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SEROPREVALENCE AND ISOLATION OF BRUCELLA SPECIES FROM CAMEL AND CATTLE WITH HISTORY OF RECENT ABORTION IN AMIBARA DISTRICT, AFAR REGIONAL STATE

MSc THESIS

BY

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Department of Microbiology, Immunology and Veterinary Public Health

JUNE, 2017

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 Yimer Gubena Hailu entitled: SEROPREVALENCE AND ISOLATION OF BRUCELLA SPECIES FROM CAMEL AND CATTLE WITH HISTORY OF RECENT ABORTION IN AMIBARA DISTRICT, AFAR REGIONAL STATE 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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DEDICATION

This piece of work is dedicated to my spiritual father, Jesus Christ, and his mother Saint Merry for everything they guide me during the terrible course of my master's degree education.

Moreover, the work is dedicated to my beloved father Gubena Hailu Shibesh who is not in life to see my success and also dedicated to my mother w/ro Emaway Temesgen Asfaw for her love, encouragement and moral support during my university life.

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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Contents

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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
C-ELISA	Competitive Enzyme Linked Immune Sorbent Assay
CFT	Complement Fixation Test
CSA	Central Statistics Agency
CVMA	College of Veterinary Medicine and Agriculture
ELISA	Enzyme Linked Immunosorbent Assay
GDP	Gross Domestic Product
IgA	Immunoglobulin A
IGAD	Intergovernmental Authority on Development
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MBM	Malachite Brucella Medium
MHC	Major Histocompatibility Complex
MZ	Modification of the Ziehl–Neelsen
NAHDIC	National Animal Health Diagnostic and Investigation Center
NVI	National Veterinary Institute
OPS	O polysaccharide
PB	Phosphate buffered saline
PR	Polymerase chain reaction
RRs	Pattern Recognition Receptors
RB	Rose Bengal Plate Test
RFLP	Restriction Fragment Length Polymorphism
RLPS	Rough Lipopolysaccharide
SDA	Serum Dextrose Agar
SLP	Smooth Lipopolysaccharide
SNNPRS	Southern Nations Nationalities and People`s Regional State
SRBC	Sheep Red Blood Cells
SSA	Sub-Saharan Africa
TLR	Toll-Like Receptors

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ABSTRACT

A cross sectional study was conducted from November 2016 to April 2017 in selected district of Afar regional state to determine the sero-prevalence of Brucellosis, isolate and identify brucella species in camels and cattle with history of recent abortion. A total of 350 blood samples were collected from camels (n=223) and cattle (n=127) based on purposive sampling technique for sero-prevalence study. In line with this, a total of 42 specimens (25 milk, 15 vaginal swab and 2 synovial fluid) were collected for isolation and identification of *Brucella* species. In addition, data related to risk factors of transmission or occurrences of brucellosis were also collected to assess the potential association of risk factors with seropositivity. The sera samples were serologically screened using classical Rose Bengal Plate Test (RBPT), Complement Fixation Test (CFT) and Competitive Enzyme Linked Immune sorbet Assay (C-ELISA). Milk, Vaginal Swab and Synovial fluid samples were properly processed and subjected to bacteriological isolation on Brucella Selective Media. Accordingly, the serological test result in the present study revealed that out of a total of 350 animals tested the overall seroprevalence of brucellosis was 8.6% using RBPT alone, 1.7% with combined RBPT-CFT and 18.3% with C-ELISA. The seroprevalence of *Brucella* infection in clinically aborted camels in the study area was estimated to be of 6.7% with RBPT alone, 1.3% with combined RBPT-CFT and 13.5% with C-ELISA; similarly the seroprevalence in cattle was 11.8% with RBPT alone, 2.4% with combined RBPT-CFT and 26.8% with C-ELISA. History and stage of abortion in cattle with Fisher`s exact test analysis and age in both cattle and camel with multivariable logistic analysis showed a statistically significant ($p< 0.05$) association with seropositivity using combined RBPT-CFT test and C-ELISA respectively. However, the association of other risk factors with seropositivity of brucellosis was not significant. Out of 42 clinical samples cultured, 5 were positive with an overall rate of isolation of 11.9% (5/42) and all the five isolates were confirmed to be *B. abortus* based on biochemical test result. *B. abortus* was isolated from vaginal swab 20% (3/15) and synovial fluid 100% (2/2) while no isolate was obtained from milk. In conclusion, Brucellosis in Ethiopia in general and in the study area in particular still remains prevalent and *Brucella abortus* was identified as causative agent of brucellosis. The isolation of *Brucella abortus* mainly from vaginal swab shows the potential risk of zoonotic transmission and hence warrants the need for further research and community awareness creation.

Key words: *Abortion, Afar, Brucella, Camel, Cattle, Isolation, Pastoralist, Seroprevalence.*

1. INTRODUCTION

Brucellosis was considered by Food and Agriculture Organization of the United Nation (FAO), World Health Organization (WHO) and Office International Epizooties (OIE) as one of the most widespread zoonoses in the world (Schelling *et al.*, 2003). According to OIE, it was the second most important zoonotic disease in the world after rabies. The disease affects cattle, swine, sheep, goats, camels and dogs. It may also infect other ruminants and marine mammals(Schelling *et al.*, 2003).

The disease poses a barrier to trade of animals and animal products, represents a public health hazard, and is an impediment to free animal movement (WHO 2006). The most common clinical manifestation of brucellosis in natural hosts is reproductive loss resulting from abortion, birth of weak offspring, or infertility. In particular, abortion, stillbirth or a weak, nonviable calf is the hallmark of brucellosis (Olsen and Tatum 2010). Loss of a calf or kid due to abortion and its sequelae frequently lead to infertility and reproductive losses. The role of specific etiologic agents such as Brucella species in causing abortion and reproductive loss has been well established, as documented by Radostits *et al.* (2000), but known causes of abortion and female infertility involve a wide range of etiologic agents.Th e disease was manifested by late term abortions, weak calves, still births, infertility and characterized mainly by placentitis, epididymitis and orchitis. *B. melitensis, B. abortus and B. suis* are zoonotic pathogenic species which can also infect humans. *B. canis* may cause infections in immune suppressed individuals (Young, 2000; Mantur &Amarnath, 2008).

Camels (*Camelus dromedarius*) were vital domestic animal species that are best adapted to harsh environments and fluctuating nutritional conditions of arid and extreme arid zones. These animals are endowed with extra ordinary features that enable them to survive and perform in such hard conditions (Habtamu *et al*., 2014). Dromedaries were versatile living assets that ensure food security even during the dry periods and also serve as means of transportation and draught power (Higgins *et al*., 1992). Africa hosts 80% of the world population of dromedary (16.5 million), of which 63% attributed to east Africa (Wilson, 1998). According to the animal population census (CSA, 2010/11), the camel population in Ethiopia was estimated to be 1.07 million. The major ethnic groups owning camels in Ethiopia are the Afar, Somali and Borana (Workneh, 2002). Camels were kept in the arid lowlands of Ethiopia which cover approximately 61-65% of the total area of the country and, were the homes to 12-13 % of the total human population (Beruk, 2003).

In spite of its vital importance particularly to the marginalized communities in the dry zone s of tropics and subtropics, studies about camel are very few. Brucellosis can affect almost all domestic species, and cross transmission can occur between cattle, sheep, goats, camels and other species (Ghanem *et al*., 2009), causing significant reproductive losses in sexually mature animals (Radostitis *et al*.,1994; Adugna *et al*., 2013).

Camelid brucellosis caused by *B. melitensis* and *B. abortus* has been reported in all camel-rearing countries except Australia and the incidence camel brucellosis appears to be closely related to breeding and husbandry practices (Richard, 1980). Omer *et al.* (2010) were also able to prove in Saudi Arabia association of camel brucellosis to husbandry practices. Camels can be infected when they are pastured together with infected sheep, goats and cattle (Musa *et al*., 2008).

Bovine brucellosis is an infectious and contagious disease known for its impact on reproductive performance of cattle and is predominantly a disease of sexually mature animals (McDermott and Arimi, 2002; Rahman *et al*., 2011; 2012; Asmare *et al*., 2013). Brucellosis in cattle is characterized primarily by abortion late in pregnancy, frequently followed by fetal membrane retention and endometritis which may be the cause of infertility in subsequent pregnancies (Ahmad *et al*. 2009; Radostits *et al*. 2007). Since the first report of brucellosis in the 1970s in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Ibrahim *et al*., 2010; Kebede *et al*., 2008; Geresu *et al*., 2016). A large number of studies on bovine have been reporting individual brucellosis seroprevalence ranging from 1.1% to 22.6% in intensive livestock management systems (Tolosa *et al*., 2010; Tesfaye *et al*., 2011).

Bovine brucellosis is usually caused by *Brucella abortus*, less frequently by *B. melitensis*, and rarely by *B. suis.* Although *Brucella abortus* is mainly associated with cattle, occasionally other species of animals such as sheep, swine, dogs, camels and horses may be infected. In horses, *B. abortus* together with *Actinomyces bovis* may be present in poll evil and fistulous withers (Radostits *et al*., 2000; OIE, 2008).

The disease occurs worldwide, except in those countries where bovine brucellosis (*B. abortus*) has been eradicated. The disease remains endemic among Mediterranean countries of Europe, Northern and Eastern Africa, Near East countries, India, Central Asia,

Mexico and Central and South America. Although *B. melitensis* has never been detected in some countries, there are no reliable reports that it has ever been eradicated from small ruminants (FAO, 2003). Furthermore, brucellosis is also considered as a re-emerging problem in many countries such as Israel, Kuwait, Saudi Arabia, Brazil and Colombia, where there is an increasing incidence of *B. melitensis* or *B. suis* biovar 1 infection in cattle (Cutler *et al.*, 2005).

Globally, this disease is under-reported because of its vague clinical symptoms, difficult laboratory diagnosis and lack of familiarity of the medical professionals (Corbel, 2006). Within Sub-Saharan Africa (SSA), many of the known infectious diseases occur commonly and are poorly controlled, both in livestock and in human populations (Mange *et al*., 2002; McDermott *et al*., 2002).It has been stated that in SSA, the epidemiology of brucellosis in humans and livestock is not well understood, and available data is limited (Schelling, 2003; McDermott *et al.*, 1999; Adugna *et al*., 2013).

Many developing countries with limited resources, including Ethiopia, are facing other priority diseases that are more spectacular and have not yet fully launched programs featuring any aspects of brucellosis intervention. Hence, brucellosis remains endemic and continues to be amajor public and animal health problem in the developing regions of the world (Godfroid *et al*., 2005). The disease can generally cause significant loss of productivity through abortion, prolonged calving, kidding, or lambing interval, low herd fertility, and comparatively low milk production in farm animals (Radostits *et al*., 2000). The disease could seriously impair socio-economic development for livestock owners, which represent a vulnerable sector in rural populations in general and pastoral communities in particular. It has a significant public health implication for a pastoral community in consequence of lifestyles, feeding habits, close contact with animals, low awareness, and poor hygienic conditions which favor infections (Schelling *et al.*, 2003). 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.

Under Ethiopian context, livestock of different species usually share pastures and dwellings. This may play a role in maintenance and transmission of endemic diseases such as brucellosis. Brucellosis is common in rural areas because farmers live in close contact

with their animals and often consume fresh unpasteurized dairy products. However, the vending of dairy products may also bring the disease to urban areas (Mantur & Amarnath, 2008; Amenu *et al.*, 2010).

