Thesis Ref. No. __________

SEROPREVALENCE, ISOLATION AND MOLECULAR DETECTION OF *BRUCELLA* SPECIES FROM CAMEL AND SMALL RUMINANTS IN TIGRAY AND AFAR REGIONAL STATES OF ETHIOPIA

BY

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JUNE, 2020

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Msc THESIS

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

BY: ABERA KEBEDE

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DEDICATION

This research paper is dedicated to my beloved family; my wife Asnakech Zegeye, our children Meron, Mahlet, Ruth, Nardos, and Yosef and to our grand children Liya, Christian, Eyosias and Barok their love, prayer and patience have encouraged me to have eneregy to complete this research work. .

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my genuine work and that all sources of material used for this thesis have been acknowledged. This thesis has been presented in partial fulfillment of the requirements for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is put at the University/College library to be made available to people who may need to have access under rules of the Library. I declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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ACKNOWLEDGEMENTS

First of all, I would like to thank my God who helped me in every direction of my life. Without his support it would have not been possible to complete this research work. Glory be to God in the name of Jesus Christ the Savior. AMEN!!!!

I would like to express my sincere gratitude to my advisors Dr. Shimeles Tesfaye from College of Veterinary Medicine and Agriculture, Addis Ababa University and Co-advisor Dr. Matios Lakew from National Animal Health Diagnostic and Investigation Center (NAHDIC) for their valuable support in providing me excellent guidance through reviewing my thesis. Their comments and suggestion were very constructive and encouraging which gave me extra energy to accomplish my research work.

I would also like to thank National Animal Health Diagnostic and Investigation Center (NAHDIC) management for supporting me to attend my MVSc by providing financial, logistic and administrative support.

Moreover I would like to extend my appreciation to American Society for Microbiology (ASM) for funding the project and CDC for the training on real-time PCR protocol for the identification of *Brucella species*.

I am also very much grateful to Dr Hagos Asgedom, Dr Biniam Tadesse, Dr Demeke Zewde, Mr Daneil Tekeste, Dr Getachew Tuli, Mr Teferi Benti, Mr Getachew Kinfe and Mrs Yeshi Biruk for their unreserved involvement from sample collection to analysis of the tests which was very cumbersome.

I do not have enough words to express my sincerely gratitude to Mr Abebe olani for his patience in processing of bacteriological samples which was time consuming.

I would like to extend my heartfelt gratitude to Mr Bayeta Senbeta, Mr Abdi Aleyi, Mr Melaku Sombo, Dr Getachew Abichu and Dr Redeat Belayneh for their keen support in testing and analyzing of samples in molecular biology laboratory even though they were very busy with testing of COVID 19 samples.

Special thanks go to Mr Asheber Abebe for his help in preparing the map of study area.

I wanted to thank and appreciate Mr Takele Worku, Mr Nega Jaeta, Ms Tigist Worede, Ms Feven Solomon and all sample reception team for their support in handling and managing the research samples.

At last but not least, I would like to extend my sincere gratitude to all NAHDIC staff for their support in one way or the other during my research work even though I did not mentioned their names.

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMOS	Abortus-melitensis-ovis-suis
BSL2	Biosafety level 2
BSL3	Biosafety level 3
Bp	Base pair
cELISA	Competitive Enzyme Linked Immuno Sorbent Assay
CFR	Code of Federal Regulation
CFT	Complement Fixation Test
Ct	Cycle threshold
DNA	Deoxy riboneucleotide Acid
dNTPs	Deoxynucleoside triphosphate
EDTA	Ethyl Diamine Tetra Acetic Acid
FAO	Food and Agricultural Organization
HOOF	Hyper Variable Octomeric Oligonucleotide Finger-Prints
iELISA	Indirect Enzyme Linked Immuno Sorbent Assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPC	Internal positive control
IU	International unit
LPS	Lipopolysaccharide
Mb	Mega base pair
MLVA	Multi locus variable number tandem repeat
MRT	Milk ring test
OD	Optical Density
OIE	Office International des Epizooties'
Omp	Omp Outer Membrane Protein
OR	Odd Ratio

PCR	Polymerase Chain Reaction
RBPT	Rose Bengal Plate Test
RFLP	Restricted fragment length polymorphism
SAT	Standard slow agglutination tube test
SDS	Software-defined storage
S-LPS	Smooth- Lipopolysaccharides
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization

ABSTRACT

A cross sectional sero- and molecular survellience was conducted in Afar and Tigray regions of Ethiopia to determine the seroprevalence, isolation and molecular identification of *Brucella* species using Real time PCR in small ruminants and camels from November 2019 to May 2020. A total of 2523 serum samples; camel (n= 1625) and small ruminants (n=898) were collected for the seroprevalence study. In addition, 15 vaginal swab samples from animals having recent history of abortion were also collected for isolation and molecular detection of *Brucella* species. Following serum harvest, the blood clot was subjected to DNA extraction for detection of *Brucella* species using PCR. In addition, data related to risk factors of brucellosis infection were collected to assess the potential association of risk factors with seropositivity. Multi-species ELISA Kit was used to detect the presence of circulating antibodies against *Brucella* species whereas; vaginal swabs were processed and cultured on *Brucella* selective media for bacterial isolation and identification. The seroprevalence of brucellosis was determined as 12.1% (95% CI: 10.1-14.5%) and 11.5% (95% CI: 8.9-11.8) for small ruminants and camels, respectively. Multivariate regression analysis revealed that sex, age and parity have significantly associated ($p < 0.05$) with *Brucella* infection in camels (OR 0.42 (95% CI: 0.27-0.64) P=0.000**,** OR 0.64(95% CI: 0.44-0.92), *P*= 0.017and OR 0.61(95% CI: 0.43-0.86), *P*= 0.004 respectively. Where as, species, abortion history, and herd size were not statistically significant (P >0.05). Concerning small ruminants, sex, age, parity, abortion history, and herd size were not statistically significant (P >0.05). Bacterial culture result showed that *Brucella* colonies were isolated from 13.3 % (2/15) of vaginal swab samples of caprine and both isolates were identified as *Brucella melitensis* using real time PCR. Similarly 295 blood clots from seropositive animals were tested with PCR and the result showed that seven camel samples were positive for *IS711* prmer that indicates *Brucella* genus. Further analysis indicated that four of them were *B. melitensis* whereas three were not amplified with both *B. melitensis* and *B. abortus* specific primers, which indicates they may be other *Brucella* species. This study showed relatively higher prevalence of brucellosis in camel and small ruminants in Tigray and Afar Regions, signaling an urgent need for intervention to control the disease and prevent zoonotic transmission of brucellosis to the community.

Keywords: *Brucella, Camel Ethiopia, Seroprevalence, Small ruminants*

1. INTRODUCTION

Brucellosis is one of the oldest and most widespread zoonotic diseases, affecting food production in the tropics and subtropics (Bekele *et al*., 2013). The disease mainly affects the reproductive organs of animals which cause infertility, retained placenta, abortion, in animals and causing economic losses due to interrupted lactation, mortality, loss of production which are the main consequence of the disease by affecting the lively hood (Kala *et al*., 2018). The main features of the disease in sexually mature and pregnant animals include localized infection in the reproductive system which leads to placentitis and abortion. *Brucella* organisms can also cause epididymitis and orchitis in male animals (Corbel *et al*., 2006).

Since *Brucellae* are intra cellular organisms they can easily invade and infect cells which enables the organism to live on for longer time and proliferate within host cells which allows the disease to be chronic (Poester *et al*., 2013). Different species of *Brucella* affects very wide range of host for instance *B. abortus*, causes bovine brucellosis; while *B. melitensis*, is the causative agent of ovine and caprine brucellosis. Similarly *B. suis*, causes swine brucellosis. *B. abortus*, *B. melitensis* and *B. suis* can cause abortion to host animals which leads to economic losses as the result of abortion. Whereas, *B. ovis* and *B. canis* are responsible to causes epididymitis and brucellosis in ram and canine respectively (Cloeckaerta *et al*., 2001).

Human brucellosis remains the commonest zoonotic disease worldwide with more than 500000 new cases annually (Godfroid, 2017). It has been known by different names among which, Malta fever, Rock fever of Gibraltar, Cyprus or Mediterranean fever, intermittent typhoid and most frequently, undulant fever are the most widely used names (Al Dahouk *et al*., 2003). As far as the virulence is concerned, *B. melitensis* and *B. suis* can cause serious disease for humans when compared to other *Brucella* species The clinical features of the disease is manifested by sudden onset or mild febrile disease usually substantiated by fever in infected patients (Corbel *et al*., 2006). The disease also causes encephalitis, meningitis, spondylitis, arthritis, endocarditis, orchitis, and prostatitis (Megid *et al*., 2010). The zoonotic nature of the disease can be a challenge and an occupational risk for farmers, veterinarians, abattoir workers, laboratory personnel, and others who work with animals and consume their products (Karadzinska *et al*., 2010).

In Ethiopia, livestock are the major sources of livelihood for a community whose life is solely depend on keeping animals and the system of production is mainly on extensive type of farming (Tadesse, 2016). Even though, Ethiopia has the largest cattle population in Africa of ~61.51 million heads, the population of small ruminants and camel are estimated to be ~72 million and ~1.761 million heads, respectively (CSA, 2019). The benefit gained from this huge resource is hampered due to various animal diseases including brucellosis and the information generated so far towards the status of the disease are not sufficient enough to support and initiate appropriate control measures. Brucellosis is among the top three priority zoonotic diseases in the country (Pieracci *et al.,* 2016). Despite some fragmented studies, there is limited information about the national and regional status of brucellosis in livestock in the country.

However, the studies conducted have showed that animal brucellosis is an endemic and widespread disease in the country (Berhe *et al*., 2007). Most of these studies were conducted on bovine brucellosis using serological methods where limited reports are available on the status of brucellosis in camel and small ruminants. There is also information gap on the isolation of the causative agent through culture and molecular characterization of the circulating *Brucella* species. Accordingly, by considering the economic and public health importance of the disease and gaps of well documented information about the current status of the disease in camel and small ruminants, it is important to conduct comprehensive study to generate information about the current status of brucellosis in northern part of Ethiopia which can be used as an input initiate and develop the national disease control strategy.

Therefore, the general objective was to study the seroprevalence, isolation and molecular identification of *Brucella* species from camel and small ruminants in Tigray and Afar regional states.

The specific objectives of the research were;

- To determine the seroprevalence of small ruminant and camel brucellosis in the study areas
- To isolate *Brucella* species from *in vitro* cultured vaginal swabs samples
- To identify molecularly a species specific *Brucella* that circulates among the study animal population in the study areas.

1. LITERATURE REVIEW

1.1. Etiology

Brucellosis is one of the oldest and most widespread zoonotic diseases affecting food production in the tropics and subtropics (Bekele *et al*., 2013).The disease was first known a by British medical officers as Malta fever which is later called Brucellosis in 1850 during the Crimean War in Malta. In 1861, a person called Jeffery Allen has explained his own case of the disease. Later in 1887, David Bruce has established for the first time that *Brucella* organisms can cause disease. But the organism was first isolated by Danish veterinarian called Bernhard Bang In 1897 (Vassallo, 1992).

Since then, the disease continues to be major health problem to humans, domestic and other animals in many countries around the world (George *et al*., 2010). The disease was described as zoonotic after the *Brucella* isolates were demonstrated in 1905 by isolating *Brucella* from goat milk. Subsequently, *Brucella* species were isolated from aborted bovine in 1895 and *Brucella* species affecting swine were detected in 1914 while *Brucella* species affecting canine were isolated from fetuses in 1966. Similarly, *Brucella species* which causes ram epididmites was identified for the first time in 1953 (Xavier *et al*., 2009).

Brucella, organisms are gram-negative, non-spore-forming, facultative, intracellular bacteria causes Brucellosis which is one of the five common bacterial zoonoses in the world (Ko and Splitter 2003). *Brucella* pathogens have the ability to survive and multiply in professional and non-professional phagocytes, and cause abortion in domestic and wild animals including humans (Cardoso *et al*., 2006).

Taxonomists have developed a classification system that recognized six *Brucella* species by considering their host preference and antigenic differences. The species categorized in this classification includes; *B. abortus, B. melitensis, B. ovis, B. canis, B. suis* which affects porcine, and *B. neotomae* (desert wood rat) (Whatmore *et al*., 2006). *B.pinnipediae* and *B. cetacea* which affect marine mammals are recently added (Moreno *et al*., 2002). Later further classification was established based on the basis of the differences in their ability to grow on media that contain certain aniline dyes, their reactions in agglutination reaction with monospecific sera and the production of hydrogen sulphide production (Liu *et al*., 2015).

So sar, 12 species of *Brucella* are described which infects different host species although each *Brucella* species has a preference for its host species (table 1). In this context, the *Brucella* species included are; *B. melitensis*, *B. abortus*, *B. canis*, *B. ceti, B. microti, B. neotomae, B. ovis, B. pinnipedialis and B.suis* and *B. inopinata* (O'Callaghan and Whatmore, 2011; Foster *et al.,* 2007), *Brucella papionis* (Whatmore *et al*., 2014) and *Brucella vulpis* (Scholz *et al.,* 2016).

Three species of *Brucella bovis* were isolated and identified in biochemical tests from placental cotyledon and vaginal swab from Asella dairy farm (Geresu *et al*., 2016). Furthermore, (Sintayehu *et al*., 2015) has able to isolate *Brucella melitensis* in small ruminants using bacteriological and molecular diagnostic methods form studies conducted in pastoral and agro-pastoral lowlands of Ethiopia. Recently, (Tekele *et al*., 2019) has isolated eight *Brucella melitensis isolates* from afar region using molecular diagnostic method.

1.1.1. Taxonomy of Brucella

The genus *Brucella* is classified under the family *Brucellaceae* of the order Rhizobiales in the alpha class of the proteobacteria. The *Brucellaceae* includes *Mycoplana*, *Ochrobactrum*, *Pseudo chrobactrum, Paenochrobactrum* and *Crabtreella* organisms. Based on the mammalian host they favored and typical antigenic and phenotypic distinctiveness relevant to each specific species, the genus *Brucella* was classified in to six species (O'Callaghan and Whatmore, 2011).

