6. Immediately dispense into all wells 100µl of the prepared conjugate solution. This gives a final serum dilution of 1/6.

7. The plate is then vigorously shaken (on the microtitre plate shaker) for two minutes in order to mix the serum and conjugate solution. Cover the plate with a lid and incubate at room temperature (21°C ± 6°C) for 30 minutes on a rotary shaker, at 160 revs/min.

If neither shaker is available the plate should be initially shaken for 30 seconds followed by 10 second hand-shakings every 10 minutes for a total of 1 hour.

Caution - care should be taken when hand shaking to keep liquid in their respective wells.

8. Shake out the contents of the plate before washing each plate 5 times with either washing solution or drinking water from a tap under low pressure (keep tap water at a steady, soft flow). Dry plate by tapping firmly onto a few layers of absorbent towel until no more liquid is removed.

N.B. Hand washing is the recommended method although a plate washer may be used if proven to be fit for purpose; the number of washes may be adjusted accordingly. Validation of the plate washer may be required to ensure the use of this equipment is suitable for the test.

9. Immediately before use, prepare the substrate and chromogen solution by dissolving one tablet of urea H_2O_2 in 12ml of distilled water. When dissolved, add the OPD tablet and mix thoroughly.

This can take a few minutes; the use of a magnetic stirrer will greatly increase the speed with which it dissolves. This solution cannot be stored.

NB OPD solution is sensitive to light and should be made up immediately before use and kept in an opaque container.

10. Add 100µl of OPD solution to all wells. Incubate the plate at room temperature (21°C ± 6°C) for a minimum of 10 minutes and a maximum of 20 minutes.

NB Gently tap the plate approximately at five minute intervals during the substrate incubation. Caution – care should be taken when tapping to keep the liquid in their respective wells.

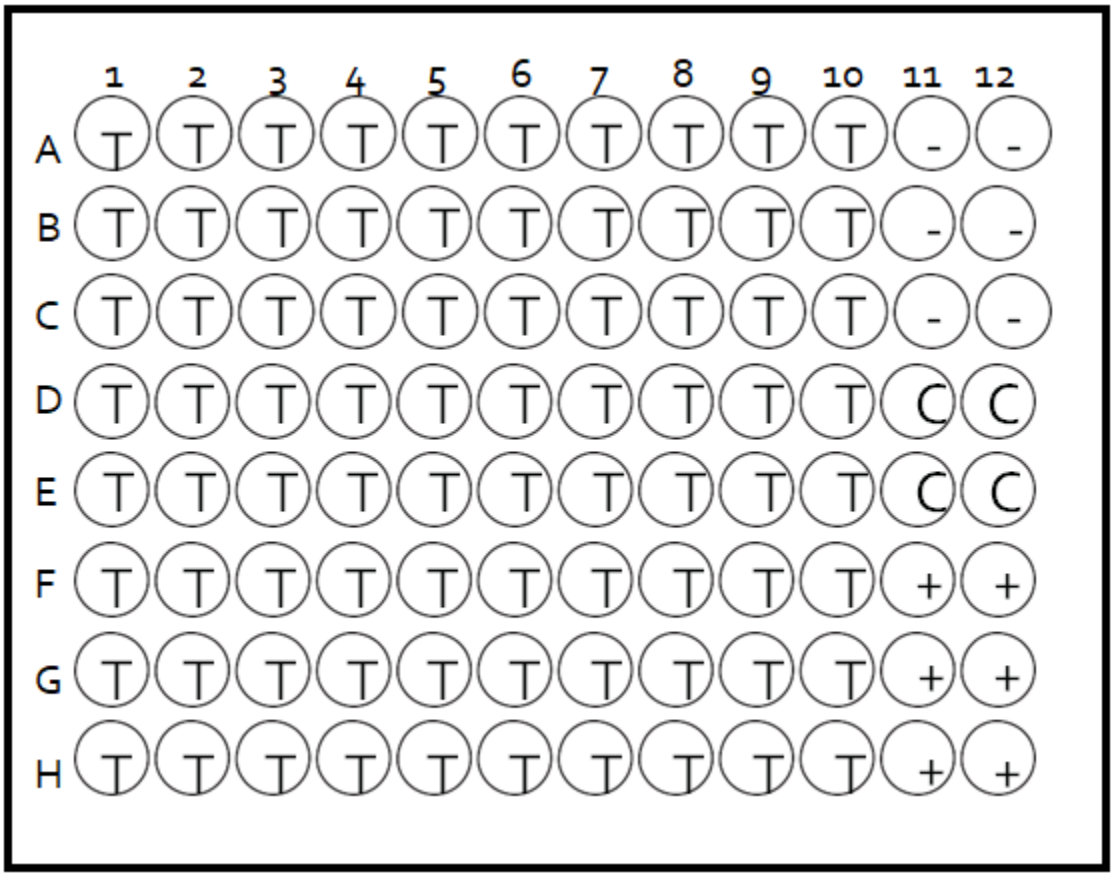
11. Switch on microplate reader and allow the unit to stabilise for 10 minutes.