Brucellosis has been reported in bovine from different parts of the world. Infection is widespread globally. Several countries in Northern and Central Europe, Canada, Japan, Australia and New Zealand are believed to be free from the agent (OIE, 2009).

The overall individual level sero-prevalence of brucellosis in cattle prevalence rates were also reported in indigenous cattle in Nigeria (32.2%) (Junaidu *et al*., 2008) and in crossbre cattle in Algeria(9.7%), (Aggad and Boukraa, 2006), and reporting individual brucellosis seroprevalence ranging from 1.1% to 22.6% in intensive management systems (Asmare *et al.,* 2007; Hailemelekot *et al.,* 2007; Tolosa *et al*., 2010; Tesfaye *et al.,* 2011) and 0.05% - 15.2% in extensive management system (Berehe *et al*., 2007; Hunduma and Regassa, 2009; Asmare *et al*., 2010; Degefa *et al*., 2011; Megersa *et al*., 2011) in Ethiopia.

Camels seroprevalence 15.4% in Kenya (Wanjohi *et al*., 2012), 3.1% in Somalia (Ghanem *et al*., 2009),30.5% in Sudan (Ahmed *et al.,* 2007), 7.61% in Egypt (Hassanain and Ahmed 2012). In Ethiopia ranging between 1.8-23.8 % (Bekele, 2004; Teshome *et al., 2003;*Musa *et al.,* 2007*;* Bekele *et al*., 2013) .7.6% (Woldegebriel, 2011), 2.1 % (Fikre *et al*., 2016) in Afar region.

Even though seroprevalence of brucellosis in Ethiopia is established in different species of animals, there were few published report on bacteriological diagnostic technique which is the most reliable and confirmatory tests for isolation of *Brucella* spp. and biovars. There was only two study carried out to isolate *Brucella* species from small ruminants and cattle in selected areas of Ethiopia (Melesse *et al.,* 2007; Minda *et al*., 2016) but there was no isolation report in camels.

Therefore, the objectives of this study were:

- To determine sero-prevalence of brucellosis in camels and cattle with a history of recent abortion.
- To isolate and identify brucella species from camels and cattle with a history of recent abortion.
- To identify risk factors associated with camel and cattle brucellosis in the study area.

2. LITERATURE REVIEW

2.1. Taxonomy of the causative agent

The etiological agent of brucellosis is a bacterium of the genus *Brucella*. Currently ten species are recognized including the better known six classical species comprised of *B. abortus, B. melitensis, B. suis, B. ovis, B. canis* and *B. neotomae*. More recently, new members to the genus include *B. ceti* and *B. pinnipedialis, B. microti* and *B. inopinata* (Godfroid *et al.,* 2011).

Brucellae are facultative intracellular coccobacilli belonging to the order Rhizobiales of the α-2 subgroup of Proteobacteria. The class alpha-proteobacteria includes organisms that are Either mammalian or plant pathogens or symbionts (Garrity, 2001; Ficht, 2010). The Proteobacteria are a major phylum of bacteria, which include a wide variety of pathogens, such as Escherichia, Salmonella, Vibrio, and Helicobacter. All proteobacteria are Gram negative, with an outer membrane mainly composed of lipopolysaccharides (Bergey *et al*., 1994). Within the family Brucellaceae, Ochrobactrum is the closest phylogenetic neighbour of Brucella. Historically, Brucellae are differentiated by host tropism, pathogenicity and phenotypic traits (Al Dahouk et al., 2013).

Brucella is taxonomically placed in the alpha-2 subdivision of the class Proteobacteria. The species of *Brucella* based on preferential host specificity: *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). Some *Brucella* specie like *B. abortus, B. melitensis, B. suis* and *B. canis* can affect a ranges of hosts in addition to their natural hosts resulting hazards on the health of animals including humans; due to this, infected countries are challenged and have been under difficulties to overcome or control brucellosis effectively. In addition to cattle, *B. abortus* can affect other animals like sheep, goats, horses, camels, swine, dogs

and humans. *Brucella melitensis* also affects other animals like sheep, horses, swine, camels, dogs and humans. *B. suis* also affects different animal species such as cattle, sheep, goats, dogs, camels, horses and humans. *B. ovis* affects only ovine while *B.canis* affects dogs and humans (FAO *et al*., 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, 8, and 5 biotypes, respectively (Whatmore, 2009). 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).

The different synonyms of Brucellosis include: undulant fever, Malta fever, Mediterranean fever, enzootic abortion, epizootic abortion, contagious abortion, Bang's disease, Gibraltar fever, Cyprus fever, Rock fever and typhomalarial fever in animal and human. It was an important zoonotic disease and causes significant reproductive losses in sexually mature animals (Forbes and Tessaro, 1996; Mantur *et al*., 2007; Wadood *et al*., 2009).

Table 1: Hosts affected by *Brucella* species

Species	*B. Abortus*	*B. Melitensis*	*B. Suis*	*B. Ovis*	*B. canis*
Cattle	+	+	(+)	-	-
Sheep	(+)	+	+	+	-
Goats	(+)	+	-	-	-
Swine	(+)	(+)	+	-	-
Dogs	+	+	(+)	-	+
Camels	+	+	-	-	-
Humans	+	+	+	-	+
Horse	+	(+)	(+)	-	-

Key: +: can be affected, - : can't be affected, (+): rarely affected;

Source: FAO, 2006.

2.2. Morphology of Brucella

Brucella species are slow-growing, Gram negative coccobacilli or short rods measuring from 0.6 to 1.5μm long and from 0.5 to 0.7μm wide, non motile, non-spore forming, non-capsulated, non-flagellated, 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 decolourisation 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^{o}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 lipopolysaccharide (RLPS) and protein antigens (Blasco *et al*., 1990) see (figure 1). Smooth lipopolysaccharide contains an immune dominant 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 atendency 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)

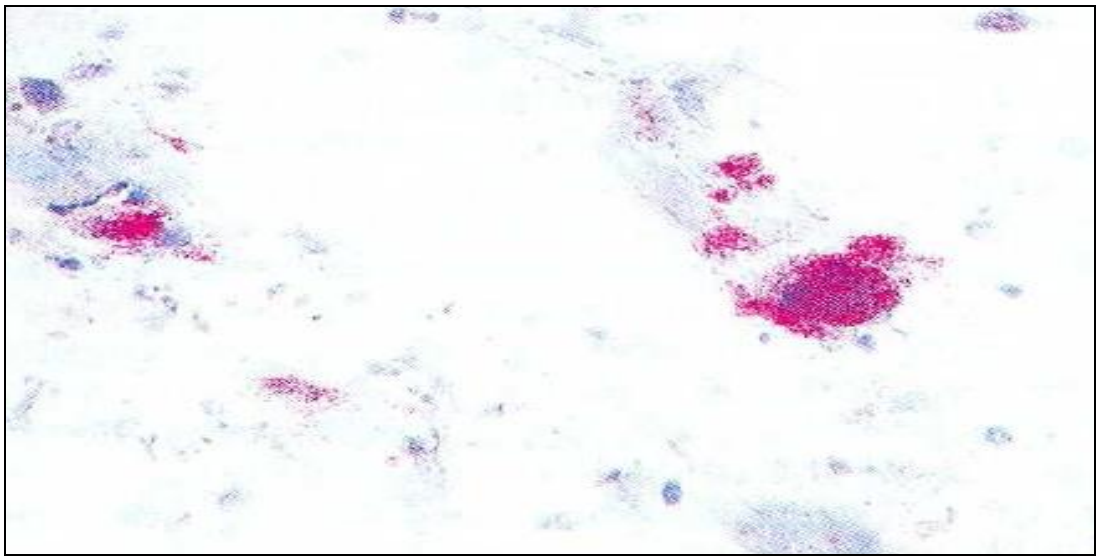

Figure 1: Brucella abortus in an MZN-stained smear of a cotyledon from a case of bovine abortion. The small red (MZN-positive) coccobacilli characteristically occur in clumps reflecting their intracellular growth.
Source: Quinn *et al*., 2004.

2.3. Epidemiology of Brucellosis

2.3.1. Source of Infection and Mode of Transmission of Brucellosis

The transmission of disease is facilitated by coming 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).

Vertical and horizontal ways of transmissions of brucellosis exist in animals. Horizontal tr ansmission occurs through ingestion of contaminated feed, skin penetration, via conjunctiva, inhalation and udder contamination during milking or by licking the discharge of an animal, newborn calf or retained fetal membrane. Fetus can be infected in uterus or suckling of infected dams. Congenital infection that happens during parturition is frequently cleared and only few animals remained infected as adult (Radostits *et al*., 2000).Venereal infections can also occur and mainly seen with *B. suis* infections. The

importance of venereal transmission varies with the species; it is the primary route of transmission for *B. ovis. B. suis and B.canis* are also spread frequently by this route. *B. abortus and B. melitensis* can be found in semen, but venereal transmission of these organisms is uncommon. Some Brucella species have also been detected in other secretions and excretions including urine, feces, hygroma fluids, saliva, and nasal and ocular secretions. In most cases, these sources seem to be relatively unimportant in transmission; however, some could help account for direct non-venereal transmission of *B. ovis* between rams (OIE, 2009; Teferi *et a*l., 2011).

Ingestion of unpasteurized dairy foods produced from unlicensed family owned flocks whose products are sold door-to-door at low prices is one of the known ways of brucellosis transmission to human. Dairy products are the main source of infection for people who do not have direct contact with animals. Transmission of infection to humans occurs through breaks in the skin, following direct contact with tissues, blood, urine, vaginal Discharges, aborted foetuses or placentas. Occupational aerosol infection in laboratories and Abattoirs has also been documented. Accidental inoculation of live vaccines (such as *B.abortus Strain 19 and B.melitensis Rev.1*) can also occur, resulting in human infections. There are also case reports of venereal and congenital infection; and it can be transmitted through transplacental transfer and breast feeding even though rarely (FAO, 2003; Kulkarni *et al*., 2009).

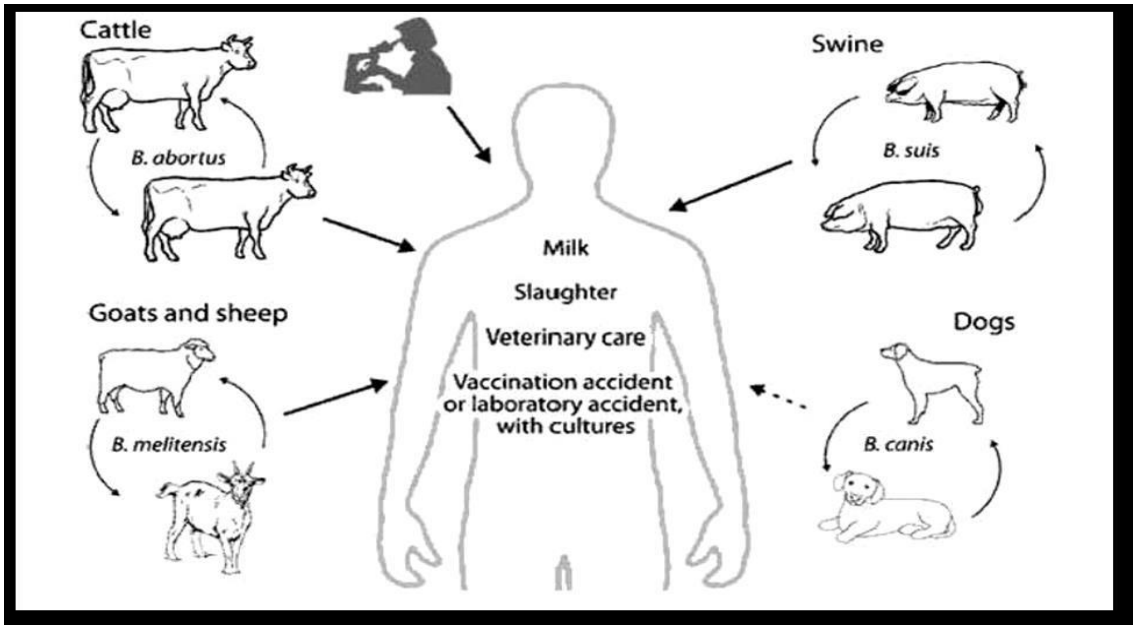

Figure 2: Transmission of *Brucella* to humans (Source: Gadaga , 2013).