Members of the class *Alphaproteobacteria* include families of organisms that are either mammalian or plant pathogens or symbionts. Among the organisms affecting mammals in the *Alphaproteobacteria* include the genera *Bartonella*, *Rickettsi*a and *Ehrlichia*, which requires arthopod vector for the transmission of the disease. Infection of mammalian cells is one of the features of *Brucella* that distinguish it from most genera within the order (Ficht, 2010).

According to taxonomical classification of the genus *Brucella,* it is divided into six classical species containing several biovars, on the basis of cultural, metabolic and antigenic characteristic they exhibit. *B. melitensis* which three biovars, *B. abortus* which have seven biovars, *B. suis* which have four biovars, *B. ovis*, *B. canis* and *B. neotomae* (Jumas *et al*, 1998). DNA–DNA hybridization has confirmed that the genus is more likely to be mono specific (Verger *et al*., 1985). There is disagreement among taxonomist concerning *Brucella* internal taxonomy, where some scientists have proposed that the DNA–DNA hybridization-based genomo-species concept be applied to the genus. According to this view, only *Brucella melitensis* should be considered as one species and the other species should be considers as biovars of *B.melitensis*. However, after critical review of the concept and analysis of genetic diversity by different methods, the scientists could not accept the concept of one species theory (Moreno *et al*., 2002; Cutler *et al.*, 2005).

Table 1: Currently described *Brucella* species and their zoonotic potential

Species	Biovars	Animal Host	Human Diseas	First description
B. abortus	1–9	Cattle, bison, buffalo, elk, yak, came	Yes	Schmidt, 1901; Meyer and Shaw, 1920
B. melitensis	1–3,	Sheep, goat, cow, camel	Yes	Hughes, 1893; Meyer and Shaw, 1920
	3	Nile catfish; dog		El-Tras ,et al 2010, Refai *et al* 1989
B. suis	1	Horse	Yes (biovars 1–4)	Cook and Kingston, 1988
	1, 2, 3	Pig, wild boar		Huddleson, 1929
	2	European hare		
	4	Caribou, reindeer		
	5	Rodents	Yes	
B. ovis		Ram	Not reported	Buddle,1956
B. neotomae		Rodent	Not reported	Stoenner and Lackman 1957
B. canis		Canines	Yes (rarely)	Carmichael and Bruner 1968
B. ceti		Whale, dolphin, porpoise	Yes	Foster *et al*. 2007
B. pinnipedialis		Seal	Not reported	Foster *et al*. 2007
B. microti		Common vole, red fox; (soil)	Not reported	Scholz *et al*. 2008
B. inopinata		Human	Yes	Scholz *et al*. 2010
Baboon isolate		Baboon	Not reported	Schlabritz-Loutsevitch *et al*. 2009
BO2		Unknown	Yes	Tiller *et al*.2010
Australian rodent strains		Rodent	Unknown	Tiller *et al*.2010

Source: Sprague *et al*., 2012. BO2 unusual *Brucella* strain

1.1.2. Brucella genome

Different researches have demonstrated that the genomes of the α-proteobacteria vary in both size from 1 to 9 Mb and organization from single circular replicons to multiple replicons, both circular and linear. Where, the genome of the common ancestor of the α-proteobacteria was determined to contain 3,000–5,000 genes of the organisms (Batut *et al.,* 2004). There is much similarity in the genome of pathogenic *Brucella* species in sequence, organization, and structure. The whole genome sequence of *B. abortus* biovar has been analyzed and it showed that the size of the genome is 3.3 Mb. *B. abortus* biovar and has composed of two chromosomes (Verger *et al*., 2000).

It is suggested that *Brucella* species having large genome size has favored them to exist in different environments that may well adapt to a number of different hosts. The differences between host species may reflect differences in cell surface structures and optimal growth conditions, as well as specialized mechanisms for uptake and intracellular growth of mammalian pathogens (Ficht, 2010).

Analysis of the genome size determination of *B. melitensis* has indicated that the species contains 3.29 Mb which is found in two circular chromosomes of 2.12 and 1.18 Mb with 57% GC content and 3,197 predicted ORFs distributed on the two chromosomes (DelVecchio *et al*., 2002). The strains of *B. suis* biovars have variable in chromosome number and size. Biovar 3 for instance has one chromosome with the size of 3.3-Mb where as biovar 1, 2, and 4 have 2 smaller size or chromosomes. This could be due to recombination events involving the three rRNA operons. When compared with other similar microorganisms, *B. melitensis* and *B. suis* genomics has showed that there are few specific open reading frames (Paulsen *et al.,* 2002).

1.1.3. Morphology

The morphology of strains of *B. abortus*, *B. suis, B. melitensis and B. neotomae* are usually in the smooth form when first isolated. Colonies of rough morphology occur in each of these species on subculture. *B. ovis and B. canis* are always in the rough form. Morphologically the colonies are characterized as dull, yellowish and opaque and when touched with an inoculation loop are found to be friable (Quinn *et al.,* 2004).

In general, the characteristics of the *Brucella* species can be demonstrated as small non-motile gram-negative cocci, coccobacilli or short rods with straight or slightly convex sides and rounded ends 0.5-0.7 µm wide by 0.6-1.5µm long. Mostly they are arranged singly, but pairs or short chains or small clusters can be found. *Brucella* organisms do not produce capsules, spores or flagella and do not have bipolar staining characteristics (Corbel and Morgan 1982).

1.1.4. Biochemical characteristics

Bichemical characteristics test performed to identify *Brucella* species have showed that they are catalase, oxidase and urease positive with the exception of *B. ovis*. Other Biochemical reactions include CO_2 need for growth, H_2S-production, sensitivity against the stains fuchsine and thionine, sensitivity against phages, and agglutination with polyvalent as well as specific antisera (Geresu *et al*., 2016).

Brucella organisms do not fix atmospheric nitrogen, but they can oxidize nitrogen compounds by encoding enzymes such as copper containing nitrate reductase, a nitrous oxide–nitric oxide reductase complex, and nitrous oxide reductase. A nitrite extrusion protein is present that transports nitrite from the cytosol to the periplasm where nitrite reeducates is found. This characteristic allows *B. melitensis* to grow easily under anaerobic conditions by respiration of nitrate (DelVecchio *et al*., 2002).

1.1.5. Growth and cultural requirements for Brucella species

Brucella species can show growth at 35-37°C and 5-10% CO_2 after 48-72 hours of incubation on blood-containing agars. Since *Brucella* organisms are slow growing in culture, incubation time should be prolonged up to three weeks before the culture is concluded negative. Young smooth (S) and older rough (R) colony can be demonstrated in the culture at the same time which resembles the culture is mixed (Schwarz *et al*., 2015). There is a range of commercially available culture media for growing *Brucella*. The most common basal media in use are: Triptcase soy, Bactotryptose, Triptic soy, and Tryptone soya. The powder media can be used to prepare either broth or agar medium. Most *Brucella* strains, particularly *B. abortus* biovar 2 and *B. ovis*, grow better in media containing 5-10% of sterile (equine or bovine) serum free from *Brucella* antibodies (Fernando *et al*., 2010).

2.2 Epidemiology

2.2.1. Occurrences

Brucellosis is the disease of livestock and humans which is widely distributed in the world. The disease is commonly distributed in the Middle East, Asia, Africa, South and Central America, the Mediterranean basin and the Caribbean which causes animal and human brucellosis (Refai *et al*., 2002). *Brucella melitensis* is particularly common in the Mediterranean basin and it has also been reported from Africa, India and Mexico. Most affected are the Eastern Mediterranean basin, the Middle East, the Arabian Peninsula, Mexico, Central and South America, Central Asia and the Indian subcontinent **(**George *et al*., 2010).

In Ethiopia the disease has been noted as one of the important livestock diseases in the country since 1970 (Domenech, 1977). Different articles published so far have indicated that brucellosis seroprevalence of 1.1% to 22.6% in intensive management systems and 0.1–15.2% in extensive management system has been reported (Asmare *et al*., 2013). However, in this review seroprevalence of 0.7% to 4.9% was recorded in cattle (Bashitu *et al*., 2015; Alehegne *et al*., 2016). Whereas, 1.9 to 16.7 % of seroprevalence was reported in small ruminants by Sintayehu *et al*. (2015); Gebremedhin, 2015) and 1.5% to 13% of seropositivity in camels were recorded by Bekele *et al***.** (2011; Tegegn *et al*., 2016).

2.2.2 Status of Brucellosis in Ethiopia

A lot of work has been done to determine the prevalence of brucellosis in cattle. Reviewed reports indicated that the seroprevalence of bovine brucellosis is found higher in intensive farms where 50% seropositivity was reported in Didituyura ranch (Yohannes *et al*., 2013, Yilma *et al*., 2016). The seroprevalence of Brucellosis in Ethiopia is indicated in table 2-4.

Table 2: Seroprevalence of Bovine brucellosis

Study area	Seroprevalence%			Reference
	RBPT	**CFT**	**ELISA**	
Eastern Ethiopia	1.3	1.3	-	Terefe *et al*., 2017
Afar and Oromia	-	-	4.8	Tschopp *et al* 2015.
Eastern Ethiopia	3.5	2.8	-	Megersa1 *et al*, 2011
Borena	8	-	-	Megersa1 *et al*, 2012
Jimma zone	1.4	-	-	Gebremichael *et al*, 2015
Alage	-	2.4	2.43	Asgedom *et al*., 2016
Debrebirhan	0.7	0.2	-	Bashitu *et al*., 2015
Exotic and cross bred cattle in Ethiopia	1.98			Asmare *et al*., 2013
western Ethiopia	1	-	0.95	Adugna *et al*., 2013
Gondar	5.4	4.9	-	Alehegne *et al*., 2016
Addis Ababa dairy Cattle	2.77	-	0.06	Edao *et al*., 2018

Table 3: Seroprevalence of camel brucellosis

Study area	Sero prevalence%			Reference
	RBPT	**CFT**	**ELISA**	
Akaki Abattoir	6.5	4.5	-	Abebe *et al*., 2017
Afar region	6.1	5.4	-	Bekele *et al*., 2013
Yabello District	3.6	3.1	-	Admasu and Kaynata.,2017
Afar region	12.2	4.1	-	Gizaw *et al*., 2017
Mehoni	6.33	3.67	-	Habtamu and Kassahun., 2014
Afar region	5.0	4.1	-	Hadush *et al*., 2013
Dire Dawa	6.1	5.4	-	Muhammed *et al*.,2011
Fafen Zone	2.3	1.53	-	(Robayo & Esubalew., 2017
Jijiga and Baile districts	3.41	2.43	-	Tilahun *et al* 2013
Dire Dawa	2	1.5	-	Warsame *et al*., 2012

Table 4 : Seroprevalence of small ruminant

Study area	Animal species	Sero prevalence%			Reference
		RBPT	CFT	ELISA	
Pastoral regions of Ethiopia	Ovine	1.9	1.9	-	Sintayehu *et al*.,2015
	Caprine	3.3	3.3	-	
Pastoral regions of Ethiopia	Ovine Caprine	1.9	-	9.7	Teshale *et al*., 2006
Ada'a-Liben, Ambo and Fentale districts	Ovine	16.7	3.57	-	Gebremedhin., 2015
Debre Ziet and Modjo	Ovine	1.99	1.76	-	Tsegay *et al*., 2015
Southern Zone of Tigray Region	Ovine Caprine	-	3.5	-	Teklue *et al*.,2013
Afar region	Ovine	8.3	7.1	-	Adugna *et al*.,2013
	Caprine	15	13.6	-	
Mirab Abaya	Caprine	6.7	-	-	Dulo, 2017
Jigjiga District	Ovine	1.5	-	-	Bekele *et al*., 2011
	Caprine		-	-	
Chifra and Ewa districts	Ovine Caprine	13%	-	-	Tegegn *et al*., 2016
Southeast Ethiopia	Caprine	9.6		-	Gumi *et al*., 2013
Oromia and Somali regional states	Ovine Caprine	8.5	3.63	-	Tsehay *et al*., 2014.

Human brucellosis

Brucellosis continues to be an important zoonosis and economical significant disease in the world. *Brucella* organisms affect human population and the disease is distributed in many developing countries including the Middle East, and Latin America where it is still endemic (Alton G. 1991). It also exists throughout sub-Saharan Africa; Most African countries where their lively hood is associated with livestock are living with and by their animal resources. The poor socioeconomic status and the limitation of intervention through surveillance and vaccination programs make the disease difficult to control (Pappas *et al.,* 2006).

Human brucellosis can cause serious complications and involvement of internal organs. The symptoms exhibited can vary depending on the site of infection. Encephalitis, meningitis, spondylitis, arthritis, endocarditis, orchitis, and prostatitis are the most clinical signs observed in infection of human brucellosis (Mohamed *et al.,* 2010). The disease has also variable incubation period which can lasts 1–4 weeks. The productions of urease by the *Brucella* organisms enable the pathogen to escape phagosome–lysosome fusion and survive within polymorpho nuclear and mononuclear phagocytes. The pathogen is then can enter into the circulation via the lymph nodes to seed different organs and body systems, ultimately manifesting the varied clinical signs and symptoms (George, 2010).

Various studies have been carried to determine the serprevalence of brucellosis in humans. Accordingly, Workalemahu *et al*. (2016) has reported 10.6% seroprevalence of human brucellosis infection in Southern part Ethiopia where possession of domestic ruminant animals, contact with ruminant animals, and husbandry practices at home were identified as associated risk factors with seropositivity. Seroprevalence of 12.7% and 4.4% by RBPT and CFT respectively was reported from human in Afar region (Zerfu *et al*., 2018).Similarly in another study conducted in Afar region has showed that the overall seroprevalence of human brucellosis was determined 16% by RBPT and 15% by CFT (Sisay and Mekonnen, 2012)..

2.2.3 Risk factors

Various authors reported different risk factors associated with *Brucella* infections in animals and human. Cattle susceptibility to *B. abortus* infection is influenced by age, sex, breed and reproductive status of the individual animal. The association of these factors with the infection has also been studied in different parts of Ethiopia (Yohannes *et al.,* 2013). Herd level analysis of the risk factors revealed that large and medium herds as well as herds kept with multiple livestock species were at higher risk of acquiring *Brucella* infection (Megersa *et al*., 2011). The possible risk factors for human brucellosis are also identified as feeding behavior, occupational exposure, contact with diseased animals or their products and discharges (Addis, 2015).

