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ISOLATION, PHENOTYPE CHARACTERIZATION AND SEROPREVALENCE SUREVY ON SMALL RUMINANTS *BRUCELLOSIS* IN ARBA MINCH ZURIA AND MIRAB ABAYA DISTRICTS OF GAMO GOFA, SOUTHERN ETHIOPIA

MSc Thesis

By

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June, 2016

Bishoftu, Ethiopia

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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

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As members of Examining Board of the final MVSc. Open defense, we certify that we have read and evaluated the thesis prepared by **Melese Yilma Zaba**, entitled: **Isolation, phenotype characterization and Seroprevalence Surevy on small ruminants *brucellosis* in Arba Minch Zuria and Mirab Abaya districts of Gamo Gofa, Southern Ethiopia**. And recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

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DEDICATION

This thesis manuscript is dedicated to my mother, Ehitenesh H/Michael (1962-2004), who was always immersed in hope about my success through death come ahead of her revels a bit before my success in joining of Addis Ababa University College of Veterinary Medicine and Agriculture.

STATEMENT OF AUTHOR

First, I declare that this thesis is my *bonafide* work and that all source of material used for this have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirement for an advanced (MVSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/ College Library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institute anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

ARRC	Areka Agricultural Research Center
BAPAT	Buffered Antigen Plate Agglutination Test
BCV	*Brucella* Containing Vacuole
BMB	*Brucella* Media Base
BoA	Bureau of Agriculture
BoLFR	Bureau of Livestock and Fish Resources
BSL	Biosafety Level
CFT	Complement Fixation Test
CI	Confidence Interval
CSA	Central Statistics Agency
CVMA	College of Veterinary Medicine and Agriculture
DC	Dendritic Cell
ELISA	Enzyme Linked Immuno Sorbent Assay
FAO	Food and Agricultural Organization
FPA	Fluorescence Polarization Assay
FPSR	False Positive Serological Reactions
g/l	gram per liter
Ig	Immunoglobulin
ILRI	International Livestock Research Institute

Kg	Kilogram
LIVES	Livestock and Irrigation Value-chain for Ethiopian Smallholders
LPS	Lipopolysacchraides
masl	meters above sea level
Mg/L	Milligram per Liter
NAHDIC	National Animal Health Diagnostic and Investigation Center
OD	Optical Density
OIE	Office International des Epizooties
OR	Odds Ratio
ORF	Open Reading Frames
PA	Peasant Association
PCR	Polymerase Chain Reaction
RBPT	Rose Bengal Plate Test
RFM	Retained Fetal Membrane
RHP	Reproductive Health Problem
SARI	Southern Agricultural Research Institute
SNNPR	Southern Nations Nationalities and Peoples Region
Spp	Species
SPSS	Statistical Package for Social Sciences
T4SS	Type IV Secretion System

TLR	Toll Like Receptor
TSA	Tryptone Soya Agar
VNTR	Variable Number of Tandem Repeat
VP	Voges Proskauer
WADU	Wolaita Agricultural Development Union
WHO	World Health Organization

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ABSTRACT

Brucellosis is an important infectious disease causes significant reproductive losses in sexually mature animals and zoonotic hazard. A cross sectional seroprevalence survey was conducted from November 2015 to March 2016 in Arba Minch Zuria and Mirab Abaya districts of Gamo Gofa zone with the objective of isolation and biochemical characterization of Brucella agent from seropositive clinical cases in small ruminants of the area. The districts were selected purposively following community based breed improvement program in the vicinity by the ILRI, 120 representative households from the breeding cooperative were randomly selected to generate information on flock reproductive health disorder. A total of 1000 serum samples (Goat, n=889 goats and sheep, n=111) were collected and screened using mRBPT, from screened test positive animals (11.3%) isolation rate were recorded. The five isolates were from vaginal swab, one isolate from placental cotyledon and suspected for two Brucella spp. through biochemical characteristic nature. On the basis of serological tests, the individual animal level seroprevalence of brucellosis was 4.3% (95%CI: 30.41, 55.59), 3.8% (95%CI: 26.13, 49.87) and 3.7% (95% CI: 25.3, 48.72) using m-RBPT, ELISA and CFT respectively. Flock level seroprevalence across the two districts was 10.81% (95% CI: 0.96, 7.90), 37.84% (95%CI: 6.81, 21.2) and 51.4% (95% CI10.64, 27.4) for small, medium and large flock size respectively. The kappa statistics almost perfect agreement (0.922 and 0.958) was record among the test mRBPT and ELISA respectively with reference to CFT. The identified putative risk factors for small ruminants Brucellosis in this study were fit using multivariable logistic regression. Odds of the risk factors at 95% confidence interval district (OR=10.35, 95%CI: 3.99, 26.8), flock size (OR=3.45, 97%CI: 1.12, 10.27), parity (OR=6.3195%CI: 1.82, 21.8), age (OR=3.43, 95%CI: 1.12, 10.51), history of reproductive health problems (OR=6.21, 95%CI: 3.16, 12.21) and source of breeding animals (OR=2.6, 95%CI: 1.33-5.06) were recorded. Also in the survey results show there was poor management of reproductive health problem and which could contribute to spread of brucellosis infection in the production system since breeding was not controlled. Therefore, particular in breeding programs strategic control measures should be implemented, such as regular testing of breeding animals, to reduce brucellosis economic impact and risk of zoonosis in the community.

Key word: *Brucella, Gamo Gofa, Isolate, seroprevalence, Small ruminants*

1. INTRODUCTION

Small ruminants which account for more than half of the domesticated ruminants in the world are an important component of the farming systems in most developing countries (Kosgey, 2004; Tedeschi *et al.*, 2011). They have a unique niche in smallholder agriculture from the fact that they require small investments; have shorter production cycles, faster growth rates and greater environmental adaptability as compared to large ruminants (Gebremedhin *et al.,* 2015).

Among many of the constraints to small ruminants' production, scarcity of feed, slow growth rate and high mortality has been the major limiting factors (Shapiro *et al.,* 2015). In Ethiopia veterinary service delivery constraint and diseases associated with reproductive tract have impact on production and productivity of small ruminants. This is typically due to small ruminants' breeding in Ethiopia is non controlled, health and nutrition management are very poor (Mirkena *et al.,* 2011). Also the extent of economic loss due to reproductive health problems and its associated risk factors were not clearly known.

Brucellosis is an important infectious disease and causes significant reproductive losses in sexually mature animals and has a significant zoonotic hazard (Radostits *et al.,* 2000). The disease is manifested by late term abortions, weak lambs and kids, still births, infertility and characterized mainly by placentitis in pregnant female animals, epididymitis and orchitis in males, with excretion of the organisms in uterine discharges and milk in female animals (Roth *et al.,* 2003). It also causes considerable loss of productivity through high morbidity (Radostits *et al.,* 2000).

Identification of breeding objective traits pertinent to specific production environments with the involvement of target beneficiaries is crucial for breed improvement program implementation (Mirkena *et al.,* 2011). The success of breeding program mainly relay on health trait so that, attention must given to health trait associated with reproduction for the successful implementation of small ruminant breeding program. Since etiology of reproductive disease like *Brucella* can transmit both horizontally and vertically and contribute lot for the production loss.

In Ethiopia the first published cases of brucellosis in animals date back to 1970s (Domenech and Lefever, 1974). Since then, brucellosis prevalence studies have been conducted in different localities of the country based on seroprevalence studies. But, until to date there is information gap on the causative agents' identification and characterization also the disease dynamics within small ruminant population and agroecological of the country was not clearly known. To my knowledge until now Asfaw, (2014) have tried to found *B. abortus* isolate from placenta cotyledons tissues of rose Bengal screened dairy caws in selected farms of Bishoftu and Asela town. But, no attempt was made to isolate the agents from small ruminants in Ethiopian. Hence, bacteriological culture isolations identifying the agent circulating in the vicinity were priority issue for the control and prevention of the diseases to enhance the reproductive performance of the breeding animal and zoonosis control.

Therefore, the general and specific objectives of this study were:

General objectives:

- Isolation, seroprevalence survey and risk factors investigation on *Brucella* infection in small ruminants

Specific objectives:

- To determine seroprevalence and associated risk factors for *Brucella* infection in small ruminants in the districts;
- To isolate and phenotype characterization of *Brucella* agent from screened and clinically suspected animals

2. LITERATURE REVIEW

2.1.Historical Background

In the year 1853, Jeffery Allen Marston made the first accurate description of the disease in British army troops serving in Malta during the Crimean war (Evans and Brachman, 2009). In 1887, David Bruce isolated Gram negative coccobacilli, named later *B. melitensis,* from spleens of fatal cases. In 1895, Bernard Bang isolated *B. abortus* from placental tissues of cattle. In 1897, M.L. Hughes published a review on the clinical and pathological features of the disease and suggested the name undulant fever (Mantur and Amarnath, 2008; Evans and Brachman, 2009). In 1897, Wright and Semple succeeded in applying serum agglutination method for differentiating brucellosis from other febrile illnesses. In 1904, the Commission of Mediterranean Fever was established (Evans and Brachman, 2009).

Between 1904 and 1907, several reports on epidemiology, bacteriology and pathology of brucellosis were published. In 1905, Themistocles Zammit identified a Maltese goat as the animal host of brucellosis. In 1918, Alice Evans published data on the antigenic relatedness between *B. melitensis* and *B. abortus.* Subsequently, the genus was named *Brucella* to honor David Bruce. In 1924, human infection with *B. abortus* was documented by Orpen in the U.K. Similar studies on *B. abortus* were performed by Morales-Otero in Puerto Rico (Mantur and Amarnath, 2008; Evans and Brachman, 2009). In 1914, Jacob Traum isolated *B. suis* from an aborted swine fetus (Mantur and Amarnath, 2008; Evans and Brachman, 2009). In 1909, Hutyra and Marek might have recovered the organism in Hungary (Mantur and Amarnath, 2008; Evans and Brachman, 2009).

In 1953, van Drimmelen made identification of *B. ovis* in sheep. In 1957, Stoenner and Lackman identified *B. neotomae* in rodents (Table 1) (Pappas *et al.,* 2005). In 1964, Carmichael and Bruner identified *B. canis* in the canines (Pappas *et al.,* 2005; Evans and Brachman, 2009). In 1994, Ewalt, Ross and colleagues identified *B. pinnediae* and *B. cetacear* in Minke whales, dolphins, porpoises as well as seals (Table 1) (Pappas *et al.,* 2005; Evans and Brachman, 2009).

In 1979, the WHO established a specialized program with a unit coordinating and managing activities (The Mediterranean Zoonoses Control Centre) operating from Athens in Greece

(Seimenis *et al.,* 2006). In 1986, the WHO and FAO recommended treatment for acute brucellosis in adults with a combination regimen composed of rifampicin and doxycycline orally for 6 weeks (Hasanjani *et al.,* 2006). In 2004, WOAH (World Organization for Animal Health) joint consultation on emerging zoonotic diseases held in Geneva defined an emerging zoonosis as a pathogen that is newly recognized or newly evolved or that has occurred previously but shows an increase in incidence or expansion in geographical, host or vector range (Cutler *et al.,*2010). In November 2006, a panel of international experts met in Ioannina in Greece and they made a number of therapeutic recommendations that included treatment of uncomplicated *brucellosis* using a combination of oral doxycycline for 6 weeks and parenteral streptomycin for 2 to 3 weeks or oral rifampicin for 6 weeks (Elzer *et al.,* 2002).

Brucellosis is a zoonotic disease that leads to considerable morbidity and characterized by abortion in females and epididymitis and orchitis in males (Smits and Kadri, 2005). Primary clinical manifestations of brucellosis among livestock are related to the reproductive tract. In highly susceptible pregnant animals, abortion at late pregnancy is cardinal feature of the disease (Radostits *et al.,* 2000). In general brucellosis can cause significant loss of productivity through abortion, still birth, low herd fertility and comparatively low in lamb and ked crops. The public health impact of brucellosis remains of concern in developing countries (Roth *et al.,* 2003).

Brucellosis occurs worldwide and remains endemic among Mediterranean countries of Europe, Northern and Eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America (FAO , 2003). Also it is considered as a reemerging problem in many countries where there is an increasing incidence of *B. melitensis, B. suis* biovar$_1$ infection (Cutler *et al.,* 2005). According to WHO , (1997) *B. melitensis* is considered to have the highest zoonotic potential, followed by *B. abortus*, and *B. suis* on those endemic regions.

Goats are the classic and natural hosts of *B. melitensis* and, together with sheep, are its preferred hosts. The main clinical manifestations of brucellosis in small ruminants are abortion and stillbirths, which usually occur in the last third of the pregnancy following infection and usually only once in the animal's lifetime (Blasco and Flores, 2011; Elzer *et al.,* 2002). Healthy animals can be exposed to *Brucella* infection in many ways, as a large number of bacteria are shed in the birth fluids or fetus, placenta and abortion secretions of infected females (Banai, 2007). The bacteria have the ability to survive several months outdoors, especially in cold, wet conditions, where they remain infectious to other animals, mainly through ingestion (Blasco, 2010).

Although females calve apparently normally in pregnancies following the first abortion, they continue to shed large numbers of bacteria into the environment. *B. melitensis* can be transmitted congenitally but only a small proportion of lambs and kids are infected in this way and most latent infections of *B. melitensis* are probably acquired by ingesting colostrum or milk (Grillo *et al.,* 1997).