2.3.2. Mechanism of entry into the host and 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 Brucellacannot 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 surv ival 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 (Debagües *et al*., 2004; Lapaque *et al*., 2005).

Brucella has developed mechanisms to avoid innate immunity by minimizing stimulation o f pattern recognition receptors (PRRs) of the host.The *Brucella* cell envelope has high hydr ophobicty and its LPS has a non canonical structure that elicits a reduced and delayed. Infl ammatory 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 (bruc ellosome).*Brucella* initially localize within acidified phagosomes (Rittig *et al.,* 2001), where they are exposed to free oxygen radicals generated by the respiratory burst of phago cytes. *Brucellae* have multiple mechanisms to detoxify free radicals. *Brucella* expresses 2 s uperoxide 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).

2.3.3. Risk factors for brucellosis

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).

2.3.4. 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).

The disease occurs worldwide, except in those countries where bovine brucellosis (B. abortus) has been eradicated. This is defined as the absence of any reported cases for at least five years. These countries include Australia, Canada, Cyprus, Denmark, Finland, The Netherlands, New Zealand, Norway, Sweden and the United Kingdom. The Mediterranean Countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America are still not brucellosis free. While B. melitensis has never been detected in some countries, there are no reliable reports that it has ever been eradicated from small ruminants in any country (Robinson, 2003). 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).

2.3.5. Distribution in Africa

Brucellosis exists throughout sub-Saharan Africa, but essentially nothing is known about its prevalence. Most African countries are of poor socioeconomic status, with people living with and by their livestock, while health networks and surveillance and vaccination programmes are virtually non-existent. Moreover, there are far more morbid endemic infectious diseases, particularly malaria. Most febrile patients in these countries are initially empirically diagnosed as suffering from malaria, and only a small part of non-responders may be further tested for brucellosis. Most of the data are derived from small

sero epidemiological studies of patients with fever or high-risk populations (McDermott and Arimi, 2002).

According to data from OIE for 2004, Cameroon, Ethiopia, Kenya, Nigeria, Tanzania, and Uganda reported the existence of human cases of brucellosis, while in 2003 similar reports indicated that Ghana, Togo, and Chad are probably also endemic according to sero epidemiological studies (Schelling *et al*., 2003).

2.3.6. Status of brucellosis in Ethiopia

Both husbandry systems as well as environmental conditions greatly influence the spread of Brucellan infection (WHO, 1997). Ethiopia owns immense diverse but largely untapped livestock resources scattered over agro ecologies (Solomon et al., 2003). Ethiopia`s agro - ecology can be broadly divided into highlands (1500 m above sea level) 39% and lowlands (1500 m below sea level) 61% (Tegegne *et al*.,2009). The lowlands, which are commonly referred to as "pastoral areas," are found in the Eastern, South-Eastern and Southern parts of the country (Tegegne *et al*., 2009). In Ethiopia 40%of livestock population was kept under the pastoral lowland (CSA, 2000). Since the first report of brucellosis in the 1970s in Ethiopia, the disease has been noted as one of the important livestock diseases in the country (Asfaw, 1998; Eshetu *et al.*, 2005; Kebede *et al.*, 2008; Ibrahim *et al*., 2010).

The pastoral and agro-pastoral production system represent approximately 45-55% of the cattle, 75% of the small ruminants, 20% of the equines and 100% of the camels of the total national livestock population. The main mobile pastoralists in Ethiopia are the Somalis (Somali region) in the east, the Afars (Afar region) in the northeast, the Borena Oromos (Oromiya region) in the south and south-east and the Southern Omo people (SNNPS region) in the south and partly in the Gambela and Benishangul regions and around the Dire Dawa Administration. Despite the large size of the regional livestock population, its economic contribution to the regional and national economy is not significant, mostly due to natural and human limitations (Amaha, 2006).

The status of brucellosis of small ruminants in Ethiopia is not well known or is not more than mere report. This may be due to the lack of attention and absence of research activity in animal disease, poor veterinary development, lack of awareness and zoonotic impact of t

he disease have contribute to the less amount of information observed. Through limited ser osurveillances carried out sofar indicated that, brucellosis may be one of the important diseases in goat raising community. Reported prevalence of small ruminant in different parts of Ethiopia in various times and there is similarity between results and this indicated in Table-2 below. Hence, it was almost similar results were documented on small ruminant brucellosis in some areas of Ethiopia.

Table 2: Prevalence of Small ruminant Brucellosis in some parts of Ethiopia

Study area	Prevalence	References
South Wollo	1.5%	Mohamed *et al*. (2010)
South Omo Zone	4.2%	Tigist *et al*. (2011)
Bahir Dari	0.4%	Yeshiwas *et al*. (2011)
Jijiga	1.5%	Mihreteab *et al.(* 2011)
Afar	5.8 %	Ashenafi *et al*. (2007)
Yabello	1.56 %	Dabassa *et al*. (2013)
Metema	3 %	Teshale *et al.* (2006)

Table 3: Prevalence of Bovine Brucellosis in some parts of Ethiopia

Study area	prevalence	references
Tigray Region	3.19%	Gebretsadik *et al*. (2007)
East Showa Zone, Oromia	11.2%	Hunduma & Regassa (2009)
Central Oromia	2.9%	Jegerfa *et al*. (2009)
Jimma Zone	3.1%	Nuraddis *et al*. (2010)
Arsi zone	0.05%	Teferi *et at.* (2011)
Jijiga Zone	1.38%	Hailu *et al. (*2011)
East Wollega Zone	1.9%	Moti *et al.* (2012)

Table 4: Prevalence of Camel Brucellosis in some parts of Ethiopia

Study area	Prevalence	References
Camel rearing regions of Ethiopia	4.2%	Teshome *et al*. (2003)
Borana Low Land	9.5%	Bekele (2004)
Dire Dawa	1.6%	Omer *et al*. (2011)
Dire Dawa	1.5%	Ismail *et al*.(2012)
Amibara district of Afar	7.6%	Woldegebriel (2011)
Afar selected district	5.42%	Wesinew *et al.(*2013)
Tigray	3.56%	Habtamu and Fisseha (2014)

Prevalence of cattle and small ruminant brucellosis at the area, nature of management, com mingling different animal species and herd size are the key factors for occurrence of camel brucellosis.Camelbrucellosis different areas of Ethiopia where camels are reared. Its preval ence was reported Animal brucellosis constitutes significant public health importance for a pastoral community where close intimacy with animals, raw milk consumption and low aw areness on zoonoses facilitate zoonotic transmission of the disease. Milk is a major staple food, and is an important source of protein and vitamins for households. Raw milk, which is the mode by which almost all the pastoral community consume it, is also a source of infection with milk-borne zoonoses such as brucellosis (Schelling *et al*., 2003).

Table 5: Prevalence of Human Brucellosis in some areas of Ethiopia

Study Area	Prevalence	References
Addis Ababa	4.8%	Jiksa (2003)
Jimma University Hospital	3.6%	Tadele *et al*. (2007)
North Western Ethiopia	2.6%	Abebe *et al*. (2009)
Borana	34.9%	Genene *et al*. (2009)
Hamer	29.4%	Genene *et al*. (2009)
Metema	3.0%	Genene *et al*. (2009)
Afar	16.5%	Yimer *et al*. (2008)

2.3.7. Pastoralism and role of its livestock in Ethiopia

Pastoralism is a traditional livelihood system based primarily on livestock production for subsistence and characterized by different degree of migration of the community in search of grazing pasture and water for their livestock. Pastoralism is a successful strategy to support a population on less productive land, and adapts well to the environment. It is one of the key production systems in the arid and semiarid dry land of the world (FAO, 2001; HPG, 2009).

Pastoralism most likely developed from agriculture as people migrated into areas of low productivity and /or regions of unreliable rainfall (Weber and Horst, 2011). As a result, these people came to rely upon domesticated animals for subsistence instead of agricultural crops (Salzman, 2004). The most typical forms of pastoral production are transhumance and nomadic forms (Yalcin, 1986). Transhumance includes the seasonal movment of animals and from valley bottoms to mountain pastures (Yalcin, 1986) while nomadic pastoralism may have developed in response to recurring and widespread drought and is ty pified by livestock being moved in constant search for forage (Salzman, 2004). Nomadic f orm differs from transhumance in that no permanent base (home or village) is developed and likewise,no pre-defined series of movments are used. In pastoralism, mobility is a key strategy used by pastoralists to utelize available resourses, notably pasture and water (Kaimba *et al.*,2011; Mamo, 2014).

Pastoralism covers about 25% of the earth`s terrestrial surface and it is an important economic and cultural way of life for about 200 million people throughtout the world surface (WISP, 2008). In developing countries,pastoralism accounts for the livelihood of 50-100 million people and approximately 60% of this population lives in more than 21 African countries confined to the most arid regions of the continent (Sheik-Mohamed and Velema, 1999; UNDP, 2007).I

In East Africa, Ethiopia has the largest pastoralist population (7-8 million), representing ar-ound 20 ethnic groups and constitute around 14-18% of the total Ethiopia population (Ma-rkakis, 2004). Pastoralist in Ethiopia are found in seven regions including Afar,Somali,SN NP, Oromia, Dire Dawa, Benshangul Gumuz and Gambella Regional States. The major ethnic group in Ethiopia are Somali, Afar, Kereyu and Borena pastoral communities

occuping the Eastern, Northeastern and southern lowlands of the country (PEF, 2010a). Ethiopia`s total livestock population has reached more than 88 million inhead count, and is the largest in Africa (Shitarek, 2012; Mamo,2014). The livstock sub-sector contributes an estimated 12% to total GDP and over 45% to agriculture GDP (Shitarek, 2012).on average, the pastoral livestock population accounts for an estimated 40% of the total livestock population of the country (Pantuliano and Wekesa, 2008) and the sector plays a crucial role for livelihood of the pastoralist communities.

