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SEROPREVALENCE OF BRUCELLOSIS AND ISOLATION OF *BRUCELLA* FROM
SMALL RUMINANTS THAT HAD HISTORY OF RECENT ABORTION IN SELECTED
KEBELES OF AMIBARA DISTRICT, AFAR REGION, ETHIOPIA

MSc THESIS



BY

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Microbiology, Immunology and Veterinary Public Health

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

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by Muluken Tekle Gebretsadik entitled: **Seroprevalence of Brucellosis and Isolation of *Brucella* From Small Ruminants That Had History of Recent Abortion In Selected *Kebeles* of Amibara District, Afar Region, Ethiopia** and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Science (MVSc) in Microbiology.

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STATEMENT OF THE AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements 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 institution anywhere for the award of any academic degree, diploma, or certificate.

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
ALIPB	Aklilu Lemma Institute of Pathobiology
AMOS PCR	Abortus-Melitensis-Ovis-Suis Polymerase Chain Reaction
BSL3	Biosafety Level three
CFT	Complement Fixation Test
CSA	Central Statistics Agency
CVMA	College of Veterinary Medicine Agriculture
DAGRIS	Domestic animal genetic resources information system
ELISA	Enzyme Linked Immunosorbent Assay
EPAIAT	Ethiopian Participatory Applied Assessment Team
ESGPIP	Ethiopian Sheep and Goat Productivity Improvement Program
FAO	Food and Agriculture Organization
ILRI	International Livestock Research Institute
m.a.s.l.	Meter above sea level
m.b.s.l	Meter below sea level
MoARD	Ministry of Agriculture and Rural Development
mRBPT	Modified Rose Bengal Plate Test
MZN	Modification of the Ziehl–Neelsen
OIE	Office International des Epizooties
OPS	O polysaccharide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFE	Pastoralist Forum Ethiopia
PRRs	Pattern Recognition Receptors
SDA	Serum Dextrose Agar
SLPS	Smooth Lipopolysaccharide
SRBC	Sheep Red Blood Cells
SSA	Sub-Saharan Africa
TLR	Toll-Like Receptor
WHO	World Health Organization

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ABSTRACT

Brucellosis is one of the most frequent contagious neglected bacterial diseases with a serious veterinary and public health importance through out the world. A cross-sectional study was conducted from October 2015 to April 2016, to determine the seroprevalence of brucellosis and isolate Brucella from small ruminants (sheep and goats) that had history of recent abortion in selected kebeles of Amibara District, Afar Region. Ethiopia. Blood samples were collected from a total of 226 small ruminants that had recent history of abortion for serological test. Subsequently, 65 specimens (28milk, 28 vaginal swabs, 2 fatal abomasal contents and 7 fetal membranes) were investigated from seropositive animals for Brucella species isolation. All serum samples collected were screened serologically using the modified Rose Bengal Plate Test (mRBPT) and sera positive with mRBPT were confirmed with Complement Fixation Test (CFT). An overall seroprevalence among small ruminants estimated at 7.52% (17/226; 95% CI: 7.41–1.36) were positive by combined mRBPT and CFT analysis, while 12.4% (28/226; 95% CI: 1.22–16.44) were found positive by mRBPT alone. Reproductive characteristics such as the stage of abortion ($\chi^2=11.26$; $P < 0.001$) and history of retained fetal membrane ($\chi^2=16.53$; $P<0.05$) were significantly associated with increased seropositivity result. A multivariable logistic regression analysis revealed that age (OR=14.57, 95% CI: 1.72–123.6), parity status (OR=8.08, 95% CI: 1.11–58.57) and stage of abortion (OR=8.94, 95% CI: 1.86–42.90) were significantly associated with Brucella infection in clinically aborted small ruminants ($P < 0.05$). Brucella was isolated from 9 (13.8%) samples out of the 65 bacteriological samples cultured on Brucella Selective Agar. Among the overall isolates, 3(10.7%) and 6 (21.4%) were from milk and vaginal swabs, respectively. In this study all the isolates were obtained from seropositive goats milk and vaginal swab. The isolates were B. melitensis based on biochemical test result, though further test is required to biovariant level. In conclusion, the present serological test revealed that brucellosis is moderately prevalent among aborted small ruminants in the study area. The isolation of B. melitensis from an aborted goats milk and vaginal swabs was not substantially reported in Ethiopia until now, this report may be considered one of the indicative studies. Therefore, further extensive molecular studies of the isolates and appropriate control strategies are required to reduce its economic impact and risk of zoonotic infection in the area.

Key words: Abortion, Afar Region, B. melitensis, Brucellosis, isolation, seroprevalence, small ruminants

1. INTRODUCTION

Brucellosis is a contagious bacterial zoonotic disease of veterinary and public health importance in developing countries. The disease affects domestic animals (cattle, sheep, goats camels and pigs), humans and wildlife. It is caused by various *Brucella* species such as *B. melitensis* in small ruminants, *B. abortus* in cattle, *B. suis* in swine and *B. canis* in dogs, while all the species are known to zoonotic importance. *Brucella* species are slow-growing, Gram negative, small coccobacilli and intracellular bacteria that is capable to survive and multiply within epithelial cells, placental trophoblasts, dendritic cells and macrophages (Gorvel, 2008).

Brucella melitensis is considered to have the highest zoonotic potential followed by *B. abortus* and *B. suis*. According to the Office for International des Épizooties (OIE), the disease is also classified as one of the neglected zoonoses with a serious veterinary and public health importance through out the world (WHO, 2006; OIE, 2009). Globally, it is estimated that nearly 500,000 cases of brucellosis would occur in humans every year (Pappas *et al.*, 2006), and often persists in the poorest and most vulnerable populations (FAO, 2003).

The economic and public health impact of brucellosis remains of concern in developing countries (Roth *et al.*, 2003). The disease poses a barrier to trade of animals and animal products, an impediment to free animal movement (Zinsstag *et al.*, 2011). It also causes losses due to abortion or breeding failure in the affected animal population, diminished milk production and in human brucellosis causing reduced work capacity through sickness of the affected people (FAO, 2003). In Africa and central Asia, the incidence of brucellosis is generally considered higher in pastoral settings. However, because of the difficulty to access pastoral communities, the occurrence and the control of brucellosis is poorly understood both in humans and their animals in the pastoral settings of the sub-Saharan Africa where the burden of the disease could be high (Mcdermott and Arimi, 2002).

According to the Central Statistics Agency (CSA), Ethiopia is one of the developing countries with domestic small ruminant population estimated to be 27.35 million sheep and 28.16 million goats (CSA, 2014). Small ruminants are the chief source of cash income to small holders (EPAIAT, 2003; Akabarmehr and Ghiyamirad, 2011). This is because sheep and goat provide

rapid cash turnover (OIE, 2009; Godfroid *et al.*, 2011). Most of the sheep and goat populations in Ethiopia are raised under pastoral conditions. These small ruminants and their milk/meat products represent an important export commodity, which significantly contributes to the national economy. At optimum offtake rates, Ethiopia can export 700,000 sheep and 2 million goats per year and at the same time supply 1,078,000 sheep and 1,128,000 goats for the domestic market (Alemu and Markel, 2008).

Even though these animals contribute much to the national economy, however, their production is hampered by different constraints in Ethiopian pastoral areas. Among many factors that limit economic return from small ruminants, reproductive diseases including brucellosis are the major disease affecting pastoral areas (ILRI, 2006). Studies also showed 3.6–16% seroprevalence of brucellosis in small ruminants in pastoral areas of Afar, Oromia and Somalia regional state (Yibeltal, 2005; Ashenafi *et al.*, 2007; Tsehay *et al.*, 2014). Recent study in Ethiopia by Tschopp *et al.* (2015) showed poor community's knowledge about brucellosis and high risk for *Brucella* infection among pastoralist communities adjacent to Awash National Park.

In Ethiopia, a number of studies shows individual seroprevalence ranging from 0.1–15.2% in different parts of the country (Berehe *et al.*, 2007; Regassa, 2009; Asmare *et al.*, 2010; Hailesillasie *et al.*, 2010; Megersa *et al.*, 2011) and most of them are largely confined to serological surveys. Though, isolation of *Brucella* species is the gold standard of identification and confirmation of animal brucellosis, there is no research done to get evidence by bacteriological isolation of the causative agent.

Therefore, the objectives of this study were to:

- ◆ Determine the seroprevalence of Brucellosis in small ruminants that had history of recent abortion.
- ◆ Isolate and characterize *Brucella* species from sheep and goats with a history of recent abortion.
- ◆ Evaluate the degree of association of potential risk factors and seropositivity of *Brucella*.

2. LITERATURE REVIEW

2.1 Brief Historical Overview and Taxonomy of Brucellosis

The history of brucellosis does not begin with the isolation and identification of *Brucella melitensis* (*Micrococcus melitensis*) in the 1880s. Many historical accounts of diseases before this time could actually be describing brucellosis including abortion epidemics in animals and fever in humans. Other than biblical references to animal abortions, one of the earliest recorded descriptions of brucellosis was made by Marston in 1859 (Vassalo, 1992). The paleo-pathological evidence from the partial skeleton of the late Pliocene *Australopithecus africanus* suggests that brucellosis occasionally affected our direct ancestors 2.3–2.5 million years ago (D'anastasio *et al.*, 2011).

The pathological, molecular (DNA analysis) and electron microscopy findings from the human skeletal remains (D'anastasio *et al.*, 2011), buried cheese remains (Capasso, 2002) also suggested the presence of brucellosis long time ago (3000-1200 B.C.) in Bahrain, Persian Gulf, 2100–1550 B.C. in Palestine and Jordan, 79 A.D. in Roman town Pompeii and Herculaneum. 1260–1020 A.D. in Butrint, Albania.

However, brucellosis is named after Sir David Bruce, who in 1886 isolated the causative agent from a soldier in Malta where the disease caused considerable morbidity and mortality among British military personnel. During the 19th century, brucellosis was known as Malta or Mediterranean fever. In 1897, Danish veterinarian Bernhard Bang isolated *B. abortus* as the agent; and the additional name "*Bang's disease*" was assigned. The popular name "*undulant fever*" originates from the characteristic undulance (or "wave-like" nature) of the fever, which rises and falls over weeks in untreated patients. Also different synonyms of Brucellosis include: Malta fever, Mediterranean fever, Gibraltar fever, Cyprus fever, Rock fever enzootic abortion, epizootic abortion, contagious abortion, and typhomalarial fever in animal and human (Charters, 1980).

Brucella is taxonomically placed in the alpha-2 subdivision of the class Proteobacteria. There are 10 species of *Brucella* based on preferential host specificity: *B. melitensis* (goats), *B. abortus*

(cattle), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep), *B. neotomae* (desert wood rats), *B. cetacea* (cetacean), *B. pinnipedia* (seal), *B. microti* (voles), and *B. inopinata* (unknown) (O’Callaghan and Whatmore, 2011). *B. melitensis* (small ruminants), *B. abortus* (cattle), *B. suis* (swine), and *B. canis* (dogs) are known to cause human disease. *B. neotomae* (desert wood rats) and *B. ovis* (sheep) are not pathogenic to humans. The majority of human cases worldwide are attributed to *B. melitensis* (pappas, 2006). In general, *B. melitensis* and *B. suis* are more virulent for humans than *B. abortus* or *B. canis* (WHO, 2006). *B. melitensis*, *B. abortus*, and *B. suis* have 3, 7, and 5 biotypes, respectively (Alton *et al.*, 1988; Lindquist *et al.*, 2007). Sequencing and annotation of the genomes of *B. suis*, *B. melitensis*, and *B. abortus* has been completed; the majority of the open reading frames share greater than 99 percent sequence similarity between species (Paulsen *et al.*, 2002; Halling *et al.*, 2005).

2.2 Etiology

Brucella infection is caused by species of the bacterial genus *Brucella* (Morgan and MacKinnon, 1979; Halling and Young, 1994). Livestock like cattle, goats, sheep, pigs, buffaloes, camels, reindeer and other mammals are affected by brucellosis (Charters, 1980). *Brucellae* are Gram-negative coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide, nonmotile, non-spore forming, non-capsulated, non-flagelated, aerobic, facultative intracellular bacteria capable of invading, survive and multiply within epithelial cells, placental trophoblasts, dendritic cells and macrophages (Gorvel, 2008).

The bacteria are usually arranged singly, and less frequently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. 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. On suitable solid media, *Brucella* colonies can be visible after 2–3 days incubation at 37°C. After 4 days’ incubation, *Brucella* colonies are round, 1–2 mm in diameter, with smooth margins. They are translucent and a pale honey colour when plates are viewed in the daylight through a transparent medium. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker (OIE, 2009).