The significance of Risk factors associated in *Brucella* infections were determined by different authors. Accordingly, (Terefe *et al*., 2017) reported large herd size, intensively managed herds, experience of dairy handlers, pregnancy, and having brucellosis exposure have significant influence in infection of bovine brucellosis. In another study conducted, sex, age parity, and reproductive status of animals have significance effect in bovine brucellosis (Asgedom *et al*., 2016; Alehegne *et al*., 2016). Furthermore, sex, age, species, parity, body condition, and flock size are identified and reported to have significance influence in small ruminant brucellosis (Adugna *et al*., 2013; Sintayehu *et al*., 2015; Tegegn *et al*., 2016). According According to Tassew and Kassahun, (2014); Abebe *et al*. (2017); Gizaw *et al*. (2017) report, age, history of abortion, parity number, body score condition, herd size, and contact with other ruminants are identified as having significance influence in camel brucellosis infection.

In contrast to previous reports, study conducted around Debrebirhan area indicated that sex does not have significant influence in infection of bovine Brucellosis (Bashitu *et al*., 2015). Teshale *et al*. (2006) has also reported that sex does not have significance importance in Ovine brucellosis. According to Gebremedhin, (2015), district, history of still birth, and neonatal losses do not have significant effect in Ovine Brucellosis as well. Moreover, Dulo, (2017); Bekele *et al***.** (2011); Tsehay *et al*. (2014) have reported that age, sex, species, and herd size do not have significance effect in caprine brucellosis. In addition, studies conducted in Camels have also showed that, District, sex, age, herd size, camel rearing experience, parity, and contact with other ruminants do not have significant influence in the infection of brucellosis (Tilahun *et al.,* 2013; Warsame *et al*., 2012; Petros and Geremu, 2017).

Infection of brucellosis in cattle, sheep, goats and swine is high in sexually mature animals. Young animals are often resistant although it should be noted that latent infection can occur and such animals may present a hazard when mature (Corbel *et al*., 2006). According to WHO report, the opinion of the reviewer on the association of risk factors towards *Brucella* infection is that, since the disease is characterized and associated with abortion, still birth, sterility, a longer calving interval and lower milk yield, sex and age would have significance influence where mature females are showing clinical symptoms of brucellosis when compare to young and male animals although it needs further investigation.

2.3 Socio -economic importance of Brucellosis

2.3.1 Public health significance

Brucellosis is an old disease which causes low mortality in human. Human brucellosis remains the commonest zoonotic disease worldwide with more than 500 000 new cases annually (Pappas *et al.,* 2006). Brucellosis in humans is characterized by multi systemic, acute to chronic disease and the symptoms include fever, headache, joint pains, musculo-skeletal pains, sweating, malaise and body wasting. Because of having various symptoms, it is very difficult to diagnose brucellosis in human case. In Africa, because of malaria is highly prevalent, brucellosis is usually misdiagnosed as malaria (McDermott and Arimi 2002).

The outcome of human brucellosis is not only on the disease it causes but the consequence it results is considerably very high health care costs when compared to the average patient looking for medical care. Usually the cost of expenses will be covered by the patient or his families depending on the health care system of the country Medications, diagnostic procedures, and laboratory tests are among the higher costs of patients should pay after diagnosis of brucellosis (Vered *et al*., 2015).

2.3.2 Economic importance

Brucellosis is one of economically important animal diseases in the world. The production losses which comes from stillbirth, sterility, a longer calving interval and lower milk yields affect the economy of people whose subsistence are depend on livestock. At the same time, the economy of countries practicing intensive livestock farming can also be affected due the trade barrier nature of the disease (Akakpo *et al.,* 2010). The outcome of brucellosis is not only on due to the morbidity or mortality of animals but it is more due to reduce of production. Brucellosis causes an estimated economic loss of 377.93 million USD per annum in cattle despite the perceived low prevalence. The challenge is more sever to people who practicing mixed crop livestock and urban/peri-urban production systems than people who are practicing other production systems (FAO, 2018).

Studies on the assessment of economical impacts of brucellosis with emphasis on the low-income countries of Africa and Asia, is structured in three main approaches. The first approach describes an overall framework for economic assessment of disease burdens and the impacts of potential control programs. Whereas, systematically reviews available animal, human and joint burden estimates from studies conducted in these regions will be the second approach. Finally the third approach provides estimates, when available, of different costs associated with brucellosis illness and its control (Pal *et al*., 2017).

The impact of brucellosis is considerably significant in sub-Saharan Africa because it affects multiple animal species and humans. However it was found difficult to evaluate the economical impacts. The major direct losses are on reproduction abortion and impaired fertility and thus also milk production. Since the economic loss from brucellosis is due to morbidity, mortality and treatment costs, the impacts on livestock species can be estimated (McDermott and Arimi 2002).

Usually, contact following an abortion is one of the main means of transmission of *Brucella* organisms from animal to animal in cattle and other bovidae animals. With regard to human brucellosis transmission from one person to the other can be effected through contaminated environment, professional exposure usually occurring from direct contact with infected animals, and food borne transmission are possible means of acquiring of brucellosis (Corbel *et al*., 2006). Humans are almost exclusively exposed to brucellosis through contact with animals and food of animal origin or transmitted via human contact with secretions, predominantly through calving and abortions (Pal *et al*., 2017).

Although passive venereal transmission via the ewe appears to be the most frequent route of infection, but ram-to-ram transmission is also very common. Animals can acquire infection simply through the ingestion of contaminated feed, through respiratory system, or conjunctival way. But drinking of unpasteurized milk products can be the main source of infection for community where the disease is endemic and consumption of raw milk is practiced (OIE, 2018).

2.3.3 Clinical manifestation

Clinical manifestation in animals include chronic infection, replicating preferentially within chorioallantoic trophoblast of the placenta which results placentitis, fetal death and abortion are the major clinical signs observed in pregnant cows. Whereas infected bulls may develop systemic sign of infection. Orchitis associated with seminal vesiculitis and epididymitisis is the most significant lesion produced by *B.abortus* (Poester *et al*., 2013). *Brucella melitensis* infection can causes abortion, reduced milk yield, and orchitis in small ruminants (Xavier *et al*., 2009).

Brucella ovis infects sheep causing genital lesions and infertility in rams, placentitis, abortions and infertility in ewes, and increased prenatal mortality in lambs. *Brucella ovis* is usually excreted in semen in infected rams (OIE 2018). Though many infected herds may have no signs, manifestations of swine brucellosis are abortion, birth of weak piglets, infertility, orchitis, epididymitis, spondylitis of especially the lumbar and sacral regions, arthritis, paralysis of hind limbs, and lameness, but. Abscesses of different sizes frequently occur in organs and tissues (Megid *et al*., 2010).

Manifestations of swine brucellosis are abortion, birth of weak piglets, infertility, orchitis, epididymitis, spondylitis of especially the lumbar and sacral regions, arthritis, paralysis of hind limbs, and lameness, but many infected herds may have no signs. There is no pyrexia, and death is rare. Abscesses of different sizes frequently occur in organs and tissues (Megid *et al*., 2010).

Figure 1: Left: unilateral testicular enlargement due to *Brucella suis* infection in Boar. Right: Bubalin aborted fetus due to *Brucella abortus* biovar infection. Source: (Megid *et al*., 2010).

2.3.4 Virulence

According to several studies conducted, outer membrane of *Brucella* organisms is considered as the main components of virulence factors. The outer membrane contains Lipopolysaccharide (LPS), which is the major virulence factor of *Brucella*. It possesses a peculiar non-classical LPS as compared to the classical LPS from Enterobacteria, such as *Escherichia* (Christopher *et al*., 2010). Rough strains containing less or no O polysaccharide (OPS) are less virulent than smooth strains and less resistant to complement attack even though virulent strains of *B. ovis* and *B. canis* are rough strains (Ko and Splitter, 2003). Even though the *brucella* pathogen has the ability to replicate in a wide variety of mammalian cell types, *they* exhibit strong tissue tropism and multiply within the vacuoles of macrophages, dendritic cells (DCs), and placental trophoblasts (Figueiredo *et al*., 2015). *Brucella* species do not have typical bacterial virulence factors like flagella, fimbriae, exotoxins, cytolysins, and other virulence factors which are responsible for virulence. However, LPS plays an important function in *Brucella* virulence because it prevents complement-mediated bacterial killing and give resistance against antimicrobial peptides (Poester *et al*., 2013).

2.4 Immunity of Brucellosis

2.4.1 Humoral immunity

Regading the humoral immunity of brucellosis, Although the humoral immune response of T helper cell type 2 efficiency is not clearly understood, passive transfer experiments conducted suggests that antibodies produces against LPS (O-polysaccharide) may give to defense mechanism to be effective . The effectiveness of rough *Brucella* vaccines currently in use challenges the responsibility of anti-LPS antibodies in defensive immunity against infection (Figueiredo *et al*., 2015). The Functions of the adaptive immune response in brucellosis can be classified into three main mechanisms. The first mechanism production IFN by CD4, CD8, and T cells which activates the bactericidal function in macrophages to hamper the intracellular survival of *Brucella*. Then cytotoxicity of CD8 and T cells kills the infected macrophages. Finally, Th1-type antibody isotypes such as IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis (Ko and Splitter 2003).

2.4.2 Cellular immunity

Brucellosis is one of the zoonotic diseases which can affect humans. The pathogenic strain of *B. abortus*, *B. suis*, *B. melitensis* which infects humans can carry a smooth LPS which are involved in the virulence of these bacteria. The LPS O-chain is a key molecule for *Brucella* survival and replication in the host by protecting the bacteria from cellular cationic peptides, oxygen metabolites and complement-mediated lysis (Cardoso *et al*., 2006).The intracellular nature of *Brucella* organisms makes it difficult for these bacteria to be completely eliminated by the host cellular responses or be eradicated by antimicrobial drugs (Elfaki *et al*., 2015).

The production of T helper type 1 (Th1) and cytokines through the introduction of *brucella* antigen is one of the mechanism of removing *Brucella* organisms from the body of infected animals (Skendros and Boura 2013). The involvement of other cellular populations such as NK cells and non-conventional T-lymphocytes, like Vg9Vd2 T cells and CD4þ invariant NKT cells (iNKT), have contribution to cellular immunity against *Brucella* (Skendros *et al*., 2011).

2.5 Diagnostic methods

Safety considerations: since all *Brucellae* are currently classified as biosafety group three microorganisms, appropriate biosafety measures should be followed when handing materials contaminated with the pathogen despite the virulence for humans and the means of transmission is not the same for all classical species (Moreno *et al*., 2002). Care should always be taken when handling blood samples and biopsy material for either serological or bacteriological diagnosis because these biological samples could contain *Brucella*e organisms in sufficient numbers to present a significant risk to personnel handling them. Biosafety level 2 laboratory should always be used to handle the organism (Corbel *et al*., 2006). Laboratory exposures to *Brucella* species is a common practice in a public health laboratories. Therefore, system should be implemented to minimize exposure and infection prevention solutions which protect against other laboratory acquired infections (Traxler *et al*., 2013). The attack rate has been reported to be 30%–100%, depending on the inoculums involved, the physical location of the workers, and the source at the moment of the exposure. Because aerosolization is the primary mechanism of transmission in this setting, it is recommended that the organism be handled according to level 3 biosafety precautions (Robichaud *et al*., 2004).

2.5.1 Bacteriological method

The method involves growing of bacterial isolate in artificial media. It requires facilities and competent personnel. Isolation of the organism is considered as the gold standard diagnostic method for brucellosis since it is specific and allows biotyping of the isolate which is relevant under an epidemiological point of view (Juliana *et al*., 2012). It is possible to isolate pathogens from different organs and tissues. The most common samples used for the isolation of *Brucella* pathogens include bone marrow, cerebrospinal fluid, synovial fluid, abscess material, spleen, and liver tissue or from consumables like milk, cheese or raw meat (Schwarz *et al*., 2015). There are wide ranges of commercially available culture media for growing *Brucella* pathogen*s.* The most common used basal media include: Triptcase soy, Bactotryptose, Triptic soy, and Tryptone soya. Broth or agar medium can be prepared from commercially available powder media for isolation purpose.

Most *Brucella* strains, particularly *B. abortus* biovar 2 and *B. ovis*, require media containing 5-10% of sterile equine or bovine serum free from *Brucella* antibodies to sustain growth of bacterial isolates (Fernando *et al*., 2010). Farrell medium which contains antibiotics that does not allows the growth of other bacteria is most commonly used medium to isolate the *brucella* organisms from suspected samples (Godfroid *et al*., 2010; Minda *et al*., 2016). Modified Farrell's medium can also be used for primary isolation of *Brucellae* which contains serum dextrose agar with 5% horse serum and 1% dextrose adding six different antibiotics to one Liter medium (Shahzad *et al*., 2015).

Blood sample can also be cultured for the isolation of *Brucella* organisms from clinical samples using tryptose broth supplemented with 5% of bovine fetal serum. *Brucella* isolates can also grow well in blood agar plates containing 5% (v / v) defibrinated sheep blood (Ilhan *et al*., 2008; Batinga *et al*. 2018). *Brucella* species show optimal cultural growth at 35-37°C and 5-10% CO2 (Schwarz *et al*., 2015). With regard to the requirement of CO_2, *Brucella* species like some wild type *B. abortus* strains require availability of CO_2 environment for their optimum growth, while other wild type *B. abortus* strains, *B. abortus B.melitensis*, and *B. suis,* do not require CO_2 at all for replication (Godfroid *et al*., 2010).

2.5.2 Serological diagnostic methods

Currently a lot of serological tests are developed and applied to detect the presence of circulating antibodies against *Brucella* infection in animals and humans. Traditional and well documented techniques for serological diagnosis include the Rose Bengal plate agglutination test (RBPT), serum agglutination test, and more recently, the indirect ELISA and competitive ELISA being put into more regular use (McGiven *et al*., 2003). Among the many serological diagnostic tests, most commonly used ones are selected and indicated in this review.