IGAD estimated in 2010 that pastoralist livestock makes up 30% of the nation`s cattle, 70 % of the goats and sheep and all camels in the country (PFE, 2010b; Shitarek, 2012). The p astoral population occupies a disproportionately large area of Ethiopia and produces much more than its share of nationa livestock output. The Ministry of Agriculture estimates that pastoralists use 60% of the country`s land area ,though exact figures of the pastoral livestock population in Ethiopia are unknown(Mamo,2014).The pastoralist livelihoods are highly vulnerable to the effect of climatic changes including the recurring drought and epidemics of disease affecting livestock and humans with huge negative impact on their survival, animal productivity and health of the pastoralist population (Perry *et al*., 2002; Mamo, 2014)

Table 6: The species, biotypes, host preferences and zoonotic potentials of *Brucella*

Species	Biovars	Colony type	Host tropism	First reported country	Zoonotic potential
B. meiltensis	1-3	Smooth	Goat, sheep, camels, cows	Malta	High
B. abortus	1-7,9	Smooth	Cattle, buffalo, camels, bison, elk, yaks	Denmark	High
B. suis	1-5	Smooth	Pigs (biotypes 1-3), wild boar and European hares (biotype 2)	USA	High
B. neotomae	-	Smooth	Desert woodrat	USA	Unknown
B. pinnipedialis	-	Smooth	Seal	Scotland	Mild
B. ceti	-	Smooth	Dolphin, porpoise, whale	Scotland	Mild
B. microti	-	Smooth	Vole, fox, (soil)	Czech Republic	Unkown
B. inopianata	-	Smooth	Unknown	Australia	Mild
B. ovis	-	Rough	Sheep	New Zealand	No
B. canis	-	Rough	Dog	USA	Mild
Future species					
B. papionis sp. nov.	-	Smooth	Baboon	USA	Unknown
BO2	-	Smooth	Unknown	Austria	Mild
Frog isolate (exceptionally motile)	-	Smooth	Bullfrogs	Germany	Unknown

2.4. Growth and Biochemical Nature of Brucella Species

2.4.1. 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 over growing 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, polymixin B, nystatin and cycloheximide onto sucrose dextrose agar for the isolation of *Brucella species* from the clinical sample (Farrell, 1974).

These antibiotic supplements of the Farrell's medium are commonly used, in different combinations and proportions onto any one of the basal media such as *Brucella* medium base (Oxoid), Tryptone soya agar (Oxoid), Serum dextrose agar (Oxoid), Columbia blood agar (Bio Merieux) and other medium bases, for the formulation of selective media for isolation of *Brucella* spp. Moyer and Holocomb, (2005) reported the use of chocolate agar containing selective supplements for the isolation of *Brucella* spp. Similarly, the use of new media such as rifampin *Brucella* medium and malachite *Brucella* medium (MBM), together with TSA, was found to enhance the recovery of *B. abortus* RB 51 (Hornsby *et al.*, 2000). For the isolation of *Brucella* spp. from milk samples although solid media have been used successfully (Farrell, 1974), the use of enrichment media such as serum dextrose, tryptone soy or *Brucella* broth containing selective supplements of at least amphotericin B and vancomycin should be used because the microorganisms are usually present in too low numbers to be detected on solid media (OIE, 2004).

2.4.2. Growth temperature

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

2.4.3. 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 3 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.

Growth 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, CO_2- independent Brucella strains produce uniform turbidity from surface down to 3 millimetres depth while CO_2-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^oC. 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).

2.4.4 .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). In semi-solid media, CO2-dependent strains produce a disc of growth few millimetres below the surface, whereas CO2- independent strains produce uniform turbidity from the surface down to a depth of a few millimetres (Alton *et al*., 1988). On solid media, growth is not apparent until about 3 to 5 days of incubation (Quinn *et al*., 1999).

B. abortus however does require 5 to 10% CO2 or serum and can be isolated on containing brucella selective supplement solid media under aerobic and anaerobic conditions at 37^oC (Alton *et al*., 1988). 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.

2.4.5. 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 catalysed and oxidase positive and they reduce nitrate to nitrite except *B. canis* strains (European Commission, 2001). The production of H_2S from sulphur containing amino acid varies.(European Commission, 2001).

Urease activity of *Brucella* species varies from fast to very slow. Indole and acetyl methyl carbinol are not produced from tryptophan 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 characterstics and biochemical tests used to identify *Brucella* species from other bacteria is given in Tables 7 and 8.

Table 7: Differential characteristics of *Brucella* species

Test	*Brucella*	*Bordetella Bronchoseptica*	*Campylobater fetus*	*Moraxella Species*	*Acinetobacter species*	*Yersinia Enterocolitica*
Morphology	Small coccobacilli	Small coccobacilli	Coma shaped	Diplococcoid	Diplococcoid	Rods
Motility at 37^{o}C	-	+	+	-	-	-
Motility at 20^{o}C	-	-	-	-	-	+
Lactose fermentation	-	-	-	Va	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	-

Positive and negative species within the genus;

B. neotomae may show some fermentation;

Except *B. ovis*, *B. neotomae* and occasional *B. abortus* strains which are negative;

Except *B. ovis* and occasional *B. abortus* strains which are negative;

Except *B. ovis* which does not reduce nitrates to nitrites; Va, variable

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

Table 8: Differentiation of the species and biovars of the genus *Brucella*

Characterstic	*B.melitensis* biovars	*B. abortus* biovars	*B.suis* biovars	*B.ovis*
	1 2 3	1 2 3 4 5 6 7 9	1 2 3 4 5	
Catalase	+ + +	+ + + + + + + +	+ + + + +	+
Oxidase	+ + +	+ + + + + + + +	+e + + + +	–
Urease	+ + +	+ + +f + + + + +	+ + + + +	–
CO_2 req.	– – –	[+] [+] [+] [+] – – – –	– – – – –	+
H_2S prod.	– – –	+ + + + – [–] [+] +	+ – – – –	–
Growth on Media containing Dyes				
Thionin	+ + +	– – + – + + + +	+ + + + +	+
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

(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).

2.4.6. Antigenic characteristics

Brucella has lipopolysaccharide (LPS) as a major component of their outer membrane and an important virulence factor (Cardoso *et al*., 2006) like other Gram-negative bacteria. Thus, their colonial 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 significant role in typing (Godfroid *el 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).

Brucella cross-react with unabsorbed polyclonal antisera. All smooth *Brucella* cross-react with one another in agglutination tests. 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 multophilia, 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).

2.4.7. 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 T bilisi (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 B*rucella* (*B.ovis*, *B. canis*) (Alton *et al.*, 1988).

Table 9: Differential characteristics of *Brucella* species to *Brucella* phages involved in ruminant brucellosis

Species	Tbilisi	Izatnagar	Rough culture
B. melitensis	–	+	–
B. ovis	–	–	+
B. abortus	+	+	–

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

2.4.8. Susceptibility to dyes

The effect of the dyes thionin and basic fuchsin on various *Brucella* species and biovars varies (Anon, 2001). *Brucella abortus* grows in presence of basic fuchsin but does not grow in presence of thionin at the mentioned concentrations (Alton *et al*., 1988).

2.4.9. 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. Rev.1 vaccine is sensitive to streptomycin while the field virulent strain is resistant to streptomycin (Alton *et al*., 1988).

On primary isolation, *Brucella*e are usually susceptible *in vitro* to gentamicin, tetracyclinea nd rifampicin. Most strains are also susceptible to ampicillin, chloramphenicol, cotrimoxax ole, erythromycin, spectinomycin and streptomycin. Most strains of *Brucella* are resistant t o β lactans, cephalosporins, polymixin B, bacitracin, cycloheximide, clindamycin, linomyc in, nystatin and vanco-mycin at therapeutic concentrations (Anon, 2001).

2.5. Pathogenesis

Brucella may enter the host via ingestion or inhalation, or through conjunctiva or skin abrasions. After infecting the host, the pathogen becomes sequestered within cells of the reticuloendothelial system. The smooth lipopolysaccharides that cover the bacterium and proteins involved in signaling, gene regulation, and transmembrane transportation are among the factors suspected to be involved in the virulence of Brucella. The smooth, non endotoxic lipopolysaccharides help to block the development of innate and specific immunity during the early stage of infection; it protects the pathogen from the microbicidal activities of the immune system and has a role in cell entry and immune evasion of the infected cell (Porte *et al*., 2003 & Lapaque *et al*., 2005).

The lipopolysaccharides are thought to alter the capacity of the infected cell to present foreign antigens to the Major Histocompatibility Complex (MHC class II) antigen presenta tion system, hence preventing attack and killing of the infected cell by the immune system. Additionally, smooth lipopolysaccharide in Brucella may be involved in the inhibition of apoptosis of infected cells, since resistance to apoptosis of infected cells has been observed in patients with acute and chronic disease (Lapaque *et al*., 2005 & Maria *et al*., 2007).

Lipopolysaccharide is vital to the structural and functional integrity of the Gram-negative bacteria outer membrane (Cardoso *et al*., 2006). The smooth phenotype of *Brucella* is due to the presence in the outer cell membrane of a complete LPS, which is composed of lipid A, a core oligosaccharide, and an O side- chain polysaccharide. Rough (vaccine) strains

(i.e., strains with lipopolysaccharide lacking the O-side chain) are less virulent because of their inability to overcome the host defence system. The LPS of *Brucella* exhibits properties distinct from other LPSs. In contrast to classical entero bacterial LPS, those of *Brucella* are several hundred-times less active and less toxic than Escherichia coli LPSs (Lapaque *et al*., 2005).

VirB is thought to be essential for intracellular survival; however, the transported effector substrate in *Brucella* has not yet been identified and it is very unlikely that the transported molecule is a classic virulence factor. The VirB pumping system is built from a series of proteins encoded by the VirB operon. Many attenuated *Brucella* strains show mutations within the VirB operon, indicating that an intact VirB is essential for virulence (Celli *et al*., 2005). VirB seems to have a role in adherence of the bacterium to the host cell, cell entry, and it modulates the intracellular trafficking and replication of the bacterium (Arenas *et al*., 2000; Boschiroli *et al*., 2002).

2.6. 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 supra mammary and genital lymph nodes, with constant or intermittent shedding of the organisms in milk and genital secretions (Fensterbank, 1987).

Abortion after the 5^{th} month of pregnancy is cardinal feature of the disease in highly susceptible non-vaccinated pregnant cattle (Radostits *et al*., 2000) and other clinical signs are mainly the calving-associated problems and breeding-associated problems such as repeat breeding, a retained placenta and metritis (Walker, 1999; Acha and Szyfres, 2003;

Shareef *et al.,* 2006). The infected cows usually abort only once after which a degree of immunity develops and the animals remain infected. At subsequent calvings, the previously infected cows excrete huge numbers of *Brucella* in the fetal fluids (Silva *et al*., 2000). Brucellosis does not usually result gross organic lesions (Schlafer and Miler, 2007), but sometimes a mild interstitial inflammatory reaction in the mammary gland may be observed ,which is associated with elimination of bacteria in the milk (Xavier *et al*., 2009a).

Bulls can be infected but they do not readily spread the disease. *B. abortus* is a common cause of orchitis that is often associated with a vesiculitis and epididymitis. Infection in males may result in either temporary or permanent infertility, depending on the intensity of the lesions (Eaglesome and Garcia, 1992; Megid *et al*., 2010). Orchitis is occasionally manifested, 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).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).

2.7. Diagnostic Techniques of Brucellosis

Brucellosis signs are non-pathognomonic in livestock, and definitive diagnosis depends on laboratory testing. Laboratory diagnosis includes indirect tests that can be applied to milk or blood, as well as direct tests (classical bacteriology and direct polymerase chain reaction or PCR based methods). The choice of a particular testing strategy depends on the prevailing epidemiological situation of brucellosis in susceptible animals (livestock and wildlife) within a country or region (Godfroid *et al*., 2013).

2.7.1. Bacteriological diagnosis

Isolation of the organism is considered the gold standard diagnostic method for brucellosis since it is specific and allows bio typing of the isolate, which is relevant under an epidemiological point of view (Bricker, 2002a; Al Dahouk *et al*., 2003). However, in spite of its high specificity, culture of *Brucella* spp. is challenging. *Brucella* spp. is a fastidious bacterium and requires rich media for primary cultures. Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory and it requires BSL3 facilities which is not available in most developing countries (Refai, 2003; Seleem *et al*., 2010; Hadush and Pal, 2013)

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 foetuses (Stamp, 1950).