The cellular and colonial morphology of the *Brucella* species are similar in most respect. All *Brucella* species possess smooth lipopolysaccharide (SLPS) in their outer cell wall except *B. ovis* and *B. canis*, which have rough lipopoly-saccharide (RLPS) and protein antigens (Blasco *et al.*, 1990). Smooth lipopolysaccharide contains an immunodominant O-polysaccharide which has been chemically defined as a homopolymer of 4, 6- dideoxy-4-formamide-alpha-D mannose linked through glycosidic linkages.

Smooth *Brucella* cultures, especially *B. melitensis* cultures, have tendency to undergo variation during growth, especially with subcultures, and dissociate to rough (R) forms, and sometimes mucoid (M) forms. Colonies are then much less transparent with more granular, dull surface (R) or a sticky gelatinous texture (M), and range in colour from matt white to brown in reflected or transmitted light. Intermediate (I) forms between S, R and M forms may occur in cultures undergoing dissociation to the non-smooth state. Changes in the colonial morphology are generally associated with changes in virulence, serological properties and phage sensitivity (OIE, 2009).

2.3 Epidemiology of Brucellosis

2.3.1 Geographical distribution

The geographical distribution of brucellosis is constantly changing, with new foci emerging or re-emerging. The epidemiology of human brucellosis has drastically changed over the past few years because of various sanitary, socioeconomic, and political reasons, together with increased international travel. New foci of human brucellosis have emerged, particularly in central Asia, while the situation in certain countries of the Middle East is rapidly worsening (Pappas *et al.*, 2006).

Brucellosis is a disease of worldwide distribution occurring in domestic as well as wild animals. It has been reported wherever animals are raised all over the world (Seifert, 1996). Although some of the industrialized countries in Europe and America have achieved eradication of brucellosis in domestic animals through intensive control and eradication schemes, the disease is still a serious problem in developing countries (Warner, 2001; Ragan, 2002).

Brucella melitensis is the most virulent species of the *Brucella* genus and has three biovars, with biovars 1 and 3 being the ones isolated most frequently in small ruminants in the Mediterranean, the Middle East and Latin America (Blasco and MolinaFlores, 2011; Lucero *et al.*, 2008). Brucellosis is a barrier to trade in animals and animal products and causes significant losses from abortion, as well as being a serious zoonosis (Benkirane, 2006; Banai, 2007; Seleem *et al.*, 2010).

2.3.2 Risk factors for brucellosis

The prevalence of brucellosis is influenced by a number of risk factors related to production systems, biology of the individual host and environmental factors. These include age, herd size and composition, hygienic status of the farm, rate of contact between infected and susceptible animals, farm biosecurity and climate (McDermott and Arimi, 2002; Radostits *et al.*, 2007).

The prevalence of brucellosis can vary according to climatic conditions, geography, species, sex and age (Gul and Khan, 2007). Brucellosis occurs in sexually mature animals, the bacteria localizing mainly in the reproductive tract especially in pregnant animals; there is also evidence that mammary gland may be even more favored for localization than the reproductive tract (Anonymous, 2007).

The proportion of people relying on livestock for some or their entire livelihood is very high in Africa, ranging from 20 to over 90%, depending on the livestock production system and country in focus (McDermott and Arimi, 2002). In the rural parts of Ethiopia, for instance, human life is highly associated with livestock population in the different livestock production systems. In both pastoral and mixed livestock production systems people live very closely with livestock having a high incidence of brucellosis and thus, are at higher risk of acquiring the infection (Gebretsadik *et al.*, 2007).

Brucellosis in human is common in rural areas because farmers live in close contact with their animals and often consume fresh unpasteurized dairy products (Habtamu *et al.*, 2015). In Ethiopia pastoral communities, the traditional habits of raw milk consumption, handling of aborted materials, manipulation of reproductive excretions with bare hands and herding of a large number of animals mixed with other animals are widely practiced. This strong dependence of the

pastoralists on their livestock would favour the transmission of zoonotic diseases like brucellosis, bovine tuberculosis and anthrax (Bekele *et al.*, 2013).

Risk factors for human brucellosis include the handling of infected animals, ingestion of contaminated animal products such as unpasteurized milk and milk products (including cow, goats and camel milk), meat, history of travel to endemic areas and improper handling of cultures of *Brucella* species in laboratories. Other risk factors include: abattoir workers, veterinarians, slaughterhouse workers and dairy workers (Corbel, 2006).

However, recent study conducted by Tschopp *et al.* (2015) showed poor community's knowledge about brucellosis and risk for brucellosis among pastoralist communities adjacent to Awash National Park Ethiopia. Hence, community based study of knowledge of brucellosis would be worthwhile to create awareness about the disease, improve of knowledge, attitudes and practices among livestock owners, and which could have significant impact on the reduction of the transmission of the disease from animals to humans.

2.3.3 Transmission

The mode of transmission of *B. melitensis* in sheep and goats is similar to that in cattle but sexual transmission probably plays a greater role. The transmission of disease is facilitated by comming of flocks and herds belonging to different owners and by purchasing animals from unscreened sources. The sharing of male breeding stock also promotes transfer of infection between farms. Transhumance of summer grazing is a significant promoting factor in some areas as is the mingling of animals at markets or fairs. In cold climates, it can be the custom to house animals in close space and this also facilitates transmission of infection (Habtamu *et al.*, 2015).

In cattle and other bovidae, *Brucella* is usually transmitted from animal to animal by contact following an abortion. Pasture or animal barn may be contaminated and the organisms are probably most frequently acquired by ingestion but inhalation, conjunctival inoculation, skin contamination and udder inoculation from infected milking cups are other possibilities. The use of pooled colostrums for feeding newborn calves may also transmit infection. Sexual transmission usually plays little role in the epidemiology of bovine brucellosis. However,

artificial insemination can transmit the disease and semen must only be collected from animals known to be free of infection (OIE, 2009).

Brucellosis in human also known as “undulant fever”, “Mediterranean fever” or “Malta fever” is a zoonosis and the infection is almost invariably transmitted by direct or indirect contact with infected animals or their products. It affects people of all age groups but those less than 14 ages are less susceptible and of both sexes (Corbel, 2006). Human brucellosis is caused mainly by *B. abortus*, *B. melitensis* and *B. suis*, also the main causes of brucellosis in cattle, goats/sheep and pigs respectively (WHO, 2006; Makita *et al.*, 2011). Wildlife animals are also equally affected and these may act as reservoirs to both domestic animals and humans (Godfroid *et al.*, 2011). Globally, over 500,000 human cases of brucellosis per year are reported (Pappas *et al.*, 2006). The humans’ exposure to infected animals and animal products increases the risk of acquiring brucellosis (Makita *et al.*, 2011; Corbel, 2006). Therefore, people in pastoral communities are at high risk of infection due to constant contact with animals and their products.

The possible means of acquisition of brucellosis include: infection from a contaminated environment, occupational exposure usually resulting from direct contact with infected animals, and food-borne transmission. Occasional cases have been reported in which circumstantial evidence suggests close personal or sexual contact as the route of transmission. More potential significance is transmission through blood donation or tissue transplantation. Certain occupations are associated with a high risk of infection with brucellosis. These include people who work with farm animals, especially cattle, sheep, goats and pigs. Farmers, farm labourers, animal attendants, stockmen, shepherds, sheep shearers, goatherds, pig keepers, veterinarians and inseminators are at risk through direct contact with infected animals or through exposure to a heavily contaminated environment (WHO, 2006).

Foodborne transmission is usually the main source of brucellosis for urban populations. Ingestion of fresh milk or dairy products prepared from unheated milk is the main source of infection for most populations. Cow, sheep, goat or camel milk contaminated with *B. melitensis* is particularly hazardous as it is drunk in fairly large volume and may contain large numbers of organisms (FAO, 2003).

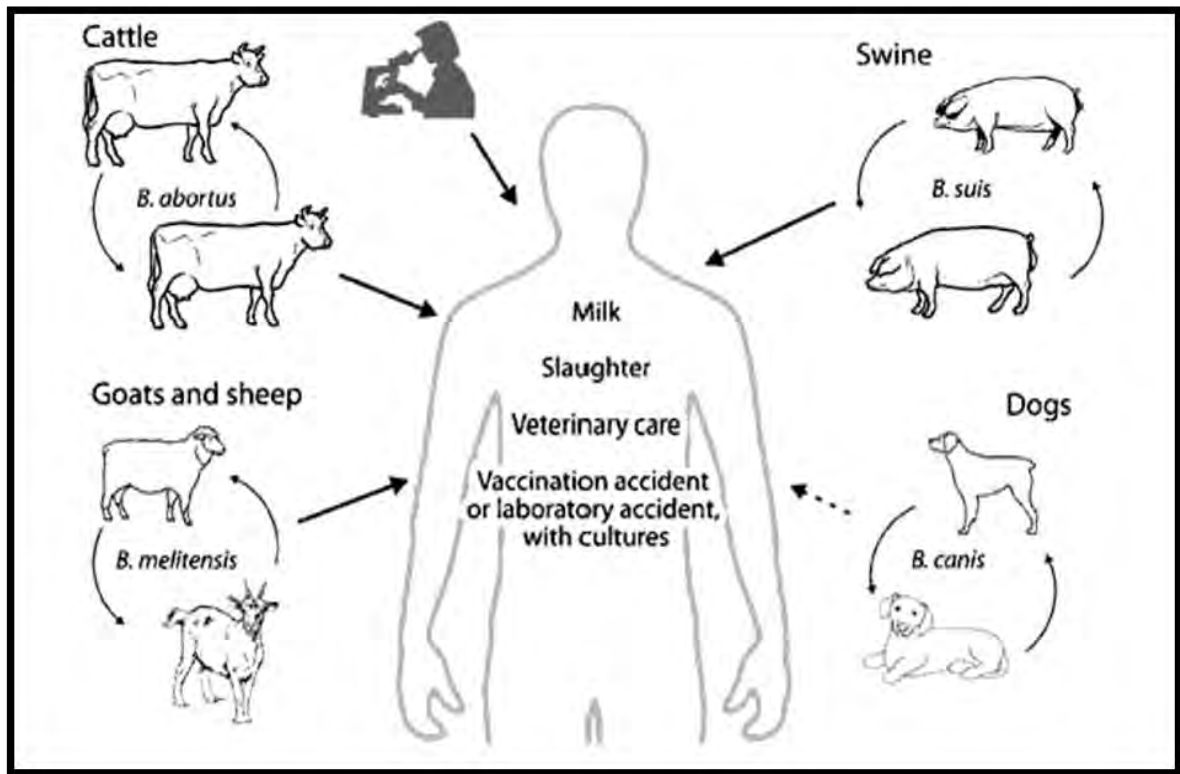


Figure1: Transmission of *Brucella* to humans

Source: Gadaga (2013)

2.3.4 Mechanism of evading the immune system

The most common portals of entry for *Brucella* in animals and humans are mucous membranes of the respiratory (aerosol) (Franz *et al.*, 2001) and digestive tracts, and in the natural host, also the conjunctiva and membranes covering the sexual organs. Bacteria are eventually taken up by phagocytic cells (macrophages, dendritic cells) and reach the regional lymph nodes, leading to subsequent systemic dissemination (Ackermann *et al.*, 1988; Salcedo *et al.*, 2008). As *Brucellae* can't multiply outside their mammalian hosts, the most important aspect of *Brucella* ecology is their ability to establish an intracellular replicative niche and remain protected from the host immune responses (Bargen *et al.*, 2012).

Brucellae lack classic virulence factors like toxins, fimbriae and capsules which raises the possibility that they might have unique and subtle mechanisms to penetrate host cells, elude host defenses, alter intracellular trafficking to avoid degradation and killing in lysosomes and modulate the intracellular environment to allow long-term intracellular survival and replication

(Delrue *et al.*, 2004). The *Brucella* LPS O-polysaccharide appears to be a key molecule for cellular entry, to prevent complement-mediated bacterial lysis and apoptosis (programmed cell death) of the macrophages within which they reside allowing them to extend their longevity (Debagüés *et al.*, 2004; Lapaque *et al.*, 2005).

Brucella has developed mechanisms to avoid innate immunity by minimizing stimulation of pattern recognition receptors (PRRs) of the host. The *Brucella* cell envelope has high hydrophobicity and its LPS has a non-canonical structure that elicits a reduced and delayed inflammatory response compared with other Gram-negative bacteria and has lower stimulatory activity on TLR4 receptors (Rittig *et al.*, 2001, 2003). The “O” side chain on the LPS can form complexes with the major histocompatibility complex class II molecules that interfere with the ability of macrophages to present exogenous proteins. *Brucella* ornithine-containing lipids and lipoproteins in the outer membrane are poor activators of innate immunity. The rough (vaccine) strains (strains with lipopolysaccharide lacking the “O” side chain) are less virulent because of their inability to overcome the host defense system. However, under *invitro* conditions, up to 90% of virulent *Brucella* and 99% of non-virulent *Brucella* may be killed following intracellular entry (Porte *et al.*, 1999; Rittig *et al.*, 2003).