12. Slow the reaction by adding 100µl of stopping solution to all wells.

13. Remove condensationfrom the bottom of the plate withabsorbentpapertowel. Read plate at 450nm. N.B. Read plate within 10 minutes.

If a microtitre plate reader is not available, a visual inspection of the plate may be used to determine whether a sample is positive or negative. (See Analysis of Results section).

Microtitre Plate Layout



C - Conjugate control

T - Test sample

\+ Positive control

\- Negative control

Analysis of Results

The lack of colour development indicates that the sample tested was positive. A positive/negative cut-off can be calculated as 60% of the mean of the optical density (OD) of the 4 conjugate control wells. Any test sample giving an OD equal to or below this value should be regarded as being positive.

Plate Acceptance Criteria

The results should be considered valid if the following apply;

The mean OD of the 6 negative control wells is greater than 0.700. (The optimal mean negative OD is 1.000).

The mean OD of the 6 positive control wells is less than 0.100

The mean OD of the 4 conjugate control wells is greater than 0.700 (the optimal mean conjugate control OD is 1)

The binding ratio is greater than 10.

$$\text{Binding Ratio} = \frac{\text{Mean of 6 negative control wells}}{\text{Mean of 6 positive control wells}}$$

Appendix 12: Culture Media Preparation

Brucella selective agar media preparation procedure

1. Suspend 21.55 grams *Brucella* Agar Base in 500 ml distilled water.
2. Boiling to dissolve the medium completely.
3. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Cool to 45-50°C and aseptically add sterile 5% v/v inactivated Horse Serum (inactivated by heating at 56°C for 30 minutes) and rehydrated contents of one vial of *Brucella* Selective Supplement (FD005) was added
5. Media is then poured in to sterile Glass petridishes.

Appendix 13: Biochemical characteristics of *Brucella* species

Species	Biotype	Fuchsin	Thionin	Safranin Inhibition	H2S production	Urease	CO2 growth
B. mlitensis	1-3	+	+	-	-	+ (24hrs)	-
B. abortus	1-6,9	+(Except biotype 2)	-(Except biotype1, 2, 4)	-	+ (Except biotype5)	+(24hrs)	+
B. suis	1-5	-(Except biotype3)	+	+	+	+ (15min)	-
B. canis	--	+/-	+	-	-	+(15min)	-
B. ovis	--	-(some strains)	-	-	-	-	+
B. neotomae	--	-	-	-	+	+(15min)	-
B. pinnipediae	--	+	+	-	-	+	-
B. cetaceae	--	+	+	-	-	+	+

Source (Yasmin and Lone, 2015)

Appendix 14. RT-PCR Protocol

Detection of *Brucella* spp. DNA using the AB® 7500 Fast or Fast Dx Real-time PCR Instrument

Purpose and Assay Principle

This assay is intended for the qualitative detection of *Brucella* spp. DNA extracted from clinical specimens or culture isolates. This real-time PCR assay has been modified from a previously published assay (1).