A previously unreported fact is that *B. melitensis* was successfully isolated from the vaginal discharge of a goat that had aborted but tested seronegative for brucellosis, making the animal a potential risk for spread undetectable by serological diagnosis (Herrera *et al.,*2011).While orchitis and epididymitis are uncommon in rams and billy goats, they do occur (Chand *et al.,* 2002). *B.melitensis* biovar 3 has been isolated from a testicular hygroma of a ram (Musa and Jahans, 1990).

2.2.Etiology of Brucellosis

Brucella belongs to α-2 subdivision of proteobacteria (Mantur and Amarnath, 2008). *Brucella* species are small, gram-negative, non-motile, non-spore forming bacteria is function as facultative intracellular parasites, causing chronic disease which usually persists for life (Radostits *et al.,* 2000; Akhvlediani *et al.,* 2010). *Brucellas* are coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly and less frequently in pairs or small groups.

They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamp's modification of the Ziehl–Neelsen's method and false positive results in the Stamps's method because other organisms that cause abortions *Chlamydophila abortus* (formerly *Chlamydia psittaci*) and *Coxiella burnetii* are difficult to differentiate from *Brucella* organisms hence, positive or negative result should be confirmed by culture (OIE , 2012). The organism is aerobic, oxidase, catalase, nitrate reductase and urease positive (Mantur and Amarnath, 2008). It is localized predominantly in organs with numerous macrophages of lungs, liver, spleen, bone marrow and synovium (Akhvlediani *et al.,* 2010).

Brucella melitensis is the most virulent and invasive species; it usually infects goats and occasionally sheep (Mantur and Amarnath, 2008). Currently ten *Brucella* species are recognized including the better known six classical species comprised of *B. abortus* (cattle, biovars 1-6, and 9), *B. melitensis* (goats, sheep, biovars 1-3), *B. suis* (pigs, reindeer and hares, biovars 1-5), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (desert wood rats). More recently, new members to the genus include *B. ceti* and *B. pinnipedialis* (dolphins/porpoises and seals respectively), *B. microti* (voles) and *B. inopinata* (reservoir undetermined) was identified (Godfroid *et al.* 2011).

Table 1 *Brucella* species, ideal host and pathogenicity for humans

Species	**Biovars**	**Preferential host(s)**	**First Description**	**Pathogenicity for humans**
B. abortus	1-6, 9	Cattle	Bang, 1897	High
B. melitensis	1-3	Sheep and Goat	Bruce, 1887	High
B. suis	1-3	pig	Traum, 1914	High
	2	Wild boar, hare		No
	4	Reindeer, Caribous		High
	5	Rodents		No
B. ovis	-	Ram (sheep)	Van Drimmelen, 1953	No
B. canis	-	Dogs	Carmichael and Bruner, 1968	Moderate
B. neotomae	-	Desert wood rats	Stoenner and Lackman, 1957	No
B. ceti	-	Cetaceans	-	Unknown
B. pinnipedialis	-	Pinnipeds	Ewart and Ross 1994	Unknown
B. microti	-	Soil, vole, fox	-	Unknown
B. inopinata	-	Unknown	-	High

(Source: Godfroid *et al.* 2011)

Of these species, *B. melitensis* has the greatest risk for human infection followed by *B. suis* and *B. abortus*, however several of the other species have been shown to be virulent for humans (Godfroid *et al.* 2011). Bovine brucellosis is usually caused by *B. abortus*, less frequently by *B. melitensis*, and rarely by *B. suis,* Although *B. abortus* is mainly associated with cattle, occasionally other species of animals such as sheep, swine, dogs and horses may be infected. In horses, *B. abortus* together with *Actinomyces bovis* may be present in poll evil and fistulous withers (Gul and Khan, 2007).

2.3.Clinical manifestation

In sheep and goats, brucellosis is mainly caused by *B. melitensis* (Radostits *et al.,* 2000). This organism is a facultative intracellular pathogen. The predominant symptoms in naturally infected sheep and goats are abortions, stillbirths and the birth of weak offspring. Animals that abort may retain the placenta. Sheep and goats usually abort only once, but reinvasion of the uterus and shedding of organisms can occur during subsequent pregnancies. Some infected animals carry the pregnancy to term, but shed the organism (Mar´ın *et al.,* 1996a).

Milk yield is significantly reduced in animals that abort, as well as in animals whose udder becomes infected after a normal birth. However, clinical signs of mastitis are uncommon. Acute orchitis and epididymitis can occur in males and may result in infertility. Arthritis is seen occasionally in both sexes. Many non pregnant sheep and goats remain asymptomatic (Navarro *et al.,* 1999; Mar´ın *et al.,* 1996a).

2.4.Diagnosis

Brucellosis presents with many clinical manifestation that make its diagnosis a difficult task (Kaltungo *et al.,* 2014). Mar´ın *et al.* (1996a) reported that a presumptive bacteriological diagnosis of *Brucella* can be made by means of the microscopic examination of smears stained with the Stamp modification of the Ziehl-Neelsen staining method. However, morphologically related microorganisms, such as *Chlamydophila abortus, Chlamydia psittaci* and *Coxiella burnetti* can mislead the diagnosis because of their superficial similarity (Mar´ın *et al.,* 1996a; Poester *et al.,* 2010). More recently, the polymerase chain reaction (PCR) has been found to be a useful and more sensitive test, but has not been validated for standard laboratory use (Kaltungo *et al.,* 2014).

The only unmistakable method for diagnosing sheep and goat infection due to *Brucellosis* is isolation on appropriate and selective culture media. PCR based different molecular markers has been shown to be a valuable method for detection *Brucella* DNA and providing promising option. For large scale surveillance purpose, the Rose Bengal plate test and complement fixation test remain the most reliable serological tests. Also enzyme linked immno sorbent assay is promising test for large scale sample, but need further evaluation and standardization before being used at large scale (Bricker, 2002; Garin-Bastuji *et al.,* 2006).

2.4.1. Direct method of diagnosis

Brucellosis definitive diagnosis depended on laboratory test and chose for particular laboratory testing depends on the prevailing epidemiology situation of brucellosis in susceptible animal within the region (Bricker, 2002). The most reliable and clear method for diagnosing animal brucellosis is isolation of *Brucella* spp. (Alton *et al.,* 1988). Since it is specifically allows biotyping of the isolate, which is relevant from epidemiological point of view (Bricker, 2002). However, in spite of its high specificity, culturing for Brucella species is challenging because the organism is fastidious growth, require rich media for primary cultures, the requirement of large number viable laboratory specimen and high biosafety level facilities which is not available in most developing country (Pal and Jain 1986).

Isolation of *B. melitensis* on appropriate culture media is recommended for accurate diagnosis. Vaginal excretion of *B. melitensis* is usually copious and persists several weeks after abortion (Alton, 1990). Thus, taking vaginal swabs samples is the best way to isolate *B. melitensis* from sheep and goats. The spleen and lymph nodes (iliac, supramammary and prefemoral) are the best sites for obtaining samples for isolation during post-mortem examination (Mar´ın *et al.,* 1996b). *B. melitensis* does not require serum or CO_2 for growth and can be isolated on ordinary solid media under aerobic conditions at 37^oC (Quinn *et al.,* 1999; OIE , 2012). Nevertheless, due to the overgrowing contaminants usually present in field samples, selective media are needed for isolation purposes. The Farrell selective medium, developed for isolation of *B. abortus* (Farrell, 1974), is also recommended for *B. melitensis* (Garin-Bastuji and Blasco, 2004). However, nalidixic acid and bacitracin, at the concentration used in that medium, have inhibitory effects for some *B. melitensis* strains (Mar´ın *et al.,* 1996b) and isolation rate increases significantly by simultaneous use of both the Farrell and the modified Thayer-Martin media (Mar´ın *et al.,* 1996a; Mar´ın *et al.,* 1996b). While culturing is a specific method, its sensitivity depends on the viability of *Brucella* in the sample, the kind of sample and the number of specimens tested from the same animal (Mar´ın *et al.,* 1996b).

Immunohistochemistry is an alternative technique for direct diagnosis of brucella species infection (Kaltungo *et al.,* 2014). It has been extensively used to studies pathogenesis and diagnosis without requiring viable specimen but, several factors may affect the result including fixation protocol and selection of primary antibody (Santos *et al.,* 1998; Schwarz *et al.,* 2015). Because of this protocol challenge and other factor this method was not used routinely in most diagnostic laboratory (Mar´ın *et al.,* 1996b).

Molecular techniques are important tools for diagnosis and epidemiology studies, providing relevant information for identification of species and biotypes of brucella allowing difference between virulent and vaccine strains (Lopez *et al.,* 2008; Hoover and Freidlander, 1997). PCR assay has been shown to be a valuable method for detecting DNA from different microorganisms and provides a promising option for diagnosis of brucellosis. Several authors reported good sensitivity of PCR, based on different molecular markers (16S rRNA, bscp31, IS 6501/711) (Halling *et al.,* 1993; Ouahrani *et al.,* 1993) for detecting of *Brucella* DNA with pure cultures (DaCosta *et al.,* 1996). However, few studies have been performed with clinical or field samples (Richtzenhain *et al.,* 2002) and even fewer have evaluated the PCR as a diagnostic tool (Romero *et al.,* 1995a; Romero *et al.,* 1995b).

The possibility of using the PCR technique to detect the DNA of dead bacteria or in paucibacillary samples and even in samples highly contaminated with other microorganisms, could increase the rate of detecting infected animals. However, up to now, no technique appears sensitive enough to replace classical bacteriology on all kinds of biological samples. Several methods, mainly restriction fragments length polymorphisms and Southern-blot analyses have been employed to find DNA polymorphism to differentiate some *Brucella* species and biovars (Fayazi *et al.,* 2002; Richtzenhain *et al.,* 2002). Specific molecular markers have specifically been developed for distinguishing the vaccine strain from wild strains (Bardenstein *et al.,* 2002; Cloeckaert *et al.,* 2002). Recently, a new method has been described for fingerprinting *Brucella* isolates based on multi-locus characterization of a variable number, 8-base pair and tandem repeat (Cloeckaert *et al.,* 2002). The technique is highly discriminatory among *Brucella* species or strains (Bricker *et al.,* 2002). Molecular based diagnosis is limited in developing country because of cost for specific primer and laboratory facility (Garin-Bastuji *et al.,* 2006).

2.4.1.1.Isolation media nature for *brucella* organisms

Brucella agent are chemoorganotrophic microorganisms requiring complex media containing several amino acids, thiamin, biotin, nicotinamide and magnesium salts. The growth of *Brucella* in simple nutrient liquid medium is usually poor unless these are supplemented with blood, serum or tissue extracts (Moyer and Holocomb, 2005). *Brucella* base medium rich in nutritive elements and growth factors like beef extract and Peptone provide nitrogen, vitamins, minerals and amino acids essential for growth, glucose is the fermentable carbohydrate providing carbon and energy, Sodium chloride supplies essential electrolytes for transport and osmotic balance and bacteriological agar is the solidifying agent (Alton *et al*., 1988; Holt *et al*., 1994). Also growth is inhibited on media containing bile salts, tellurite or selenite and the addition of the antibiotic supplement enhances the mediums to selectivity for the growth of *Brucella* (Alton *et al*., 1988). *Brucella* spp. are more fastidious than other. Most *Brucella* spp. can be isolated in non supplemented, enriched peptone based media (Alton *et al*., 1988; Quinn *et al*., 1999). Good growth is obtained on *Brucella* medium base, sucrose dextrose agar, tryptone soy agar or glycerol dextrose agar supplemented with 5% horse serum (OIE , 2012; Moyer and Holocomb, 2005; Al Dahouk *et al*., 2013).

Brucella spp. are slow growing and the use of selective media is recommended for primary isolation from most clinical specimens because of the high numbers of overgrowing contaminants (Mar´ın *et al*., 1996a). Such selective media are prepared by incorporating antibiotics and bacteriostatic dyes onto basic enriched media such as *Brucella* medium base (Farrell, 1974). Farrell's medium was found not to be an ideal medium for the isolation of *B. melitensis*, because the concentrations of nalidixic acid and vancomycin in this medium have inhibitory effects on some strains (Mar´ın *et al.,* 1996a). Therefore, the use of modified Thayer-Martin'medium supplemented with haemoglobulin (10g/l), colistin methanesulphonate, vancomycin, nitrofurantoin, nystatin and amphotericin B in tandem with Farrell's medium is believed to enhance the chances of isolating *B. melitensis* (OIE , 2012). Recently, due to its carcinogenicity, cycloheximide has been removed from the *Brucella* selective supplements used in the Farrell's medium (Anon, 2005).

These antibiotic supplements of the Farrell's medium are commonly used, in different combinations and proportions onto any one of the basal media such as *Brucella* medium base (CONDA), Tryptone soya agar (TSA), Serum dextrose agar, Columbia blood agar (Bio Merieux) and other medium bases, for the formulation of selective media for isolation of *Brucella* spp. Other types of selective media have at some stage been used in the isolation of *Brucella* spp. Selective BCYE (polymyxin, anisomycin, cefamandole) is commercially available (Raad *et al*., 1990; Moyer and Holocomb, 2005). Moyer and Holocomb, (2005) reported the use of chocolate agar containing selective supplements for the isolation of *Brucella* spp.

Terzolo *et al*., (1991), used Skirrow's agar to isolate *B.abortus, B. suis, B. melitensis, B. canis* and *B. ovis* from contaminated vaginal exudates and milk. Hornsby *et al*., (2000) also found Skirrows agar, together with Modified Kuzdas medium and Tryptone soya agar (TSA) suitable for the recovery of the vaccine strain *B. abortus* RB 51, while Farrell's, Ewalt's and Kuzdas and Morse's medium were not suitable. Similarly, the use of new media such as rifampin *Brucella* medium (RBM) and malachite *Brucella* medium (MBM), together with TSA, was found to enhance the recovery of *B. abortus* RB 51 (Hornsby *et al.,* 2000).