After entering into the host cell, smooth *Brucella* quickly traffic through the early endosomal compartment and depart the phagosome to form the modified phagosome (brucellosome). *Brucella* initially localize within acidified phagosomes (Rittig *et al.*, 2001), where they are exposed to free oxygen radicals generated by the respiratory burst of phagocytes. *Brucellae* have multiple mechanisms to detoxify free radicals. *Brucella* expresses 2 superoxide dismutases (SodA and SodC), which detoxify superoxide anions generated by the respiratory burst of phagocytes. *Brucellae* require acidification of the phagosomal compartment to pH <4.5 before they display wild-type intracellular replication in initial stages of intracellular infection. Localization in an acidified environment induces expression of the VirB operon (virB 1–10), which controls expression of genes associated with type IV secretion system. The VirB operon interacts with the endoplasmic reticulum to neutralize the pH of the phagosome (Anderson *et al.*, 2008).

The *Brucella*-induced modifications of the phagosome prevent fusion with the lysosome. Virulent *Brucella* strains express a cyclic glucan synthase (cgs) that produces and secretes low molecular weight cyclic glucans. These molecules disrupt the lipid raft microdomain structures within intracellular membranes surrounding the bacteria. This modification of lipid raft distribution in phagosomal membranes inhibits phagosome maturation, prevents fusion with lysosomes (Arellano *et al.*, 2005).

2.3.5 Brucellosis in small ruminants

Brucellosis is a disease of many animal species including humans but especially of those that produce food: cattle, sheep, goats, camels and other species (Corbel, 2006; Ghanem *et al.*, 2009). In sexually mature animals the infection localizes in the reproductive system and typically produces placentitis followed by abortion in the pregnant female, usually during the last third of pregnancy and epididymitis and orchitis in the male. So the disease causes significant losses in reproduction and productivity of sexually mature animals through high morbidity (Pappas *et al.*, 2006; Radostits *et al.*, 2007). *B. melitensis*, *B. abortus* and *B. suis* are zoonotic pathogens which can infect humans. *Brucella canis* may cause infections in immune-suppressed individuals.

Brucellosis in sheep and goats (excluding *Brucella ovis* infection) is primarily caused by one of the three biovars of *B. melitensis*. Sporadic infections caused by *B. abortus* or *B. suis* have been observed in sheep and goats, but such cases are rare. In most circumstances, the primary route of transmission of *Brucella* is the placenta, fetal fluids and vaginal discharges expelled by infected ewes and does when they abort or full-term parturition. Shedding of *Brucella* is also common in udder secretions and semen and *Brucella* may be isolated from various tissues, such as lymph nodes from the head, spleen and organs associated with reproduction (uterus, epididymides and testes), and from arthritic lesions (OIE, 2009).

Goats are the classic and natural host of *B. melitensis* and together with sheep are its preferred hosts. In pathological and epidemiological terms, *B. melitensis* infection in small ruminants is similar to *B. abortus* infection in cattle: the main clinical manifestations of brucellosis in 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 (Elzer *et al.*, 2002; Blasco and Molina, 2011).

Table 1: The species, biotypes, host preferences and zoonotic potentials of *Brucella*

Species	Biovars	Colony type	Host tropism	First reported country	Zoonotic potential
<i>B. melitensis</i>	1-3	Smooth	Goat, sheep, camels, cows	Malta	High
<i>B. abortus</i>	1-6,9	Smooth	Cattle, buffalo, camels, bison, elk, yaks	Denmark	High
<i>B. suis</i>	1-5	Smooth	Pigs (biotypes 1-3), wild boar and European hares (biotype 2)	USA	High
<i>B. neotomae</i>	-	Smooth	Desert woodrat	USA	Unknown
<i>B. pinnipedialis</i>	-	Smooth	Seal	Scotland	Mild
<i>B. ceti</i>	-	Smooth	Dolphin, porpoise, whale	Scotland	Mild
<i>B. microti</i>	-	Smooth	Vole, fox, (soil)	Czech Republic	Unknown
<i>B. inopianata</i>	-	Smooth	Unknown	Australia	Mild
<i>B. ovis</i>	-	Rough	Sheep	New Zealand	No
<i>B. canis</i>	-	Rough	Dog	USA	Mild
Future species					
<i>Brucella papionis</i> sp. nov.	-	Smooth	Baboon	USA	Unknown
BO2	-	Smooth	Unknown	Austria	Mild
Frog isolate (exceptionally motile)	-	Smooth	Bullfrogs	Germany	Unknown

Adapted from Adams (2002), Xavier *et al.* (2009) and Neta *et al.* (2010)

2.4 Brucellosis in Pastoral Systems

In Africa, the occurrence of brucellosis in sub-Saharan countries (either prevalence or incidence) is not well documented and reports submitted to the World Organization for Animal Health, Office International des Epizooties (OIE) are largely confined to serological surveys, and mainly conducted for cattle and less for sheep and goats. McDermott and Arimi (2002) referred to a great variation in prevalence in sub-Saharan Africa (ranging from 4.8 to 41%) in pastoral systems. In comparison with bovine brucellosis, brucellosis in sheep and goats caused mainly by *B. melitensis* has with only a few exceptions a low or sporadic degree of incidence throughout the African continent (McDermott and Arimi, 2002).

There are about 50 to 100 million pastoralists globally and the majorities are confined to Africa. Ethiopia has the largest pastoral population of 7 to 8 million and the majorities are living in the Afar Region. The Afar pastoralists live in arid and semi-arid parts of the country and they have been suffered from political and geographical marginalization in the past (Bekele *et al.*, 2013). The pastoralists of Afar Region depend on livestock and their products for their livelihood as milk and meat from cow, camel and goat are the main sources of their food.

Goats and sheep are important domestic animals in tropical livestock production systems (Devendra and McLeroy, 1990), accounting for 21% of the global small ruminant population. According to statistics from the Central Statistical Agency (CSA, 2014), Ethiopia has over 27 million head of sheep and 28 million goats (CSA, 2014). 25% of the sheep and 73% of the national goat population inhabit the lowlands (mostly pastoral areas). Also according to Pastoralist Forum Ethiopia (PFE, 2004), most goat populations in Ethiopia are raised under pastoral communities. These small ruminants and their milk/meat products represent an important export commodity, which significantly contributes to the national economy.

However, brucellosis is widely distributed in Afar region and the disease poses a barrier to livestock trade and is an impediment to free animal movement (Zinsstag *et al.*, 2011). Previous work in different districts of the region based on questionnaire survey has revealed that the prevalence in small ruminants range from 1.7-13.6% (Teshale *et al.*, 2006; Ashenafi *et al.*, 2007; Wesinew *et al.*, 2012).

Most of the studies conducted on brucellosis were entirely based on estimation of seroprevalence of the disease. There are very few reports on the seroprevalence of brucellosis, which are conducted on different livestock species and human in pastoral and agro-pastoral areas of the Ethiopia. Additionally, pastoral households often keep a diverse composite of livestock species as part of a coping mechanism for uncertainties and risks. Such conditions certainly increase aggregation and interaction of different animals at villages, grazing fields and water points, thus, facilitate transmission of the disease.

The dynamics and frequent migration of pastoral flocks might increase the chance of coming into contact with other potentially infected flocks and exposure to geographically limited or seasonally abundant diseases. Mobility also increases the opportunity of interactions with wild animals. This has already been confirmed by (Samui *et al.*, 2007; Megerssa *et al.*, 2011) in that flocks coming into contact with wildlife had higher likelihood of acquiring infection than those without contact.

However, there is paucity of information on an integrated community/health facility-based parallel study of brucellosis in animals and humans in Ethiopia. For instance, study conducted in Afar Region to determine the prevalence of brucellosis in camels and to identify risk practices that would facilitate the transmission of zoonoses to humans was failed to carry out parallel study on the prevalence of brucellosis in owners. Another study which was conducted in Oromia region to assess the prevalence of brucellosis in cattle and to assess potential risk factors of the disease to humans also failed to assess the seroprevalence of *Brucella* infection in owners (Tschopp *et al.*, 2013).

Table 2: The prevalence of small ruminant brucellosis in pastoralist areas of Ethiopia

Region	Zone	District	Species	Number Tested	Number positive (%)		Source
					RBPT	CFT/iELISA	
Oromia	Borana	Yabello	Small ruminants	384	9 (2.34)	6 (1.56)	Dabassa <i>et al.</i> , 2013
			Sheep	171	3 (1.75)	2 (1.17)	
Oromia and Somali		Liben and Flitu	Goats	510	48 (9.6)	40 (7.8)*	Gumi <i>et al.</i> , 2013
Dire Dawa			Small ruminants	384	36 (9.38)	35 (9.11)	Negash <i>et al.</i> , 2012
			Sheep	171	15 (8.77)	15 (8.77)	
			Goats	213	21 (9.88)	20 (9.39)	
SNNP	South Omo	Hammer and Dasenech	Goats	384	20 (5.2)	16 (4.2)	Ashagrie <i>et al.</i> , 2011
Somali		Jigjiga	Small ruminants	730	12 (1.6)	11 (1.5)	Bekele <i>et al.</i> , 2011
			Sheep	421	5 (1.2)	5 (1.2)	
			Goats	309	7 (2.3)	6 (1.9)	
Afar	All (five zones)	Eighteen Districts	Small ruminants	1,568	147 (9.4)	76 (4.8)	Ashenafi <i>et al.</i> , 2007
			Sheep	563	-	18 (3.2)	
			Goats	1,005	-	58 (5.8)	
Afar and Somali			Sheep	928	10 (1.1)	52 (5.6)*	Teshale <i>et al.</i> , 2006
			Goats	1,072	28 (2.6)	141 (13.2)*	

*iELISA-Indirect enzyme linked immunosorbent assay

2.5 Growth and Biochemical Nature of *Brucella* Species

2.5.1 Selective media

Brucella species are slow growing and the use of enriched selective media is recommended for primary isolation from most clinical specimens because of the high numbers of overgrowing contaminants may inhibit the isolation (Marin *et al.*, 1996). Such selective media are prepared by incorporating antibiotics and bacteriostatic dyes onto basic enriched media such as *Brucella* selective medium base (HiMedia). Prepared by adding six antibiotics; bacitracin, vancomycin, nalidixic acid, polymyxin B, nystatin and cycloheximide onto sucrose dextrose agar for the isolation of *Brucella species* from the clinical sample (Farrell, 1974).

2.5.2 Carbon dioxide and pH requirements

Members of the genus *Brucella* are aerobic but some strains require atmosphere containing 5 to 10% CO₂ added for growth especially on primary isolation (Alton *et al.*, 1988). *B. melitensis*, however does not require CO₂ or serum and can be isolated on ordinary solid media under aerobic conditions at 37°C (Garin, 2006). On repeated culture, isolates of *Brucella* can loose the requirement for added CO₂ for growth and may grow in air alone (Eze, 1981). The optimum pH for the growth of *Brucella* species is from 6.6-7.4 and culture media should be adequately buffered near pH 6.8 for optimum growth.

2.5.3 Growth temperature

The optimum growth temperature for *Brucella* organisms is 36-38°C, but most strains can grow between 20°C and 40°C (Anon, 2001; European Commission, 2001).

2.5.4 Colonial behaviour

Brucella requires biotin, thiamin and nicotinamide and the growth are improved by serum or blood. On suitable solid media *Brucella* colonies are visible after 2 days incubation. After four days, *Brucella* colonies become round, 1-2mm in diameter, with smooth (S) margins, transparent and pale honey colour when plates are viewed in transmitted light and have a bluish translucent appearance in reflected light (Alton *et al.*, 1988). When viewed from above, colonies appear

convex and fairly white. Later, colonies become larger and slightly darker. Rough *Brucella* isolates produce similar colony size and shape but are more opaque off-white in colour with a rather granular surface.

On blood agar, growth is slower than on Serum Dextrose Agar (SDA) with the production of non-haemolytic, greyish- white glistening colonies after 72 hours incubation. Growth in liquid media is usually poor unless the culture is vigorously shaken. On semi-solid media, CO₂-independent *Brucella* strains produce uniform turbidity from surface down to 3 millimetres depth while CO₂-dependent strains produce a disk of growth 2 millimetres below the surface of the medium (Corbel *et al.*, 2006). Little or slow growth is produced by many *Brucella* strains on MacConkey agar, even after five days at 37°C. The growth of most *Brucella* strains is inhibited by media containing bile salts, tellurite or selenite and does not require haeme (V-factor) and NAD (Alton *et al.*, 1988).

2.5.5 Biochemical characteristics

Brucella metabolism is oxidative and cultures show no ability to acidify carbohydrate media in conventional tests (Alton *et al.*, 1988). *Brucella* species are usually catalase and oxidase positive and they reduce nitrate to nitrite except *B. canis* strains (European Commission, 2001).