Real-time polymerase chain reaction (PCR) assays use a fluorescently-labeled probe or

intercalating dye to visualize a PCR reaction and monitor the quantity of double-stranded product that is produced. The fluorogenic 5' nuclease assay (TaqMan® assay) is a real-time PCR assay which uses a fluorogenic probe, consisting of an oligonucleotide with a reporter dye attached to the 5' end and a quencher dye attached at or near the 3' end. The probe anneals to a specific target sequence located between the forward and reverse primers. During theextension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe causing the reporter dye to separate from the quencher dye and a fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes and the fluorescence intensityis monitored during the PCR. The Taq polymerase used in this assay is inactive at room temperature. It must be activated by incubation at 95°C, which also minimizes the production of nonspecific amplification products.

Biosafety

At a MINIMUM these procedures should be performed using BSL-2 facilities and BSL-3 practices. Perform all sample manipulations within a Class II (or higher) biological safety cabinet (BSC). Refer to Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th edition. http://www.cdc.gov/biosafety/publications/bmbl5/index.htm

Materials

Reagents

1. *Brucella* spp. real-time PCR primer and probes includes 6 primers and 3 FAM labeled probes. RNase P primers and probes are included to assess extraction efficiency when DNA has been extracted from human clinical specimens. Store 20 µM working stocks in the dark at 2–8°C. Store stock concentrations of oligonucleotides a -20°C to -70°C.

a. Primer/probe set IS711 contains

i. IS711 forward primer (IS711-F)

ii. IS711 reverse primer (IS711-R)

iii. IS711 probe (IS711-P)

b. Primer/probe set Melitensis contains

i. Mel forward primer (Mel-F)

ii. Mel reverse primer (Mel-R)

iii. Mel probe (Mel-P)

c. Primer/probe set Abortus contains

i. Abortus forward primer (Abortus-F)

ii. Abortus reverse primer (Abortus-R)

iii. Abortus probe (Abortus-P)

d. RNase P (RP) Real-time PCR Primer and Probe

i. RNase P forward primer (RNaseP-F)

ii. RNase P reverse primer (RNaseP-R)

iii. RNase P probe (RNase-P)

2. Real-time PCR Positive control DNA.

a. *Brucella canis* (IS711 PCR)

b. *Brucella ovis (B. melitensis* PCR)

c. *Brucella abortus* plasmid (*B. abortus* PCR)

3. RNase P positive control: Either of the following two products may be used:

a. Human Genomic DNA from human blood (buffy coat); Roche Applied Science, Catalog #11691112001

b. Human Genomic DNA; Promega Corporation, Catalog #G3041

4. 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach). This product eliminates RNases and DNA.

5. TaqMan Universal Master Mix, no AmpErase UNG (Life Technologies, 4324018) store at 2–8°C. Refer to manufacturer's instructions for expiration information.

6. TaqMan® Exogenous Internal Positive Control (IPC) (Life Technologies, 4308323)

7. PCR grade (nuclease free) water.

Supplies

1. Laboratory marking pen

2. Disposable gloves

3. Ice bucket and ice or freezer rack

4. Racks for 1.5 mL (or 1.7 mL) micro-centrifuge tubes

5. P2/P10 aerosol barrier (plugged) pipette tips
6. P200 aerosol barrier (plugged) pipette tips
7. P1000 aerosol barrier (plugged) pipette tips
8. Sterile 1.5 mL (or 1.7 mL) micro-centrifuge tubes, nuclease-free
9. Optical 96-Well Fast Thermal-Cycling Plates (Applied Biosystems; catalog #4346906 or #4366932)
10. MicroAmp® Optical Adhesive Film (Applied Biosystems; catalog #4360954 or #4311971)

Equipment
1. Vortex mixer
2. Micro-centrifuge
3. P2 or P10 pipette
4. P200 pipette
5. P1000 pipette
6. Applied Biosystems 7500 Fast Dx Real-time PCR Instrument (Applied Biosystems; catalog #4406985 or #4406984) or Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems; catalog #4351106 or #4351107)
7. MicroAmp® Adhesive Film Applicator (Applied Biosystems; catalog #4333183)