For the isolation of *Brucella* spp. from milk samples, although solid media have been used successfully (Farrell, 1974), the use of enrichment media such as serum dextrose, tryptone soy or *Brucella* broth containing selective supplements of at least amphotericin *B* and vancomycin should be used because the microorganisms are usually present in small numbers to be detected on solid media (OIE , 2012).

2.4.2. Indirect method of diagnosis

Due to the cost and low individual sensitivity of culture and PCR techniques, the indirect diagnosis of disease is recommended for large-scale surveillance and/or eradication purposes. Although several serological methods are available, these tests can be classified as screening test (buffered antigen plate agglutination test), monitoring and epidemiological surveillance test (milk ring test) and complement arty or confirmatory test (2-mercaptoethanol, complement fixation, ELISAs and fluorescence polarization assay) (Nielsen, 2002; Poester *et al.,* 2010).

Detection of antibodies against relevant *Brucella* epitopes is the more practical approach. However, precise antigens and adequate tests have to be used for a proper efficacy and reliability. Particularly relevant is the problem of the specificity of serological tests since antibodies against *Brucella* epitopes may be present in the animal population due to vaccination and/or of contacts with other Gram-negative bacteria (*Yersinia enterocolitica* O:9) sharing cross-reactive epitopes with *Brucella*. Although the Rev-1 vaccine is an essential tool to control small ruminants' brucellosis, when applied under standard conditions, it induces long lasting serological responses that interfere with subsequent serological screening (Alton, 1990; MacMillan, 1990).

There is no agreement on what should be the nature and characteristics of a universal antigen for diagnosing brucellosis. One of the most critical and controversial points concerning serological diagnosis of *B. melitensis* infection in small ruminants is related to which *Brucella* species and biovars are used in production of antigens. The Rose Bengal test (RBT) and the complement fixation test (CFT) are the most widely used tests for the serological diagnosis of sheep and goats brucellosis (Farina, 1985; MacMillan, 1990); they are also the official tests for international trade (Collective , 2004). The antigenic suspensions or whole cells used in both tests are made with A dominant *B. abortus* biovar-1 Alton *et al.* (1988) and theoretically infections due to M dominant strains (*B. melitensis* biovar-1) could be misdiagnosed (Alton *et al.,* 1988; MacMillan, 1990). However, existence of a common (C) epitope in the immunodominant smooth lipopolsacchraides (S-LPS) can account for the high sensitivity of the *B. abortus* biovar 1 antigen to detect *B. melitensis* biovar 1 infections and vice versa (D´ıaz-Aparicio *et al.,* 1993). In fact, no significant differences have been found in the sensitivity of the classical *B. abortus* 1 RBT antigen (AC) between ovine populations infected either with *B. melitensis* biovar-1 (MC) or 3 (AMC) (Blasco *et al.,* 1994a; Blasco *et al.,* 1994b). Moreover, the indirect ELISA sensitivity in sheep, goats and cattle is not affected by the epitopic composition (AC or MC) of the antigens used (Alonso-Urmeneta *et al.,* 1998).

There is limited information on the value of outer membrane (OMP) and inner cytoplasmic proteins for diagnosis of brucellosis in sheep and goats. The immunoelectrophoretical pattern of cytoplasmic proteins, considered specific for the genus *Brucella* (D´ıaz *et al.,* 1968) shows little differences between the several *Brucella* species when assayed with polyclonal sera (D´ıaz *et al.,* 1967). The cytoplasmic antigens have been reported to be sensitive and specific enough when used in precipitation tests (D´ıaz *et al.,* 1968; D´ıaz-Aparicio *et al.,* 1994). In contrast, when used in ELISA, there was high background IgG reactivity in sera from *Brucella* free animals (Debbarh *et al.,* 1996a).

An important drawback of tests using uncharacterized cytosolic proteins is the lack of specificity when testing Rev-1 vaccinated animals, although a partially purified soluble protein of 26 kDa (CP26) from the cyto soluble protein extract (CPE) of *B. melitensis* has been reported as specific when used in an ELISA (Debbarh *et al.,* 1995, Debbarh *et al.,* 1996b). However, this test is significantly less sensitive than both RBT and CFT tests to diagnose infected ewes (Debbarh *et al.,* 1996a). A competitive ELISA (cELISA) using CPE extracts and some of the above monoclonal antibodies improved sensitivity in infected sheep, with no antibody responses being detected in Rev-1 vaccinated animals (Debbarh *et al.,* 1996b). Several authors have attempted to identify the main specificities of the antibody response to OMP extracts of *B. melitensis* by using either immunoblotting or cELISAs with specific monoclonal antibodies (Debbarh *et al.,* 1996b; Tibor *et al.,* 1996).

However, OMPs of 10, 16, 19, 25–27 and 31–34 kDa were found suitable as potential antigens by immunoblotting or ELISA, the antibody responses detected in infected sheep were scanty and heterogeneous (Zygmunt *et al.,* 1994a; Zygmunt *et al.,* 1994b). Further research is needed on the identification, isolation, and characterization and cloning from both inner and outer membrane proteins to be used as diagnostic antigens of improved sensitivity and specificity. As a second step, the development of subunit or live antigen-deleted vaccines, able to protect animals without interfering with diagnostic tests could be an interesting research approach (Zygmunt *et al.,* 1994b).

2.4.2.1. Serological tests

It is widely assumed that serological tests used for *B. abortus* infection in cattle are also adequate for diagnosis of *B. melitensis* infection in small ruminants. Accordingly RBT and CFT are the most widely used tests for the serological diagnosis of brucellosis in ruminants (Alton, 1990; MacMillan, 1990). Serological methods of diagnosis measure the ability of the serum antibody to agglutinate a standardized amount of killed *B. abortus* antigen containing O-side chain. These tests are most commonly used because they are safe.

However, they are prone to false-positive results due to other cross-reacting bacteria and also, they are not useful in the detection of *B. canis* and *B. ovis* which lack the O-side chain (Kaltungo *et al.,* 2014). Traditionally, screening tests are inexpensive, fast and highly sensitive, but most of the time, lack specificity. However, confirmatory tests are required to be both sensitive and specific, thereby eliminating some false positive reactions. Most confirmatory tests are more complicated and more expensive to perform than the screening tests (Díaz*,* 2013).

2.4.2.1.1. Rose Bengal test (RBT)

Despite the scanty and sometimes conflicting available information (MacMillan, 1990; Blasco *et al.,* 1994a; Blasco *et al.,* 1994b), this test is internationally acknowledged as the choice for the screening of brucellosis in small ruminants (Garin-Bastuji and Blasco, 2004). However, standardization conditions suitable for diagnosing cattle infection are not adequate in sheep and goats (Blasco *et al.,* 1994a; Blasco *et al.,* 1994b) and account for the low sensitivity of RBT antigens in small ruminants (Blasco *et al.,* 1994a; Falade, 1978; Falade, 1983) along with the fact that a high proportion of animals in infected areas give results negative in RBT, but positive in CFT (Blasco *et al.,* 1994a), question the efficacy of the present RBT as an individual test. A simple modification increasing slightly the amount of sera for the test dose from 25µl to 30µl and 75µl to 90µl at the same time maintaining the antigen volume (25 to 30µl), increases significantly the sensitivity without affecting specificity (Blasco *et al.,* 1994a; Ferreira *et al.,* 2003).

2.4.2.1.2. Complement fixation test (CFT)

CFT is the most widely used confirmatory test. Despite its complexity and the heterogeneity of the techniques used in the different countries, there is agreement that this test is effective in small ruminants (MacMillan, 1990). However, CFT lacks sensitivity and does not fully discriminate between infection and Rev-1 vaccination (Marin *et al.,* 1999). When testing a limited number of sera obtained from *B. melitensis* culture positive and *Brucella* free goats, CFT provided the same sensitivity than those of RBT and ELISA (D´ıaz-Aparicio *et al.,* 1994). However, Blasco (*et al.,* 1994ab) reported the sensitivity of CFT has been lower for sheep in field conditions (88.6%) than those of RBT (92.1%) and ELISA (100%). On the other hand, CFT has many drawbacks, such as complexity, variability of reagents, prozones, anticomplementary activity of sera, difficulty to perform with haemolysed sera and subjectivity for the interpretation of low titres (Blasco *et al.,* 1994ab).

Therefore, while sensitivity of the currently used unmodified RBT seems to be sufficient for the surveillance of free areas at flock level, RBT and CFT should be used together in infected flocks to obtain a more accurate individual sensitivity in latter stages of testand slaughter eradication programs (Alton, 1990). However, this procedure can lead to a considerable lack of specificity in case of countries affected by false positive serological reactions (FPSR) due to cross-reacting bacteria, a problem that also seems to affect sheep and goats (Garin-Bastuji *et al.,* 1998). As commented above, the CFT test has a very low specificity when testing sera from sheep and goats vaccinated subcutaneously with Rev-1 (D´ıaz-Aparicio *et al.,* 1993). However, when Rev-1 vaccine is administered conjunctively, the problem of interferences is significantly reduced in all serological tests, including the CFT (D´ıaz-Aparicio *et al.,* 1994; Marin *et al.,* 1999).

2.4.2.1.3. Enzyme linked immunosorbent assays (ELISA)

Good diagnostic results have been obtained in sheep and goats with iELISA or, at a lesser degree, cELISA using various antigens, but generally those with a high content of smooth lipopolysaccharide (LPS) are the most reliable. These ELISA provide similar or better sensitivity than both RBT and CFT, but like classical tests, ELISA are unable to differentiate infected animals from animals recently vaccinated with the Rev-1 vaccine (Ferreira *et al.,* 2003) or animals infected with cross-reacting bacteria.

However, the association of the conjunctiva vaccination procedure and the presence of a moderate interval after vaccination minimize or abrogate the specificity problems. A similar indirect technique has been also proposed for diagnosing sheep brucellosis in individual or pooled milk samples (Alonso-Urmeneta *et al.,* 1998), but the test lacks sensitivity when compared with serological tests. A highly immunogenic periplasmic protein from *B. abortus* (Rossetti *et al.,* 1996) or *B. melitensis* (Cloeckaert *et al.,* 1996a) has been applied to brucellosis diagnosis in different host species.

Indirect and competitive ELISA with this antigen could be sensitive and specific tests for diagnosing *B. melitensis* infection in sheep and have been reported to be useful in differentiating Rev-1 vaccinated from infected animals (Cloeckaert *et al.,* 1996b). All these ELISA have potential advantages in sensitivity and specificity with respect to both RBT and CFT, but their diagnostic efficacy at large scale is unknown and a great deal of standardization work is still required (Garin-Bastuji and Blasco, 2004).

2.5.Epidemiology

Brucellosis infection occurs naturally in sheep and goats in the Mediterranean region, but is widespread worldwide. North America is free of this disease, as are Northern Europe, Southeast Asia and Oceania (FAO, 1997). Pathologically and epidemiologically, this infection is very similar to *B. abortus* infection of cattle and the main clinical manifestations are reproductive failure, orchitis and epididymitis. *B. melitensis* biovars 1 and 3 are the most frequently isolated in Mediterranean countries (Mar´ın *et al.,* 1996a). In most circumstances, the primary excretion sources are foetal fluids and vaginal discharges after abortion or full-term parturition. Then, excretion of *B. melitensis* is important in milk and is also common in semen. As it happens in cattle, *B. melitensis* can be transmitted congenitally from ewes to lambs, these animals remaining infected but sero-negative (Grillo *et al.,* 1997).

There is paucity of published data on the status of small ruminant brucellosis in Ethiopia. Study information indicates that seropositivity for small ruminants brucellosis ranges 0.8 -9.6 % for RBPT and 0.4 – 5.1% for CFT in different agro-ecology. And within Species *B. melitensis* high seroprevalence was reported in goat. Using iELISA only Tedeschi (2006) reported tested sera from 2000 sheep and goats in pastoral regions of Ethiopia and documented 1.9% positive using RBPT and 9.7% positive by iELISA. Higher proportion of the existing report was using RBPT and CFT tests in different region of the country.

Table 2 Reported prevalence of brucellosis in small ruminants in some endemic areas of the Ethiopia

Location	Test used	Sample size	Overall (%)	Reference
Afar and Somali pastoral of East	RBPT, iELISA	2000	1.9, 9.7	Teshale *et al.,* 2006
South East Somali and Oromia	RBPT	510	9.6	Gumi *et al.,* 2013
Bahir Dar, Northwest Ethiopia	RBPT, CFT	384	1.2, 0.4	Yeshwas *et al.,* 2011
Boku live sheep export farm	RBPT, CFT	2030	0.8, 0.6	Girmay *et al.,* 2013
East Showa	RBPT	384	1.56	Lemu *et al.,* 2014
Debre Ziet & Modjo export abattoirs	RBPT, CFT	853	1.99, 1.76	Tsegay *et al*., 2015
Southern N/N/P/R	CFT	3964	5.1	Mengistu, 2007
Yabello district	RBPT, CFT	384	2.3, 1.56	Dabassa *et al.,* 2013

CFT: Complement Fixation Test, RBPT: Rose Bengal Plate Test, iELISA: Indirect-Enzyme Linked Immunosorbent Assay

In Ethiopia report reveled that the major risk factor for animal brucellosis are increased age and parity, flock size and composition and history of reproductive health problem (RFM, abortion, etc) across agroecology and production system. Repot indicated that there was a statistically significant increase in seroprevalence to brucellosis with increasing age and as herd size increases from small to medium (Berhe *et al.,* 2007). Megersa *et al.,* (2011a) showed that abortion was more commonly reported in goats 12.4% with seropsitivity for anti Brucella antibodies 1.9% of goats. Flock species composition odds ratio (4.1, 95% CI 1.2–14.2) was found to be risk factors for seropositivity in goats (Megersa *et al.,* 2012). Using logistic regression analysis Tassew and Kassahun, (2014) reported highly significant association of positive antibody status with potential risk factors for age, history of abortion and parity number for *B. melitensis* infection Mehoni District, southeast Tigray. Similar study by Asmare *et al.,* (2013b), argued the abortion is the major risk factor for small ruminants brucellosis.