Rose Bengal Plate Test (RBPT)

RBPT is one of the common rapid screening diagnostic tests of brucellosis which is widely used method for detection of antibodies. The test is simple spot agglutination technique which does not require additional equipment. The principle is based on the use of suspension of *Brucella* antigen and Rose Bengal dye, buffered to pH 3.65. At neutral pH, this test can measure the presence of IgM, IgG1 and IgG2 (Kaltungo *et al*., 2014). Since the test is simple and easy to carry out, it can be used in laboratories or at field level to screen sera for antibodies to *Brucella.* The test may yield false negative results, although rarely in infected cattle that give positive results with the CFT (Cadmusa *et al*., 2008).

***Complement fixation test* (*CFT*)**

Complement fixation test is commonly used in many microbiology laboratories as confirmatory test following screening test for *Brucella* infections. In order to conduct the test, it needs a number of reagents and technically knowledgeable laboratory personnel. The test proper includes of *brucella* organisms whole cell antigen incubated with heat inactivated serum which allows destroying the indigenous complement and a titrated source of complement (Nielsen *et al*., 2010). The principle of the test procedure however is based on the recognition of antibodies raised against *Brucella* antigen that is able to activate complement. The immunoglobulins (Ig) that are responsible for activation of bovine complement are IgG and the IgM. Even though the sensitivity of the test is not high, it has shown an excellent specificity (Godfroid *et al*., 2010).

Indirect enzyme-linked immunosorbentassay (iELISA)

iELISA tests has been acknowledged and practiced for its better performance in *Brucella* diagnosis when compared to other serological diagnostic methods. This test is widely practiced and acceptable test in many microbiological laboratories. One of the reasons is believed to be the sensitivity of the primary binding assays which is higher for iELISA rather than RBPT and CFT (Tabasi., 2019). Most iELISAs makes use of purified *brucella* antigen but different anti-bovine Ig conjugate to detect the antigen-antibody complex while conducting the test. Even though the sensitivity of iELISA is high, the test is prone to to non-specific reactions, especially *Yersinia enterocolitica* strain (YO9) infection can cross react with the test. The problem of cross reactivity leads the development of cELISAs (Godfroid *et al*., 2010).

Competitive enzyme-linked immunosorbent assay (cELISA)

Competitive enzyme linked immunosorbent assay detects the presence of circulating *Brucella* antibodies by using monoclonal antibodies directed against specific epitopes of the *Brucella* LPS, the development of more specific cELISAs has been possible. These tests are more specific, but less sensitive, than iELISAs. This test is the selected and prescribed tests for trade by OIE (Godfroid *et al*., 2010). The specificity of multispecies cELISA assay for the detection of antibody against *Brucella* in serum allows differentiating vaccinal strains from antibodies elicited by field infection in cattle (Lucero *et al*., 1999).

Fluorescence polarization assay (FPA)

The method is simple and works based on the rotational differences between a small soluble antigen molecule in solution which is labeled with a fluorochrome and the antigen molecule complexed with its antibody. A small molecule will rotate randomly at a rapid rate resulting in rapid depolarization of light while a larger complex molecule will rotate slower and depolarize light at a reduced rate. The rate change in depolarization can be measured which give the value of the tested sample (Nielsen *et al*., 2001).

The method has been used extensively for the diagnosis of *Brucella* species infection in man and several animal species, using serum, milk or whole blood in EDTA. The advantage of the method is that the test can be performed under field condition which does not need additional equipment. Sensitivity of FPA assay varies from 87.5% and 100%, and specificity from 84% to 100%, which is similar to the levels obtained with cELISA (Juliana *et al*., 2012).

Milk ring test (MRT)

The MRT is the most practical and widely used method for diagnosing brucellosis in infected dairy animals and for screening of brucellosis free herds. The method is simple agglutination test which can be used on fresh milk, but the test does not work on pasteurized or homogenized milk (Najibullah., 2014). Immunoglobulins present in the milk will be attached to fat globules through the Fc part of the antibody. If antibody to *Brucella* species is present, the antigen will bind to it, resulting in a purple band in the cream layer. If no antibody is present, the fat layer will remain a buff colour and the purple antigen was evenly distributed throughout the milk (Nielsen *et al*., 2010). The method is available cheap, simple and requires no specialized equipment. And can be easily used to detect the presence of antibodies against *Brucella* infection in milk sample. It is also very useful method to detect the presence of anti-*Brucella* immunoglobulins attached to milk fat globules (Cadmusa *et al*., 2008).

Serum Agglutination test (SAT)

The *Brucella* serum agglutination test is simple, economical and can be applicable very easily in many laboratories for the presumptive diagnosis of human brucellosis (Lopez-Merimo and Lopez-Santiago, 1989). The method been widely used and evaluated in human brucellosis with high degree of reproducibility and accuracy even though false positive and false negative SAT results have been reported (Memisha *et al*., 2002). In order to increase the specificity of the test, the addition of EDTA to SAT has demonstrated to considerably increase the test specificity by reducing the chances of cross-reactions (Kaltungo *et al*., 2014; OIE 2018).

Coombs test

The method is a rapid serological method for detecting circulating non-agglutinating *Brucella* IgG antibodies form *Brucella* infected animals (Hayrunisa *et al*., 2017). Comb test helps for the diagnosis of brucellosis in the epidemiological survey because of the advantage of detecting incomplete antibodies of the IgG types that combine with cellular antigens but do not give rise to an agglutination reaction. The test has been adapted to micro-titer plate setup to save time. Since the test could not differentia the between vaccinated and non vaccinated animals, it is not recommended for testing vaccinated animal (Kaltungo *et al*., 2014).

Immunohistochemistry

Immunohistochemistry is an alternative technique for direct diagnosis of *Brucella* species infection in tissue samples. It has been extensively used in many laboratories in studies of pathogenesis and diagnosis of brucellosis, allowing in situ localization of the organisms within *Brucella* induced lesions. An advantage of this technique is that it does not require viable bacteria or serum to detect the presence of *Brucella* pathogens and it also allows retrospective studies (Xavier *et al*., 2009a). The method is very useful diagnostic tool to detect *Brucella* pathogens in a clinical sample. Immunohistochemistry is based on the binding of antibodies to a specific Antigen in tissue sections suspected of *Brucella* pathogens. The most common immunoglobulin (Ig) used in immunohistochemistry is IgG; IgM is less commonly used (Ramos-vara 2005).

Brucellin allergic skin test

The brucellin skin test is an alternative immunological assay which can be used for screening animals which are not vaccinated against brucellosis. The method is very effective for diagnosing of diseased animals if only purified and free of S-LPS of standardized (OIE 2018). The specificity of the skin test with brucellin can provided important information for screening false reactors. The sensitivity of the brucellin skin test is comparable to other serological tests; animals which are found positive with ELISA tests are also reactive when tested by brucellin skin test (Saegerman *et al*., 1999).

2.5.3 Molecular Methods

Advanced molecular methods for the diagnosis of brucellosis are now developed and practiced in many laboratories. The application of molecular diagnostic technology in veterinary diagnostic laboratories helps to detect specific sequence *Brucella* organisms from clinical samples.

The polymerase chain reaction (PCR) has an advantage over bacterial culture procedures for the detection of *brucella* infection because of short time used for the test and the specificity and sensitivity is high. However, it requires laboratory infrastructure to establish the system (Batinga *et al*., 2018). Several PCR based methods have been developed and practiced in many microbiological laboratories. The best validated methods for the amplification of target sequences are designed to detect specific sequences of *Brucella* species such as the bcsp31 gene, the 16S-23S genes and the *IS711* insertion sequence (Godfroid *et al*., 2010).

Conventional PCR

Conventional PCR system is gel based PCR for detecting amplified specific gene of *Brucella* species which is commonly used diagnostic test in many laboratories. Currently amplification of DNA by PCR for the diagnoses of several infectious diseases is commonly practiced method. The application of this technology in previous studies has revealed that PCR can be used to detect *Brucella* DNA (Conchi *et al*., 1995). *Brucella* DNA can also be detected by using nucleic acid based amplification techniques such as PCR which can give reliable result providing a promising option (Ilhan *et al.,* 2008). As far as the sensitivity and specificity of the test is concerned convention PCR has demonstrated 100% sensitivity and 98.3% specificity by using B4 / B5 primer and amplifying a 223-bp fragment of the *bcsp31* gene compared with 70% of blood culture (Christopher *et al*., 2010).

Table 5: Primer sequence of *Brucella* species used for conventional PCR amplification

Primer type	Specificity to *IS711* of	Fragment length	Sequence
Forward	*B. abortus*, biovars 1, 2, 4	498 bp	5'-GAC-GAA-CGGAAT-TTT-TCC-AATCCC-3'
	B. melitensis	731 bp	5'-AAA-TCG-CGTCCT-TGC-TGGTCT-GA-3'
	B. ovis	976 bp	5'-CGG-GTT-CTGGCA-CCA-TCGTCG-3'
Reverse	Above listed species / Biovars	Variable / See above	5'-TGC-CGA-TCACTT-AAG-GGCCTT-CAT-3'

Source (Schwarz, *et al*., 2017).

Real time PCR

Real time PCR is a robust diagnostic test when compared with the conventional PCR. Real-time PCR is valuable technique in determining the quantification of nucleic acids in individual samples as well as in automating the data (Wang *et al*., 2014). A number of different approaches can be used to generate the fluorescence signal. Real time PCR technology follows three approaches SYBR Green I (a double-stranded DNA intercalating dye), 5′-exonuclease enzymatically released fluors with 5' exonucleic activities, and hybridization probes (fluorescence resonance energy transfer) were evaluated for use in a real-time PCR assay to detect the presence of *Brucella isolates* (Newby *et al*., 2003).

Real Time PCR allows detection and quantitative measurement of products generated during each cycle of the PCR process that are directly proportional to the amount of the template DNA before the start of the PCR process. Such chemistry requires the use of a method to detect the product formed on each cycle and of a thermocycler that is adapted to record the results obtained on each amplification cycle in a real time manner (Ericka *et al*., 2010).

Multiplex PCR

So far a number of multiplex PCR assays have been developed in microbiology laboratories for the detection and identification of *Brucella* organisms at the species level using different primers. AMOS-PCR assay was the first species-specific multiplex PCR developed and used to identify and differentiate *B. abortus* biovars 1, 2 and 4, *B. melitensis, B. ovis and B. suis* biovar 1, based on the polymorphism arising from species-specific localization of the insertion sequence *IS711* in the *Brucella* chromosome was published in 1994 (Bricker and Halling, 1994). The advantage of multiplex PCR is it is possible to use five primers to identify *Brucella* at the species level in one reaction. This will help identify mixed infections with in short period of time (Wei and Klaus 2010). The other advantages of using multiplex PCR technique are that it minimizes expense and recognizes many pathogens at once (Wang *et al*., 2014).

Tandem repeat based typing

The method is used to identify specific *Bruclla* species with high sensitivity and specificity. Experiments done on tandem repeats were identified by using the sequences of *B. suis* and *B. melitensis* genome and make use tandem repeat finder software. This program identified a large number of tandem repeats and incorporated into the HOOF-Prints assay. A selection of these were assessed for their potential value in a typing scheme by designing PCR primers flanking the tandem repeats and assessing the size diversity of resulting PCR products by agarose gel electrophoresis across a panel of *Brucella* isolates (Whatmore *et al*., 2006).

Experimental researches have showed that, multilocus variable-number tandem-repeat analysis (MLVA) was very efficient method when it was applied to collections of *Brucella* isolates from wide geographically locations (Mireille *et al*., 2008). The research done on *Mycobacterium tuberculosis* complex, *Bacillus anthracis*, and *Yersinia pestis* has proven that the method be highly appropriate for the typing of pathogenic bacteria species with a high genetic homogeneity. Recently, a family of tandem repeats located within a repeated sequence and present in multiple loci in the *Brucella* genome was used for strain typing (Philippe *et al*., 2016).

Restriction fragment length polymorphism based Approaches

The implementation of restriction fragment length polymorphism diagnostic method is very useful method to discriminate field strains from the vaccine strains which was very difficult to identify in daily field practices (Christoforidou *et al*., 2018). This technology helps to avoid potential risk of acquiring the disease when handling live *Brucella* in the laboratory where strict biosafety measures is not practiced. In order to avoid these disadvantages, method based on the PCR-RFLP shows excellent typeability, reproducibility, stability, and epidemiological concordance. The *omp2* locus contains two gene copies (named *omp2a* and *omp2b*) coding for porin proteins and has been found particularly useful for molecular typing and identification of *Brucella* at the species, biovar, or strain level (Ebtehaj *et al*., 2015). Amplified fragment length polymorphism (AFLP) is a whole-genome fingerprinting method that relies on the selective PCR amplification of restriction fragments (Whatmore *et al*., 2005).

2.6 Treatment prevention and control of brucellosis

Treating with antibiotic of known infected animals or potentially exposed to infected animals has not been commonly practiced and it should be ruled out as an option in the control of brucellosis. Different studies carried out have shown that reductions of the incidence of disease can be reduced when infected animals or flocks are treated by antibiotics. But this is not feasible and treating of infected animals is not recommended because of the uncertainty of the outcome of the treatment which is not reliable (Corbel *et al*., 2006).

Control strategies should be in place to prevent brucellosis infection in order to device strategy for the control of brucellosis, an interdisciplinary collaboration between veterinarians and public health experts is crucial. The involvement of various stake holders, government and non-governmental institutions as well as individual farmers/farmer cooperatives is important for the effectiveness of the control strategy (Tshopp *et al*., 2013). Even though mass vaccination is important for the control of brucellosis in animals it should be supported by other measures that hamper the transmission of the disease by implementing means for identification of animals and herds, and increase the participating of the communities through awareness creation procedures (Smits 2013).

If an appropriate method for control of brucellosis is not established, it can lead to animals affected by the chronic nature of the disease especially associated with carpal hygromas and infertility. Various health problems could also be associated with brucellosis which includes multiple abortions and infertility. Aborted fetuses and discharges from infected animals contain large numbers of organisms and animals with chronic infection of brucellosis can be a source transmission via milk, reproductive tract discharges and even vertically to subsequently born normal calves (McDermott and Arimi, 2002). Improvement of the food value chain through practicing awareness creation of the public and implementation of policies to reduce the risk of infection may reduce incidence of brucellosis if vaccination of animals could not be practiced (Tadesse, 2016).