Controls/quality control testing
1. When DNA from human clinical specimens is tested, include the Ribonuclease P (RNase P) primer/probe set to serve as a control for specimen quality, the extraction process, reagents and equipment. While a poor RNase P signal may indicate inhibition, typically it indicates a problem with the DNA extraction process (e. g. low DNA yield).
RNase P positive control is used to ensure adequate DNA yield with specimen collection or the DNA extraction process for human clinical samples. The material should be diluted to 50 ng/5 μL or 10 μg/mL with 10 mM Tris pH 7.4-8.2 prior to use. An acceptable Ct range is 20-30.

2. The TaqMan® Exogenous Internal Positive Control (IPC) should be added to the

master mix and tested with each clinical specimen and control.

a. The IPC should show a positive result (Ct value <45). If the IPC is negative, the specimen may contain PCR inhibitors and should be diluted 1:10 and repeated in the reaction.

i. NOTE: if IPC is negative but samples is positive it does not need to be repeated (see figure 1)

ii. If the diluted sample is retested and is still negative for the IPC, the DNA extraction should be repeated.

b. The kit comes with a 10X Exo IPC blocking reagent that can be used in a NTC to block the IPC fluorescence if desired (we do not use this)

3. PCR grade water serves as the negative control for this assay in the no template control (NTC). Include NTCs for each signature (one each for IS711, Melitensis, Abortus, RNase P) as applicable in each run.

4. Appropriate agent-specific controls should be run concurrently with samples each time this assay is performed. The positive controls should be added after NTCs and samples have been added to the plate. Exercise caution in the addition of the positive control to minimize cross-contamination.

5. Test serial dilutions of the known *Brucella* spp. DNA to determine a dilution that produces a CT value of 30–36 for use as a positive control. A concentration of 10-100 fg/µl is an acceptable concentration for the positive control DNA. Dispense the extracted DNA into aliquots and store using the following storage conditions:

a. < 7 days at 2–8° C

b. Short term storage (< 1 year), store at -20±5°C

c. Long term storage (> 1 year), store at ≤-65°C

6. The *B. abortus* plasmid can only be used as a positive control using the primers and probes described in this protocol. The *B. abortus* plasmid contains the PCR product produced by the primers in the *B. abortus* PCR assay and will produce fluorescence in the presence of the *B. abortus* probe under thermal cycling conditions.

7.Interpretation of results: If any of the controls do not perform as expected, investigate to determine the cause, implement and document the corrective action taken and repeat the assay (see Figure and section on Troubleshooting).

Procedure

1. Extract DNA from clinical specimens following the manufacturer's procedures.

a. For blood clots, tissues, and milk pellet the Qiagen DNeasy Blood and Tissue kit is recommended.

b. For other bodily fluids such as serum, CSF (cerebral spinal fluid) and bone marrow the Qiagen DNA Blood Mini kit is recommended.

2. When testing clinical specimens, first screen samples using the IS711 pan-Brucella marker prior to moving forward with the species-specific identification (*Brucella abortus* and *Brucella melintensis*).

3. See suggested plate layout below. Create run sheet and calculate the number of reactions prior to entering the lab.

	IS711	**RP**	**IS711**	**RP**	**IS711**	**RP**	**IS711**	**RP**	**IS711**	**RP**	**IS711**	**RP**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H	NTC	NTC	EN	EN							Bru+	RN+

NTC = no template control, EN = extraction negative, RP = RNase P, Bru+= *Brucella* positive control DNA (*B. canis*), RN+ = RNAse P positive control DNA

4. Remove DNA samples and all necessary working stocks of primers and probes for the assay(s) you will be setting up and the TaqMan Universal Master Mix, no AmpErase UNG and IPC reagents from the refrigerator or freezer.