3. MATERIALS AND METHODS

3.1.Study animals

The study animals are small ruminants kept under extensive management system. Purposively adult age groups above six months (sexually matured age groups) and animals with history of recent abortion and retained placenta were targeted in the study.

3.2.Study area descriptions

The study was conducted in Arba Minch Zuria and Mirab Abaya districts, located in Gamo Gofa Zone of the Southern Nation Nationality Peoples Regional State. Gamo Gofa is named for the Gamo and Gofa peoples, whose homelands lie in this Zone. In the study districts the production systems were mixed crop livestock production principally extensive management systems. Small ruminants allowed extensively browsing in the natural pasture through “Wude” (herding of flocks in the vicinity through 1to5 farmers development group) within district flock size per “Wude” was different these, “Wude” flock size in Mirab Abaya was larger than Areba Minch Zuria district (which was about 120 to 150 animals were herd together). And breeding practices were not controlled even the owner doesn’t knew how much animals give birth and death per year in the flock. In both district breeding cooperatives were newly establishing aimed in genetic improvement of local goat population through selection in paternal (sire) line, for this representative households were selected in the Pa’s through animal’s identification.

Table 3 Basic data of Arba Minch Zuria and Mirab Abaya district

Sample district	Location	Altitude (masl)	Rainfall (mm)	Annual temp (^{o}c)	Small ruminants population	Samples size
Arba Minch Zuria	6^{0}0’0’N 37^{0}53’E	1987	800-1000	29-38	63,629	600
Mirab Abaya	6°10’N 37°00’E	1229	655 (mean)	31-35	44,597	400
Total					112,226	1000

(Source: BoLF, personal communication with office letter ref. No: ARC/189/05/2016)

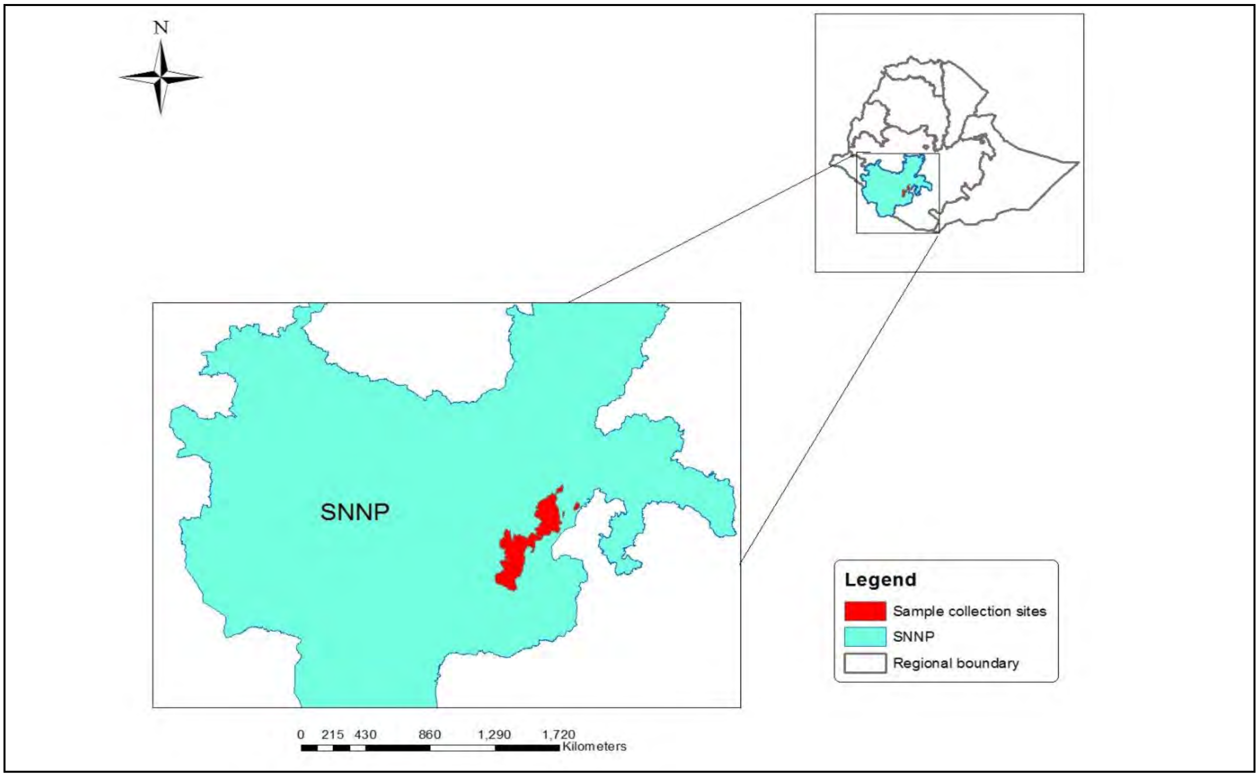

Figure 1: Location of study area

3.3.Study Design

A cross sectional study design was employed using field survey and serum sample collection and then followed by purposive bacteriological sample collection from serologically screened *Brucella* positive and animal with recent history of abortion and retained placenta.

3.3.1. Sample size determination

Bothe simple random and purposive sampling techniques were employed in the study. From the two districts following Livestock and Irrigation Value-chain for Ethiopian Smallholders project research mandate area owing to high small ruminants population and easier accessibility 10 Peasant Association (Pa's) were selected. On average 12 household (by considering 30% female head households in the breeding cooperatives) were included. A total of (n=120) representative households were randomly selected form the breeding cooperative member data list for filed survey. Data related to herd health and potential risk factors for *Brucella* was collected from each of the 120 households enrolled. Data collected include household characteristic, flock size and species composition, animal breeding practice and reproductive health problems, occurrences and history of abortion. Individual adult age group animal from household flock were randomly selected using lottery systems to collect serum samples by considering recent history of reproductive health problem. Sample size determination was based on Thrusfiled, (1995) and Toma *et al.,* (1999) formula. Assuming a 5.1% overall seroprevalence as reported by Mengistu (2007) in southern Ethiopia, with the expected precision of 5%; at 95% confidence interval.

$n= [1.96^2 * p\ (1-p)]/\ d^2$

Where, n: sample size, Confidence interval CI=95%, reported prevalence P=0.051, expected precision d^2= 0.0025. So, the sample size was calculated as 74 per PA. And to increase the precision and to allow for clustering at herd per "wude" at each Pas level, 100 serum samples were collected from each PA resulting in a total of 1000 serum samples. Data on history of abortion, still birth, parity, individual animal origin and parental line history flock size, flock composition and individual animal age by owner information and dentition (Shively, 1987; Abebe and Yami, 2008) were collected.

3.3.2. Serological diagnosis

3.3.2.1.Collection of serum Sample

Random samplings from the accessible small ruminants were used. Approximately 5-7ml of blood was collected from the jugular vein of adult small ruminants for serological examination using plain vacutainer tubes. After the identity of each animal was labeled on the corresponding vacutainer tube, the collected blood sample allowed to stand overnight in order to get the serum, then entire serum sample was centrifuged at 1500rpm for 10min and decanted aseptically according to the standard laboratory procedure and sera were separated from the clot by siphoning using plastic pipette, into the other screw caped sterile plastic tubes (1.8ml) and preserved at -20^{o}c until testing.

3.3.2.2.Modified Rose Bengal plate Test (m-RBPT)

For the mRBPT, the procedure described by Alton *et al.,* (1988) was followed with little modification by Blasco *et al.,* (1994a). Reactions were categorized as 0, +, ++, +++, according to

Nielson & Dunkan (1990), where: 0 = means no agglutination, + = barely perceptible agglutination (using magnifying glass), ++ = fine agglutination, some clearing, and +++ = clumping, definite clearing. Those samples identified with no agglutination (0) were regarded as negative, while those with +, ++ and +++ were regarded as positive. This test was performed following the procedure described by Blasco *et al.,* (1994a) mixing 75 µl of sera and 25 µl of the antigen. The plates were shaken for 4 min and any agglutination that appeared within this time was recorded as a positive reaction. The screened positive sample and the sera were preserved at -20^{o}c until CFT and ELISA test. Then mRBPT screened positive animals were traced back for bacteriological sample collection.

3.3.2.3.Enzyme Linked Immuno sorbent Assay (ELISA)

For further laboratory analysis ELISA was performed using a diagnostic INGEZEM BB COMPAC kit to detect antibodies to *Brucella* in serum sample and the protocol provided by the developers was followed precisely (Appendix XII) was conducted at NAHDIC. The test sera were analyzed at a final dilution of 1/100. The positive and negative controls were used at a dilution of 1/40 as has been indicated by the manufacturer. Following the addition of the conjugate and substrate chromogen mixture at a recommended strength, the plate was incubated and examined for the intensity of reaction with automated ELISA reader at 405nm. Color development within a well indicates that the tested serum has antibodies to *Brucella.* A positive/ negative cut-off value was calculated as 40 % of the mean of the percentage of inhibition (PI) of the positive control wells. The test serum with a percentage of inhibition value equal to or above 40% PI was considered positive.

3.3.2.4.Complement Fixation test (CFT)

Positive sera with mRBPT and 25% of the RBPT negative sera were further tested with CFT for confirmation using standard *Brucella abortus* antigen (New Haw, Addleston, and Surrey KT15 3NB, UK). The producers' outlines for CFT test proper and reagent preparation procedures were followed. Sera with strong reaction, more than 75% fixation of complement (3+) at a dilution of 1:5 or at least with 50% fixation of complement (2+) at a dilution of 1:10 and above, were classified as positive (OIE , 2012). The test was conducted at National Animal Health Diagnosis and Investigation Center (NAHDIC).

3.3.3. Bacterial isolation (culture)

Purposively selected seropositive animals with the history reproductive health problems were sampled to collect bacteriological specimen. The laboratory specimen from vaginal swab, abomasums content of aborted fetus and retained placenta tissue were collected and biosafety level 2 with personal protection was applied for culture isolation.

3.3.3.1.Bacteriology sample and microbiology procedure

Swabs samples from field were transported in semi-solid *Brucella* transport media (Stuart transport media) within cold chain to Woliyta Soddo Veterinary Regional Laboratory then to College of Veterinary Medicine Agriculture Microbiology laboratory (Bishoftu). Aborted fetuses tissues were necropsized and abomasums contents and mucosal swabs were sampled at laboratory and Retained placenta tissues samples were removed aseptically with sterile Scalpel and forceps. The tissue samples are prepared by removal of extraneous material (like fat), cut into small pieces and macerated using a 'Stomacher' with a small amount of sterile phosphate buffered saline (PBS) and samples were inoculated to *Brucella* selective growing media prepared form (CM0169®, CONDA), supplemented with *Brucella* Selective Supplement (CAT: 6060, CONDA) and 5% horse serum) to the manufacturer instruction.

The bacteriological specimen were inoculated on selective media supplement with antibiotic and incubated at 37°C for 4-12 days for growth. The plates were examined after 72hr and then daily to observe the growth of bacterial colonies. *Brucella spp*. was identified by colony morphology, and biochemical characteristic and a modified Ziehl–Neelsen's staung method was conducted.

3.3.3.2.Phenotypic characterization

Phenotyping is the most reliable technique to diagnosis of *Brucella spp.* Laboratory specimens were inoculated on agar plates and incubated in 37°C under 5% CO_2 conditions for 3-8 days for growth. Colony morphology was examined by stereomicroscope. The requirement for added CO_2, growth in the presence of basic fuchsin (20 μg/mL), production of hydrogen sulfide, the oxidase test was performed by Kovaks' method, Catalase test and urease activity of isolates was tested (Jang *et al.,* 2004; Nielson and Duncan, 1990).

3.3.4. Questionnaire survey

In parallel to the collection of serum samples, identified household heads from the breeding cooperative members were interviewed to generate information on socio-demographics (variables including age and educational level), household flock size and composition, source of breeding animals, management and breeding practice (culling) and information flock history of reproductive health problem was collected.

3.4.Data management and analysis

Data collected from the field and laboratory assay were stored on Microsoft (Ms) Excel spread sheet and analysis was done. Key informative collected through questionnaire survey was analyzed using SPSS ver. 20. Disease prevalence and odds ratio were measured using STATA.11 software. Associations between the outcome variable and its potential risk factors were first screened in a univariable analysis using logistic regression of measuring odds ratio. The significant results of the univariable analysis were included in the final model using multivariable logistic regression analysis. *Brucella* infection positive was considered as the dependent variable and the risk factors as independent variables. Similarly, herd level seroprevalence was calculated as the number of flocks with at least one positive animal by CFT divided by the total number of herds tested. Finally, odd ratios and 95% confidence interval were calculated and disease associated risk factors with a p-value less than 0.05 taken as significant small ruminants' brucellosis infection in the area.