2.6.1 Vaccination practice in Ethiopia

Concerning the vaccination of brucellosis, so far *B. abortus* S19 vaccine is used for the prevention of brucellosis in cattle. It is considered as referral vaccine when compared to other because of it is acceptance by many countries (Jonathan and John, 2017). There is no brucellosis vaccination practice in Africa; South Africa is possibly the country that has implemented a sustained effort based on a classical control and eradication strategy through vaccination. Although control and prevention strategy is in place, bovine brucellosis has not been eradicated and it appears that the situation is worsening with more than 250 outbreaks/year reported (Ducrotoya *et al*., 2015). Because of lack of sufficient information and data on the epidemiological status, the economical and public health importance of the disease for the implementation of appropriate control measures, there is no vaccination program implemented in Ethiopia so as to prevent and control animal brucellosis so far.

3. MATERIALS AND METHODS

3.1 Study areas

The current study was conducted in Afar and Tigray regions located in North-Eastern and Northern parts of Ethiopia respectively. From Tigray region, 2 districts (Raya-Alamata and Raya-azebo) from Southern zone, and 4 districts from Western zone (Tahtay qoraro, Asged-tsimbla, Tahtay-Adyabo, Adi daro) were included. A total of Seven districts; three districts (Dubti, Asaita and chifra) from zone 1, 3 districts (Ewa, Aura and Golina) from zone 4 and 1 district (Ab-ala) from zone 2 were covered from Afar region in this study. Zones and districts were selected based on availability of previous data on prevalence of small ruminants and camel Brucellosis at district and kebele level.

The study areas in Afar region are pastoral lowland areas located between $39^{0}34'$ and 42°28'east longitude and $8^{o}49'$ and $14^{o}30'$ north latitude with an altitude of 120m below sea level to 1500m above sea level (Figure 2). The annual rainfall ranges from 500 mm in the semi-arid western escarpments and decreasing to 150 mm in the dry zones to the east and the annual temperature ranges from $20^{0}C$ in higher altitudes to $48^{0}C$ in lower elevations. According to Agricultural sample survey of CSA in 2019, there are 14,450,150 small ruminants and 1,118,981 camel populations in the region (CSA 2019).

Whereas the study areas in Tigray region are also lowland areas located between 12°–15° N and 36° 30' – 40° 30' E with an altitude of highlands in the range of 2300-3200 m.a.s.l and lowland plains with an altitude range of 500-1500 m.a.s.l (Figure 2). The annual rainfall is about 646 mm of rain in a year. According to Agricultural sample survey of CSA in 2019, there are 6515426 small ruminants and 52,905 camel populations in the region (CSA, 2019; Hadgu *et al*., 2013).

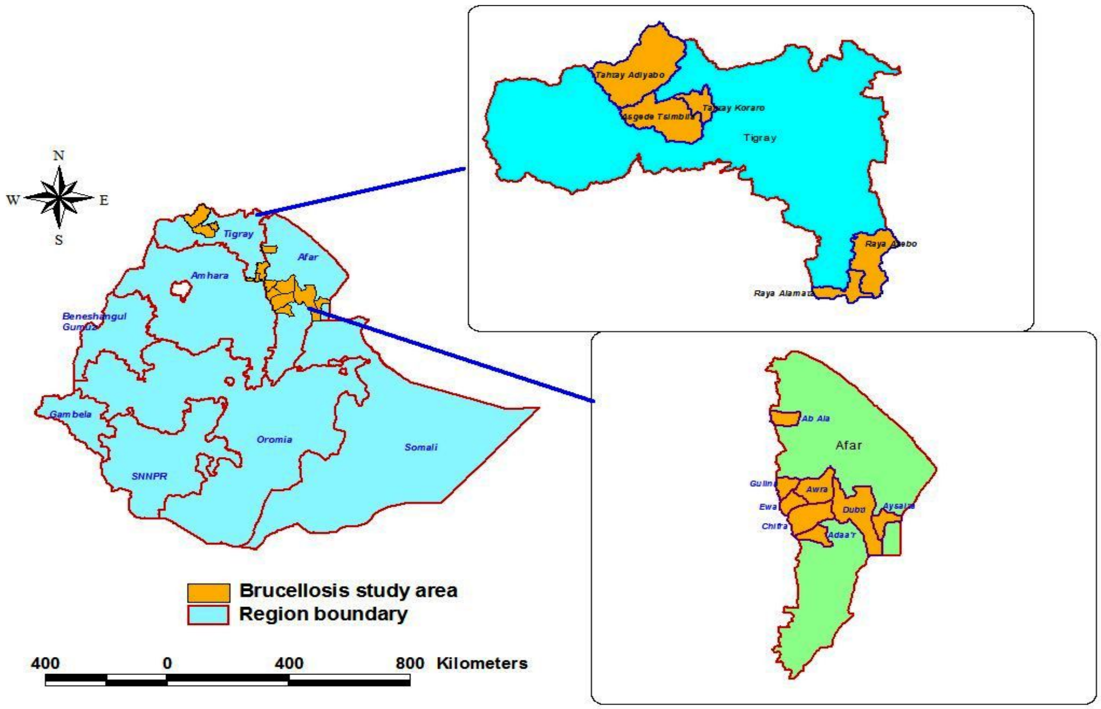

Figure 2: Map of study area

3.2 Study population and sampling strategy

The study animals were local breed small ruminants (sheep and goat) and dromedary camel managed under extensive pastoral production system and mixed crop livestock production system in Afar and Tigray regions respectively. Animals of both sex and adult and young age groups were sampled. Age of the animals was determined based on owners information and dental eruption; accordingly, animals were categorized into two age groups: > 6 months and ≤ 1 years (young), >1 years (adult) for small ruminants and > 6 months and ≤ 4 years (young), >4 years (adult) for camels (Megersa *et al*. 2011). A number of herd-level predictor variables such as herd size, were categorized as small herd size (<10), medium herd size (10-50) and large herd size (>50) according to Geresu *et al*. (2016). Below six months of age were not sampled (Belal and Ansari, 2013). Vaccination was not considered during sampling as there is no introduced brucellosis vaccine in Ethiopia so far.

Four-stage sampling method was used to collect the required sample size. Zones were selected purposively based on the previous history of brucellosis prevalence. Other administrative levels; district, kebeles and villages were randomly selected. Accordingly, a total of 7 districts from 3 zones were selected from Afar region. Similarly a total of 5 districts from 2 zones were also selected from Tigray region. Once the herd from each village was selected randomly, then all the possible individual animals from the selected herd were sampled using systematic sampling method (Ching-Ho and Kam-Wah, 1996).

3.1 Study design

A cross-sectional study was conducted in Northern and Northeastern part of Ethiopia from November 2019 to May 2020 to study seroprevalence, isolation, and molecular detection of *Brucella* species in the study areas.

3.3 Sample size

The sample size was calculated separately for small ruminants and camel brucellosis based on the previous reports of Teklue *et al*. (2013); for small ruminants and Habtamu and Kassahun. (2014) for camels in Tigray region.Where as for samall ruminants and camels in Afar region, the sample size was determined according to Adugna *et al*. (2013) and Bekele *et al*. (2013) respectively. Accordingly, the following formula was used to calculate the sample size.

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

Where: n = required sample size; Pexp = expected prevalence; d =desired absolute precision. With 5% desired precision, at 95% confidence level is considered.

To determine the number of samples in small ruminants from Tigray region, the sample size was calculated based on Teklue *et al*. (2013) who reported 3.5% seroprevalence of brucellosis in Southern Zone of Tigray region. Therefore, 104 samples were to be collected from two districts which were 52 samples from each district. How ever, to increase the precision, a total of 141 samples were collected from each identified districts and selected herds. Concerning sample collection for camels, it was collected based on Habtamu and Kassahun. (2014) report which was 6.33% seroprevalence in Mehoni area therefore based on this report, a total of 546 samples were to be sampled from all six districts which is 91 samples from each district. However, to maximize the precision, 198 sera were added to the calculated 546 samples.

Similarly, sample size determination for small ruminants from Afar region was according to Adugna *et al*. (2013) findings who reported 7% seroprevalence. Therefore, 100 samples from each identified districts and selected herds were calculated to be collected. Whereas, 88 samples were assumed to be collected from each seven districts of Afrar zone according to the report of Bekele *et al*. (2013), which is 6.1% seroprevalence. Accordingly, a total of 1628 of samples (871 from camel and 757 from small ruminants) were collected. More samples were collected than calculated in order to increase the level of precision.

In general, a total of 2523 samples were collected from all animals in the selected study areas as shown in the table 6.

Table 6 : Summary of sample size collected.

	Calculated sample size			**Collected sample size**		
Species	**Afar**	**Tigray**	**Total**	**Afar**	**Tigray**	**Total**
Small ruminant	700	175	875	**757**	141	898
Camel	616	556	1071	871	754	1625
Total	1316	630	1946	1628	895	2523

3.4 Sample collection

3.4.1 Blood Sample Collection

For serological and molecular detection, blood samples were collected aseptically from the jugular vein of individual animals. Approximately 5-7 ml of blood was collected from each study animal using sterile plain vacutainer tubes and then the serum samples were then separated from the clot. The separated serum was carefully harvested into cryovial without mixing with the clotted blood. The harvested sera and the blood clot were transported to the National Animal Health Diagnostic and Investigation Center (NAHDIC) in icebox with ice packs. The sera and blood clot was stored at -20 °C in the laboratory until processing. During the collection of blood samples, information was recorded on the attributes of individual animals such as sex, age, history of abortion, herd size and parity.

3.4.2 Bacteriological sample collection

Fifteen vaginal swab samples was collected using Stuart transport medium from animals that had a history of recent abortion at the time of sampling and transported to the bacteriology laboratory of National Animal Health Diagnostic and Investigation Center which is located at Sebeta and stored at -20 ° C until processed.

3.5 Laboratory Diagnosis

3.5.1 Serological tests

`Commercial brucellosis serum indirect multi-species ELISA Kit (BRUS-MS-5P ID Screen Brucellosis Serum Indirect, Multispecies, to detect antibodies directed against *B. abortus, B. melitensis* and *B. suis* was used for 2523 sera samples collected and the test procedure was performed as per manufacturer's instructions . OD values were measured at 450 nm and kit was verified as per kit instructions and the positive cut-off point was calculated as:

Sample positivity percentage (S/p **%)** = $\frac{^{OD}\text{sample} - ^{OD}\text{NTC mean}}{^{OD}\text{PC mean} - ^{OD}\text{NC mean}} \times 100$

Sample with a s/p%;

- Less than or equal to110% was considered negative.
- Greater than 110% and less than 120 % was considered doubtful.
- Greater than or equal to 120% was considered positive.

3.5.2 Bacteriological isolation and identification

Biosafety procedure: Proper personal protective equipment (PPE) was worn and the PPE depends on the type of specimen being sampled. Culture, staining and inactivation of bacterial isolate was performed in biosafety level 3 (BSL3) laboratory and swab extraction and Real-time PCR procedures was conducted in class II, type A2 biological safety cabinets. Additional precautions were followed during processing of samples which includes the use of powered air-purifying respirators and protective laboratory clothing. All used PPE were disposed in biohazard waste bags and then were autoclaved and all work surfaces and equipment's was decontaminated with fresh 10% bleach for 15 minutes.

Media preparation and culturing: Vaginal swabs samples were plated on to *Brucella* medium base supplemented with 5% horse serum (Oxoid, England). The plates were incubated at 37^{o}C for 48 hours for sterility check up and no bacterial colony growths were observed and considered as sterile to use for culture.

The inoculated plates were incubated at 37ºC both in the absence and presence of 5% CO2 for up to two weeks. After the incubation, the suspected colonies were examined for *Brucella* species growth. *Brucella* suspected colonies were characterized by their typical round, glistening, pinpoint and honey drop-like appearance and examined for Gram stain.

Finally, the presumptive isolates were stained using Modified Ziehl-Neelsen (MZN). CO_2 requirement and biochemical tests including catalase, oxidase and urea hydrolysis was conducted as previously described by Quinn *et al*. (2004). The isolates were then kept at -20°C until processed for PCR assay.

3.6 Detection of *Brucella* species from blood samples using PCR

3.6.1 DNA Extraction

Genomic DNA extraction from blood clot From an individual blood clot of previously known positive sample with I-ELISA, DNA was extracted using QIAMP DNA Mini Kit as follows; 1.5 gm of blood clot was taken and added to 20µl of proteinase K and 180 AL buffer and kept in 56^0C of water bath for one and half an hour vortex mixing every 30 minute for lysis of cells, after lysis 200 µl of ATL buffer was added and thoroughly mixed. After transferring the whole volume into mini spin column, two steps washing with AW1 and AW2 washing buffer was carried out. Finally DNA was eluted with AE buffer in 100 µl of volume.

Genomic DNA extraction from culture*:* DNA was extracted from solid media colonies by simple boiling method as described by Khosravi *et al*. (2006). Few colonies were taken and suspended in 500µl of sterile double distilled water in a 1.5ml micro-centrifuge tube and was kept in boiling water for 10 minutes. 5µl of supernatant was used for the PCR after centrifugation at 12000g for 3 minutes and the rest of the DNA sample was stored at -20^oC. The extraction procedure of DNA from culture using QIAMP DNA Mini Kit was the same as extraction from blood clot except instead of 1.5 gm of blood clot, 200 µl of colony suspension was used.

3.6.2 Amplification (quantitative RT-PCR)

An extracted DNA was amplified with applied biosystem 7500 PCR system using specific primers for *B.melitensis* and *B.abortus*. First, *IS711* gene (5-GCT-TGA-AGC-TTG-CGG-ACA-G-3) forward, (5'- GGC-CTA-CCGCTG-GGA-AT 3') reverse and FAM-AAG-CCA-ACA-CCC-GGC-CAT-TAT-GGT-BHQ-1 probe with internal positive control (IPC)

10x Exo IPC Mix and 50x Exo IPC DNA was used to amplify for screening *Brucella* organisms at genus level (V. Hinić *et al*., 2008).