However, morphologically related microorganisms such as *Chlamydia psittaci* and *C.burn etii* can mislead one in the diagnosis (Garin, 2006; Radostits *et al.,* 2007). So bacterial cult ure plays an important role in confirming the presence of disease and it is essential for anti microbial 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).

Samples for *Brucella* spp. isolation from cattle include fetal membranes, particularly the placental cotyledons where the number of organisms tends to be very high. In addition, fetal organs such as the lungs, bronchial lymph nodes, spleen and liver, as well as fetal gastric contents, milk, vaginal secretions and semen are samples of choice for isolation (Poester *et al*., 2006; Lage *et al*., 2008).

Vaginal secretions should be sampled after abortion or parturition, preferably using a swab with transporter medium, allowing isolation of the organism up to six weeks post parturition or abortion (Poester *et al*., 2010). Milk samples should be a pool from all four

mammary glands. Non pasteurized dairy products can also be sampled for isolation (Lage *et al.*, 2008; Poester *et al*., 2010). Samples of choice in slaughterhouses include mammary, iliac, pharyngeal, parotids and cervical lymph nodes, and spleen. Samples must be immediately sent to the laboratory, preferentially frozen at -20 ºC (+4 ºC for milk sample), and they must be identified as suspect of *Brucella* spp. infection (Poester *et al*., 2010). Vaginal swabs, semen and seminal fluid have low numbers of viable organisms, and therefore isolation is more difficult, often resulting in false negative results. Enrichment media containing selected antibiotics can improve the sensitivity in these cases (De Miguel *et al*., 2011).

Brucella spp. colonies are elevated, transparent, convex, with intact borders, smooth, and a brilliant surface. The colonies have a honey colour under transmitted light. Optimal temperature for culture is 37^{o}C, but the organism can grow under temperatures ranging from 20^{o}C to 40^{o}C, whereas optimal pH ranges from 6.6 to 7.4. Some *Brucella* spp. requires CO_2 for growth. Typical colonies appears after 2 to 30 days of incubation, but a culture can only be considered negative when there are no colonies after 2 to 3 weeks of incubation. False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low (Poster *et al*., 2010).

Solid media such as dextrose agar, tryptose agar, and trypticase soy agar usually are recommended for primary isolation of *Brucella*, but some species, i.e. *B. ovis* and *B. canis* require addition of 5-10% of sterile bovine or equine serum to the culture media. In the case of blood or milk, biphasic media such as Castaneda's medium is recommended for improving sensitivity (Poester *et al*., 2010). 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

2.7.2. 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 CO_2 for growth; production of H_2S; production of urease; growth in the presence of basal fuchsine 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.

2.7.3. Molecular methods for Brucella species genotyping

Molecular techniques are important tools for diagnosis and epidemiologic studies, providing relevant information for identification of species and biotypes of *Brucella* spp., allowing differentiation between virulent and vaccine strains (Ocampo-Sosa *et al*., 2005; Le Flèche *et al*., 2006; López-Goñi *et al*., 2008). Molecular detection of *Brucella* spp. can be done directly on clinical samples without previous isolation of the organism. In addition, these techniques can be used to complement results obtained from phenotypic tests (Bricker *et al*., 2002b). 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 satisfac torily 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 thepolymorphism arisi ng from species specific localisation of the insertion sequence IS711 in the Brucella chrom osome, 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 iden-tified erroneously as *B. suis* (López *et al*., 2011).

2.7.4. Serological tests

Serological tests are crucial for laboratory diagnosis of brucellosis since most of control and eradication programs rely on these methods. Inactivated whole bacteria or purified fractions (i.e. lipopolysaccharide or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Antibodies against smooth *Brucella* spp. (e.g. *B. abortus*, *B. melitensis*, and *B. suis*) cross react with antigen preparations from *B. abortus*, whereas antibodies against rough *Brucella* spp. (e.g. *B. ovis* and *B. canis*) cross react with antigen preparations from *B. ovis* (Nielsen, 2002b).

Serological tests can be divided broadly into two groups and these are screening tests and confirmatory tests. Some screening tests used in the field clinics or in regional laboratories, such as the Rose Bengal Plate Test. The Rose Bengal Plate Test (RBPT) has a very high sensitivity to ensure that infected animals are not missed. Confirmatory tests include Complement Fixation Tests (CFT), competitive ELISA, are very useful in distinguishing vaccinal antibody responses from those induced by field infections (FAO, 2003).

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 Pa squali, 2013; Abernethy *et al*., 2012). Serological tests cannot differentiate between *Brucel la* species and cannot therefore identify which species has induced host antibodies.

Therefore, only isolation of the species or specific DNA detection by polymerase chain rea ction (PCR), allows identification of the infectingStrain (Godfroid *et al.*, 2010; Plumb *et al* ., 2013).

a) Rose Bengal plate test (RBPT)

The RBPT is a rapid, slide-type agglutination assay performed with a stained *B. abortus* suspension at pH of 3.6-3.7 and plain serum. Its simplicity made it an ideal screening test for small laboratories with limited resources. The drawbacks of RBPT include: low sensitivity particularly in chronic cases, relatively low specificity in endemic areas and prozones make strongly positive sera appear negative in RBPT (Diaz *et al*., 2011). The overall sensitivity is 92.9%, so the use of RPBT should be considered carefully in endemic areas, particularly in individuals exposed to brucellosis and those having history of *Brucella* infection (Ruiz Mesa *et al*., 2005).

RBPT is an agglutination test that is based on reactivity of antibodies against smooth lipopolysaccharide (LPS). As sensitivity is high, false negative results are rarely encountered. To increase specificity, the test may be applied to a serial dilution [1:2 through 1:64] of the serum samples (Supriya *et al*., 2010). The present World Health Organization (WHO) guidelines recommend the confirmation of the RBPT by other assays such as serum agglutination tests (Ruiz Mesa *et al*., 2005; Diaz *et al*., 2011).

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 nonspecif ic agglutinins. The temperature of the antigen and the ambient temperature at which the rea ction takes place may influence sensitivity and specificity (Macmillan, 1990).

b) Enzyme linked immune sorbent assays test (ELISA)

The ELISA tests offer excellent sensitivity and specificity whilst being robust, fairly simple to perform with a minimum of equipment and readily available from a number of commercial sources in kit form. They are more suitable than the CFT for use in smaller laboratories and ELISA technology is now used for diagnosis of a wide range of animal

and human diseases. Although in principle ELISAs can be used for the tests of serum from all species of animal and man, results may vary between laboratories depending on the exact methodology used. Not all standardization issues have yet been fully addressed. For screening, the test is generally carried out at a single dilution. It should be noted, however, that although the ELISAs are more sensitive than the RBT, sometimes they do not detect infected animals which are RBT positive. It is also important to note that ELISAs are only marginally more specific than RBT or CFT (Corbel, 2006).

ELISA has become popular as a standard assay for the diagnosis of brucellosis, serologically. It measures IgG, IgA and IgM antibodies and this allows a better interpretation of the clinical situation. The diagnosis of brucellosis is based on the detection of antibodies against the smooth LPS. Detection of IgG antibodies is more sensitive than detection of IgM antibodies for diagnosing cases of brucellosis but specificity is comparable (Sathyanarayan *et al*., 2011; Agasthya *et al*., 2012).

Compared to the conventional agglutination methods, ELISA is more sensitive in acute and chronic cases of brucellosis and it offers a significant diagnostic advantage in the diagnosis of brucellosis in endemic areas. For case detection and an accurate diagnosis of suspected cases, the combination of ELISA IgM and IgG tests should be used as this combination of laboratory tests has been shown to be the most efficient technique in the detection and diagnosis of brucellosis. For follow-up and monitoring of prognosis, ELISA IgM and 2-mercapto ethanol (2-MET) are more promising (Mantur *et al*., 2010; Asaad and Alqahtani, 2012). It can also be used both for screening and confirmatory tests (FAO, 1996).

ELISA is an excellent method for screening large populations for *Brucella* antibodies and for differentiation between acute and chronic phases of the disease. It is the test of choice for complicated, local or chronic cases particularly when other tests are negative while the case is under high clinical suspicion. It can reveal total and individual specific immunoglobulins (IgG, IgA and IgM) within 4-6 hours with high sensitivity and specificity. In addition to the detection of immunoglobulin classes, ELISA can also detect *Brucella*-specific IgG subclasses and other *Brucella* immunoglobulins such as IgE (Agasthya *et al*., 2012).

The competitive ELISA (ELISAc) with smooth *Brucella* LPS as antigen is used for detection of anti-*Brucella* in serum samples from cattle, sheep, goats, and pigs. This test is capable of differentiating vaccine antibody response from actual infections, and its sensitivity varies from 92 to 100%, whereas the specificity ranges from 90 and 99% (Godfroid *et al*., 2010; Perrett *et al*., 2010).

c) Complement fixation test (CFT)

Complement fixation test (CFT) detects specific antibodies of the IgM and IgG1 type that fix complement.The CFT is highly specific but it is laborious and requires highly trained personnel as well as suitable laboratory facilities that makes less suitable for use in developing countries. Although its specify is very important for the control and eradication of brucellosis, it may test false negative when antibodies of the IgG2 type hinder complement fixation. The CFT measures more antibodies of the IgG1 than antibodies of the IgM type, as the latter are partially destroyed during inactivation Since antibodies of the IgG1 type usually appear after antibodies of the IgM type, control and surveillance for brucellosis is best done by CFT (Buchanan and Faber, 1980).

Complement fixation test is used as confirmatory test for *B. abortus*, *B. melitensis*, and *B. ovis* infections (Chin *et al.*, 1991) due to its high accuracy, and it is the reference test recommended by the OIE for international transit of animals (OIE, 2009 a, b). However, this method has some disadvantages such as high cost, complexity for execution, and requirement for special equipment and trained laboratory personnel. In addition, the test presents limitations with hemolysed serum samples or serum with anti-complement activity of some sera, and the occurrence of prozone phenomena (OIE, 2009a). Sensitivity of complement fixation ranges from 77.1 to 100% and its specificity from 65 to 100% (Gall *et al*., 2001; Perrett *et al*., 2010).

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, camels, 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 and its specificity is very important for the control and eradication of

brucellosis but may test negative when antibodies of the IgG2 type hinder complement fixa tion (Farina, 1985; Alton, 1990; Macmillan, 1990).

2.8. Treatment, Prevention and Control

Treatment failure and relapse rates are high and depend on the drug combination and patient compliance due to intracellular localization of *Brucella* and its ability to adapt to the environmental conditions encountered in its replicative niche e.g. macrophage (Seleem *et al*., 2008; Sriranganathan *et al.*, 2010). The optimal treatment for brucellosis is a combination regimen using two antibiotics since mono therapies with single antibiotics have been associated with high relapse rates (Pappas *et al*., 2005 and 2006a; Seleem *et al*., 2009). Seleem *et al.*, (2010) indicated, the combination of doxycycline with streptomycin (DS) is currently the best therapeutic option with less side effects and less relapses, especially in cases of acute and localized forms of brucellosis (Ersoy *et al*., 2005;Seleem *et al*., 2009; Sriranganathan *et al*., 2010). Neither streptomycin nor doxycycline alone can prevent multiplication of intracellular *Brucella*.

It is less practical because the streptomycin must be administered parenterally for 3 weeks although the DS regimen is considered as the gold standard treatment. A combination of doxycycline treatment (6 weeks duration) with parenterally administered gentamicin (5 mg/kg) for 7 days is considered an acceptable alternate regimen (Glynn and Lynn, 2008).