4. RESULT S

4.1.Seroprevalence and associated risk factors

Using cross sectional study design from November/2015 to March/2016, 1000 serum samples were collected from adult age group small ruminants to screen and confirm antibodies against brucellosis, using mRBPT, ELISA and CFT. Based on serological tests, the individual animal level seroprevalence of brucellosis was 4.3% (95%CI: 30.41, 55.59), 3.8% (95%CI: 26.13, 49.87) and 3.7% (95% CI: 25.3, 48.72) using m-RBPT, ELISA and CFT respectively. Overall herd level seroprevalence were 10.81% (95% CI: 0.96, 7.90), 37.84% (95%CI: 6.81, 21.2) and 51.4% (95% CI: 10.64, 27.4) small (< 4 head of small ruminants), medium (5-8 head of small ruminants) and large (>9 head of small ruminants) herd size respectively. In the study 111 sheep and 889 goats were sampled. Randomly 229 male and 771 female breeding age group animals were sampled for sex proportion.

Table 4 Overall seroprevalence of brucellosis across district

District	N	Seroprevalence (CI95%)		
		m-RBPT	ELISA	CFT
Arba Minch Zuria	600	0.8(2.48,13.52)	0.4(0 .085, 7.92)	0.5(0.63, 9.4)
Mirab Abaya	400	3.5(23.89, 46.10)	3.4(23.04, 44.96)	3.2(21.34, 42.66)
Overall (%)	1000	4.3(30.41, 55.59)	3.8(26.13, 49.87)	3.7(25.3,48.72)

N= number of animas tested

Individual animals animal level seroprevalence across the districts were significant with Pearson (X^2 = 8.5549, P=0.025) using CFT.

Kappa statistics reveal that there was almost perfect agreement between m-RBPT and ELISA with CFT as standard test reference. The kappa value in this study (0.922 and 0.958) were recorded for mRBPT and ELISA respectively, with the test CFT as reference test was almost in a perfect agreement and significant ($p<0.05$).

Table 5 Kappa statistic for the laboratory test agreement between RBPT, ELISA and CFT

Serological test	CFT		Kappa value	Kappa value interpretation
	Neg.	Pos.		
mRBPT				
Neg.	957	0	0.922**	perfect agreement
Pos.	6	37		
ELISA				
Neg.	961	1	0.958**	perfect agreement
Pos.	2	36		

Pos. =test positive; Nge. =test Negative; significant P<0.05**; Non Significant-P >0.05NS

Common interpretation of kappa: <0.2: slight agreement, 0.2 to 0.4: fair agreement, 0.4 to 0.6: moderate agreement, 0.6 to 0.8 = substantial agreement, >0.8 = almost perfect agreement according to the common interpretation of kappa.

Within district higher individual animal seropositive result (3.2%) were recorded for Mirab Abaya ($p<0.05$). within flock seroprevalency were 0.4%, 1.4% and 1.9% for small (< 4 head of small ruminants), medium (5-8 head of small ruminants) and large (>9 head of small ruminants) flock size respectively and significant ($p<0.05$) using chi-square test. In similarly, with the increased parity and age seropositivity positive effect significant ($P<0.05$).

Table 6 Identified risk factor for seroprevalence of small ruminate brucellosis across district using chi-square test

Parameters categories	N	Prevalence (%) CFT	X^2
District			
Arba Minch Zuria	600	0.5	34.59**
Mirab Abaya	400	3.2	
Flock size (per head of animals)			
<4	284	0.4	5.87**
5-8	312	1.4	
>9	404	1.9	
Small ruminants spp			
Sheep	111	-	4.79**
Goat	889	3.7	
sex			
Male	229	-	0.04^{NS}
Female	771	3.7	
Parity(number)			
0	392	0.9	11.35**
1-2	353	1.2	
3- 4	224	1.2	
>5	31	0.4	
Age (years)			
< 1	184	0.4	10.54**
1-2	367	0.8	
2-3	237	1.0	
>3	212	1.5	
RHP			
No	862	1.5	35.47**
Yes	138	2.2	
Origin/ source of breeding dam/sire line			
Home (birth)	607	1.5	10.63**
Market (Purchase)	357	2.2	
Other source	36	-	

N=number of flock tested; Pos. =test positive; significant P<0.05**; Non Significant-P >0.05NS; RHP= reproductive health problem; RFM=retained fetal membrane; other source (family gift, SISO...); small flock: < four head of animals; Medium flock: 5-8 head of animals; large flock: more than 9 head of animals.

Animals with the history of reproductive health problem had (2.2%) seropostivity, where as animals which have no history of RHP about (1.5%) was significant (P<0.05) using chi-square. Animals origin as a predisposing factors for seropositivity 1.5% and 2.2% were recored for home born and purchased animals respectively within the breeding stock and was significant (P<0.05) using chi-square tests.

Logistic regression analysis results revealed that among the risk factors considered in the analysis, district, flock size, parity, age and history of reproductive health problem, had statically significant effect on seropositivity ($p<0.05$), while small ruminants species and sex had no statistically significant effect on seropositivity. The individual animal level sero prevalence was significantly higher in Mirab abaya district (3.2%) than in Arba Minch zurea and the result shows that animals in Mirab Abaya district have about 10 times higher chances (OR=10.35, CI95%: 3.99, 26.8) of getting infection with *Brucella* than those reared in Arba Minch Zuria district. Large flock size (more than nine head of small ruminants per household) was found epidemiologically very important for disease circulation in the area and as flock size increase the seropositivity of *Brucella* infection will increase approximately by three times (OR=3.45, 95% CI: 1.12, 10.27) with seroprevalence of 0.4%, 1.4% and 1.9% for small, medium and large flock size respectively. Similarly, *Brucellosis* seroprevalence was significantly increased with increased parity and age; the seroprevalence significantly higher in multiple parturition (1.2%) animals than zero parity (0.9%) with the likelihood of get seropositive with increased parity was by six times than zero parity animals (OR=6.31; 95% CI: 1.82, 21.8). The odd of seropositivity in older animals age above 3 years were about three times higher than that of young animals (OR=3.43; 95% CI: 1.12, 10.51).

Table 7 Logistic regression (reporting odds ratio) on associated risk factors for small ruminants *Brucellosis* in the study area

Risk factor category	Test animal	Prevalence (CFT) %	cOR (95% CI)	aOR (95% CI)
District				
Arba Minch Zuria	600	5(0.5)	10.35(3.99-26.79)	10.35(3.99-26.8)**
Mirab Abaya	400	32(3.2)		
Flock size (per head of animals)				
<4	284	4(0.4)		
5-8	312	14(1.4)	1.61(1.04-2.488)	3.29(1.07-10.11)
>9	404	19(1.9)		3.45(1.12-10.27)**
Parity number				
0	392	9(0.9)		
1-2	353	12(1.2)	1.71(1.18-2.465)	1.5(0.62-3.59)
3- 4	224	12(1.2)		2.41(0.99-5.81)
>5	31	4(0.4)		6.31(1.82-21.8)**
Age (year)				
< 1	184	4(0.4)		
1-2	367	8(0.8)	1.60(1.18-2.31)	1.01(0.29-3.4)
2-3	237	10(1.0)		1.98(0.61-6.43)
>3	212	15(1.5)		3.43(1.12-10.51)**
History of RHP				
No	794	15(1.5)	6.21(3.16-12.204)	6.21(3.16-12.21)**
Yes	206	22(2.2)		
Animal origin/source				
Home born	602	15(1.5)		
Purchase	357	22(2.2)	1.63(0.96-2.782)	2.6(1.33-5.06)**
Other source	36	-		

OR: Odds ratio; CI: Confidence interval.RHP= reproductive health problem; RFM=retained fetal membrane; other source (family gift, SISO...); cOR= crud odds ratio; aOR=adjusted odds ratio; small flock: < four head of animals; Medium flock: 5-8 head of animals; large flock: more than 9 head of animals.

Regarding to the history of reproductive health problem (RHP) for individual animals record significant higher seroprevalence (2.2%) and the chances to test seropositive were approximately six times higher (OR:6.21, 95% CI: 3.16-12.21) compared to animals which had no history of RHP.

4.2.Bacterial isolation and identification

Field bacteriological swab and tissue collection was conducted along side with serum sample collection from screened positive and clinical suspected animals. 43 vaginal swabs from mRBPT seropositive goats and 6 placenta cotyledons tissue and 4 aborted fetal tissues samples from case animals were collected through house to house flock visit and aseptically transported to laboratory and until tests were conducted keep in -20^{o}c. Total of 53 clinical samples cultured in *Brucella* selective growth media 11.32 % (6/53) isolation rate were recorded. From vaginal swabs and placenta tissue cotyledon 11.63% (5/43) and 16.67% (1/6) respectively culture isolate were harvested. All the isolates were examined using *Brucella* organisms' specific biochemical tests and found positive for two known *Brucella* species. But, no isolate was recorded from fetal abomasums content and mucosa swab cultures.

Table 8 Clinical specimen collected bacteriological isolate record

Sample type	No. of examined samples	Positive culture isolation	Overall (%)
Vaginal swab	43	5	11.63
Fetal abomasums content & mucosa swab	4	-	-
Placental cotyledon	6	1	16.67
Total	53	6	11.32

Of the six isolate, five isolates were suspected as *B. melitensis* and one as *B. ovis* through biochemical test. These identified colonies have pinpoint, round, convex with smooth/rough margin, translucent and pale honey growth was recorded. Except SS3 sample code colonies were no growth was recorded in 5% carbon dioxide supplied incubator, agglutinated with positive control serum for small ruminants brucellosis while negative with negative control serum with *anti-Brucella* polyclonal serum test positive. The cultures smear gram negative coccobacilli in Gram's staining and red coccobacilli in modified Ziehl-Nelsen (ZN) staining and non-heamolytic on blood agar. Also colonies growths were records on dye media except SS3 sample code.

Table 9 Biochemical characteristics of species of the genus *Brucella*

Isolate cod	Sample	Colony morphology	Catalase	Oxidase	Urease activity	CO_2 requirement	H_2S production	Growth on dyesa (Basic fuchsin)	Agglutination polyclonal sera
SS3	Swab	R	-	-	-	+	-	-	+
SS13	Swab	S	+	+	+	-	-	+	+
SS14	Swab	S	+	+	+	-	-	+	+
SS24	Swab	S	+	+	+	-	-	+	+
SS25	Swab	S	+	+	+	-	-	+	+
P2	Cotyledon	S	+	+	+	-	-	+	+

P: Placenta, R: Rough, S: Smooth, SS: Swab, (-): Negative, (+): Positive

Colonies morphology was identified on the growth nature in *Brucella* selective growth medium, were found rough colonies for sample code SS3 and the other were smooth and through Gram's stain and mZN characteristic belong to *Brucella* nature. Further catalase and oxidatase test were conducted reveales and support the first result. As to the secondary biochemical test the activity of the organisms splitting and hydrolsing urea compound, requirement for CO_2, H_2S production and growth on the basic fuchsin containing media were conducted and further confirms that the isolates were belongs to the *Brucella* spp.

4.3.Questionnaire survey

4.3.1. Socio economic characteristics of study district

Alongside with the serum sample collection from the districts, randomly selected (n=120) household owner, who have small ruminants were interviewed using semi structured interviews. The results showed that from the total 120 households interviewed 69.2% were males and 30.8% were females. The average age of the respondent was (39.5±12.2) years which is statistically significant across the district. The overall average family size in the study district was (6.16±2.66) head per household and was significantly different ($p<0.05$) in the district.

Assessment of educational profile of the household heads indicated that the majority were complete grade 5-8 (42.5%), then illiterate (30.8%), followed by 9-12 high school and those who attend grade 1-4 formal elementary which is 18.3% and 8.3% respectively was significant differ across the district.

The average small ruminants' population in the study district was (8.7±5.5) head of animals per household and was not significantly differ across the district. For the species composition 86.7% of the respondent owen goat, followed by 10.8% mixed spp. of both and only 2.5% had sheep, owning mixed species is very important in reproductive associated disease epidemiology in the area and the species composition is not significantly differ within the study district. With regard to the flock composition most of the respondent Owen breeding ewes and buck (67.5%) and followed by those who own only breeding dews (18.3%) was statistically significant across the district ($P<0.05$).

Table 10 Socio-economic characteristics of households in the study woredas (Mean± SD and Frequency and Chi-square values)

Parameters (%)	Study district		Overall (N=120hh)	$\chi 2$
	Aribaminch zuria (N=70hh)	Mirab Abaya (N=50hh)		
Sex of respondents				
male	70	68	69.2	.055^{NS}
female	30	32	30.8	
Age of respondents (year)	39.2±13.3	40.1±10.6	39.5±12.2	36.509 **
Marital status				
Single	17.1	10	14.2	1.224 ^{NS}
Married	82.9	90	85.8	
Family size of respondents (persons)	6.97±2.62	5.98±2.73	6.16±2.66	8.967 **
Educational profile of respondents				
Illiterate	28.6	34	30.8	
Elementary (1-4)	10	6	8.3	1.139 **
Elementary (5-8)	41.4	44	42.5	
High school 9-12)	20	16	18.3	
Respondent occupation				
Livestock rearing	58.6	80	67.5	
Agricultural activity other than livestock rearing	30	14	23.3	6.122 ^{NS}
Other activity (non agriculture activity)	11.4	6	9.2	
Small ruminants species composition				
Only goat	82.9	92	86.7	
Only sheep	4.3	0	2.5	3.059 ^{NS}
Both sheep & goat	12.9	8	10.8	
Small ruminants flock size (head)	7.7±5.1	10.1±5.9	8.7±5.5	30.655 ^{NS}

hh = interviewed households; $\chi 2$ = chi square; significant P<0.05**; Non Significant-P >0.05NS

4.3.2. Reproductive health problem across the district

Assessments for the occurrence of reproductive health disorder in the flock overall 72.5% responds were record and which were statistically significant within districts (P<0.05). A female animal with increased stage of parity had (1.93±1.51) history of reproductive disorder record across districts. For the record history of RHP 58.3% respond mixed type (i.e. Abortion, RFM, Still birth), then followed by Abortion and RFM history 32.5% and 2.5% respectively was statistically significant across districts (P<0.05).