After screening test, amplification was performed using species specific primers for *B.abortus* using (5'-GCA-CAC-TCA-CCT-TCC-ACA-ACA-A-3') forward, (5'-CCC-GTT-CTG-CAC-CAG-ACT-3') reverse and *B.melitensis* (5'-TCG-CAT-CGG-CAG-TTT-CAA-3') forward and (5'-CCA-GCT-TTT-GGC-CTT-TTC-C-3') reverse primers. The Taqman Probe sequences, FAM-CCT-CGG-CAT-GGC-CCG-CAA-BHQ-1 and FAM-TGG- AAC–GAC-CTT-TGC-AGG-CGA-GAT-C-BHQ-1 were used for detection of *B.melitensis* and *B.abortus* respectively (Hinić *et al*., 2008; Wubishet *et al*., 2018).

The master mix components were used for three specific primers in micro centrifuge tube separately for *IS711*, *B. abortus* and *B. meltensis*. The Insertion Sequence 711(*IS711*) was for screening test of *Brucella* genus where as the other two was for *Brucella* species detection. For *IS711* the master mix was consist of (20µM Forward Primer, 20µM Reverse Primer, 20µM Probe, Taq Man Universal PCR Master mix (2x), 10x Exo IPC Mix, 50x Exo IPC DNA and Water) and for species specific master mix component was (20µM Forward Primer, 20µM Reverse Primer, 20µM, Probe, Taq Man Universal PCR Master mix (2x) and RNA free water) for both *B. melitensis* and *B .abortus*. The final volume of mixture was 25 µl. (Hinić *et al*., 2008; Wubishet *et al*., 2018).

The thermal cycler run was 95^{o}c for 10 minute to denaturation double stranded DNA, then amplification/extension was set at 95^{o}c for 15 second and 60^{o}c for 1 minute for final extension. The amplification cycle was adjusted to run for 45 cycles. A positive result was indicated by fluorescence above a threshold of 0.06 according to Hinić *et al*., (2008) and Hinić *et al*., (2009) with the auto settings used for the baseline.

3.7 Data analysis

Information collected from field and data obtained from laboratory tests were coded and stored in Microsoft Office Excel spread sheet and later transferred to STATA version 12 for Windows (STATA Corp. College Station, TX, USA) for statistical analysis. Concerning data analysis of serological test, optical density (OD) of the test was measured at 450nm using Biotek ELx 800 ELISA reader (Spectrophotometer) and the data was retrieved from Gen 5 software into Microsoft excel spreadsheet and analysis was done by calculating the percent positivity of each optical density reading as per the manufactures instruction. Prevalence of seropositive animals was determined by dividing the number of i-ELISA-positive animals by the number of animals tested.

Herd level prevalence was calculated by dividing the number of herds with at least one reactor/positive in multispecies i-ELISA by the number of all herds tested. Age, sex, parity, abortion history, and herd size were considered to determine the association of potential risk factors with seroprevalence using multivariate logistic regression. The degree of association was considered significant when a P-value of less than 0.05 was obtained or when the 95% confidence intervals of the odds ratio (OR) in the multivariable logistic regression analysis doesn‘t include 1 (Thrusfield, 2007).

Culture of *Brucella* isolates and blood clots from seropositive animals were further tested by real time PCR using applied biosystem PCR platform and 7500 Fast SDS 21 CFR software for the determination and analysis of cycle threshold (Ct) values of positive reactions.

3.8 Ethical clearance

Ethical clearance was offered by Animal Research Ethics Review Committee of the Addis Ababa University College of Veterinary Medicine and Agriculture Bishoftu, Ethiopia.

4. RESULTS

4.1 Seroprevalence of brucellosis

Serum samples were collected from a total of 2523 animals (898 small ruminants and 1625 camels) from Afar and Tigray regions of Ethiopia. As a result all the collected sera sample were tested for the presence of circulating *Brucella* antibodies using I-ELISA. The overall seroprevalance of animal level brucellosis in this study was determined as 12.1% (95% CI: 10.1, 14.5) and 11.5% (95% CI: 8.9, 11.8) for small ruminants and camels respectively. Whereas the herd level prevalence was 17.8 (95% CI: 15.3, 20.3) and 15.6% (95% CI: 13.8, 17.4) for small ruminants and camel respectively (Table 7).

Table 7 : Animal and herd level seroprevalence of brucellosis %

Species	Animal level			Herd/flock level		
	Number examined	**Number Positive**	**Prevalence% (95%CI)**	**Herd tested**	**Herd Positive**	**Prevalence% (95%CI)**
Small ruminant	898	109	12.1(10.1 14.5)	45	8	17.8(15.3 20.3)
Camel	1625	186	11.5(8.9 11.8)	64	10	15.6 (13.8 17.4)
Total	2523	295	11.7(10.4 12.9	109	18	16.5(15.1 18.0)

Table 8 : Univariable logistic regression analysis of risk factors for individual animal level ELISA positives.

Species	No of animals tested	No of Positives (ELISA)	Prevalence% (95%CI)	OR (95% CI)	*p* value
Camel	1625	186	11.5(8.9 11.8)	1.06 (0.83 1.37)	0.605
Small ruminant	898	109	12.1(10.1 14.5)		

Univariable regression for determination of risk factors for small ruminants and camel brucellosis showed that species did not have statistically significant association with seropositivity. The prevalence of brucellosis in this study has showed that camels and small ruminants are equally infected by brucellosis which indicates that sharing the same grazing area and rearing together has contributed for the transmission of brucellosis between the two species.

4.1.1 Multivariable Logistic Regression Analysis of Risk Factors Associated with Brucella Seropositivity

Anima level multivariable logistic regression analysis was done for the variables: sex, age, abortion history, parity and herd size. The analysis of the logistic regression for the determination of risk factors have shown that sex, age, abortion history, parity and herd size did not have statistically significant association with *Brucella* infection of small ruminants (Table 9). However, sex, age, and parity have significant association with *Brucella* seropositivity in camels. OR 0.42 (95% CI: 0.27, 0.64) *P* value =0.000, OR 0.64(95% CI: 0.44, 0.92) *P* value = 0.017, OR 0.61(95% CI: 0.43 0.86) *P* value =0.004 respectively whereas, abortion history, and herd size were not significantly associated (p>0.05) (Table 10).

Table 9: Multivariable logistic regression analysis of risk factors for ELISA positive small ruminants

Factors	**Category**	**No of animals tested**	**No of Positives (ELISA)**	**Prevalence % (95%CI)**	**OR (95% CI)**	***p* value**
Sex	Male	47	1	2.1(1.65 2.61)	0.16 (0.02, 1.12)	0.075
	Female	851	108	12.7(11.6 13.8)	1	
Age class	Young	98	6	6.1(5.32, 6.92)	0.47(0.20, 1.11)	
	Adult	800	103	12.9 (11.8 14.0)	1	0.087
	Primiparous	93	9	9.7(9.68 11.8)	1	
Parity	Multiparous	595	72	12.1(11.0, 13.2)	0.78(0.37, 1.64)	0.617
	Null	210	28	13.3 (12.2, 14.5)	1.14(0.71 1.83)	
Abortion	No	878	106	12.1(10.1 13.2)	1	0.587
	Yes	20	3	15 (13.8, 16.2)	1.4(0.39, 4.9)	
Herd size	Small	127	9	7.0(6.1, 8.1)	1	0.746
	Medium	389	49	12.6(11.3 , 13.9)	0.93(0.61, .1.42)	
	Large	382	51	13.4 (12, 14.7)	0.49 (0.24, 1.04)	

Table 10: Multivariable logistic regression analysis of risk factors for ELISA positives camels

Factors	**Category**	**No of animals tested**	**No of Positives (ELISA)**	**Prevalence % (95%CI)**	**OR (95% CI)**	***p* value**
Sex	Male	421	27	6.4.(5.8 7.0)	0.42 (0.27,0.64)	0.000
	Female	1204	159	13.2(12.8 14.1)	1	
Age class	Young (≤ 4yrs)	447	41	9.2(8.5, 9.9)	0.64(0.44, 0.92)	0.017
	Adult (> 4yrs)	1178	145	12.3(11.5 13.1)	1	
	Null	807	65	8.1(7.4, 8.7)	1	
Parity	Multiparous	718	97	13.5 (12.6, 14.4)	0.61(0.43 0.86)	0.004
	Primiparous	100	24	24 (22.3 25.0)	2 (1.2, 3.31)	
Abortion	No	1614	186	11.5(10.6 12.2)	1	0.648
	Yes	11	0	0		
	Small	72	3	4.2(3.4, 4.9)	1	
Herd size	Medium	1015	136	13.4(11.5, 13.1)	1.3(0.9,1.89)	
	Large	538	47	8.7(5.9.7.1)	0.41 (0.12,1.37)	0.150

4.2 Isolation of *Brucella* species

In the present study, fifteen vaginal swabs were collected and cultured for isolation of *Brucella* species. As the result, *Brucella* species were isolated from 13.3% (2/15) of the samples. Growth of colony was observed under both aerobic and 5% CO_2 incubation and the colonies were characterized as, round, smooth and translucent colonies on culture media (Figure 3a).

Microscopic examination of Gram stained smear from the colonies indicated that small Gram negative coccobacilli arranged individually and in pairs were observed while with Modified Ziehl-Neelsen (MZN) stain, the organisms of *Brucella* was appeared stained red on a white background (Figure 3b). Concerning biochemical reaction, the suspected colonies showed hydrolysis of urea (Figure 4c). No growth was observed on MacConkey agar and the colonies were non hemolytic on blood agar (Figure 4d).

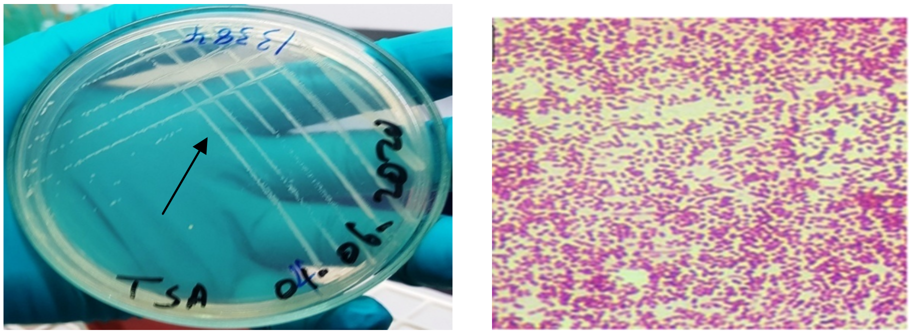

Figure 3: (A) Colonies of *Brucella* isolates on tryptic soy agar (TSA) agar and (B) Modified Zeihl Neelesen stain of *Brucella* species (Red coccobacilli aginst white back ground).

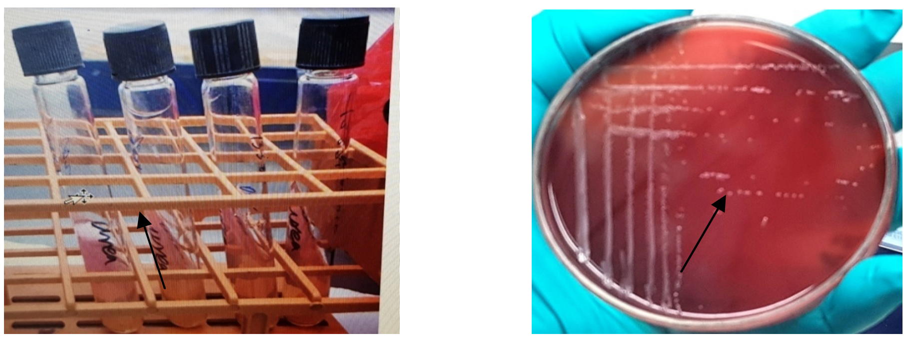

Figure 4 : (C) Hydrolysis of urea by *Brucella* isolates and (D) Non hemolytic colonies of *Brucella* isolates on blood agar.

Table 11: Summary of test results of brucellosis

Animal species	iELISA	Culture	PCR
Small ruminant	12.1% (109/898)	13.3 (2/15)	100% (2/2)
Camel	11.5% (186/1625)	-	2.4% (7/295)
Total	11.7% (295/2523)	13.3 (2/15)	3% (9/297)

4.3 Molecular detection

A total of two hundred ninety five blood clots from seropositive animals and two suspected bacterial colonies from vaginal swab samples of caprine species were tested with real-time PCR for the detection of *Brucella* species.

Intially, two *Brucella* colonies extracted by boiling were subjected to be amplified with *IS 711* primer and species specific primers for *B. melitensis* and *B. abortus*. The result showed that the suspected two *Brucella* colonies were negative for *IS711* , *B.melitensis* and *B.abortus* primers (Fig 5). The same suspected colonies from two samples were tested again after the DNA is extracted by QIAMP DNA Mini Kit together with seven blood clot extracts from camels which were positive for *IS711* primer (Fig 6).

The result revealed that colonies from two samples were found positive for *IS711* primer. The two isolate and blood clot samples found positive for *IS711* gene were tested with species specific primers of *B.melitensis* and *B.abortus* to identify at species level (Fig 7). The result showed that two culture samples from caprine species and four blood clot samples from camel were positive for *B.melitensis* primes whereas, three blood clot samples from camel which were positive for *IS711* gene were not amplified with *B. melitenesis*. None of the samples were positive for *B.abortus* primer.

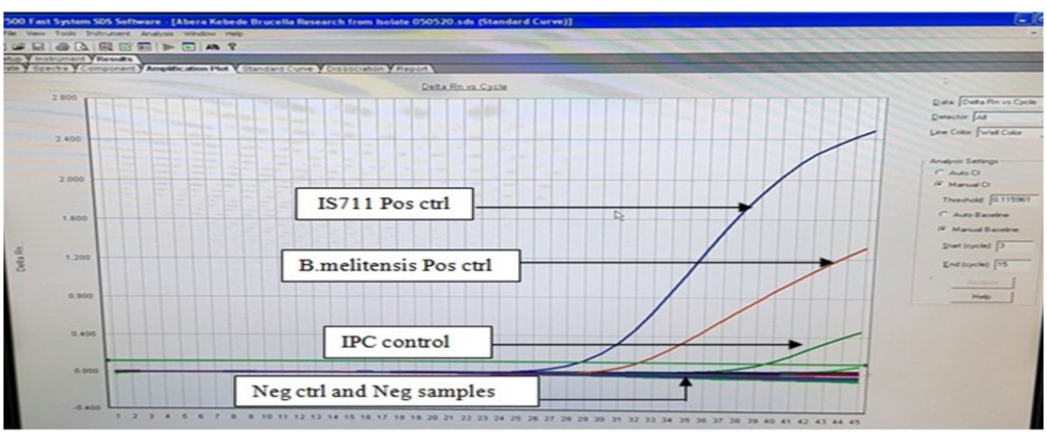

Figure 5 : *Brucella* culture samples negative for *IS 711* and *B.melitensis* primers extracted by boiling method.