The production of H₂S from sulphur containing amino acid varies. *B. melitensis* does not produce H₂S (European Commission, 2001). Urease activity of *Brucella* species varies from fast to very slow. Indole and acetyl methyl carbinol are not produced from tryptophane and glucose respectively (Anon, 2001). Methyl red and Voges-Proskauer tests are negative and *Brucella* neither liquefies gelatine nor lyses red blood cells (Alton *et al.*, 1988). A summary of the differential characteristics and biochemical tests used to identify *Brucella* species from other bacteria is given in Tables 3 and 4.

Table 3: Differential characteristics of *Brucella* species

Test	<i>Brucella</i>	<i>Bordetella</i> <i>Bronchoseptica</i>	<i>Campylobacter</i> <i>fetus</i>	<i>Moraxella</i> species	<i>Acinetobacter</i> species	<i>Yersinia</i> <i>enterocolitica</i>
Morphology	Small coccobacilli	Small coccobacilli	Coma shaped	Diplococcoid	Diplococcoid	Rods
Motility at 37°C	—	+	+	—	—	—
Motility at 20°C	—	—	—	—	—	+
Lactose fermentation	—	—	—	V ^a	V	—
Acid production	— ^b	—	—	—	V	+
Haemolysis on Blood agar	—	+	—	V	V	—
Catalase	+	+	+	V	—	—
Oxidase	+ ^c	+	+	+	—	—
Urease	+ ^d	+	—	V	V	+
Nitrate reduction	+ ^e	+	+	V	—	+
Citrate utilization	—	+	—	—	V	—

^aPositive and negative species within the genus; ^b*B. neotomae* may show some fermentation; ^c Except *B. ovis*, *B. neotomae* and occasional *B. abortus* strains which are negative; ^d Except *B. ovis* and occasional *B. abortus* strains which are negative; ^e Except *B. ovis* which does not reduce nitrates to nitrites; V^a, variable (Alton et al., 1988).

Table 4: Differentiation of the species and biovars of the genus *Brucella*

Characteristics	<i>B. melitensis</i>			<i>B. abortus</i> biovars								<i>B. suis</i> biovars					<i>B. ovis</i>	<i>B. neotomae</i>	<i>B. canis</i>
	1	2	3	1	2	3	4	5	6	7	9	1	2	3	4	5			
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+
CO ₂ req.	–	–	–	[+]	[+]	[+]	[+]	–	–	–	–	–	–	–	–	–	+	–	–
H ₂ S prod.	–	–	–	+	+	+	+	–	[–]	[+]	+	+	–	–	–	–	–	+	–
Growth on Media containing Dyes																			
Thionin	+	+	+	–	–	+	–	+	+	+	+	+	+	+	+	+	+	– ^d	+
Basic fuchsin	+	+	+	+	–	+	+	+	+	+	+	[–]	–	+	[–]	–	[–]	–	[–]
Agglutination With monospecific Anti-sera																			
A	–	+	+	+	+	+	–	–	+	+	–	+	+	+	+	–	–	+	–
M	+	–	+	–	–	–	+	+	–	+	+	–	–	–	+	+	–	–	–
R	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+

(a) Symbols: +, positive; [+], positive for most strains, [–], negative for most strains, –, negative all strains. (b) For more certain differentiation of biovar 3 and 6, thionine at 1:25, 000 (w/v) is used; biovar 3 gives a positive growth response, biovar 6 is negative. (c) Dye concentration, 1:50, 000 (w/v). (d) Growth will occur in the presence of thionine at a concentration of 1:150, 000 (w/v). (e) Rapid reaction, most strains of *B. suis* test positive within 5 minutes (f) Some field strains of *B. abortus* may be negative

Source: (OIE, 2004; and Garritty et al., 2005)

2.5.6 Antigenic characteristics

Like other Gram-negative bacteria, *Brucella* has lipopolysaccharide (LPS) as a major component of their outer membrane and an important virulence factor (Cardoso *et al.*, 2006). Thus their colony morphology is termed as either 'smooth' or 'rough' depending on the LPS structure (Baldwin and Goenka, 2006). Structural variation in the LPS of smooth strains also defines the so called A and M antigens that have some a significant role in typing (Godfroid *et al.*, 2010). These antigens reflect differential O-side chain which is linked to α -1, 2 in A dominant strains but with every fifth residue linked α -1, 3 in M dominant strains (Bundle *et al.*, 1987). The O-PS is involved in bacterial virulence. It contributes in complement resistance and more importantly critically modulates bacterial entry into cells so that its removal causes attenuation (Conde *et al.*, 2013). All smooth *Brucella* cross-react with one another in agglutination tests. They also cross react with unabsorbed polyclonal antisera. This cross-reaction does not occur with non-smooth or the rough *Brucella* strains.

Lipopolysaccharide (LPS) comprises the major surface antigens of the corresponding colonial phase involved in agglutination. The (S-LPS) molecules carry the A and M antigens, which have different quantitative distribution among the smooth *Brucella* strains (Bundle *et al.*, 1987). This is of value in differentiating biovars of the major species using absorbed monospecific A and M antisera (Anon, 2001). Serological cross-reaction has been reported between the smooth *Brucella* and various other Gram negative bacteria like, *E. coli* O: 116 and O: 157, *Salmonella* group N (O: 30) of Kaufmann-white and *Pseudomonas multophila*, *Vibrio cholerae* and especially *Yersinia enterocolitica* (O: 9). These organisms can induce significant antibodies, which cross-react with S-LPS of *Brucella* antigens in diagnostic tests (Anon, 2001; Corbel *et al.*, 2006).

2.5.7 Susceptibility to phages

There are about 40 phages which are lytic and specific to the genus *Brucella* and they are not known to be active against any other bacteria that have been tested. Therefore, lysis by *Brucella* phages is a useful test to confirm the identity of *Brucella* species and for speciation within the genus (Anon, 2001). *Brucella* phages currently in use for typing are Tbilisi (Tb), Weybridge (Wb), Izatnagar (Iz) and Rough Culture (R/C) (Alton *et al.*, 1988). The first three phages are used for differentiation of smooth *Brucella* and R/C is used for rough *Brucella* (*B. ovis*, *B. canis*) (Corbel *et al.*, 1988).

Table 5: Differential characteristics of *Brucella* species to *Brucella* phages involved in ruminant brucellosis

Species	Tbilisi	Izatnagar	Rough culture
<i>B. melitensis</i>	–	+	–
<i>B. ovis</i>	–	–	+
<i>B. abortus</i>	+	+	–

Source: (Alton *et al.*, 1988)

2.5.8 Susceptibility to dyes

The effect of the dyes thionin and basic fuchsin on various *Brucella* species and biovars varies (Anon, 2001). The susceptibility of *Brucella* species to dyes at standard concentrations of 20µg/ml to 40µg/ml is used as routine typing test for *Brucella* species (Alton *et al.*, 1988). *Brucella melitensis* strains all grow in the presence of both dyes while *Brucella abortus* grows in presence of basic fuchsin but does not grow in presence of thionin at the mentioned concentrations.

2.5.9 Susceptibility to antibiotics

Brucella species are sensitive to a wide range of antibiotics. Penicillin is used for the routine differentiation of the vaccinal strain of *B. abortus* species biovar 1 strain 19, used for the immunization of cattle from its respective field strain. This is because the S19 vaccine strain is sensitive to penicillin while the field virulent strain is resistant. Similarly, streptomycin is used for routine differentiation of *B. melitensis* biovar 1 strain, the vaccine widely used for the immunization of small ruminants from its virulent field strains. This is because Rev.1 vaccine is sensitive to streptomycin while the field virulent strain is resistant to streptomycin (Alton *et al.*, 1988). On primary isolation, *Brucellae* are usually susceptible *in vitro* to gentamicin, tetracycline and rifampicin. Most strains are also susceptible to ampicillin, chloramphenicol, cotrimoxazole, erythromycin, spectinomycin and streptomycin. Most strains of *Brucella* are resistant to β -lactams, cephalosporins, polymyxin B, bacitracin, cycloheximide, clindamycin, lincomycin, nystatin and vancomycin at therapeutic concentrations (Anon, 2001).

2.6 Pathogenesis

The major route of infection is through mucous membranes of the oropharynx and upper respiratory tract or conjunctiva (Tabak *et al.*, 2008). Another route is through the mucous membrane of the male and female genital tract. On entering into the body of the host, the organism encounters the cellular defences of the host but generally succeed in arriving via the lymph vessels at the nearest lymph node after escaping the cellular defenses (Ko and Splitter, 2003). The fate of the invading bacteria is mainly determined by cellular defences of the host chiefly macrophage and T-lymphocytes though specific antibody also plays apart (Radostits *et al.*, 2007). The outcome depends on the ruminant species infected, age, immune status of the host, pregnancy status, and the virulence and the number of invading *Brucella* (Seifert, 1996).

When the bacteria prevail over the host's defences, a bacteraemia is generally established. The bacteraemia is always detected after 10 to 20 days and persists from 30 days to more than two months. If the animal is pregnant, bacteraemia often leads to the invasion of the uterus (Olsen, 2010). At the same time, infection becomes established in various lymph nodes and organs, often in the udder and sometimes in the spleen (WHO, 2006).

The main lesions which appear in the male animals are orchitis and epididymitis, as well as inflammation of the joints and bursa. Abortion may also occur in the females presenting the typical yellowish sticky layers on the placenta. The consequences of brucellosis in small ruminants are infertility, high mortality rate in calves, mastitis and reduced milk production (Oyedipe *et al.*, 1981; Radostits *et al.*, 2007).

2.7 Clinical Manifestation of Brucellosis

Brucellosis could be suspected in any herd with history of abortion during the last stage of pregnancy, infertility, orchitis, epididymitis, stillbirths, neonatal mortality and hygroma (Poester *et al.*, 2010). The major clinical sign in the first stage of the disease is abortion, but other signs due to localization of the organism may be observed. These signs include orchitis, epididymitis, hygroma, arthritis, metritis and subclinical mastitis among others (Radostits *et al.*, 2007). However, numerous animals develop self-limiting infection or they may become asymptomatic latent carriers and potential excretors (WHO, 2003). Infection is not established if the female is exposed to the organism at the end of the pregnancy. The second stage is

characterized by either elimination of *Brucella* or more frequently, by persistent inflammation of mammary gland and supramammary and genital lymph nodes, with constant or intermittent shedding of the organisms in milk and genital secretions (Fensterbank, 1987). Animals generally abort once during the mid- third of gestation but re-invasion of the uterus occurs in subsequent pregnancies with shedding in fluids and foetal membranes.

The pregnancy can also get to full- term (Oyedipe *et al.*, 1981). Females that are born into an infected area and get infected generally abort less than others. This explains the high level of abortions in newly infected herds and their relatively low frequency in herds where infection is enzootic. The udder is a very important predilection site for *Brucella* organisms. Infection in lactating, nonpregnant goats is likely to lead to colonization of the udder with excretion of *Brucella* organisms in the milk (Radostits *et al.*, 2007). Retention of placenta and metritis are common sequels to abortion. Females usually abort only once, presumably due to acquired immunity. In general, abortion with retention of the placenta and the resultant metritis may cause prolonged calving interval and permanent infertility (Walker, 1999).

Infection in males may result in either temporary or permanent infertility, depending on the intensity of the lesions (Megid *et al.*, 2010). Orchitis is occasionally manifested, that is often associated with a vesiculitis and epididymitis and when it occurs it is usually unilateral, but both testicles may be affected. Scattered foci of necrosis coalesce to produce total testicular necrosis (Foster and Ladds, 2007). Hygromas, usually involving leg joints, are a common manifestation of brucellosis in some tropical countries and may be the only obvious indicator of infection; the hygroma fluid is often infected with *Brucella* (OIE, 2009).

Human brucellosis has a wide clinical spectrum, presenting various diagnostic difficulties because it mimics many other diseases for example malaria, typhoid, rheumatic fever, joint diseases and other conditions causing pyrexia (Andriopoulos *et al.*, 2007; Kunda *et al.*, 2007). The disease manifests with continued, intermittent or irregular fever (hence the name undulant fever), headache, weakness, profuse sweating, chills, arthralgia, depression, weight loss, hepatomegaly, and splenomegaly and generalized aching. Cases of arthritis, spondylitis osteomyelitis, epididymitis, orchitis, and in severe cases neurobrucellosis, liver abscesses, and endocarditis with infection of the aortic valves and other multiple valves with *Brucella* has been reported in human (María *et al.*, 2007).

2.8 Diagnostic Techniques of Brucellosis

The most reliable and the only unique method for diagnosing animal brucellosis is isolation of *Brucella* species (Alton *et al.*, 1988). In the history of microbiology, very few diseases have more diagnostic tests than brucellosis. Diagnostic tests are applied for the following purposes: confirmatory diagnosis, screening or prevalence studies, certification, and, surveillance in order to avoid the reintroduction of brucellosis (in countries where brucellosis is eradicated) through importation of infected animals or animal products (Godfroid *et al.*, 2010). The diagnostic methods include direct tests, involving isolation of organism or DNA detection by polymerase chain reaction (PCR)-based methods and indirect tests, which are applied either in vitro (mainly to milk or blood) or in vivo (allergic test). Isolation of *Brucella* species or detection of *Brucella* species DNA by PCR is the only method that allows certainty of diagnosis.