Table 11 Reproductive Health Problem in the study woredas (Mean± SD and Frequency and Chi-square values)

Parameters (%)	Study district: Aribaminch zuria (N=70hh)	Study district: Mirab Abaya (N=50hh)	Overall (N=120hh)	$\chi2$
History of RHP within flock				
No	32.9	20	27.5	2.418**
Yes	67.1	80	72.5	
History of RHP and parity stage within flock	1.63±1.43	2.34±1.53	1.93±1.51	9.743**
Record history on RHP type				
Dystocia record	7.1	0.5	4.2	
Abortion record	30	35.5	32.5	
Uterine prolepses record	0	2	0.8	11.584**
RFM record	0	6	2.5	
Still birth	1.4	2	1.7	
Mixed type (Abortion, RFM, Still birth)	61.5	54	58.3	
Respondent consent on identifying cause of RHP				
No	80	66	74.2	2.984**
Yes	20	34	25.8	
Etiology for RHP (abortion, RFM & still birth)				
Mechanical	11.4	16	13.3	
Disease (infection)	5.7	18	10.8	7.292**
Feed poisoning	4.3	0	2.5	
Unknown cause	78.6	66	73.3	
History of flock with RHP during parturition				
No	30	24	27.5	0.527^{NS}
Yes	70	76	72.5	
Abortion history (past two breeding seasons)				
No	47.1	30	40	3.571**
Yes	52.9	70	60	
Abortion history and gestation length (month)	1.82±1.84	2.34±1.51	2.03±172	14.026**
Abortion record history per female flock (head)	0.77±0.88	1.28±1.16	0.98±1.04	7.5001**

RFM= retained fetal membrane; RHP=reproductive health problem; hh = interviewed households; $\chi2$ = chi square; significant P<0.05**; Non Significant-P >0.05NS

Across the district only 25.8% respondents identified the cause of flock reproductive health disorder but, the rest did not knew the route, which was record significant (p<0.05), among those mechanical trauma contribute 13.3% and followed by disease history (infection) on pregnant female animals by 10.8% which is statistically significant to the district. Overall 60% of the respondents reported abortion history in the flock for the past two breeding season which is statistically significant across the district, for the record history abortion average gestation length was about 2.03±172 month, were very important both biologically and epidemiologically for reproductive disease circulation like *brucellosis*. Overall 0.98±1.04 average breeding female head animals give abortion per flock in both district which is statistically significant ($p<0.05$).

4.3.3. Source of breeding animals and management practice

In both districts mainly two sources for breeding dam and sire line were identified 51.7% were sourced from home (birth) and the rest 48.3% purchase from the purchase (local market) which was record significant ($p<0.05$) for the districts. Culling as management practice for replacement stock by average 54.2% respond conduct culling significant ($p<0.05$).

Table 12 Source of breeding animals and management practice in the study woredas (Mean± SD and Frequency and Chi-square values)

Parameters (%)	Study district		Overall (N=120hh)	$\chi2$
	Aribaminch zuria (N=70hh)	Mirab Abaya (N=50hh)		
Source of breeding dam& sire line				
Market (through purchase)	42.9	56	48.3	2.017**
Home (through birth)	57.1	44	51.7	
Culling practice as management				
No	52.9	36	45.8	3.338**
Yes	47.1	64	54.2	
Methods of culling				
Sale	75.7	68	72.5	
Slaughter	18.6	14	16.7	4.669**
Other (mixed)	5.7	18	10.8	
Reason for culling				
Increase age	15.7	16	15.8	
Increase parity	17.1	14	15.8	4.698**
RHP	5.7	18	10.8	
Other (mix of age, parity, RHP etc.)	61.4	52	57.5	
Owen breeding ram/buck				
No	44.3	38	41.7	0.474NS
Yes	55.7	62	58.3	
Source of breeding ram/buck				
Home born (birth)	58.6	50	55	0.866NS
Community (through selection)	41.4	50	45	
History of programmed vaccination for flock				
No	91.4	100	95	4.511**
Yes	8.6	0	5	

hh = interviewed households; $\chi2$ = chi square; significant P<0.05**; Non Significant-P >0.05NS

Method of culling 72.5% through sale (market), then 16.7% slaughter and followed by mixed type (use both market and slaughter) 10.8%, which was significant across the district. In both district identified reason for culling was not statistically significant. Flock programmed vaccination in advance for reproductive health improvement only 5% practice over the two districts which is statistically significant ($P<0.05$).

5. DISCUSSIONS

5.1.Seroprevalence and associated risk factors

In the present study individual animal level of seroprevalence of (3.7%, 95% CI: 2.49, 4.66) was record using CFT and thus is in line with the reported study by (5.1%) Mengistu, (2007) in selected districts of southern region higher (0.4%)Yeshwas *et al.,* (2011) Bahir Dar Northwest Ethiopia, and (0.6%) Girmay *et al.,* (2013) Baku live sheep export farm and (1.56%) Dabassa *et al.,* (2013) Yabello district the difference was due to flock management and agro ecology variation. Overall highest herd level seroprevalence 10.81% (95% CI: 0.96, 7.90), 37.84% (95%CI: 6.81, 21.2) and 51.4% (95% CI: 10.64, 27.4) were recorded within small, medium and large flock size respectively which is in agreement with the past study report in other part of Ethiopia by (Asmare *et al.,* 2013a). The recoded seroprevalence in the present study in the absence of *Brucella* vaccination program in Ethiopia indicates that the disease is endemic wide spreading in the vicinity.

Among the risk factors considered in this study district, flock sizes, parity stage, age, and history of reproductive health problem and source of breeding animals were found to predispose small ruminants acquiring *Brucella* infection fit using multivariable logistic regression analysis. Study district found significantly associated with seropositivity (OR=10.35, CI95%: 3.99, 26.8) and was found the higher in camper with the study report OR= 2.37 by Abay, (1999). This difference was due to traditional “wude” herding practice and ago-ecology difference of the study area with poor reproductive health management of breeding animals.

Brucellosis is disease of sexually matured animals (Radostits *et al.,* 2000). In this finding old adult age (above three years) category were three times more likely to be seropositive than young animals (less than one year of age) (OR=3.43; 95% CI: 1.12, 10.51) and in agreement with report from Afar (Asenafi *et al.,* 2007), Borana (Megersa *et al*., 2011), South Omo (Ashagrie *et al.,* 2011), Jigjiga (Mihretab *et al.,* 2011) and South region, Oromia region (Asmare *et al.,* 2013a). Despite the inconsistency in the demarcation of adult age, all the authors reported the recovery of more sero reactors in adult age group. This increased susceptibility with increased sexual maturity is due to the influence of sex hormones and erythritol on the pathogenesis of Brucellosis (Radostits *et al.,* 2000).

In the present study average household flock size 8.7±5.5 were recorded and based on this fact the household flock size was categorized in three and using multivariable logistic regression larger the flock size (flock which have more than nine head animals) the chance of being seropositive was approximately three times higher than small and medium (3.45, CI: 1.12, 10.27) was agree with the study report (2.7, CI: 1.4, 5.1) by Asmare *et al.,* (2013a) and inconsistence with (1.06, CI: 0.52, 2.13) Gebremedhin, (2015). This difference was due to poor flock management. In most research output parity stage was not reported but, with the increased parity stage above five (6.31, CI: 1.82, 21.8) was significantly ($p<0.05$) associated with seropositivity higher than (1.71, CI: 1.185, 2.465) Asmare *et al.,* (2013a) report and this was typically due to reproductive physiology and hormone effect on individual animals genetic performance and animals breed/ population difference in their respective niche.

Regarding to the history of reproductive health problem for individual animals record significant higher seroprevalence (2.2%) and the likelihood of infection increase by six times (OR=6.21) of which have no history of RHP and was in line with the survey output. This is might be due to reproductive and genetic performance of the population. To the reproductive health problem animals with the history of abortion and retained fetal membrane had significant association with seropositivity with odds ratio (6.21, CI: 3.16, 12.21) while history of still birth is not. This due to sexual hormone effect on the pathogenesis of the disease on small ruminants animal (Radostits *et al.,* 2000). Animals sourced from market (purchase) found epidemiologically very important (OR=2.6, 95% CI: 1.33, 5.06) being seropositive two times higher than home (sourced by birth) the fact was due to unrestricted movement of animals through multi channels of trade within the neighborhood zone around the studies districts.

For serological test agreement kappa statistics shows almost perfect agreement between mRBPT and ELISA with CFT, according to the common interpretation of kappa (Dohoo *et al.,* 2003). This finding agree with Asfaw, (2014) and Abay, (1999) for mRBPT and CFT but, inconsistent with Asfaw, (1998) moderate agreement (k=0.44) report between CFT and RBPT. This was might be due to the laboratory condition during the work time here in this study all the test were conducted through OIE ISO standard at SNAHIC. In similarly the test result (k=0.958) almost perfect agreement was agree with Asfaw, (2014) for ELISA and CFT.

5.2.Bacterial culture, isolation and biochemical identification

Brucella isolation has not be done so far in small ruminants of Ethiopia hence, the present study have aimed to isolate *Brucella* spp. from seropositive small ruminants of the study area. Therefore, this work is a significant contribution to improve the understanding of circulating *Brucella* species in Ethiopia. Fifty three (53) clinical suspected samples from seropositive cases were cultured and 6 (11.32 %) isolation rates were recorded. Of which 43 were vaginal swabs 11.63% (5/43) and 6 placenta tissue cotyledon 16.67% (1/6) culture isolate were record. To best of our knowledge this the first report of isolation of *Brucella* species from small ruminants of Ethiopia. The present study from seropositive animals with the history of reproductive disorder is in agreement with the previous reports from other countries (Bricker *et al.,* 2002; Ocholia *et al.,* 2004 and Poester *et al.,* 2010; Shemesh and Yagupsky, 2012).

While the low isolation rate of 11.32% was due to the slow growing and fastidious nature of *Brucella* organisms (Seleem *et al.,* 2010; Kaltungo *et al.,* 2014) and comparable the existing study reports in *Brucella* endemic regions. 11.63% isolation rate obtained from vaginal swabs was higher than Iranian report 2.77% of isolation rate from goats (Ebrahimi *et al.,* 2014). This might be due to in the present study the swab samples were collected from screened *Brucella* positive animals with history of reproductive health problem while the Iranian study used only goats with history of abortions.

In contrast few studies had a higher rate of isolation of small ruminants brucellosis was reported (27.76%) Sahin *et al.,* (2008) and (33.8%) Buyukcangaz *et al.,* (2009) from animals with the history of reproductive health problems for vaginal swab samples. This difference may be related to the usage of more than one selective culture media in their study while in the present study only *Brucella* selective media was used. Isolation of *Brucella* spp. can be improved if more than one selective culture medium is used (Buyukcangaz *et al.,* 2009; Ali *et al.,* 2014).

Biochemical test result for *Brucella spp*. was basis for culture, morphological and biochemical characteristics (D′ıaz *et al.,* 1968; Alton *et al.,* 1988; D′ıaz-Aparicio *et al.,* 1994; Blasco *et al.,* 1994a; Blasco *et al.,* 1994b). In the present study bacteriological culture, colon morphology, modified ZN test and biochemical tests result suggested that isolate might be *B. melitensis* and *B.ovis* and agree with OIE , (2012) and need further molecular characterization.

Shedding of *Brucella* organisms through body secretion was an important source of infection in humans (Radostits *et al.,* 2000). In present study isolate of *Brucella* was harvested form vaginal swabs and placental tissue cotyledons which are very important in disease epidemiology. Since, farmers assist delivery without any personal protection which aggravates the disease circulation and need coordinated work brucellosis prevention strategies for human and animals.

5.3.Respondent consent about reproductive health problem

Form the seroprevalence study history of reproductive health problem and increased parity stage (1.93±1.51) with age increase were biologically important risk factor for the infection of brucellosis in small ruminants. Since the disease was by nature problem of reproductively mature animals (Radostits *et al.,* 2000). Within district 72.5% (n=120) of small ruminants had history of RHP and this result was inconsistent with Adugna *et al.,* (2013); (Asmare *et al.,* 2013a); Gebremedhin, (2015) this typically due flock breeding management difference, sample size, ago ecology and production system difference. And in close agreement with study report by Hadush *et al*., (2013) in center Ethiopia and Dawite and Ahmed, (2013) in northern Ethiopia who reported 44.3%and 40.3% respectively.

A female animal with overall increased stage parity (1.93±1.51) had history of reproductive disorder record across districts. For the record history of RHP 58.3% respond mixed type (i.e. Abortion, RFM, Still birth), then followed by Abortion and RFM history 32.5% and 2.5% respectively was statistically significant for the districts. Due to the sex hormone affect erytritol and abortion in late pregnancy stage was path gnomonic sign for brucellosis infection (Hafez, 1993; Radostits *et al.,* 2000). Within district female animals with abortion history had record of overall gestation length 2.03±172 month was statistically significant ($p< 0.05$) and within female flock overall 0.98±1.04 head of animals had history of abortion which is agree with the seroprevalence report in this study.