It is nearly always more economical and practical to prevent diseases than to attempt to control or eliminate them. Careful selection of replacement animals should be one of the measures to prevent the occurrence of brucellosis. Animals that are either purchased or produced from existing stock should originate from Brucella-free herds or flocks. Pre-purchase tests are necessary unless the replacements are from populations in geographically circumscribed areas that are known to be free of the disease. Isolation of purchased replacement animals should be done for at least 30 days. In addition, a serological test prior to commingling is necessary. Prevention of contacts and commingling with the herds or flocks of unknown status or those with brucellosis should be carried out. If possible, laboratory assistance should be utilized to diagnose causation of abortions, premature births, or other clinical signs. Suspect animals should be isolated until a diagnosis can be made. Herds and flocks should be included in surveillance

measures such as periodic milk ring tests in cattle (at least four times per year), and testing of slaughtered animals with simple screening serological procedures such as the RBT. Proper disposal (burial or burning) of placentas and non-viable foetuses should be given prime importance. Disinfection of contaminated areas should be performed thoroughly with the cooperation of public health authorities to investigate human cases (Corbel, 2006).

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. Unpasteurized dairy products and raw or undercooked animal products (including bone marrow) should not be consumed. Good hygiene and protective clothing/equipment are very important in preventing occupational exposure. Precautions should be taken to avoid contamination of the skin, as well as inhalation or accidental ingestion of organisms when assisting at a birth, performing a necropsy, or butchering an animal for consumption. Particular care should be taken when handling an aborted fetus or its membranes and fluids. Risky agricultural practices such as crushing the umbilical cord of newborn livestock with the teeth or skinning aborted fetuses should be avoided (OIE, 2009).

The Strain 19 *B. abortus* vaccine and *B. melitensis* Rev-1 vaccine must be handled with caution to avoid accidental injection or exposure. Adverse events have also been reported with the *B. abortus* RB51 vaccine, although it is safer than Strain 19. Persistent infections with vaccine strains have occasionally been reported in vaccinated animals. These strains can be shed in the milk or aborted fetuses and can infect humans. Obstetricians should also take precautions when assisting at human births, particularly in regions where brucellosis is common. Recently, an obstetrician became infected by ingesting amniotic fluid and secretions from a congenitally infected infant. In the laboratory, *Brucella* spp. should be handled under biosafety level 3 conditions or higher. Human vaccines are not available (OIE, 2009).

The aim of an animal control programme is to reduce the impact of a disease on human health and the economic consequences. A major issue is that control measures should continue for a long period of time and be complemented with a monitoring system that may be hard to keep in place once the number of cases begins to decrease In many countries, methods for the control of brucellosis are backed by governmental

regulation/legislation. Further classified under the general categories of test and isolation/slaughter, hygiene, control of animal movement, vaccination *(*Lundervold *et al.*, 2004*).*

Hygienic methods, to the control of brucellossis are also applied, to reduce exposure of susceptible animals to those that are infected. Owners should be informed about disease transmission and recommendations, such as separation of parturient animals, pasteurization of milk for consumption, avoidance of handling of parturient materials. Unauthorized sale or movement of animals from an infected area to other areas should be forbidden. Similarly, importations into clean areas must be restricted to animals that originate from brucellosis-free areas, that have a herd/flock history of freedom from the disease and that have given negative reactions to recently performed diagnostic tests. In practice, it is much more difficult to control the movement of livestock kept under pastoral and agro pastoral conditions than that of beef or dairy cattle kept under intensive conditions because the own ers of herds and flocks may be accustomed to seasonal migrations which may cross nationa l boundaries (Corbel, 2006).

There is a general agreement that the most successful method for prevention and control of brucellosis in animals is through vaccination. While the ideal vaccine does not exist, the attenuated strains of *B. melitensis* strain Rev.1 for sheep and goats and *B. abortus* strain 19 and the non-agglutinogenic *B. abortus* strain RB51 proven to be superior to all others. It is often recommended that vaccination with strains 19 and Rev.1 should be limited to sexually immature female animals. This is to minimize stimulation of post vaccinal antibodies which may confuse the interpretation of diagnostic tests and also to prevent possible abortions induced by the vaccines (Corbel, 2006).

3. MATERIALS AND METHODS

3.1. Description of the Study Area

The study was conducted in the Amibara District of the zone three of Afar Regional State which is located in the Middle Awash Valley about 260 km in Ethiopia to the North East of Addis Ababa. The District has 18 *kebeles* (the smallest 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).

Afar National Regional state is one of the 9 regions of Ethiopia and geographically located in the north-east of the country between $39^{0}34'$ and 42°28'east longitude and $8^{o}49'$ and $14^{o}30'$ 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).

The Region is characterized by an arid and semi-arid climate with low and erratic rainfall. The altitude of the region ranges from 120m below sea level to 1500m above sea level. Temperatures vary from $20^{o}C$ in higher elevations to $48^{o}C$ 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 increas ingly 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 po -pulation of 1.5 million and administratively, divided into five zones, which are further sub divided into 32 weredas (administrative districts) and 358 pastoral associations (CSA, 2014

The Region is a great potential resource of livestock resources comprising of 2336488 heads of cattle, 4267969 goats, 2463632 sheep, 85216 camels and 187287 equines that support the region and contributes to the national economy (CSA, 2010). The Region during May/June is the driest season of the year, **'hagay'**. It is said to be unsuitable for

browsing since bushes dry up. The main rainy season **'Karma'**, accounts for above 60% of the annual total rainfall are from July to September. This is followed by the best grazing season of **'Kayra'** that occurs from September to November. Another minor rainy season is Sugum and appears during March and April. **'Gilal'** is less severe dry season with relatively cool temperatures (November to March). Occasional rainfalls called **dada** may interrupt **'Gilal'** (ARFEB) (2007).

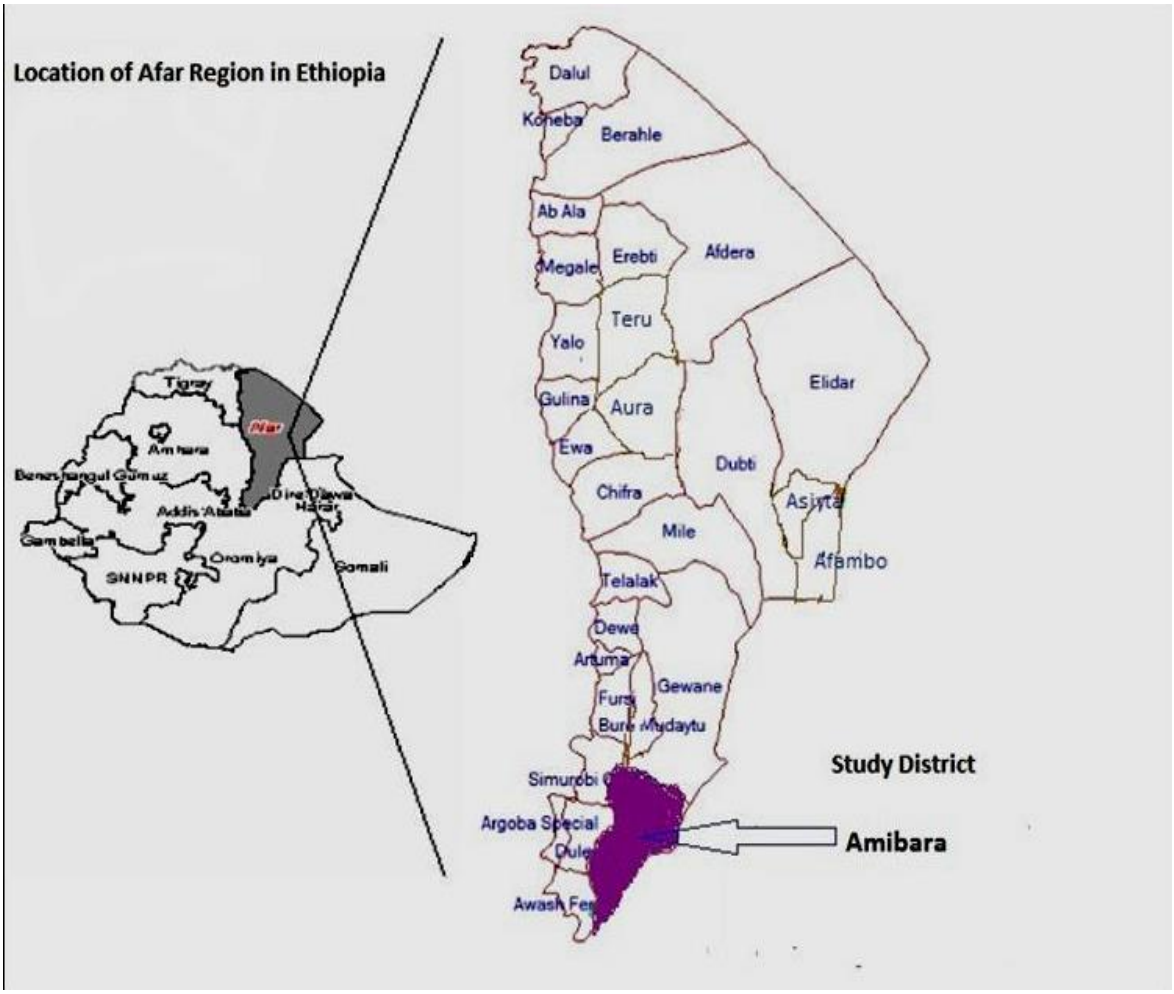

Figure 3: Map showing the Afar Regional State, Ethiopia.

Source: CSA (2014).

3.2. Study Design

A cross-sectional study was conducted from Nov. 2016 to April, 2017 to estimate the seroprevalence of brucellosis and to isolate *Brucella* species in cattle and camels with recent history of abortion. Recent history of abortion was defined for the purposive of this study as those animals experienced abortion in less than one and half month at the time of the study and was verified based on information obtained from the owners.

Six Kebeles in Amibara district were selected purposively selected for serological survey based on accessibility and population of cattle and camels. From the selected kebeles those herds with history of abortion cases in cattle and camels were selected purposively to collect samples including vaginal swab, foetus material, retained foetal membrane, milk and synovial fluids for bacteriological culture in order to isolate Brucella species.

The sample size for serological study was calculated using the formula described by Thrusfield (2007) with defined precision of 5 % and 95% level of confidence interval.

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

Where,

n=required sample size,
*P*ex=expected prevalence, and
d=desired absolute precision

Hence, based on the above formula and taking into account the previous prevalence of brucellosis in camel as 7.6% (Weldegebreal, 2011) the minimum sample size would be:

$$n=\frac{1.96^2 \text{ X } 0.076 \text{ X } (1-0.076)}{(0.05)^2} \qquad n= 107.$$

$$n = \frac{3.8416 \text{ x } 0.076 \text{ x } 0.924}{0.0025} = 107.$$

Therefore, the minimum sample size calculated was 107. However, to increase the level of precision, the sample size was increased to 350. Hence, a total of 350 animals (127 cattle and 223 camels) were considered for this study from selected kebeles of Amibara district.

3.3. Study Animals

The study animals comprised camels and cattle that had a history of recent abortion. Study animals related traits such as species, sex, age, body condition score, lactation and reproductive status, parity number, period of abortion and history of abortion were collected and recorded at the time of sampling.