Definitive diagnosis of brucellosis is based on culture, serologic techniques or both. Presumptive evidence of brucellosis is provided by the demonstration by modified acid-fast staining of organisms of *Brucella* in abortion material or vaginal discharge, especially if supported by serological tests. Whenever possible, *Brucella* species should be isolated using plain or selective media by culture from uterine discharges, aborted fetuses, udder secretions or selected tissues, such as lymph nodes and male and female reproductive organs. Species and biovars should be identified by phage lysis, and by cultural, biochemical and serological criteria. Polymerase chain reaction (PCR) can provide both a complementary and biotyping method based on specific genomic sequences (Alton *et al.*, 1988; OIE, 2009).

2.8.1 Serological Diagnosis

Despite the development of numerous serological tests, no single test identifies all infected animals and a wide variation exists in estimates of their diagnostic accuracy (Adone and Pasquali, 2013; Abernethy *et al.*, 2012). The current serological tests used for the diagnosis of *B. melitensis* and *B. ovis* in sheep and goats were initially developed for the diagnosis of *B. abortus* in cattle (OIE, 2012). Although not formally validated for use in sheep and goats, these tests, especially RBPT, CFT and more recently ELISA, have been widely used for the serological diagnosis of brucellosis in sheep and goats (Macmillan, 1990; Farina, 1985). They are also the official tests for international trade (European Commission, 2001; OIE Collective Manual, 2004).

Serological tests cannot differentiate between *Brucella* species and cannot therefore identify which species has induced host antibodies. Therefore, only isolation of the species or specific DNA detection by polymerase chain reaction (PCR), allows identification of the infecting strain (Godfroid *et al.*, 2010; Plumb *et al.*, 2013).

a) Rose Bengal plate test (RBPT)

This test was developed by Rose and Roekpe (1957) for the diagnosis of bovine brucellosis to differentiate specific *Brucella* agglutinins from non-specific factors. When the antigen was buffered at pH 4.0 they observed that agglutination of *B. abortus* cells by non-specific agglutinins of bovine serum was inhibited whereas the activity of specific *Brucella* antibodies was not affected. Despite the scanty and sometimes conflicting information available (Alton, 1990), this test is internationally acknowledged as the test of choice for the screening of brucellosis in cattle as well as in small ruminants (Garin and Blasco, 2004; WHO, 2006).

However, the standardization conditions suitable for diagnosing cattle infection (European Commission, 2001; Garin and Blasco, 2004) are not adequate in sheep and goats and account for the low sensitivity of RBPT in small ruminants. If the antigen is standardized differently, to give a higher analytical sensitivity, the diagnostic sensitivity to *B. melitensis* infection will be improved. The RBPT is based on the detection of specific antibodies of the IgM and IgG types but more effective in detecting antibodies of the IgG1 type than the IgG2 and IgM types. Also the low pH (3.65) of the antigen enhances the specificity of the test by inhibiting nonspecific agglutinins. The temperature of the antigen and the ambient temperature at which the reaction takes place may influence sensitivity and specificity (Macmillan, 1990).

The RBPT could be modified for testing of sera in endemic, low prevalence areas to increase the sensitivity of the test. This simple modification is achieved by increasing slightly the amount of sera for the test dose from 25 µl to 75 µl, at the same time maintaining the antigen volume at 25 µl. This results in significantly increase in the sensitivity of the test without affecting the specificity (Blasco *et al.*, 1994; Ferreira *et al.*, 2003).

b) Complement fixation test (CFT)

Complement fixation test is the most widely used confirmatory test and recommended by OIE (Garin *et al.*, 2006). As in cattle brucellosis, there is agreement that this test is effective for the serological diagnosis of brucellosis in sheep and goats despite the complexity and the

heterogeneity of the techniques used in different countries. The CFT is based on the detection of specific antibodies of the IgM and IgG1 that fix complement. It is highly specific but laborious and requires highly trained personnel as well as suitable laboratory facilities. Its specificity is very important for the control and eradication of brucellosis but may test negative when antibodies of the IgG2 type hinder complement fixation (Farina, 1985; Alton, 1990; Macmillan, 1990).

2.8.2 Microscopic examination of stained smears

Smears of placental cotyledon, vaginal discharge or fetal stomach contents may be stained using modified Ziehl-Neelsen (Stamp) method. The presence of large aggregates of intracellular, coccobacillus red organisms is presumptive evidence of brucellosis. It is still often used, even though this technique is not specific as other abortive agents such as *Chlamydomphila abortus* or *Coxiella burnetii* are also stained red (Alton *et al.*, 1988; FAO, 2006).

2.8.3 Cultural isolation

The only ‘gold standard’ method for the diagnosis of brucellosis is the cultural isolation or detection of *Brucella* organisms from the infected host (Alton *et al.*, 1988; OIE, 2009; Smirnova, *et al.*, 2013). This can be made by means of microscopic examination of smears stained with the modified Ziehl-Neelsen method from vaginal swabs, placenta, or aborted fetuses (Stamp, 1950).

However, morphologically related microorganisms such as *Chlamydia psittaci* and *C. burnetii* can mislead one in the diagnosis (Garin, 2006; Radostits *et al.*, 2007). So bacterial culture plays an important role in confirming the presence of disease and it is essential for antimicrobial susceptibility, biotyping and molecular characterization which provide valuable epidemiological information to know the sources of infection in outbreak scenarios and the strain diversity in endemic regions (Kattar *et al.*, 2008).

Important clinical samples include aborted fetuses (stomach, spleen, and lung), fetal membranes, vaginal secretions, colostrum, milk, sperm, and hygroma fluid. *Brucella* may also be isolated post-mortem from supra-mammary, internal iliac and retropharyngeal nodes, spleen, udder tissue, testes and gravid uterus. Care should be taken to minimize the fecal and

environmental contamination of the material to give the greatest chance of successfully isolating *Brucella*.

However vaginal swabs and milk from aborted animals are the best materials/samples for the isolation of *Brucella* species, while spleen and lymph nodes (iliac, mammary and prefemoral) are the most reliable samples for isolation purposes in necropsied animals (Marin *et al.*, 1996). For the isolation of *Brucella* species the most commonly used medium is Brucella Selective Medium (HiMedia) with sterile inactivated horse serum, which contains antibiotics able to inhibit the growth of other bacteria present in clinical samples.

2.8.4 Biotyping

The identification of *Brucella* involves Stamps modified Ziehl-Neelson's Gram's reaction, colonial and cellular morphology and routine biochemical tests (Corbel *et al.*, 2006). Species are distinguished on the basis of lysis by bacteriophages and oxidative reactions on amino acids and carbohydrate substrates.

Biotyping of *Brucella* species is performed using different tests, like agglutination tests with antibodies against rough (R antigen) or smooth LPS (against the A or M antigens); lysis by phages, dependence on CO₂ for growth; production of H₂S; production of urease; growth in the presence of basal fuchsin or thionine; and the crystal violet or acriflavine tests (Alton *et al.*, 1988). These techniques must be carried out using standardized procedures by experienced personnel and usually performed only in reference laboratories.

2.8.5 Molecular typing

Despite the high degree of DNA homology within the genus *Brucella*, several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP) and Southern blot, have been developed that allow, to a certain extent, differentiation between *Brucella* species and some of their biovars (OIE, 2009).

Brucella biotyping and distinguishing vaccine strains by PCR can be accomplished satisfactorily but there has been limited validation of the PCR for primary diagnosis. The first species-specific multiplex PCR assay for the differentiation of *Brucella* was described by Bricker & Halling. The assay, named AMOS-PCR, was based on the polymorphism arising

from species-specific localisation of the insertion sequence IS711 in the *Brucella* chromosome, and comprised five oligonucleotide primers that can identify without differentiating *B. abortus*, biovars 1, 2 and 4 but could not identify biovars 3, 5, 6 and 9. Modifications to the assay have been introduced over time to improve performance, and additional strain-specific primers were incorporated for identification of the *B. abortus* vaccine strains, and other biovars and species (OIE, 2009).

A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of *Brucella*. The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most *Brucella* species as well as the vaccine strains *B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev.1. In contrast to other PCRs, Bruce-ladder is able to detect also DNA from *B. neotomae*, *B. pinnipedialis* and *B. ceti*. In addition, *B. abortus* biovars 3, 5, 6, 7, 9, and *B. suis* biovars 2, 3, 4, 5 can be identified by this new multiplex PCR. The only minor inconvenience of the Bruce-ladder is that some *B. canis* strains can be identified erroneously as *B. suis* (López *et al.*, 2011).

2.9 Treatment, Prevention and Control

One of the most successful methods for prevention and control of livestock brucellosis is through vaccination. In different parts of the world both live vaccines, such as *B. abortus* S-19, *B. melitensis* Rev1, *B. suis* S-2, rough *B. melitensis* strain M111 and *B. abortus* strain RB51 and killed vaccines, such as *B. abortus* 45/20 and *B. melitensis* H.38 are available. Use of the RB51 attenuated live vaccine has recently gained popularity for control of brucellosis in cattle (Cheville *et al.*, 1996).

Hitherto, no vaccine has been approved for the prevention of human brucellosis. Therefore, human brucellosis is usually prevented by controlling the infection in animals. Pasteurization of dairy products is an important safety measure where this disease is endemic. Treatment regimes for human brucellosis require combination of antibiotics like rifampicin or gentamicin and doxycycline twice daily is the combination most often used, and appears to be efficacious (Yohannes *et al.*, 2013). The combination of doxycycline with streptomycin is currently the best therapeutic option with less side effects and less relapses, especially in cases of acute and localized forms of brucellosis (Seleem *et al.*, 2010).

Implementation of measures to reduce the risk of infection through personal hygiene, adoption of safe working practices, protection of the environment and food hygiene should minimize risks of further infection in nomadic populations where people travel in search of green pasture and water, the proper handling and burying of abortion materials to prevent contamination of water sources and pasture is of paramount importance. Furthermore, the common practice of feeding abortion materials to dogs should be avoided as this increases the risk of transmission to other animals. It is imperative to education on risks for infection to these populations in order to influence behavioral practices that will reduce risks of transmission (Yohannes *et al.*, 2013).

The development of a national veterinary extension services in the country, is essential to promote awareness about brucellosis, its impact on livestock production and zoonotic risks, would provide a valuable prevention measure. This would help to unify both community/dairy cattle producers to control and eliminate brucellosis. Currently, many dairy cattle producers hide or dispose of animals with a history of abortion, potentially facilitating disease transmission between farms and regions. This seriously undermines efforts of controlling and preventing the disease (Yohannes *et al.*, 2013).

3. MATERIALS AND METHODS

3.1 Description of the Study Area

Afar National Regional state is one of the 9 regions of Ethiopia and geographically located in the north-east of the country between 39°34' and 42°28' east longitude and 8°49' and 14°30' north latitude. The total geographical area of the region is about 270,000 km². The region shares common international boundaries in the North-East with Eritrea and in the east with Djibouti, as well as regional boundaries in the North-West with the Regional States of Tigray, in the South-West with Amhara, in the South with Oromia Regional State and in the South-East with Somali Region of Ethiopia (CSA, 2014).

Afar National Regional State is characterized by an arid and semi-arid climate with low and erratic rainfall. The altitude of the region ranges from 120 m below sea level to 1500 m above sea level. Temperatures vary from 20°C in higher elevations to 48°C in lower elevations. Rainfall is bi-modal throughout the region with a mean annual rainfall below 500 mm in the semi-arid western escarpments and decreasing to 150 mm in the arid zones to the east. Afar is increasingly drought prone. The production system of the Afar region is dominated by pastoralism (90%) from which agro-pastoralism (10%) is now emerging following some permanent and temporary rivers on which small scale irrigation is developed. The region has a total population of 1.5 million and administratively, divided into five zones, which are further subdivided into 32 weredas (administrative districts) and 358 pastoral associations (CSA, 2014).

The study was conducted in the Amibara District of the zone three which is located in the Middle Awash Valley about 260 km to the North East of Addis Ababa. The District has 18 *kebeles* (the least administrative units). The district has a total population of ~63,378, of whom 35,374 were men and 28,004 women. The livestock populations of the Amibara district are composed of 103,959 cattle, 122,526 goats, 48,043 sheep, 3,888 donkeys and 39,995 camels (CSA, 2008).

The sample size for serological study was estimated based on the previous questionnaire report of 18 % seroprevalence of *Brucella* infection in aborted small ruminant in pastoral region (Yohannes *et al.*, 2013). Therefore, the sample size was calculated using the formula described by (Thrustfield, 2007), with defined precision of 5 % and level of confidence interval of 95%.