The study shows different management practice had add up high seroprevalence. Sources for breeding dam and sire line were identified 51.7% was sourced from home born and followed by 48.3% purchase from the local market which was record significant ($p<0.05$) for the districts. And most scholar were not reported association of breeding animals source with seroprevalence but, here in this study had clear risk factor in the study area.

Culling as management practice for replacement stock by average 54.2% respond significant. Of the identified reason for culling in study area increase in age and parity add up for seroprevalence was significant across the district ($p<0.05$). Epidemiologically very important for brucellosis infection in small ruminants and agree with (Adugna *et al.,* 2013; Asmare *et al.,* 2013a; Gebremedhin, 2015).

It is important for Ethiopia to introduce molecular epidemiological studies and reemphasis should be given to isolate and characterize the agent circulating at country level through advanced method of testing. So far only Asfaw (2014) had tried to isolates the agent from dairy and this work is the first report in the area and at national level, in further epidemiological study emphasis should be given to animals with recent abortion history with RBT positive that could enhance isolation rate in combination of using more than one selective media. Therefore, one many more samples are collected; geographical distribution of strains can be studied allowing bettering understanding the epidemiology of brucellosis in Ethiopia.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study indicated that small ruminants' brucellosis was endemic in the study area and was confirmed by serology and culture isolation. Overall 3.7% seroprevalence was recorded using CFT. In bacteriological iolation from seropositive animals with history reproductive health problems *Brucella spp* were isolated and 11.32% isolation rate was recorded in clinical suspected and seropositive animals. This study report on isolation *Brucella spp* in Ethiopia was the first in line with serological study to confirm brucellosis in the study area. In the present study the isolation *Brucella* from retained placenta and vaginal swab showed the significance of these clinical materials in transmission of the organism and indicate their role in the epidemiology and zoonotic significant. The serology results in the present study indicated that the disease was widespread and well established infection on small ruminants in area. Also the putative risk factors considered were district (animal location), flock sizes, parity, age, history of reproductive health problem, and source of breeding animals fit using multivariable logistic regression and found to predispose factor for seropositivity of small ruminants in the study area and also identified through checklist survey. In conclusion, bacteriological isolation combined with prevailing *Brucella* seropositivity confirm that small ruminants brucellosis infection has been endemic and potential public health implication in the areas so, strategies small ruminants breeding plan setting is priority issue to enhance productivity and zoonosis control.

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

- Further study on wider setting epidemiology and bacterial isolation combined with molecular biotyping on circulating *Brucella* agent should be conducted;
- Regular screening test of candidate dam and sire line in breeding programs through recording on pedigree information;
- Feasible national and regional brucellosis control strategies must be designed in further

7. REFERENCE

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8. APPENDIXES

Appendix I: Modified Rose Bengal Plate Test Procedure

Rose Bengal plate test is a simple spot agglutination test using antigen stained with rose Bengal and buffered to a low PH, usually 3.65± 05.

Material employed

- Reagents: RBPT brucella antigen, test sera, positive and negative control serum.
- Apparatus: plat, micropipette of 30µl, applicator stick and magnifying glass.

Procedures (Alonso *et al.,* 1988)

- The sera and antigen were removed from the refrigerator and left at room temperature for at least 30 minutes before the test was commenced.
- Briefly, 75µl of the sera sample dispensed on to the plate and volume of 25µl of RBPT antigen were dropped along the side of the sera to produce a zone of approximately equal to 0.2cm in diameter.
- By using applicator sticks the antigen and sera were mixed.
- The mixture then rocked gently for four minutes at ambient temperature and examined for agglutination.
- For interpretation of results both positive and negative control were employed.

Interpretation: Reaction were interpreted as "0", "+", "++" and "+++"

Where: - 0 = No agglutination

\+ = barely visible agglutination, using magnifying glass.

++ = Fine agglutination, somewhat clearing

+++ = Coarse clumping, definite clearing

Those samples with no agglutination were recorded as negative while those with any visible reaction ("+", "++" and "+++") were recorded as positive (MacMillan, 2012).

Appendix II: Complement fixation test Procedure

Principle: the complement system consists of series of protein that, if triggered by an antigen-antibody complex react in a sequential manner to cause cell lysis. The test has two steps, in the first step antigen, test serum and complement are mixed and incubated, and in the second step an indicator system which consists of sheep red blood cells (SRBC) and amboceptor (sensitized RBC to the action of complement) is added. If the test serum contains antibodies to *Brucella* an antigen-antibody complex is formed (positive reaction) and the complement is used up, so it cannot react in the hemolytic system. Therefore, no lysis of SRBC and will occur intact as sediment. If the test serum does not contain *Brucella* antibodies complement will not fixed and lysis of SRBC. Indicator system SRBC and amboceptor forms immune complex.

Procedure

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

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

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

- Repeat steps ii and iii above for each serum to act as anti-complementary serum controls (see below).

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

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

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

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

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

Interpretation

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

Appendix III: Enzyme Linked Immuno sorbent Assay Procedure

The diagnostic INGEZEM BB COMPAC kit is designed to detect antibodies specific for LPS of *Brucella* spp. Principles of the test is the sold plates coated with purified LPS of *B. abortus*. After adding the sample to the well, if it contains specific antibodies against *Brucella*, they will bind to the antigen absorbed on the plate while if the sample does not contain specific antibody they will not bind to the antigen. If we add a specific monoclonal antibody (conjugated peroxidase) against LPS antigen coated to the plate, it will compete with the antibody of the serum. If the serum sample contains specific antibodies, they will not permit the binding of the labeled Mab to the antigen whereas if it does not contain specific antibodies the Mab will bind them to the antigen on the plate. After washing the plate to eliminate all non fixed material, we can detect the presence or absence of labeled Mab by adding the substrate (TMB) that in presence of the peroxidase will develop a colorimetric reaction.

Procedure

- Allow all the reagents to come to room temperature ($21^0c \pm 5^0c$) before use. Homogenize all reagents by inversion or vortex.
- Add 25µl of buffer 13 to each well. 25µl of the positive control to wells A1 and B1. 25µl of the negative control to wells C1 and D1 and 25µl of each sample to be tested to the remaining wells. Mix and seal. Incubate 1 hr at room temperature (20 - 25^oc);
- Wash each well 4 times with approximately 300 micro litter of the wash solution. Avoid drying of the wells between washings.
- Prepare the conjugate 1X by diluting the conjugate 10X to 1/10 in dilution buffer 4. Add 100 µl of the conjugate 1X to each well. Incubate 1 hr at room temperature (20 - 25^oc);
- Wash each well 3 times with approximately 300 micro litter of the wash solution. Avoid drying of the wells between washings.
- Add 100 micro litter of the substrate solution to each well. Incubate 10 min ± 2min at room temperature (20 - 25^oc);
- Add 100 µl of the stop solution to each well in order to stop the reaction.
- Read and record the O.D result.

Interpretation of the Results

Calculate the percentage of inhibition (PI) of each sample as follow:

PI = 100 [1-(OD sample / OD negative control)]

The samples with PI ≥ 40% should be considered as positive for *Brucella* antibodies and vice versa will be negative for *Brucella* antibodies.

The test is validated if:

- The mean value of the negative control O.D. is greater than 1.
- The mean value of positive control is less than 0.35

Appendix IV: Gram's Stain Procedure

Organisms are classified according to their Gram staining reaction. Gram positive bacteria have thicker and denser **peptidoglycan layers** in their cell walls. Iodine penetrates the cell wall in these bacteria and alters the blue dye to inhibit its diffusion through the cell wall during decolourisation. Gram positive bacteria must have an intact cell wall to produce a positive reaction. Gram negative cells which do not retain the methyl/crystal violet are stained by a counterstain. Neutral red, safranin or carbol fuchsin may be used as the counter stain (Duguid 1996).

Procedure

Form a liquid culture, take a loop full of bacteria emulsify it in a small drop of water or saline on the slide.

- Prepare a smear and heat gently to fix
- Flood the slide with 0.5% methyl/crystal violet and leave for 30s
- Tilt the slide; pour on sufficient (1%) Lugol's iodine to wash away the stain, cover with fresh iodine and allow to act for 30s
- Tilt the slide and wash off the iodine with 95-100% ethanol, or acetone, until colour ceases to run out of the smear
- Rinse with water
- Pour on 0.1% counterstain (neutral red, safranin or carbol fuchsin) and leave to act for about 2min
- Wash with water and blot dry

Interpretation of the result: Gram positive organisms stain deep blue/purple. Negative Result: Gram negative organisms stain pink/red.

Appendix V: Modification of the Ziehl–Neelsen's method (Brucella staining)

Dilute carbol fuchsin preparation- as Gram stain, Acetic acid (decolorizer), concentrated acetic acid -1.0ml, Distilled water 200.0ml and Methylene blue solution- as Ziehl neelsen counter stain

Procedure

- Fix a smear by heat
- Overlay the slide completely with diluted carbol fuchsin for 15 minutes
- Differentiate the smear for 15 seconds 0.5% acetic acid and wash in with tap water
- Counter stain with 3% malachite green or methylene blue solution for 2 minutes, was again with tap water and dry it

Interpretation

- Brucella species appeared red coccobacilli or short rods
- Other bacteria and background appear green.

Appendex VI: Bacteriology tissue sample preparation form aborted fetus material

Material: scissors, forceps, sterile pipit and sterile swab, transport media

Procedure:

- Place aborted fetus on the table and follow the ventral side
- Made incision on the ventral side behind the umbilical cored
- Expose and put out the abomasums
- Put small opening on the abomasums
- Holed the opening with sterile forceps

Collection:

- Aspirate the abomasums content with sterile pipit and put on transport tube
- Take abomasums mucus by sterile swab and put to transport media for further processing
- Take piece of placental cotyledon tissue and put transport tube for further processing

Preparation for culture:

- Tissues were dissolved in the sterile saline water at the same concentration and homogenized with vortex mixer and centrifuged at high revelation to take sediment for inoculation on solid media.
- Abomasum contents and homogenates removed for culture were inoculated on agar plates

- incubated in 37 °C and with or without 5% CO_2 conditions for 3-8 days for growth

Appendix VII: Oxidase test

Principles: Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein).

These are available in the form of impregnated oxidase test discs/strips or ready to use bottled reagents/droppers Bacteriological straight wire/loop (platinum) or disposable alternative Filter paper

- Soak a piece of filter paper in a sterile petri dish with the reagent solution
- Scrape some fresh growth from the culture plate with a disposable loop or stick and smear onto the treated filter paper OR
- Touch a colony with the edge of the moist treated filter paper
- Observe for colour change within 10s

Interpretation for result all reaction times listed are based upon freshly made reagents without stabilising agents. If commercially prepared reagents are used, it should be noted that these often contain stabilising agents and therefore manufacturer's instructions should be followed. Positive Result Development of a deep purple-blue/blue colour indicates oxidase production. Negative Result No purple-blue colour/No colour change. Note: Microorganisms are oxidase positive when the colour changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the colour changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the colour does not change or it takes longer than 2 minutes.

Appendix VIII: KOH test

Principles: In the presence of potassium hydroxide, Gram negative cell walls are broken down, releasing viscid chromosomal material which causes the bacterial suspension to become thick and stringy. Gram positive organisms remain unaffected. Hence the alternative name for this procedure, the "String Test".

Discrete colonies growing on solid medium, 3% potassium hydroxide in water, Microscope slide Bacteriological straight wire/loop or disposable alternative

Procedure

- Place one drop of 3% potassium hydroxide solution on a clean microscope slide
- Emulsify a few colonies of the suspect organism to the drop of potassium hydroxide to make a dense suspension
- Stir continuously for 60sec and then gently pull the loop away from the suspension
- Observe any changes - a string of the suspension will follow the loop when it is raised

Interpretation Positive Result: Organisms become thick, stringy and form long strands within the first 30sec. This is seen in Gram negative bacteria. Negative Result: Organisms leave the suspension unaltered or absence of stringing. This is seen in Gram positive bacteria.

Appendix IX: Catalase test

Principle: The catalase test is used to detect the presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen

Discrete bacterial colonies on solid medium, inoculated pure agar slant culture Hydrogen peroxide solution 3–6 % commercial preparations are available. Clean capped test tubes (plastic or glass) or Bijoux bottles Bacteriological straight platinum wire/loop or disposable alternative

- Place 4 to 5 drops of hydrogen peroxide solution in a test tube or bijoux bottle
- Carefully pick a colony to be tested with a wire/loop or disposable alternative
- Rub the colony on the inside wall of the bottle just above the surface of the hydrogen peroxide solution
- Cap the tube or bottle and tilt it to allow the hydrogen peroxide solution to cover the colony
- Observe for immediate bubble formation

Result and interpretation: Positive Result Vigorous bubbling indicates the presence of catalase. Negative Result No bubbling indicates the absence of catalase. Note: Both positive and negative control must be tested alongside the test organism.

Appendix X: Urease test

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

Procedure

- Prepare the urea concentrated solution by dissolving 29g of urea in 100ml of distilled water.
- Sterilize by filtration (using filter Millipore 0.22µm).
- Dissolving 15g of Bacto agar in 900ml of distilled water and heat it to dissolve the agar; adjust the PH to 6.8-6.9
- Autoclave at 115^{o}c /20min and cool the agar in Bain marie at 45^{o}c
- Add 100 of the concentrated urea solution and mix carefully, then distribute the media in sterile slope
- Inoculate with loop full of pure culture of the test organisms and incubate at 35±2^{0c} for 18-24hrs.