3.4. Sample Collection

3.4.1. Blood sample collection

Blood samples of 7-10ml after disinfecting the site of jugular vein were collected in sterile plain vacutainer tubes from each cattle and camel included in the study. The blood samples were kept in a slanting position overnight at room temperature to separate the serum according to OIE manual (2009). Then sera were gently decanted into sterile screw cupped Eppendorf tubes (1.8ml), labeled and transported in cold chain to Addis Ababa University, College of Veterinary Medicine and Agriculture (AAU, CVMA), Microbiology, Immunology and Veterinary Public Health (MIVP) Laboratory, Bishoftu, Ethiopia and stored at -20^{o}C until tested for antibodies against natural *Brucella* exposure analysis using RBPT, CFT and C- ELISA.

3.4.2. Bacteriological sample collection

Specimens were taken purposively from 223 camels and 127 cattle that had a history of recent abortion (less than one and half month period before the time of sampling) in selected kebeles of Amibara district for isolation and identification of *Brucella species*. The specimens like milk from cattle and camels (n=17) (n=8) and vaginal swab from cattle and camels (n=9) and (n= 6) respectively and synovial fluids only from cattle (n=2) were collected parallel to blood sample according to recommendations of OIE (2009).

A total of 25 camels and cattle vaginal swab samples were collected with sterile applicator stick in Amies with Charcoal Transport Medium (HiMedia, Mumbai, India) and transporte d to AAU, CVMA, MIVP laboratory in cold chain and stored at -20^{o}C until processed for

culturing and isolation under Biosafety level II (BSL2) for personal protection (safety measures).

Milk samples (10-20ml mid stream) were similarly collected aseptically from each teat into sterile 50ml screw-capped falcon tubes after cleaning the teat. The Samples were transported chilled to AAU, CVMA, MIVP laboratory samples in ice packs and stored at 4^{o}C until processed for isolation of *Brucella* species using *Brucella* Selective Agar (HiMedia, Mumbai, India).

The synovial fluid (5-10ml with a sterile syringe) was collected aseptically after cleaning the knee of the cow. The fluid samples were poured into a transport media (Amies) and the transported in ice packs and stored at -20^{0}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 (RBPT, CFT and competitive ELISA) were carried out at AAU, CVMA and National Veterinary Institute (NVI) and National Animal Health Diagnostic and Investigation Center (NAHDIC) laboratories, in Bishoftu and Sebeta, Ethiopia respectively.

3.5.1.1. Classical Rose Bengal Plate Test (RBPT)

All sera samples collected were screened using RBPT 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 Institute (NVI) Bishoftu, Ethiopia (Annex1). Briefly, for the method, 30µl of serum and 30µl 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.

3.5.1.2. Complement Fixation Test (CFT)

Sera that positive for RBPT 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 2). 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 he molysis was considered as negative.

3.5.1.3. Competitive ELISA

Commercial multispecies competitive ELISA kit (SVANOVIR *Brucella*-Ab C-ELISA), batch No, A51031, produced by the Veterinary Laboratory Agency Boehringer Ingelheim, Svanova, SE-751 45 Uppsala, Sweden, for Diagnostics,Sweden and Pharmachem International, Sweden. This kit uses the detection of specific antibodies to *B. abortus*, *B. melitensis, B. suis* in bovine, ovine, caprine and porcine serum respectively. The kit was validated according to the kit instructions, the validation guidelines of the OIE (2000) (Annex 3). The test was performed according to the kit instructions. The positive cut off point was calculated as the mean OD values for each of the controls and samples and the OD measured at 450 nm. Additionally, the percent inhibition (PI) was also calculated from the formula:

$$PI = 100 - \frac{\text{OD sample or control X100}}{\text{OD Conjugate Control }^{Cc}}$$

3.5.2. Microbiological isolation of Brucella species

Isolation and identification of *Brucella* were done as described in Bergey's manual and (OIE, 2009).

3.5.2.1. Brucella Microbiological culture

The specimens like milk, vaginal swab and synovial fluids collected parallel to blood sampling were processed and subjected to isolation according to OIE (2009). Briefly, the samples were inoculated on *Brucella* Selective Agar Base with Selective Antibiotics supplement active ingredients (FD161) (HiMedia, Mumbai, India), under Biosafety Level II (BSL2) facilities with proper personal protections (Alton *et al*., 1988; Lopez *et al*., 2006) (Annex 4).

Vaginal swabs and synovial fluids were also streaked directly from Amies with Charcol transport medium to Brucella Selective Agar while the milk samples were centrifuged at 3000 rpm for 10 minutes to concentrate the organism to obtain the sediment-cream mixture. The deposit was spread on *Brucella* Selective Agar Base with supplement (Himedia Mumbai, India). Caution was made to avoid the risk of aerosol contamination to working laboratory personnel

The inoculated plates with specimens were incubated at 37^{o}C both in the absence and presence of 5% to 10% CO_2 (using AnaeroGas Pack 1.5L use 2 or 3 sachets in proportion to the jar volume by disposable oxygen-absorbing and carbon dioxide-generating agent for use in anaerobic jars for cultivation under conditions of less than 45^{0}c) for up to two weeks. Colonies were checked every 24 hrs for growth of *Brucella* species. *Brucella*-suspected colonies were characterized based on their typical round, glistening, pinpoint and honey drop-like appearance according to Alton *et al*. (1988).

3.5.2.2. Brucella microscopic examination

The *Brucella* suspected 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 5). *Brucella* species were identified based on Gram negative, very tiny appearance and coccobacili shape that was arranged mostly in single but some in pairs and also in clusters according to Alton *et al*. (1988).

3.5.2.3. Biochemical tests of Brucella

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 characterizatio-*n* of the organism was done using (Oxidase test, catalase test, urea hydrolysis, nitrate reduction test, hydrogen sulphide (H_2S) production and hemolysis on blood agar), growth in the presence of thionin and basic fuchsin dyes incorporated with Trypicase Soya agar at 20 to 40 ug/ml concentrations in separate petridishes, CO_2 requirement and slide agglutination test with an anti-*Brucella* polyclonal serum were also checked according to Alton *et al*. (1988) (Annex 6). However, *B. abortus* require CO_2 or serum and can be isolated with and without 5-10 % CO_2 on solid media under aerobic or anaerobic conditions at 37^oC as described by Alton *et al*. (1988).

3.6. Data management and analysis

Data collected from field and serological test were coded and stored in Microsoft Office Excel spread sheet and transferred to STATA version 11 for Windows (Stata Corp. College Station, TX, USA) for statistical analysis. The seroprevalence for animal level was calculated on the basis of RBP, CFT and C-ELISA positivity dividing the number of *Brucella* reactors by total number of tested animals. To identify association of seropositivity with the potential risk factors, (PA, age, sex, body condition, parity, abortion history, stage of abortion) were considered. Descriptive and analytic statistics were computed and Fisher`s exact test was employed to see the association of risk factors with that of seropositivity to *Brucella* antibody. The degree of association was considered significant when a P-value of less than 0.05 was obtained or when the 95% confidence intervals of the odds ratio (OR) in the multivariable logistic regression analysis doesn't include 1. (Thrusfield, 2007).

The agreement between RBPT, CFT with C-ELISA, considering as standard test, was done using kappa test and interpreted according to the recommendations of Dohoo *et al.* (2003).

4. RESULTS

4.1. Seroprevalence of brucellosis

The present result revealed out of a total of 350 animals tested (223 camels and 127 cattle) with history of recent abortion animals 30 using RBPT alone, 6 were confirmed with CFT and 64 by C-ELISA were positive giving the overall seroprevalence of Brucella to be 8.6% (95% 5.8-12), 1.7%,(95% 0.6-3.6), 18.3% (95% 14-22) respectively.

4. 3. Comparison of Serological Test Agreement

The kappa statistics showed that there was moderate agreement between RBPT with C-ELISA as standard test while almost fair agreement was observed between CFT and C-ELISA (Table 11).

K = 0: no agreement; K = 0 – 0.20: slight agreement; K = 0.21 – 0.40: fair agreement; K = 0.41 – 0.60: moderate agreement; K = 0.61 – 0.80: substantial agreement; K > 0.81: almost perfect agreement; K = 1: perfect agreement.

Table 10: Kappa test for agreement between RBPT, CFT and C-ELISA

	C-ELISA		Kappa	Kappa value	p-value
	-	+	value	interpretation	
RBPT			0.432	moderate agreement	0.000
Negative	300	20			
Positive	14	16			
CFT			0.224	fair agreement	0.000
Negative	0	314			
Positive	6	30			

4.4. Association of the Putative Risk Factors with *Brucella* Seropositivity.

Fisher`s exact test analysis for association between *Brucella* infection using combined RBPT and CFT and C-ELISA and were carried using Fisher's exact tests (Table 12 and Table 13). In the analysis abortion history and stage of abortion were significantly associated ($P< 0.05$) with seropositivity of bovine brucellosis while other factors were not statistically significant ($P > 0.05$). (Table13).

Similarly, in camels association of host risk factors including sex, age, parity, history of abortion, stage of abortion and body condition with brucella seropositivity showed no statistically significant association ($P >0.05$).

All female camels and cattle that were seropositive to *Brucella* infection were found to have a history of retained placenta.

Table 11: Association of risk factors with Fisher`s exact test result in cattle of Amibara District Afar Region using CFT test.

Risk factors	**tested Animals**	**Prevalence (%)**	**p-value**
Kebeles			1.000
Halidege	74	3 (4)	
Hulis	11		
Bonta	20	0 (0)	
Hasoba		0 (0)	
Buri	7		
	15	0(0)	
		0(0)	
Sex			0.720
Female	118	3 (2.54)	
Male	9	0 (0)	
Age			0.316
≤4 years	58	0 (0)	
≥5-12 years	65	3 (4.62)	
>12 years	4	4 (1)	
Parity (n=200)*			0.233
Null	56	1(.017)	
≤ 3 =1	32	0 (0)	
>3=2	30	2 (.067)	
Body condition			0.314
Poor	27	0 (1.04)	
Medium		2 (0.92)	
	88		
Good	12	1 (8.33)	
History of abortion* (n=200)			0.004
No abortion	99	0 (0)	
Abortion	19	3 (0.158)	
Stage of abortion (n=200)*			0.004
Early abortion	99	0 (0)	
Late abortion	19	3 (0.158)	

*Male animals were not included in the total numbers.

Table 12: Association of risk factors with Fisher`s exact test result in camel of Amibara District, Afar Region using CFT test

Risk factors	**tested Animals**	**Prevalence (%)**	**p-value**
Kebeles			0.175
Halidege	52	0(0)	
Hulis	14		
Bonta	78	1(7.1)	
Hasoba		1(1.3)	
Buri	10		
Angelele	47	0(0)	
		0(0)	
	22	1(4.6)	
Sex			0.801
Female	200	3 (1.5)	
Male	23	0 (0)	
Age			0.617
≤4 years	88	1(1.13)	
≥5-12 years	84	2 (2.38)	
>12 years	51	0 (0)	
Parity (n=200)*			0.616
Null	94	2(0.21)	
≤ 3 =1	51	1(0.01)	
>3=2	33	0 (0)	
Body condition			0.314
Poor	96	1 (1.04)	
Medium	108	1(0.92)	
Good	19	1 (5.3)	
History of abortion(n=200)*			0.430
No abortion	166	2(0.12)	
Abortion	34	1(0.29)	
Stage of abortion (n=200)*			0.388
Early abortion	170	2(1.2)	
Late abortion	30	1(3.3)	

*Male animals were not included in the total numbers.