5. When using the IPC for the IS711 and RNase P PCRs, combine the reagents for the master mix as follows:

Reagent	Final Conc.	1 Rxn [µl]
TaqMan Universal PCR Master Mix (2x)	1x	12.5
Water	******	3.9
10x Exo IPC Mix	1x	2.5
50x Exo IPC DNA	1x	0.5
Primer F (20 µM)	20 µM	0.2
Primer R (20 µM)	20 µM	0.2
Probe (20 µM)	20 µM	0.2
Total		20

*Dilute 15 µl of 10x IPC Block into 15 µl water to make 5x and add 5 µl to each reaction.

6. When performing the species specific PCRs (*B. melitensis* and *B. abortus*) the IPC will not be used, combine the reagents for the master mix as follows:

Reagent	Final Conc.	1 Rxn [µl]
TaqMan Universal PCR Master Mix (2x)	1x	12.5
Water	******	6.9
Primer F (20 µM)	20 µM	0.2
Primer R (20 µM)	20 µM	0.2
Probe (20 µM)	20 µM	0.2
Total		20

7. Gently mix the master mix then briefly spin in a micro-centrifuge.

8. Aliquot 20 µl of each master mix into pre-designated wells of a MicroAmp 96-well microtiter plate.

9. Add water to the 'NTC' positions.

10. In a designated DNA handling space such as a Clean Spot, add 5 µl of your extracted DNA and extraction blank to their designated position.

11. Add 5 µl of your positive control DNA to correct positions for each assay. It is best to position your positive control DNA as far away as possible from your test specimens on the 96 well plate.

12. Apply the adhesive film or optical strips and seal the plate tightly using the film applicator or cap roller.

13. Centrifuge the plate briefly to collect reagents and DNA in the bottom of the wells.

Instrument set-up and running PCR for ABI 7500

1. Turn on ABI 7500 Real Time PCR System instrument.
2. Open plate tray and place test plate into instrument. Close tray.
3. Launch the Applied Biosystems 7500 Fast System software.
4. Click “Create New Document”.
5. Change “Run Mode” to “Standard 7500”. Click Next.
6. Select the appropriate detector for the assay(s) you are running and add them to the Detection in Document window. NOTE: Refer to 7500 Programming document to create the following detectors.

a. IS711 FAM
b. Melitensis FAM
c. Abortus FAM
d. RNAse P FAM
e. ABI IPC VIC

7. Next to “Passive Reference”, select “ROX”. Click Next.
8. Using the mouse, select the well boxes corresponding to the wells containing samples.
9. Select the corresponding ‘detector’ associated with each well. i.e IS711 and IPC and RNAase P and IPC
10. Next to the detector box, check the “Use” box for the appropriate detector(s).

a. When using the ABI internal positive control, select both IS711 (FAM) and ABI IPC (VIC) detectors since the IPC is plexed with your FAM reactioin.

11. Change the task menu to mark “U” for unknown, “NTC” for no-template controls or “S” for you positive controls. Enter sample names into appropriate wells. Click Finish.
12. You can right-click on a well and select well inspector to label each well with what reaction is being run and the name of the sample
13. Select the Instrument tab. Modify the thermal cycling conditions to reflect the conditions below:

Thermal cycling conditions:			
Denature	95°C	10 min	
Amplification	95°C	15s	45 cycles
	60°C	1 min	
Settings:			
Reactions:	25 µl reactions		
Mode:	7500 Standard mode		
Passive reference:	ROX		

14. Other settings:

a. Increase the number of cycles from 40 to 45

b. Change the sample volume from 20 µl to 25 µl

c. Verify that "Run Mode" is "Standard 7500"

15. Click File, Save As, and name and save the file in an appropriate project folder.

16. Click Start to begin the run. The run typically takes 1.5 hours.

Data Analysis

1. When the run is complete, click on the Results tab.

2. Click on the Amplification Plot tab to view the raw data.

3. Highlight all the samples in the order below and select on that detector so the curves can be viewed before analysis:

a.IPC (VIC/HEX): confirm that all samples that include the IPC are positive, the human DNA control for RNAseP should have a delayed (higher Ct) curve due to inhibition

b.RNAseP (if run, FAM): all human samples and the control should be positive

c.Controls (FAM): look at your respective positive and negative controls for the IS711, B. abortus or B. melitensis assays to confirm the controls worked appropriately