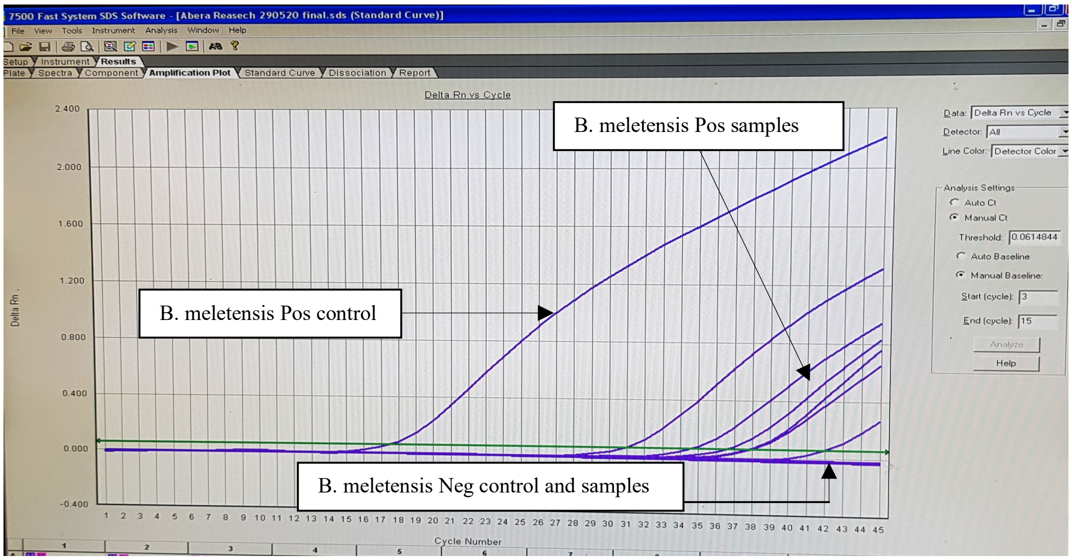

Figure 6: *Brucella* culture and blood clot samples amplified by *B.melitensis* primer extracted by QIAMP DNA Mini Kit.

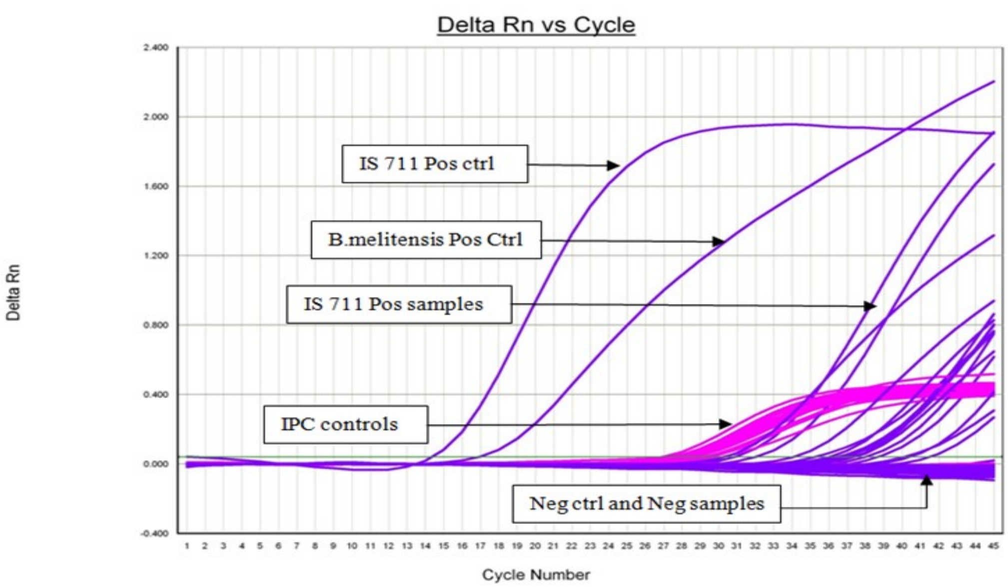

Figure 7: *Brucella* culture and blood clot samples amplified by *IS711* and *B.melitensis* primers extracted by QIAMP DNA Mini Kit.

5. DISCUSSION

5.1 Brucellosis seroprevalence

The present study showed 12.1% (95% CI: 10.1, 14.5) and 11.5% (95% CI: 8.9, 11.8) animal level sero-prevalence of brucellosis in small ruminants and camels respectively. The prevalence rate of 12.1% brucellosis observed in small ruminants was in close agreement with 12.35% reported by Tegegn *et al*. (2016). The study finding was quite higher than prevalence rates of 1.9% by Gumi *et al*. (2013), 5.6% by Sintayehu *et al.* (2015), 3.57% by Gebremedhin, (2015), 1.9% by Tsegay *et al.* (2015) and 4.8% by Ashenafi *et al*. (2007) reported in small ruminants. Similarly slightly lower prevalence rates of 9.7 % and 9.1% were reported by Teshale *et al.* (2006) and Yohannes *et al*. (2013) respectively.

The camel brucellosis sero- prevalence of 11.5% observed in this study was higher than 7.6% reported by Sisay and Mekonnen, (2012), 5.2% by Teshome *et al.* (2003), 5.42% by Adugna *et al*. (2013), 5.4% by Muhammed *et al*. (2011), 0.9% by Gumi *et al*. (2013), 1.5% by Warsame *et al*. (2012), 1.53% by Robayo and Esubalew, (2017), 2.43% by Tilahun *et al*. (2013), and 2.09% by Zeru *et al.* (2016) in different areas of Ethiopia. The current high seroprevalence rate could be attributable to sample size, management and sensitivity and specificity of test method.

According to Getachew *et al.* (2016), the sensitivity of RBT, I-ELISA, and CFT was determined as 89.6% (95% PI: 79.9–95.8), 96.8% (95% PI: 92.3–99.1), and 94% (95% PI: 87.8–97.5) and similarly, specificity was determined as 84.5% (95% PI: 68–94.98), 96.3% (95% PI: 91.7–98.8), and 88.5% (PI: 81–93.8), respectively. However, compared to the current finding a higher prevalence rate 12.1% in Jordan (AL-Majali*et al*., 2008) and 12.84% in Iraq (Al-Hassani *et al*., 2018)and the highest 23.8%, 30.5% and 37.5% Musa *et al*. (2008); Mokhtar *et al*. (2007) and Omer *et al*. (2010) were reported respectively.

Among the risk factors considered predisposing to brucella sero-positivity of small ruminants, sex and age did not have significant association ($p > 0.05$) with *Brucella* infection. This was comparable to the result reported by Tsehay *et al*. (2014); Teshale *et al.* (2006); Bekele *et al*. (2011) where the association of age and sex with brucella sero-positivity was not significant (P>0.05). In contrast, Sintayehu *et al.* (2015) have reported sex and age have significant association in seropositivity of brucellosis ($P<0.05$). While, Adugna *et al*. (2013) and Tegegn *et al*. (2016) have reported sex has significant association with seropositivity (P<0.05). Even though sex and age did not have significant association with seropositivity, prevalence of female animals were (12.7%) which was higher than male animals (2.1%) OR = 0.16 (95% CI: 0.02, 1.12) where males are 16% less affected by brucellosis than females. Similarly the prevalence of adult animals was higher than young animals which were 12.9% and 6.1% respectively OR = 0.47 (95% CI: 0.20, 1.11) which indicates that young animals are 47% less affected by brucellosis than adult animals.

Even though the current prevalence study of small ruminants brucellosis showed that abortion history and parity did not have statistically significant association, multiparous animals have got higher seropositivity 12.1% (72/595) when compared to primiparous animals 9.7% (9/93) OR= 0.78 (95% CI: 0.37, 1.64). In this study, it was observed that primaparous animals were 78% less affected by brucellosis than multiparous animals. The nature of the *brucellae* organisms which are intracellular bacteria and the ability of these bacteria to invade, survive for long periods of time and multiply within host cells might be the reason why multiparous and adult animals have higher positivity when compared to young and primaparous animals (Poester *et al*., 2013).

Statistical analysis of risk factors like herd size has shown that it did not have significant association with *brucella* seroposistivity. This result concurs with the earlier reports of Tilahun *et al.* (2013); Dulo, (2017); Robayo and Esubalew, (2017) and Admasu and Kaynata, (2017). However, when prevalence rate was compared, higher rates (13.4%) and lower rate 7% was determined in large and small herd respectively which might be attributable to contact rate between infected and non infected animals.

Analysis of risk factors for *brucella* sero-positivity in camels revealed that sex, age, and parity have significant association with *Brucella* infection OR= 0.42 (95% CI: 0.27, 0.64)

P value=0.000, OR =0.64 (95% CI: 0.44, 0.92) *P* value=0.017 and OR = 0.61(95% CI: 0.43, 0.86) *P* value=0.04 respectively. It was observed that male camels were 42% less affected and young camels were 64% less affected by brucellosis when compared to females and adult animals respectively. The reason female have high prevalent than male could be due to sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms tend to increase in concentration with age and sexual maturity (Radostits *et al*., 2007).

The current result was in agreement with reports of Habtamu and Kassahun. (2014) who reported age and parity have significant association with seropositivity of brucellosis. In another study, Habtamu *et al.* (2015) has also reported age has significant relation with seroposistivity. In contrast, Tilahun *et al.* (2013) has reported sex, age, and parity did not have significant association with seropositivity. Similarly, Robayo and Esubalew, (2017) has recorded that sex and age did not have significant association. The current prevalence study of camel brucellosis showed that no animals were found positive which have abortion history. The reason could be due to the sample size or the cause of abortion may be due to other infectious diseases.

In this study, the overall herd level brucellosis prevalence rate of 17.8 %. was determined in small ruminant which was in agreement with the findings of Gebremedihn, (2015)16.74% in East and West Shewa Zones of Oromia regional state and 18% by Tegegne *et al.* (2016) in Chifra and Ewa districts of Afar regional state. Higher records for herd level prevalence were reported in studies at different areas of Afar and Tigray regions where seropositivity of 28% and 22% Teklue *et al.* (2015) in southern zone of Tigray region and Adugna *et al.* (2013) in Afar region were reported respectively. Similarly herd level brucellosis prevalence rate of 15.6 % was determined in camels which is comparable to 15.14% reported by Tilahun *et al.* (2012) in eastern Ethiopia.

From this study it was revealed that the prevalence of camel and small ruminant brucellosis was high in extensive farms of pastoral areas as compared to intensive farms as reported by different authors. For instance Edao *et al.* (2018) have reported 2.77 % and

0.06% prevalence of brucellosis in catlle using RBPT and c-ELISA respectively in Addis Ababa dairy farms while, Asmare *et al*. (2013) reported 1.98% sero prevalence using RBPT in exotic and cross bred cattle in Ethiopia. Therefore, the higher prevalence of brucellosis in the study area would have zoonotic importance implication since consumption of raw milk is common practice.

5.2 *Brucella* Isolation and detection

In the present study, the isolation of *Brucella* species from vaginal swabs collected from animals with history of recent abortion was 13.3% (2/15) which is in agreement with the result reported by Sintayehu *et al*. (2015) who reported 14.3% (2/14) from small ruminants. In contrast, the current result is lower than the report of Tekle *et al. (*2019) who isolated 18.5% (5/27) from vaginal swab samples. Based on cultural, morphological, and biochemical characteristics, the two isolates were identified as *Brucella* organism isolates (Alton et al., 1988). For further analysis the isolates were subjected to DNA extraction with boiling method and commercially available extraction kit (QIAMP DNA Mini Kit) to detect the isolates at species level using real time PCR assay.

The two colonies where the DNA was extracted by boiling method were allowed to be amplified with *IS 711* primer using real time PCR assay and the result has shown that no amplification was observed. This could be due to the fact that heat lysis method yields low concentration of DNA as compared to commercial kits based DNA extraction method which consequently results in false negative PCR reaction (Tabibnejad *et al*., 2016).Whereas, the same two samples extracted by QIAMP DNA mini Kit showed positive reaction for *IS 711* primers. Further amplification using species specific primer for *B.melitensis* and *B.abortus* revealed that the two isolates were identified as *B.melitensis* species. No amplification was detected with *B.abortus* primer.

The molecular detection of blood clot samples indicated that out of 295 seropositive animals, seven camel samples 2.4% (7/295) were found positive for *Brucella* at genus level. The positive samples were then tested for species specificity using *B.melitensis* and

B.abortus primer. Out of seven *Brucella* positive blood clot samples, four samples were identified as *B.melitensis* species while the rest three camel blood clot samples were not amplified with *B.melitensis* primer. None of the samples were positive for *B.abortus* primer. Those samples which were positive for *IS 711* primers and negative for *B.melitensis* and *B.abortus* primers could be another species of *Brucella* which needs further investigation.

The PCR results of *Brucella* culture isolates were higher than reported by Bayeta, (2019) who reported 36.4% (4/11) and Tekle *et al*. (2019) who reported 37% (3/8) of *B.melitensis* from vaginal swabs of small ruminants. Whereas, the PCR results of blood clot samples obtained in this study was lower than the results reported by Wubishet *et al*. (2018) who reported 16.7% (2/18) positive for *B.abortus* in blood clot samples of cattle using real time PCR assay. In another study, 15.3% *Brucella* DNA was detected and reported by Karthik *et al*.(2014) and 13.6% (27/199) by Hinić *et al.*(2009) from blood samples using *IS 711* primer in real time PCR assay.