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

Where,

n =required sample size,

P_{exp} =expected prevalence, and

d =desired absolute precision

Hence, based on the above formula, taking the expected prevalence of brucellosis in aborted cases as 18% (Yohannes *et al.*, 2013), a desired absolute precision of 5% and and 95% confidence level, 226 animals are required.

$$\begin{aligned} n &= \frac{3.84 \times 0.18 (1-0.18)}{(0.05)^2} \\ &= 226 \end{aligned}$$

Therefore, a total of 226 aborted small ruminants were considered for this study from selected *kebeles* of Amibara district.

3.3 Study Animals

The animals under study comprised 226 clinically aborted (with a period of less than one month after abortion) indigenous small ruminants were included for both serological test and bacteriological isolation. Study animals related information (such as age, body condition score, lactation and reproductive status, parity number, period of abortion, history of retained fetal membrane) were collected and recorded at the time of sampling.

3.4 Sample Collection

3.4.1 Blood sample collection

After shaving and disinfecting the site of jugular vein, (3–5 ml) of blood samples were collected in sterile plain vacutainer tubes from each study animals. 226 blood samples were kept in a slanting position overnight at room temperature to separate the serum and the clotted red blood cells according to OIE manual (2009). Then sera were gently decanted into sterile screw capped Nunc tubes (1.8ml), labeled and transported in cold chain to Addis Ababa University, College of Veterinary Medicine and Agriculture (AAU, CVMA), Immunology Laboratory, Bishoftu, Ethiopia and stored at -20°C until screened and tested for antibodies against natural *Brucella* exposure analysis using modified Rose Bengal Plate Test (mRBPT) and Complement Fixation Test (CFT).

3.4.2 Bacteriological sample collection

During the study period for isolation and identification of *Brucella* from 226 small ruminants that had a history of recent abortion or that had aborted at the time of sampling in selected *kebeles* of Amibara district specimens (Milk, Vaginal swab, fetal abomasal content and retained fetal membrane) were collected parallel to blood sample according to (OIE, 2009).

Swab samples were collected with sterile applicator stick in Ames with Charcol Transport Medium (HiMedia, Mumbai, India) and transported to Addis Ababa University (AAU), College of Veterinary Medicine and Agriculture (CVMA), Microbiology laboratory in cold chain and stored at -20°C until processed for culturing and isolation under Biosafety level two (BSL2) with high personal protections using *Brucella* Selective Agar (HiMedia, Mumbai, India) with antibiotic supplement according to Alton *et al.* (1988) Lopez *et al.* (2006).

Similarly, Milk Sample was collected aseptically after washing, drying and disinfecting the whole udder and teats. 10-20ml mid stream milk samples were collected from each teat into sterile 50 ml screw capped falcon tubes. The samples were transported chilled to AAU, CVMA, Microbiology laboratory in ice packs and stored at +4°C until processed for isolation of *Brucella* species using *Brucella* Selective Agar (HiMedia, Mumbai, India).

3.5 Laboratory Diagnosis

3.5.1 Serological tests

The serological tests (mRBPT and CFT) were carried out at AAU, CVMA and National Veterinary Institute (NVI) laboratories, respectively, Bishoftu, Ethiopia.

a) Modified Rose Bengal Plate Test (mRBPT)

All serum samples collected were screened using mRBPT at AAU, CVMA, according to the procedures described by Alton *et al.* (1988), the World Organization for Animal Health (OIE, 2004) and manufacturers' instruction. The antigen used was Rose Bengal antigen, which constitutes a suspension of *B. abortus* purchased from National Veterinary Insitute (NVI) Bishoftu, Ethiopia (Annex 6). Briefly, for the modified method, 75 µl of serum and 25 µl of antigen were mixed on a test plate and rocked for 4 minutes. After four minutes of rocking, visible agglutination was considered as positive. Agglutinations were recorded as 0, +, ++ and +++, according to the degree of agglutination (Nielsen and Duncan, 1990). A score of 0 indicates the absence of agglutination; + indicates barely visible agglutination; ++ indicates fine agglutination, and +++ indicates coarse clumping.

The presence of agglutination was considered positive reaction while the absence of agglutination was considered negative. *Brucella* positive and negative control sera were also tested along with the test sera to guide in the reading of the results. The results were recorded and stored in Microsoft Excel.

b) Complement Fixation Test (CFT)

Sera that positive for mRBPT were further tested using CFT for confirmation using standard *B. abortus* antigen S99 (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom). Preparation of the reagent was evaluated by titration and performed according to protocols recommended by World Organisation for Animal Health (OIE, 2009) (Annex 7). 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 considered as positive and lack of fixation/complete hemolysis was considered as negative.

3.5.2 *Brucella* isolation from milk and swab samples

Isolation and identification of *Brucella* were done as described in Bergey's manual and (Alton *et al.*, 1988; Corbel, 1988; OIE, 2009).

a) Media preparation and culturing

Media was prepared by suspending 21.75gm of *Brucella* Selective agar (HiMedia, Mumbai, India) in 500 ml distilled water and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 min and cool to 45-50°C. Finally sterile 5% V/V inactivated horse serum (RM1239) and rehydrated contents of one vial of *Brucella* selective supplement, (FD005) were aseptically added (Annex 1) (Alton *et al.*, 1988).

From serologically positive by mRBPT, sixty five (65) specimens (milk, vaginal swab, fetal abomasal content and fetal membrane) were selected and cultured. Briefly, the samples were inoculated on *Brucella* Selective Agar Base with Selective Antibiotics supplement (FD005) (HiMedia, Mumbai, India), under Biosafety Level two (BSL2) facilities with proper personal protections (Alton *et al.*, 1988; Lopez *et al.*, 2006).

Milk samples, fetal abomasal contents and fetal membrane were processed according to (OIE, 2009). The milk samples were centrifuged at 6000 rpm for 15 minutes to concentrate the organism under conditions that reduce the risk of aerosol contamination to personnel, and the cream and deposit were spread on *Brucella* Selective Agar Base with supplement (HiMedia Mumbai, India) while vaginal swabs were streaked directly from Amies with Charcol transport medium to *Brucella* Selective Agar.

Similarly, Tissues samples were processed aseptically by removing extraneous material and chopped into small pieces, and macerated using a 'Stomacher' or tissue grinder with a small amount of sterile phosphate buffered saline (PBS), before being inoculated on to solid media (OIE, 2009).

The inoculated plates with specimen were incubated at 37°C both in the absence and presence of 5% to 10% CO₂ for up to two weeks and colonies were checked every 24 hrs for *Brucella* species growth. *Brucella*-suspected colonies were characterized by their typical round, glistening, pinpoint and honey drop-like appearance according to Alton *et al.* (1988).

b) Microscopic examination

Suspected *Brucella* colonies were picked using sterile wire loop and mixed with a drop of sterile distilled water on a clean glass slide to make a smear. The smear was heat fixed on the slide and air-dried. The slide stained by Stamp's modification of the Ziehl–Neelsen's and Gram staining method for subsequent microscopic identification of the organisms (Annex 2). *Brucella* species were identified based on Gram negative, very tiny appearance and coccobacilli shape that was arranged mostly in single but some in pairs and also in clusters according to Alton *et al.* (1988).

c) Biochemical tests

Brucella shows no ability to acidify carbohydrate media in conventional tests. *Brucella* species are usually urease, catalase and oxidase positive and they reduce nitrate to nitrite except *B. canis* strains (European Commission, 2001). Further biochemical characterization of the organism was done using (Oxidase test, catalase test, urea hydrolysis, nitrate reduction test, hydrogen sulphide (H₂S) production and hemolysis on blood agar), growth in the presence of thionin and basic fuchsin dyes incorporated with Trypticase Soya agar at 20 to 40 ug/ml concentrations in separate petridishes, CO₂ requirement and slide agglutination test with an anti-*Brucella* polyclonal serum were also checked according to Alton *et al.* (1988) (Annex 3).

However, *B. melitensis* does not require CO₂ or serum and can be isolated on ordinary solid media under aerobic conditions at 37°C as described by Garin, (2006).

3.6 Data Analysis

The data were entered into a computer on a Microsoft Excel spreadsheet. Statistical analysis (multivariate logistic regression) was performed using 'STATA version 11.0 for MA Windows (Stata Corp. College Station, USA). Prevalence was computed by dividing the number of test positives by the total number examined multiplied by 100. The Chi-square (χ^2) and logistic regression tests were employed to identify possible association between risk factors and reproductive characteristics with seropositive to *Brucella* infection. The degree of association was considered significant when a *P*-value of less than 0.05 is obtained or when the 95% confidence intervals of the odds ratio (OR) in the multivariable logistic regression analysis doesn't include 1 (Thrusfield, 2007)

4. RESULTS

4.1 Seroprevalence of Brucellosis

In the present study, among 226 serum samples from clinically aborted small ruminants (goats and sheep) tested, 28 were found positive for *Brucella* infection by the mRBPT and 17 were confirmed by CFT tests. Thus, the overall seroprevalence of *Brucella* infection in clinically aborted small ruminants in Amibara district of Afar Region is estimated to be 12.4% (95% CI: 1.22 – 16.44) using the mRBPT test and 7.52% (95% CI: 7.41 – 11.36) by the combined mRBPT and CFT tests.

4.2 Association of Risk factors and Reproductive Characteristics with *Brucella* Seropositivity

Analysis for association between environmental factor and *Brucella* infection using combined mRBPT and CFT was carried using Pearson's chi-square and Fisher's exact tests (Table 6). However, there was no significant association between *kebele* and seroreactivity to *Brucella* infection ($P > 0.05$). Similarly, host associated risk factors including species, age and body condition of the sheep and goats were insignificantly associated with *Brucella* infection ($P > 0.05$). Nonetheless, a relatively higher proportion of seropositivity was observed in Andido (16.13%) and Hasoba (11.67%) kebeles, goats (8.04%) and > 5 years of age (13.46%) while null small ruminants with poor condition.

All small ruminants that were seropositive to *Brucella* infection were found with a history of retained placenta. Besides the stage of abortion and history of retained placenta were significantly associated to *Brucella* seropositivity by combined mRBPT and CFT tests ($P < 0.05$). Whereas, parity and number of abortion were not significantly associated with *Brucella* infection ($P > 0.05$).

Table 6: Association of risk factors with *Brucella* seropositivity in small ruminants, Amibara District, Afar Region

Variables	Tested	Seropositive	Prevalence (%)	χ^2 - value	P-value
Kebeles				8.56 ^a	0.135
Andido	31	5	16.13		
Arba	32	2	6.25		
Bonta	27	1	3.70		
Halidege	31	0	0.00		
Hasoba	60	7	11.67		
Sheleko	45	2	4.44		
Species				0.64 ^a	0.701
Caprine	199	16	8.04		
Ovine	27	1	3.70		
Age				3.68 ^b	0.159
≤2 years	102	5	4.90		
≥3-5 years	72	5	6.94		
> 5 years	52	7	13.46		
Body condition				0.17 ^a	1.000
Poor	2	0	0.00		
Medium	196	15	7.65		
Good	28	2	7.14		
Parity				1.45 ^b	0.485
Null	88	6	6.82		
1-2	96	6	6.25		
≥3	42	5	11.90		
Stage of abortion				11.26 ^a	0.001*
Early abortion	115	2	1.74		
Late abortion	111	15	13.51		
Frequency of abortion				0.69 ^b	0.452
Once	128	8	6.25		
≥2 times	98	9	9.18		
Retained placenta				16.53 ^a	<0.001*
Absent	107	0	0.00 ^a		
Present	119	17	14.29		
Total	226	17	7.52		

χ^2 : Chi-Square; ^aFishe's exact value; ^bPearson's chi-square vlaue; *Significant;

According to the multivariable logistic regression model fitted (Table: 7) for the overall effect within small ruminant to *Brucella* seropositivity revealed that small ruminants in age group greater than 5 years (OR=14.57, 95% CI: 1.72-123.6) are more likely to be at higher risk for *Brucella* infection than animals in ≤ 2 and ≥ 3 -5 years age group.

The multivariate analysis also revealed that increased parity of sheep and goats was more likely to be associated with an increasing risk of getting *Brucella* infection when evaluated collectively for other factors. Thus animal with multiple parturition were at higher risk of encountering *Brucella* infection (OR=8.08, 95% CI: 1.11-58.57) than animals with 1-2 parity and non-parous.

Additionally, *Brucella* infection with in small ruminants were found significantly high in animals aborted in late gestation (OR=8.94, CI: 1.86- 42.90) than in early gestation.