4. Select on wells that are testing for IS711 and RNAseP (if applicable) (or B. melitensis and B. abortus) and make sure all the appropriate detectors are selected, look at the NTC samples to confirm they are negative

5. Click the radio button next to "Manual Ct" and enter a number corresponding to the scale on the y axis to get the baseline onto the graph that is above the NTC.

6.Using the mouse, click and drag the red threshold line until it lies above any background noise, all NTCs curves and within the exponential phase of the fluorescence curves.

7. Click the green triangle to "Analyze". The red threshold line will turn to green, indicating the data has been analyzed.

8. Click File, Save to save the file again after analysis.

9. Click the Report tab to display the Ct values. Either print or export the data to a portable data storage device for data analysis.

a. On the file tab select export and then select report.

Interpretation of Results (Also see Appendix A and B)

a.Positive: Ct value ≤ 45 for both IS711 and RNAseP and an IPC Ct value of <40, and appropriate results for positive and negative controls in the run.

b.Negative: Ct value negative for IS711 with a positive IPC (Ct value of <40) in a clinical sample, along with appropriate positive and negative controls in the run.

c.Inconclusive: Clinical specimens showing inhibition in the IPC (Ct value of >40) despite repeated testing using diluted DNA and/or with new reagents.

d.Follow-up testing: Samples that are positive on the IS711 PCR should be further tested by the species specific PCRs (Brucella melitensis and B. abortus).

Guidelines for Reporting and Notification

1. Results should be saved on the machine and/or exported on a USB drive and saved in a results file.

2. For each test sample the test sample's unique identification number, testing date, plate number, Ct results for each target, and overall interpretation and reported species (if applicable) should be recorded in the PCR testing logbook (see Appendix C for a PCR testing logbook page template).

3. Any discrepancies noticed during the testing process must be recorded in the comments field of the PCR testing logbook and brought to the attention of the Team Lead or designee.

References

1."Novel identification and differentiation of *Brucella melitensis, B. abortus, B. suis, B. ovis, B. canis, and B. neotomae* suitable for both conventional and real-time PCR systems" published by V. Hinić, I. Brodard, A. Thomann, Ž. Cvetnić, P.V. Makaya, J. Frey, C. Abril in the Journal of Microbiological Methods 75 (2008) 375–3

PCR testing logbook page template

[1]Sample ID#	Test date	[2]Plate #	[3]IS711	[4]RNAseP	[5]IPC	[6]Interpretation	[7]Melitensis	[8]Abortus	Comments	[9]Storage location			
										Fridge or Freezer #	Rack #	Box #	Grid #

[1]**Sample ID# =** unique identification number
[2]**Plate # =** unique number of the plate
[3]**IS711 =** Ct value for IS711
[4]**RNAseP=** Ct value for RNAseP
[5]**IPC =** Ct value for IPC
[6]**Interpretation for screening=** positive, negative, repeat
[7]**Melitensis =** Ct value for *B. melitensis* PCR (if applicable, screening must be positive)
[8]**Abortus =** Ct value for *B. abortus* PCR (if applicable, screening must be positive)
[9]**Storage location** = refrigerator# or freezer#, rack#, box#, grid#

Appendix 15. Informed Consent form

I _____, the undersigned have read and understand this letter of invitation to participate in the research study: "**Seroepidemiology and Molecular Identification of *Brucella* Species in Livestock and Humans in South Omo Zone, South Ethiopia Regional State, Ethiopia**". I have received adequate information regarding the nature of the study and understand what will be requested of me. I am aware of my right to withdraw at any point during the study without penalty. I hereby consent to participate in this research study.

Participants Signature: _____ Date: _____

Researchers Signature: _____ Date: _____