In this present study, it was evident that detection of *Brucella* organisms from blood clot was very low (2.4%) as compared to culture and serological tests. This is due to the fact that *Brucella* is an intracellular pathogen and the presence of PCR inhibitors in the blood could further complicate the detection (Leal-Klevezas., 1995). This study revealed that the detection of *B.melitensis* in camel species would be for the first time in Ethiopia to the best of the knowledge of the author.

The isolation of *B. melitensis* from the vaginal swabs of small ruminant and from blood clots of camels has great significant as far as public health importance is concerned. The habit of consumption of raw unpasteurized milk among pastoralist communities is one of the predisposing factors to zoonotic consequences. Similarly, the practice of unsafe handling and disposal of animals and contaminated retained placenta could lead to contracting brucellosis (Habtamu *et al*., 2015).

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, this study showed relatively higher prevalence of brucellosis in extensive production/pastoral areas as compared to the prevalence reported in intensive dairy farms in the country and requires urgent intervention to control or possibly reduce further transmission of brucellosis in the northern part of Ethiopia. The prevalence of brucellosis in this study has showed that camels and small ruminants are equally infected by the disease. Higher prevalence of brucellosis was observed in adults and female animals in both species. In this particular study, *Brucella melitensi*s isolates were found in blood clots of camel samples. *Brucella* isolate at genus level were also identified from blood clots of camel samples which needs further investigation. The findings of *Brucella* isolates indicated that there is high risk of public health importance for human population in the study areas which needs attention where people are consuming raw milk and in appropriate handling of aborted materials is practiced.

Therefore, based on the above conclusion the following recommendations were forwarded:

- Still much studies to be done on brucellosis with special emphasis to camels where information are lacking as compared to bovine and small ruminants,
- Strengthening and implementing the diagnostic capacity of veterinary and public health laboratories which enables to isolate and characterize *Brucella* pathogens should be one of the national agenda in Ethiopia, since it is one of the global public health issue and export constraint factor,
- Further sequencing and characterization of the PCR positive DNA samples to identify the circulating *Brucella* strains in the area.
- There should be a strong plan to strengthen the epidemiological surveillance system which helps to indicate the national status of the disease and sketch disease distribution mapping on brucellosis in Ethiopia,
- The observed higher prevalence of brucellosis in the study area, needs further investigation about its zoonotic implication for the pastoral community in the area, Hence Interdisciplinary collaboration between Veterinarians and public health experts

needed to compile organize and formulate strategies based on the information so far generated so as to produce documents which helps for the prevention and control measures

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8. ANNEXES

Annex 1: **Multispecies i-ELISA procedure**

All reagents should equilibrate to room temperature at 21°c ± 5°c before use and homogenized by inversion or vortexing.

1. Add;

A. 190 µl Dilution Buffer into all wells.

B.10 µl of the Negative control to A1 and B1

C.10 µl of the Positive control to C1 and D1

D. 10 µl of each sample to be tested to the remaining wells.

2. Incubate 45 min (plus or minus 4°c min) at 21°c (plus or minus 5°c).

3. Empty the wells.wash each well 3 times with approximately 300 µl of the wash solution.avoid drying of the wells between washings.

4. Prepare the conjugate by diluting the concentrated10x to 1/10 (short incubator) or to 1/20 (over night incubator) in dilution Buffer 3

5. Add100 µl of the conjugate 1x to each well.

6. Incubate 30 min plus or minus at 21°c (plus or minus 5°c). 7. Empty the wells.wash each well3 time with approximately 300 µl of the wash solution. 8. Add 100 µl of the substrate solution to each well. 9. Incubate 15 min plus or minus 2min at 21°c (plus or minus 5°c) in the dark. 10. Add 100 µl of the stop solution to each well in order to stop the reaction. 11. Read and record the O.D.at 450nm

Interpretation For each sample, calculate the (s/p %) as follows using the sample and control values.

$$\text{Sample positivity percentage (S/p\%)} = \frac{{}^{OD}\text{sample} - {}^{OD}\text{NTC}}{{}^{OD}\text{PC} - {}^{OD}\text{NC}} \times 100$$

Sample with a s/p%;

Less than or equal to110% was considered negative.

Greater than 110% and less than 120 %

Greater than 110% and less than 120 % was considered doubtful.

Greater than or equal to 120% was considered positive.

Validation: The test is validated if; the mean value of the positive control OD (ODpc) is greter than 0.350. ODpc >0.350 the ratio of the mean value of the positive and Negative contrl (ODpc and ODNC) is greater than 3. ODPC/ODNC>3

Annex 2: Media preparation and culturing

Procedure

1. Suspend 22.5gm in 500 litre of distilled water and bring to the boil to dissolve completely.
2. sterile by autoclaving at 121oc for 15 min.
3. Cool to 50oC and add 5% of inactivated horse serum(i.e.horse serum held at 56oC for 30 min).mix well before pouring
4. Add rehydrated one vial of *Brucella* selective supplement (SR083A) to sterilized *Brucella* medium base and homogenize before plating
5. Pour 15-20ml of the medium on the petridish
6. Incubate the plate at 37^0C for sterility check up for 48 hrs.

Annex 3: **Modified Ziehl-Neelsen Staining procedure for *Brucella***

Procedure

1. Fix a smear by heat
2. Overlay the slide completely with dilute carbolfuchsin for 15 minutes
3. Dicolorize the smear for 15 seconds in 0.5% acetic acid and wash it with tap water
4. Counter stain with methylene blue for 2 minutes, wash again with water and dry it.
5. Examine under 100x oil immersion objective microscope Interpretation *Brucella* species appeared red, small coccobacilli arranged single, pair or sometimes graphe. Other bacteria appear blue.

Annex 4: Urea Test

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 35^0Cfor 18-24 hrs.

Interpretation; Organisms that hydrolyse urea rapidly may produce positive reaction within 1-2 hrs.less active species 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

Annex 5: **Genomic DNA extraction from blood clot procedure**.

1. In biosafety cabinet, cut up to 25 mg of blood clot by using sterile forceps and scaple in sterile petridish and place in 1.5 ml screw- cap micro-centrifuge tube.
2. Add 180 µl of ATL lysis buffer and 20µl of proteinase K to the tissue
3. Incubate the sample suspention at 56^{o}C for 1-3hr in the water bath.vortexing occationally to disperse the sample
4.Vortex for 15 min .Briefly spin samples in a mini-centrifuge to remove droplet from under cap.Add 200 µl buffer Alto the sample and mix thoroughly by vortexing.Then add 200 µl ethanol (96-100%),and mix again thoroughly by vortexing.
5.Pippet the mixture (incliding any precipate) in to the Dneasy Mini spin colomn placed in a 2ml collection tube.centrifuge at 8000rpm for 1 min.Discardflow-through and collection tube.
6. Place the DNeasy Mini spin colomn in a new 2ml collection tube, add 500µl BufferAW1, and centrifuge for 1min at 8000rpm. Discard flow-through and collection tube.
7.Place the DNeasy Mini spin colomn in a new 2ml collection tube,add 500µl Buffer AW2 and centrifuge for 3min at 14,000rpm to dry the DNeasy membrane.Discardflow-through and collection tube.
8. Place the DNeasy Mini spin colomn in a clean 1.5ml or 2ml microcentrifuge tube and pipet 100 µl Buffer AE directly on to the DNeasy membrane.incubate at room tempereturefor 1 min.and then centrifuge for 1min at 8000rpm to elute.
9. The eluted DNA is ready to use for PCR

Annex 6: Format used for individual Animal sampling

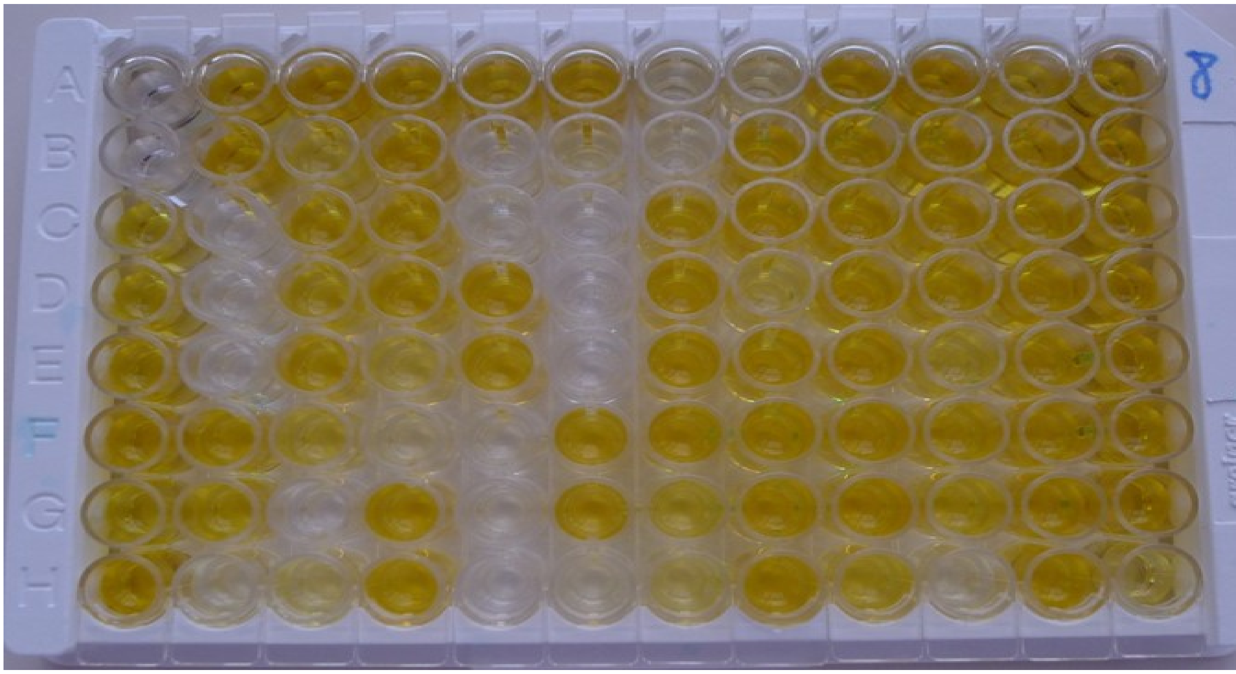
Field ID	Animal ID	District	Kebele	Species	Sex	Age	Sample type	Abortion history	Stage of abortion	Parity

Annex 7: Plate lay out for iELISA test

	1	2	3	4	5	6	7	8	9	10	11	12
A	Neg Ctrl	S-5	S-13	S-21	S-29	S-37	S-45	S-53	S-61	S-69	S-77	S-85
B	Neg Ctrl	S-6	S-14	S-22	S-30	S-38	S-46	S-54	S-62	S-70	S-78	S-86
C	Pos Ctrl	S-7	S-15	S-23	S-31	S-39	S-47	S-55	S-63	S-71	S-79	S-87
D	Pos Ctrl	S-8	S-16	S-24	S-32	S-40	S-48	S-56	S-64	S-72	S-80	S-88
E	S-1	S-9	S-17	S-25	S-33	S-41	S-49	S-57	S-65	S-73	S-81	S-89
F	S-2	S-10	S-18	S-26	S-34	S-42	S-50	S-58	S-66	S-74	S-82	S-90
G	S-3	S-11	S-19	S-27	S-35	S-43	S-51	S-59	S-67	S-75	S-83	S-91
H	S-4	S-12	S-20	S-28	S-36	S-44	S-52	S-60	S-68	S-76	S-84	S-92

S= Sample

Annex 8: I-ELISA test result for highly seropositive animals



Annex 9: Master Mix preparation for amplification with *IS711* primer

REAGENT/STOCK CONCENTRATION	FINAL CONCENTRATION	µL per REACTION
Rnase free water	/	3.9 µl
IS711 Forward Primer (5-GCT-TGA-AGC-TTG-CGG-ACA-G-3) (20µM)	0.16 µM /25µl	0.2µl
IS711 Reverse Primer(5'-CCT-ACC-GCT-GCG-AAT-3') (20µM)	0.16 µM/25µl	0.2µl
Probe: FAM-AAG-CCA-ACA-CCC-GGC-CAT-TAT-GGT-BHQ-1) (20µM)	0.16 µM/25µl	0.2µl
Exo IPC Mix (10x) IPC primers and probe	1x/25µl	2.5µl
Exo IPC Mix (50x) IPC template DNA	1x/25µl	0.5 µl
Taq man universal PCR master mix(2x)	1x /25µl	12.5µl
VOLUME		20µl
DNA		5µl
FINAL REACTION VOLUME		25 µl

Annex 10: Master mix preparation for amplification with *B, abortus* primer

REAGENT/STOCK CONCENTRATION	FINAL CONCENTRATION	µL X 1 REACTION
Rnase free water	/	6.9 µl
Forward Primer (5'-GCA-CAC-TCA-CCT-TCC-ACA-ACA-A-3') (20µM)	0.16 µM /25µl	0.2µl
Reverse Primer (5'-CCC-GTT-CTG-CAC-CAG-ACT-3') (20µM)	0.16 µM/25µl	0.2µl
Probe: FAM-TGG- AAC–GAC-CTT-TGC-AGG-CGA-GAT-C-BHQ-1 (20µM)	0.16 µM/25µl	0.2µl
Taq man universal PCR master mix(2x)	1x /25µl	12.5µl
VOLUME		20µl
DNA		5µl
FINAL REACTION VOLUME		25 µl

Annex 11: Master mix preparation for amplification with *B.meleitensis* primer

REAGENT/STOCK CONCENTRATION	FINAL CONCENTRATION	µL X 1 REACTION
Rnase free water	/	6.9 µl
Forward Primer (5'-TCG-CAT-CGG-CAG-TTT-CAA-3') (20µM)	0.16 µM /25µl	0.2µl
Reverse Prime (5'-CCA-GCT-TTT-GGC-CTT-TTC-C-3') (20µM)	0.16 µM/25µl	0.2µl
Probe: FAM-CCT-CGG-CAT-GGC-CCG-CAA-BHQ-1 (20µM)	0.16 µM/25µl	0.2µl
Taq man universal PCR master mix(2x)	1x /25µl	12.5µl
VOLUME		20µl
DNA		5µl
FINAL REACTION VOLUME		25 µl