Interpretation

The organism that hydrolse urea rapidly urea split (red/pink color through the media) my produce positive reaction within 30 min to 1hrs and less active spp may require more time (even more than days).

Appendix XI: Questionnaire format prepared for animal owners

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

Checklist format prepared for flock owners (at smallholder level)

1. **Purpose:** The purpose of this survey is to assess risk factors for brucellosis infection in SR rearing.
2. **Address:**
   District_____________________________________PA________________________________
3. **Demography**

Respondents name	
Sex	[1].Male, [2].Female
Age	# ………..
Marital status	1.Single, 2.Married
Family size	# ………..
Level of Education	[1].Illiterate,[2].Elementary, [3].High school, [4].College, [5].other
Working area & Occupation	[1]. Livestock rearing, [2].other agricultural activity, [3]. Other
Wife's or husband occupation	[1]. Livestock rearing, [2].other agricultural activity, [3]. Other

4. **Flock information**

I. Small ruminants flock size and composition

Animal composition	**Category**	**Flock size #**
Do you have goat? [1]. Yes, [0]. No	Total	
	Breeding dews	
	Breeding buck	
Do you have sheep? [1]. Yes, [0]. No	Total	
	Breeding ewes	
	Breeding buck	

5. **RHP (reproductive health problem)**

I. Have you observed RHP in your flock? [1]. Yes [0]. No, If yes how many RHP have you encountered during the last 2 breeding season in your farm?

Cod	**RHP**	**Occurrence [1].Yes, [0]. No**	**Parity stage (0, 1, 2, 3….)**
[1]	Dystocia		
[2]	Abortion		
[3]	Uterine prolepsis		
[4]	RFM		
[5]	still birth		

II. Did you know the cause of RHP in your flock? [1]. Yes, [0]. No**,** If yes, which is the cause of RHP [1] Mechanical injury, [2] infection, [3] Feed poison [4] unknown

III. Did your flock be tested for any RHP? [1]. Yes, [0]. No, If yes please tell us for which disease & what was the rest result ________________________

IV. Is there any problem on your ewe during parturition? [1]. Yes, [0]. No**,** If Yes, please mention the problem ______________________

V. Do any of your ewes aborted in the past two breeding season? [1]. Yes, [0]. No, If Yes, please mention the time of abortion (period of gestation in month) and how many ewe aborted at time _______________(month) and _____________

VI. Did you identify what was /are the cause of abortion? _________________________________

6. **Risk factor for brucellosis**

a. Source for small ruminants breeding dam/sire line:

Source	**Source** [1]. Yes, [0]. No	**Rank (1, 2, 3)**
Market (Purchase)		
Birth		
Gift		
Research institute		
BoLF aid		
NGO support		
Other (specify)		

b. Did you practice culling of small ruminants (breeding dam/sir line) **1.Yes, 2.No**.

c. Ways of culling & reason of culling?

Reason for culling	[1]. Yes, [0]. No	**Ways of culling**	[1]. Yes, [0]. No
[1]Aging		[1]Sale	
[2]Increase parity (above 4)		[2]Slaughter	
[3]Reproductive health problem		[3]Other (specify)	
[4]Disease /ill			
[5]Other (specify)			

d. Management practice as major risk factor for the occurrence of brucellosis & other disease

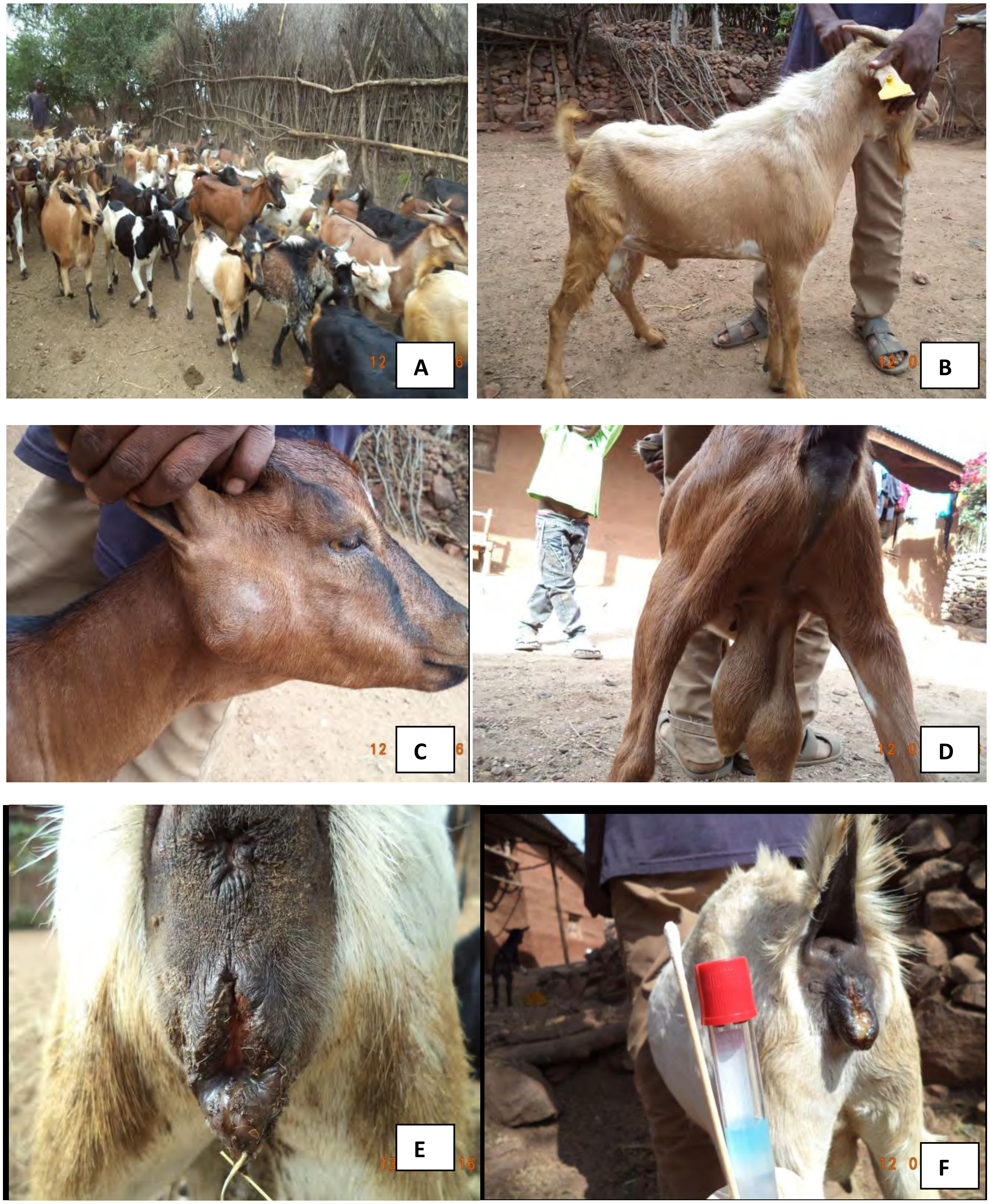
Management practice	**Rank**
Do the animals have their own shelter (housing)	[1]. Yes, [0]. No
Are the animals kept indoors (feeding)	[1]. Yes, [0]. No
Do your animals make close contact with other herd animals neighboring	[1]. Yes, [0]. No

e. Do you use your Owen breeding ram [1]. Yes, [0]. No**,** if so please tell us the source of breeding ram?

Source	[1]. Yes, [0]. No	**Rank (1, 2, 3)**
Home born (owned ram)		
Community (via selection)		
Aid ram by NGO, RI, BoLFR		

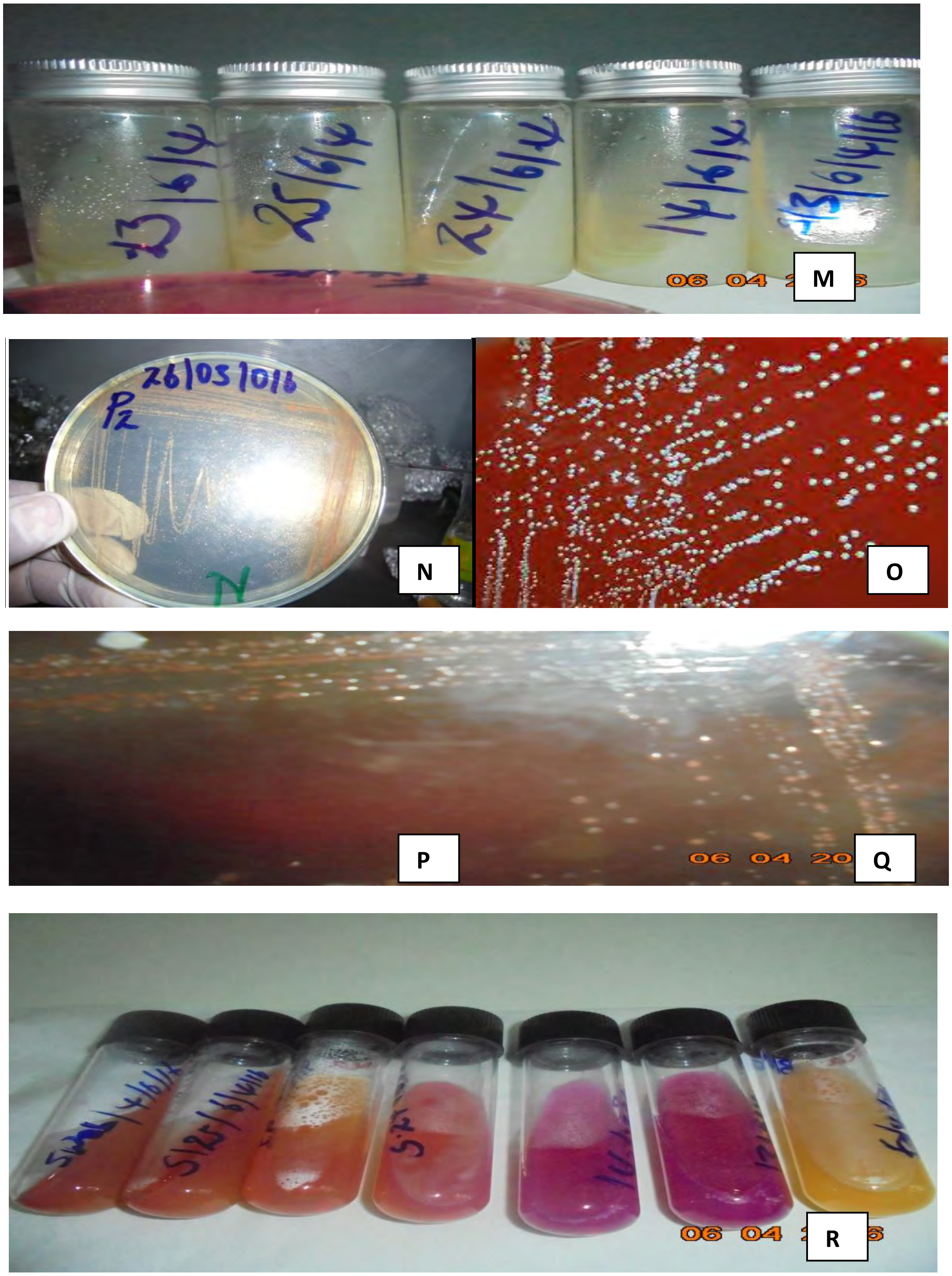
f. Did you practice vaccination to your flock? [1]. Yes, [0]. No, if yes when did you vaccinate? For which disease was the vaccine given__________________

Appendix XII: Picture taken during field and laboratory work



[A] Flock (Fetele Pa's); [B] initial candidate buck for selection; [C] Dews with history of reproductive disorder; [D] buck with poor breeding value due to scrotal position; [E] Dew with the history of recent abortion; [F] vaginal swab collection case

[G] Aborted fetus sample; [H] postmortem of fetus abomasums and content; [I] serum sample; [J] mRBPT result with plate; [K] CFT result with plate; [B] ELISA test plate coated with Anti-gene and test serum



[M] preserved brucella suspected colonies with gycerol in slnte; [N] pine and small colonies growth on brucella selective conda base medium; [O] non hemloytic smooth colone of brucella on blood agar; [P] grwth on dey medium; [Q] non hemolytic rough colone on blood agar; [R] Urease test

Appendix XIII Biographical Sketch

The author, MELESE YILMA ZABA was born on 19/Jun/1987 in Areka town of Boloso Sore Woreda, Wolaita Administrative zone. He attended his elementary education in Areka WADU Elementary School from 1991 to 1994 and his junior education at Areka Junior School from 1995 to 1998. He also attended his senior seconadary education at Areka Preparatory and Senior Secondary School from 1999 to 2004. He joined Addis Ababa University college of Veterinary Medicine and Agriculture and was awarded a Diploma in Doctor of Veterinary Medicine (DVM) in 2010. Soon after graduation, he was employed by the former Ministry of Agriculture now Ministry of Livestock and Fish Resource as Animal health, District level Southern Tsetse Eradication Program (STEP) focal person and health team leader, at Wolaita zone Agricultural and Rural Development Main Department at Damote Wode (Bedessa) Woreda and served for six month. Then he was employed by Southern Agricultural Research Institute as *Ass. Researcher II* in Livestock Research Director at Areka Research Center and at the research center delighted to coordinate livestock research process alongside with his research activity until he joined back to school of Graduate study of Addis Ababa University to pursue degree of Master of Science in Veterinary Microbiology in September 2015. He has published four research output in intentional accepted pre reviewed and reputable journals during his stay in Research institute.