Table 7: Multivariables logistic regression analysis of factors associated with *Brucella* Seropositivity

Variables	Number of animal tested	Infected (%)	Crude OR (95% CI)	Adjusted OR (95% CI)	P-Value
Kebeles					
Andido	31	5 (16.13)	1 ^a	1 ^a	—
Arba	32	2 (6.25)	0.35 (0.06–1.94)	0.26 (0.03–2.48)	0.241
Bonta	27	1 (3.70)	0.20 (0.02–1.83)	0.04 (0.00–0.60)	0.019
Halidege	31	0 (0.00)	—	—	—
Hasoba	60	7 (11.67)	0.69 (0.20–2.37)	0.47 (0.09–2.33)	0.353
Sheleko	45	2 (4.44)	0.24 (0.04–1.34)	0.32 (0.04–2.34)	0.263
Species					
Caprine	199	16 (8.04)	1 ^a	1 ^a	—
Ovine	27	1 (3.70)	0.36 (0.05–2.81)	1.75 (0.11–27.28)	0.690
Age (years)					
≤2	102	5 (4.90)	1 ^a	1 ^a	—
≥3-5	72	5 (6.94)	1.45 (0.40–5.20)	4.38 (0.64–29.72)	0.131
> 5	52	7 (13.46)	3.02 (0.91–10.03)	14.57 (1.72–123.6)	0.014*
Body condition					
Poor	2	0 (0.00)	1 ^a	1 ^a	—
Medium	196	15 (7.65)	0.93 (0.26–3.36)	0.80 (0.11–5.68)	0.826
Good	28	2 (7.14)	—	—	—
Parity					
Null	88	6 (6.82)	1 ^a	1 ^a	—
1-2	96	6 (6.25)	0.91 (0.28–2.94)	0.63 (0.13–3.10)	0.886
≥3	42	5 (11.90)	1.85 (0.53–6.44)	8.08 (1.11–58.57)*	0.039*
Stage of abortion					
Early	115	2 (1.74)	1 ^a	1 ^a	—
Late	111	15 (13.51)	8.83 (1.97–39.58)	8.94 (1.86–42.90)	0.006*
Frequency of Number					
Once	128	8 (6.25)	1 ^a	1 ^a	—
> one times	98	9 (9.18)	1.52 (0.56–4.09)	0.40 (0.09–1.80)	0.230
Total	226	17 (7.52)			

*Significant; OR: Odds Ratio; CI: Confidence Interval, ^aReference

4.3 Isolation of *Brucella*

From a total number of clinical samples collected (n=65) seropositive with mRBPT specimens were subjected to *Brucella* selective cultural isolation and confirmation of *Brucella* isolates by biochemical tests (Annex 3) in present study, 13.84% (9/65) over all *Brucella* isolates were recovered from goats. Among the overall isolates 10.71% (3/28) and 21.43% (6/28), were from milk and vaginal swabs respectively, while no isolates was obtained from fetal abomasal content and fetal membrane (Table: 8).

Morphology and Growth Characteristics: The isolates were initially recognized on the basis of colony morphology a characteristic of *Brucella* growth with very small, glistening, smooth, round and pin-point like colonies with honey like appearance were observed on *Brucella* selective agar plates after 96 hrs or 4 days incubation at 37°C in without CO₂ incubator.

Staining and Biochemical Characteristics: Microscopic examination were performed immediately after the primary isolation on *Brucella* selective agar and Gram stained cultures revealed small Gram negative coccobacilli arranged singly and in pairs and on Modified Ziehl-Neelsen (MZN) stain, the *Brucella* organisms were stained red against a blue background. On different biochemical reactions, the present *Brucella* species were found to be positive for catalase, oxidase, urea hydrolysis, nitrate reduction tests and all the colonies were grown without 5-10% carbon dioxide supplied incubator.

The isolates were further differentiated biochemically using parameters such as CO₂ requirement, H₂S production, and growth on thionin and basic fuchsin dyes incorporated into tryptic soy agar at different concentrations were tested and also all the 9 isolates were checked for agglutination by standard polyclonal antiserum obtained from National Veterinary Institute (NVI), Bishoftu, Ethiopia.

Table 8: *Brucella* isolates recovered from aborted seropositive goats milk and vaginal swabs

Type of Sample	Number		Percentage (%)
	Sample cultured	Isolates	
Milk	28	3	10.71%
Vaginal swab	28	6	21.43
Fetal abomasal content	2	0	0
Fetal membrane	7	0	0
Total	65	9	13.84

Accordingly, biochemical test, CO₂ requirement and sensitivity to dyes (Table: 9) all the nine (9) cultural isolates obtained in present study from seropositive goats milk and vaginal swabs sample revealed *B. melitinensis*.

Table 9: Staining and biochemical test results of *Brucella* from seropositive goats milk and vaginal swabs

<i>Brucella</i> Isolates	Animal species	Gram's Stain	MZN	Biochemical Properties					Growth on dyes		Agg. With pl.clonal serum	
				Cat.	Oxd.	Ure.	CO ₂ Req.	H ₂ S pro.	Nitr.Red	Thionin		Basic Fuchsin
Milk 1	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Milk 2	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Milk 3	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Vaginal swab 01	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Vaginal swab 02	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Vaginal swab 03	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Vaginal swab 04	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Vaginal swab 05	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+
Vaginal swab 06	Goat	Gram negative	+	+	+	+	—	—	+	+	+	+

Cat, Catalase; Oxi, Oxidase; Ure, Urea hydrolysis; MZN, Modified Zeihl Neelsen stain; CO₂ Req, Carbon Dioxide requirement; Agg, Aggultination; H₂S pro, Hydrogen Sulphide production; Nitr.Red, Nitrate reduction.

5. DISCUSSION

The present study revealed that the overall seroprevalence of brucellosis in small ruminants that had history of recent abortion was 12.4% and 7.52% with mRBPT and CFT, respectively. This is fairly comparable to the seroprevalences of small ruminant bucellosis reported elsewhere in Ethiopia incuding 12.35% reported in Afar region (Anteneh *et al.*, 2014), 9.6% in Yabello pastoral Area (Yohannes *et al.*, 2013) and 9.11% in Dire Dawa (Negash *et al.*, 2012). It is also consistent with the report of Denis *et al.* (2015) who reported 8% and 11% prevalences in sheep and goats with a history of abortion and retained placenta respectively in Soroti towns of Uganda. This could be due to the similarities of animal husbandry, communal grazing of range lands and watering areas and possibly similar climatic conditions (Teshale *et al.*, 2006).

However, the prevalence obtained in this study by CFT is higher than the prevalence of 4.8% in small ruminants reported by Ashenafi *et al.* (2007) in the same study area. Teshale *et al.* (2006) also reported a seroprepvalence of 1.7% in Goat and 1.6% in sheep in Somali Pastoral Area. Other studies revealed seroprevalence of 1.3% in goats and 1.5% in sheep (Teklay and Kasali, 1990) in central highlands of Ethiopia. These differences could be mainly due to the current study was carried out on animals that had history of recent abortion and this may show the strong association of abortion and *Brucella* infection (Radostits *et al.*, 2007; OIE, 2009). It could be also due to variation in sensitivity and specificity imparted by the various tests, agro-ecological location and amount of sampled study population, management and production systems. Most of the above findings used standard Rose Bengal Plate Test (RBPT) for screening, but in this study mRBPT (Modified Rose Bengal Plate Test) was used. This simple modification is achieved by increasing slightly the amount of sera for the test dose from 25 µl to 75 µl, at the same time maintaining the antigen volume at 25 µl this may significantly increase in the sensitivity of the test without affecting the specificity (Blasco *et al.*, 1994; Ferreira *et al.*, 2003; WHO/ FAO, 2006).

The present study revealed that stage of abortions and retained placenta was significantly associated with seropositivity for *Brucella* infection in the study animals. This indicates that abortions or stillbirths and retained placenta are typical outcomes of brucellosis (Radostits *et al.*, 2007).

The distribution of Brucellosis in small ruminants having history of recent abortion among the different pastoral *kebeles* the highest seroprevalence was recorded in Andido 15.6%, followed by 10.8%, 9.1%, 5.7% and 3.3% and in Hasoba, Arba, Sheleko and Bonta, respectively. However, no statistically significant difference ($P>0.05$) was observed in the prevalences among the six pastoral *kebeles* of the district.

Age, stage of abortion and parity status remained significant in multivariable logistic regression analysis. Multivariable logistic regression analysis revealed that seropositivity for *Brucella* infection increases approximately 15 times in > 5 years old animals compared to ≤ 2 and $\geq 3-5$ years old. This might be the frequency of exposure to *Brucella* infection could be high in mature animals (European commission, 2001). Several previous studies have indicated that higher seroprevalence of brucellosis in adult age group of small ruminants (Ashenafi *et al.*, 2007; Bekele *et al.*, 2011). Sexually mature animals are more susceptible to *Brucella* infection than sexually immature animals of either of sex, which is due to the fact that sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organism, tend to increase in concentration with age and sexual maturity (Quinn *et al.*, 2004; Radostits *et al.* 2007).

The seropositivity for *Brucella* infection in small ruminants found to be high in animals with a history of abortion in late stage of gestation than in early stage both in invariable and multivariable logistic regression analysis. This could be explained by the presence of higher concentration erythritol (2R, 3S) - butane- 1, 2, 3, 4, tetraol, a low calorie sugar alcohol produced naturally by the developing foetus may favours multiplication of *Brucella* where it causes degeneration and necrosis of the cotyledons leading to abortion from about the last months of gestation (Smith *et al.*, 1972; Coetzer and Tustin, 2004; Radostits *et al.*, 2007). In addition, in highly susceptible non vaccinated pregnant small ruminants, abortion on the last month of pregnancy is cardinal feature of the disease (Radostits *et al.*, 2000).

Similarly, multivariable logistic regression revealed the risk of seropositivity was approximately 8 times higher in (≥ 3) parity compared to (1-2) and null parity group. Higher parity was also significantly associated with the disease which agrees with the finding of Ashagrie *et al.* (2011). This might be animals generally abort once during the mid-third of gestation but re-invasion of the uterus occurs in subsequent pregnancies with shedding in fluids and foetal membranes (Fensterbank, 1987).

Isolation of *Brucella* species is the gold standard for identification and confirmation of animal brucellosis. Previous studies in various parts of Ethiopia indicated that the disease is widespread among small ruminant populations. However, most surveys of brucellosis in Ethiopia rely on serological test only, and there is no evidence for bacteriological isolation of *Brucella* species except by Minda *et al.* (2015) (unpublished MSc thesis) who reported isolation of *B.abortus* from aborted dairy cattle in Asela.

To our information, isolation and biochemical characterization of *Brucella* from aborted seropositive goats' milk and vaginal swab were carried out for the first time in Ethiopia. The present, 13.84% *Brucella* isolates from seropositive animals with a history of recent abortion is in agreement with previous report from elsewhere 14.2% of *B.melitensis* (Čekovska *et al.*, 2010).

The isolation rate of the *Brucella* from seropositive goat's milk and vaginal swab in the present study is lower than 33.8% of *B. melitensis* isolated from aborted sheep and goats in Turkey (Esra *et al.*, 2009). This variation might be because of the sensitivity of *Brucella* culture media used, facilities and culture techniques employed (WHO/FAO/ 2006). In addition, *Brucella* species, stage of the disease and quantity of circulating bacteria could affect the isolation rate (Marin *et al.*, 1996).

In present study among the overall isolates three isolates were from milk and six isolates from vaginal swabs recovered, while no isolates was obtained from placenta and foetal abomasal content. This might be due to the slow growing and fastidious nature of the pathogen and fast-growing contaminants would usually suppress and overwhelm the growth of *Brucella* species if present, since it is slow-growing (Koneman *et al.* 1997; Erdenlig and Sen, 2000; Seleem *et al.*, 2010).

Shedding of *Brucella* in the milk of infected animals is an important source of transmission of the disease to humans if raw milk is consumed. However, in pastoral areas drinking of raw milk is common (Sözen, 1996; Erol, 1997; Tantillo *et al.*, 2003).

Based on biochemical characterization among the overall isolates 3 (10.34%) from milk revealed *B. melitensis* which is fairly similar to the findings of different studies, *Brucella* isolated from milk of cattle with rate of 3.2% (Ali *et al.*, 2014) in Pakistan, 4.4% (Celebi and Otlı, 2011) and 4% (Recep, 2013) in Turkey. This might be in goats, about two thirds of acute infections acquired naturally during pregnancy leads to infection of the udder and excretion of

the bacteria in milk during subsequent lactation and there is also evidence that mammary gland may be even more favored for localization than the reproductive tract (Alton, 1990; Anonymous, 2007).

Generally, this finding revealed that high risk of transmission of the disease in the small ruminant and people of the studied area because *Brucella* organisms are usually shedded through the milk of infected goats. And beside this there is a strong cultural belief among the pastoralists that favour the consumption of raw milk against pasteurized milk. The most common means of transmission of brucellosis from animals to humans is through the consumption of unpasteurized or raw milk and milk products (Radostits *et al.*, 2000).

The isolation of *B. melitensis* from the vaginal swab is also of great public health significance. This is because in cases of retained placenta, the pastoralists usually use their bare hands to pull the placenta out of the vulva. These habits and practices expose them to high risk of contracting brucellosis (Bekele *et al.*, 2013; Habtamu *et al.*, 2015).

6. CONCLUSION AND RECOMMENDATIONS

Small ruminant brucellosis caused by *B. melitensis* has a major impact on human health, besides its significant economical losses in small ruminant industry. Based on growth characteristics combined with biochemical test analysis all the nine (9) *Brucella* isolates from seropositive goats milk and vaginal swabs in this study revealed *B. melitensis*. This may be the first report to show that the possible prevailing *Brucella* circulating among small ruminants with a history of recent abortion in the study area. Moreover, the serological test revealed that the prevalence rates of brucellosis among small ruminants were moderately high in the study area. In addition, age, history of late abortion period, parity status and occurrence of retained placenta among small ruminants were significantly associated with *Brucella* seropositivity. In conclusion, the bacteriological isolation of presumptive *B. melitensis* from milk and vaginal swab combined with the prevailing *Brucella* seropositivity in small ruminants that had history of recent abortion indicate the importance of brucellosis in small ruminants and represents a potential threat to animal production and public health in the study areas.

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

- ◆ Active infection control measures such as proper disposal (burning or buried) of aborted fetus, pasteurization or boiling of milk before consumption should be carried out to reduce risk of infection and transmission of the disease in livestock and human in the study area.
- ◆ Public health educational programmes should be carried out targeting brucellosis in the areas to alert, not only the livestock owners also the general public on the danger of possible disease transmission both in animals and humans.
- ◆ Since the isolation of *Brucella* carried out in this study was based on biochemical test level, further molecular characterization of the isolates should be carried out to species and biovariant level in order design effective vaccine for small ruminants
- ◆ A similar work should be initiated in the study area to determine the prevalence of brucellosis in other livestock (cattle, camel, and dog) and also in humans.
- ◆ Case-control studies, not only *brucella* but also other abortion causing pathogens investigation should be carried out in the area.

- ♦ Interdisciplinary collaboration and joint efforts among veterinary and public health professionals should be encouraged to control this disease.

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8. ANNEXES

Annex 1: Preparation of media

a) Brucella selective agar

Procedure

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

Annex 2: Preparation of staining solutions and procedure of staining

a) Stamps Modified Ziehl-Neelsen Staining procedure for Brucella

Reagents:

Diluted Carbol Fuchsin

Concentrated Carbol Fuchsin.....10.0 ml

Distilled Water90.0 ml

Acetic Acid (Decolorizer)

Concentrated Acetic Acid.....1.0 ml

Distilled Water.....200.0 ml

Methylene Blue (Counter Stain)

Methylene blue.....8.0 g

Ethanol 95% v/v300.0 ml

Distilled water.....1300.0 ml

Potassium Hydroxide0.13 g

Procedure

1. Fix a smear by heat
2. Overlay the slide completely with dilute carbolfuchsin for 15 minutes
3. Dicolorize the smear for 15 seconds in 0.5%acetic acid and wash it with tap water
4. Counter stain with methylene blue for 2minutes, wash again with water and dry it.
5. Examine under 100x oil immersion objective microscope.

Interpretation

Brucella species appeared red, small coccobacilli arranged single, pair or sometimes graphe. Other bacteria appear blue.

b) Gram's staining**Procedure:**

From a fresh colony take a loop full of bacteria and emulsify it in a small drop of water or saline on the slide. This should be a thin, not milky, suspension or it will not stain properly. Air dries the slide.

1. Fix the smear by passing on Bunsen burner
2. Pour crystal violet for 1 minute
3. Flood with tap water
4. Pour Gram's iodine (Mordant) for 1 minute
5. Flood with Tap water
6. Decolorize with 95% Ethanol alcohol for 5-10 seconds
7. Rinse with tap water
8. Pour safranin (counter stain) for 1 minute
9. Rinse with Tap water
10. Examine the slide under 100x oil immersion microscope

Interpretation

Gram positive bacteria appear blue/ violet

Gram negative bacteria appear red/ pink

Annex 3: Biochemical tests**a) Catalase Test**

This test detects the enzyme catalase that converts hydrogen peroxide to water and gaseous oxygen.

Procedure:**Slide method:**

With an inoculating needle/loop pick a pure colony and place on a clean glass slide.

Add a drop of 3% H₂O₂ over organisms on slide

Test cannot be applied if blood agar is used

Do not reverse the order of procedure as false positive result may occur.

Do not mix with inoculating needle or loop.

Mixing of culture and H_2O_2 is not necessary

Tube methods:

Directly add 1.0ml of H_2O_2 to an 18 to 24 hrs heavily inoculated pure agar slant culture.

Observe for immediate bubbling and record the result.

Interpretation

Positive result: immediate bubbling, easily observed

Negative: no bubbling

b) Oxidase test

Oxidase reagents:

Tetramethyl-p-phenylenediamine dihydrochloride (1% water solution) – **Kovac's** reagent

Dimethyl-p-phenylenediamine dihydrochloride (1% water solution) – **Gordon and McLeod's** reagent.

Procedure:

Direct plate procedure

1. Add 2-3 drops of oxidase reagent directly to a few suspected colonies growing on plate medium. Do not flood the entire plate.
2. Do not invert the plate
3. Observe color change after 15-30 seconds (Kovac's reagent)

Indirect paper procedure

Place a 6cm² pieces of Whatman no. 1 filter paper in petri dish

Add 2-3 drops of Kovac's reagent to a center of the paper

Smear loop full of a suspected colony on to the reagent impregnated paper in a line 3-6 cm long

A positive color reaction occurs within 5-10 seconds

Interpretation

Oxidase positive: the colonies form dark blue color after few seconds

Oxidase negative: no color formation

c) Urease Test

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

Procedure

Urea agar/broth inoculated with a loop full of pure culture of the test organisms and incubates at the 35°C for 18-24 hrs.

Interpretation

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

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

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

No urea hydrolysis – Medium used original yellow colour.

d) Lead acetate paper test to detect H₂S production

When a sensitive technique for detecting H₂S production is required, the lead acetate paper test is recommended. For this, you have to:

Inoculate a tube or bottle of sterile trypticase soy broth with the test organism.

Insert a lead acetate paper strip in the neck of the bottle or tube above the medium, and stopper well.

Incubate the inoculated medium at 35-37°C, and examine daily for a blackening of the lower part of the strip.

Expected Results

Blackening ————— Positive test H₂S produced

No blackening ————— Negative test No H₂S produced.

Annex 4: Preparation of dyes (thionin and basic fuchsin)

Weigh 0.1g (0.1%) of thionin or basic fuchsin and dissolve in 100ml of distilled water to obtain a stock solution of thionin and basic fuchsin respectively and 1g in 100ml (1%) of safranin to obtain stock solution of safranin.

Heat the solutions in fowing steam for 20 minutes or in boiling water for 1 hour.

Leave for at least 48 hours before using them for the first time.

Store the stock solution of dyes at 4°C and discard after 3 months.

Before preparing the dye media, it is advisable to remove the stock solutions of dyes from the refrigerator and allow them to rest at room temperature for 4 hours.

Preparation of dye medium

40g of Trypticase soy agar (TSA) is dissolved in 1 litre of distilled water. The suspension is autoclaved and cooled to 45-56°C and 5% of calf or horse serum is added.

The appropriate amount of the dye solutions is then added.

The media is then poured into petri

Annex 5: Agglutination with positive and negative *Brucella* sera

For antigen production, the seed culture is used to inoculate a number of potato-infusion agar slopes that are then incubated at 37°C for 48 hours. SDA and TSA, to which 5% equine or newborn calf serum and/or 0.1% yeast extract may be added, are satisfactory solid media provided a suitable seed is used as recommended above. The growth is checked for purity, re-suspended in sterile PBS, pH 6.4, and used to seed layers of potato-infusion agar or glycerol–dextrose agar in Roux flasks. These are then incubated at 37°C for 72 hours with the inoculated surface facing down. Each flask is checked for purity by Gram staining samples of the growth, and the organisms are harvested by adding 50–60 ml of phenol saline (0.5% phenol in 0.85% sodium chloride solution) to each flask. The flasks are gently agitated, the suspension is decanted, and the organisms are killed by heating at 80°C for 90 minutes. Following a viability check, the antigen is stored at 4°C.

The culture is harvested by centrifugation to deposit the organisms, which are re-suspended in phenol saline. The organisms are killed by heating at 80°C for 90 minutes and are stored at 4°C. They must form stable suspensions in physiological saline solutions and show no evidence of auto-agglutination. A viability check must be performed on the suspensions and no growth must be evident after 10 days' incubation at 37°C. The packed cell volume (PCV) of the killed suspensions can be determined by centrifuging 1 ml volumes in Wintrobe tubes at 3000 *g* for 75 minutes.

Procedure and Interpretation

A loopful of *Brucella* suspension was added to a drop of both positive and negative *Brucella* sera on a clean glass slide. They were mixed using wire loop, rocked and observed for agglutination. Agglutination confirmed an isolate positive for *Brucella* while absence of agglutination indicated negative result.

Annex 6: The Rose Bengal Plate Test

Procedure

Sera (control and test sera) and antigen for use were left at room temperature for half an hour before testing, since active materials straight from the refrigerator react poorly

1. 25µl serum was mixed with 75ul volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.
2. The antigen and serum were mixed thoroughly using an applicator stick (a stick being used only once)
3. Rock plate by hand for about 4 minutes
4. Examine for agglutination in a good light
5. Use magnifying glass when micro agglutination suspected

Interpretation

0 = no agglutination

+ = barely perceptible

++ = fine agglutination, some clearing

+++ = coarse clumping, definite clearing

Annex 7: Complement Fixation Test

Procedure

1. Test sera and appropriate working standards are diluted with an equal volume of veronal buffered saline in small tubes and incubated at 58°C for 50 minutes in order to inactivate the native complement.
2. Using standard 96-well U-bottom microtitre plates, 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.
3. Serial doubling dilutions are then made by transferring 25 µl volumes of serum from the second row onwards continuing for at least four dilutions.
4. Repeat steps ii and iii above for each serum to act as anticomplementary serum controls (see below).
5. Volumes (25 µl) of complement at 1.25 MHD, are added to each well and 25 µl of antigen, diluted to working strength, are added to all wells excluding those of the anticomplementary controls. These latter wells receive 25 µl of veronal buffered saline instead.

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

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

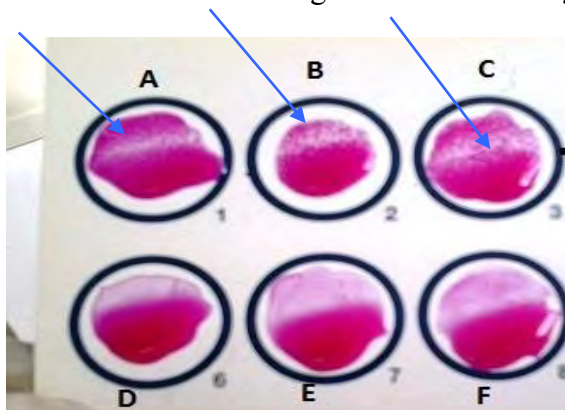
8. Volumes (25 µl) of sensitised SRBC suspension are added to each well, and the plates are reincubated at 37°C for 30 minutes with agitation at least for the first 10 minutes.

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

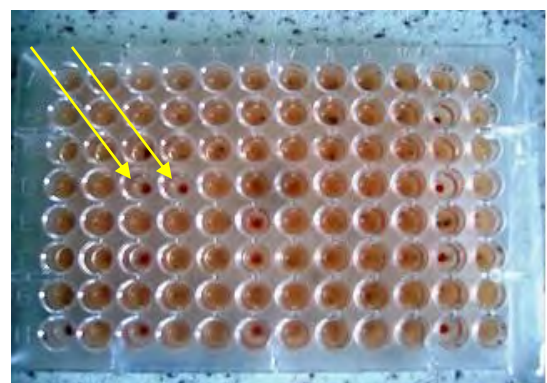
Interpretation

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

Annex 8 : Pictures on serological and bacteriological result



a) mRBPT test, Arrows indicate positive
Test result

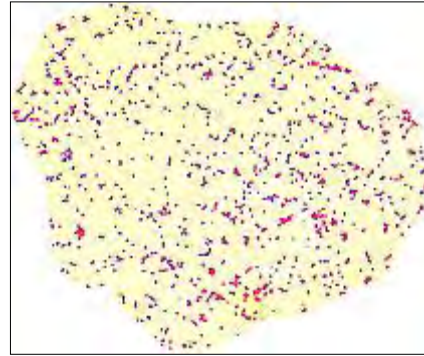


b) CFT result test, Arrows indicate
positive test result



c) *Brucella* Species Colony Cultured on Brucella Selective agar after (94 hrs) 4 day's incubation





d) Photo taken from Microscope, MZN staining (Left) and Gram's stain (Right) of *Brucella* (red, short coccobacilli)



e) Non-hemolysis on blood agar



f) Oxidase positive test Result



g) Growth on Basic dye



h) Urease Test Result positive (right) negative (left)