4.5. Multivariable Logistic Regression Analysis of Risk Factors Associated with Brucella Seropositivity

The logistic regression analysis of the putative risk factors indicated that cattle were more likely to be significant (P< 0.05) of camels with age in both species (OR= 14, 95 % CI: 2.09 – 96.9 and OR=12, 95% CI: 2.23-65.6) with *Brucella* respectively (Table 14 and Table 15).

Table 13: Multivariables logistic regression analysis of factors associated with *Brucella* Seropositivity in camel by C-ELISA.

Variables	**Tested**	**seropositive**	**Std err**	**OR(95%CI)**	**(*P*-value)**
Kebeles					
Halidege	43	20.53	1	1	-
Hulis	8	75	4.60	5.04 (0.84,30.21)	0.076
Bonta	75	4	0.10	0.11(0.02,0.67)	0.016
Hasoba	10	0	-	-	-
Buri	40	17.5	2.48	3.22(0.71,14.56)	0.126
Angelele	17	29.4		2.58(0.49,13.49)	0.260
Sex					
Female	174	26	1	1	-
Male	19	4	11.85	12.59(1.99,79.71)	0.007
Age					
≤4 years	80	8	1	1	-
≥5-12 years	79	5	10.43	12.10(2.23,65.64)	0.04*
>12 years	49	2	31.60	32.93(5.04,215.72	0.000*
Body condition					
Poor	91	5	1	1	-
Medium	99	9	0.85	1.27(0.34,4.7)	0.722
Good	18	1	0.00	1.11(0.19,6.53)	0.900
Parity					
Null	90	4	1	1	
1-3	40	11	0.79	0.93(0.17,4.97)	0.936
>3	53	2	0.39	0.31(0.02,3.68)	0.357
History of abortion					
Once	150	16	1	1	-
≥ 2 times	24	10	1.61	1.57(0.21,11.67)	0.655
Stage of abortion					
Early abortion	154	16	1	1	-
Late abortion	20	10	2.67	2.74(0.40,18.48)	0.298
Total	174	26	3.67		

Table 14: Multivariables logistic regression analysis of factors associated with *Brucella* Seropositivity in cattle by C-ELISA.

Variables	**Tested**	**Seropositive**	**Std err.**	**OR(95%CI)**	**(*P*-value)**
Kebeles					
Halidege	48	26	1	1	-
Hulis	10	1	0.82	0.67 (6.0,750)	0.748
Bonta	16	4	0.06	0.35(0.1, 30)	-
Hasoba	4	3	0.21	(9.0,140)	0.141
Buri	15	0	0	0(0,21)	-
Age					
≤4 years	55	3	1	1	-
≥5-12 years	38	31	70.56	14.07(209,9690)	0.015
>12 years			-	-	-
Body condition					
Poor	20	7	1	1	-
Medium	65	23	0.54	0.80(21,305)	0.748
Good	8	4	0.26	0.22(16,931)	0.846
Parity					
Null	51	5	1	1	-
1-3	18	14	0.42	0.29(17,498)	0.398
>3	15	15	0.60	0.42 (2, 695)	0.549
History of abortion					
Once	73	26	1	1	-
≥ 2 times	11	8	0.35	0.53(15,193)	0.342
Stage of abortion					
Early abortion	160	10	1	1	-
Late abortion	28	2	-	-	-
Total	188	12			

*Significant; OR: Odds Ratio; CI: Confidence Interval, 1, Reference

4.2. Isolation of Brucella from cattle and camel cases with recent history of abortion

In the current study, out of 42 clinical sample cultured 5 were positive with an overall rate of isolation of 11.9% (5/42) was found and all the five isolates were *B. abortus* based on biochemical tests result. *B. abortus* was isolated from vaginal swab 20% (3/15) and synovial fluid 100% (2/2) while no isolate was obtained from milk (Table 16).

Table 15: *Brucella* isolates recovered from aborted seropositive cattle vaginal swabs and synovial fluid

The examination of the isolates with Gram's staining showed Gram negative coccobacilli and red stained coccobacilli in modified ZiehlNeelsen staining the *Brucella* organisms w -ere stained red against a blue back ground. The isolated colonies were not grown on MacConkey agar and non-haemolytic on blood agar. Growth was noticed in plate with basic fuchsin. The detailed result of basic biochemical and metabolic profiles of field *B.abortus* isolated from the study area were depicted in (Table 17).

Table 16: Staining and biochemical test results of *Brucella* from seropositive cattle vaginal swab and Synovial fluid.

Biochemical properties										**Growth on dyes**		
***Brucella* Isolates**	**Animal Species**	**Gram's Stain**	**MZN**	**Cat.**	**Oxd.**	**Ure.**	**CO_2 Req.**	**H_2S pro.**	**Nitr.Red**	**Thionin**	**Basic Fuchsin**	**Agg. With pl. Clonal serum**
Vaginal swab 01	Cattle	Gram negative	+	+	+	+	-	-	+	+	+	+
Vaginal swab 02	Cattle	Gram negative	+	+	+	+	-	-	+	+	+	+
Vaginal swab 03	Cattle	Gram negative	+	+	+	+	-	-	+	+	+	+
Synovial fluid 01	Cattle	Gram negative	+	+	+	+	-	-	+	+	+	+
Synovial fluid 2	Cattle	Gram negative	+	+	+	+	-	-	+	+	+	+

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

5. DISCUSSION

5.1. Brucella Isolation

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 different animal populations. However, most surveys of brucellosis in Ethiopia are based on serological test and there is no evidence for bacteriological isolate on of *Brucella* species except few studies conducted to isolate *Brucella* species from cattle and small ruminants in selected areas of Ethiopia (Minda *et al*., 2016; Melesse *et al.*, 2007 ; Muluken, 2016), respectively.

In the present study, isolation of *B. abortus* from seropositive cattle with history of abortion was carried out and the present study confirmated the isolation of *B. abortus* from clinically aborted cattle vaginal swab 20% (3/15) and synovial fluid 100% (2/2) and while no isolate was obtained from milk. This isolation of *Brucella abortus* form cattle was the first report in pastoral area of Ethiopia.

The isolation rate of the *Brucella* from seropositive vaginal swab and synovial fluid in the present study (11.9%) was greater than previous report of 6.52% (Minda *et al*., 2016) and 6.4% (Celebi and Otlu, 2011). This variation might be because of the slow growing and fastidious nature of the pathogen (Seleem *et al.*, 2010). In addition, *Brucella* species, stage of the disease and quantity of circulating bacteria could affect the isolation rate (Marin *et al*., 1996).

A higher rate of isolation of *B. abortus* was reported by Gulhan *et al.* (2011) who reported 26.7%, Ali *et al. (*2014) who reported 40%, Unver *et al. (*2006) who reported 55.6% from aborted cattle foetuses in Turkey and Pakistan. This difference may be related to the usage of more than one selective culture media in their study. On the contrary, in the present study utilized only Himedia medium. Isolation of *B. abortus* can be improved if more than one selective culture medium is used (Ali *et al.*, 2014).

Bacteriological cultural, morphological and biochemical tests in the present study confirmed that all the five isolates obtained from the cases of vaginal swabs and synovial

fluids of aborted cattle were *B. abortus*. Similar to the earlier reports all the *Brucella* isolates found in this study were positive for catalase, oxidase and urea hydrolysis and negative for indole production, citrate utilization, methyl red, and voges-proskauer tests revealing them to be *Brucella* spp. (Koneman *et al*., 1997). Other reports have also indicated cultural, morphological, and biochemical characteristics could enable to identify *Brucella* spp. (Alton *et al*., 1988; Koneman *et al*., 1997).

Drinking of raw milk is common in pastoral areas (Sozen, 1996; Erol, 1997; Tantillo *et al.,* 2003). Shedding of *Brucella* in the milk of infected animals is an important source of transmission of disease to humans if raw milk is consumed. In contrary to this fact, there was no recovery of *Brucella* species from 25 milk samples collected from RBPT positive cows and camels in the present study. This result might be due to the secretion of an organism in the milk a few days (2 to 5 days) after abortion, small number of sample cultured and use of only single media (Himedia). The isolation of *Brucella* from milk samples may be improved if more than one culture medium is used (Ali *et al*., 2014). Hence, the result should not underestimate the risk of consuming milk as source of *Brucella* infection in the study areas. The fact that *Brucella* spp were isolated from milk of cattle from different studies with rate of 3.2% (Ali *et al.,* 2014) in Pakistan and 4% (Recep, 2013) and 4.4% (Celebi and Otlu, 2011) in Turkey showed the need for further investigation in Ethiopia.

In general, this finding revealed that the isolation of *B. abortus* 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). 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).

5. 2. Brucellosis Seroprevalence

The present study revealed that the seroprevalence of brucellosis in camels and cattle with history of recent abortion was 6.7%, 6.7% and 1.3% and 11.8% 12.6% and 2.4% with RBP -T, C-ELISA and CFT, respectively.

The current study revealed that the overall seroprevalence of camel brucellosis was 1.3% (3/223). This finding is in close agreement with the earlier reports of 1.5% in Dire Dawa town by Ismail *et al*. (2012), 1.8% and 1.7% in Borana and Tigray, respectively by Bekele *et al*. (2011), 0.4-2.5% and 0.9% by Gumi *et al*. (2013) in Borena, Oromia region, respectively. However, it is lower when compared to 5.7% by Teshome *et al*. (2003) and 5.8% by Moustafa *et al*. (1998), in Afar region, 7.6% by Woldegebrel (2011), and 5.42% by Wesinew *et al*. (2013).

The seroprevalence result of the current study moreover was also lower than reports in other African countries. For instance a prevalence of 2.0 to 15.4% was reported in Kenya (Tilahun *et al*., 2013), 3.1% in Eritrea (Omer *et al*., 2000), and 3.1% in Somalia (Ghanem *et al*., 2009) 30.5% in Sudan (Ahmed *et al*., 2007), 7.61% in Egypt (Hassanain and Ahmed, 2012), 3% in Iraq (Yawoz *et al*., 2012), 19.4% in Jordan (Dawood, 2008). Generally, the results of the present study fall in between the range of previous reports ranging from 1.2% (Teshome *et al*., 2003) to 5.5% (Richard, 1980) in Ethiopian camels.

The difference in seroprevalence between the current study and the previous study results might be due to differences in sample size, imparted by the various tests, agro-ecology, study population. According to Radostits *et al*. (2007), herd size and management conditions determine the rate of transmission of *Brucella* infection in different study areas.

There was also no statistical significance ($P>0.05$) in seroprevalence of camel brucellosis among sex, age, body condition, parity status, history of abortion and stage of abortion in Fisher`s exact analysis of the present study. Fisher`s exact analysis revealed that seropositivity of infection may occur in animals of all age groups but persists commonly in sexually matured animals. Younger animals tend to be more resistant to infection and frequently clear infection although few latent infections may occur (Radostits *et al*. 2000). The present study showed that there was slightly higher significant association with the

occurrence of the disease in that 2.38% in adult of > 4 years old than young camels of 6 month to 4 years old. The low seroprevalence in young camels might be because of maternal immunity. Susceptibility appears to be more commonly associated with sexual maturity and risk of infection increases with pregnancy and as the stage of pregnancy advances the chance of acquiring infection increases (Crawford *et al* 1990).

Age, of status remained significant in multivariable logistic regression analysis. Multivariable logistic regression analysis by C-ELISA revealed that seropositivity for *Brucella* infection increases approximately 12.6 times in ≥5-12 years old animals comp ared to >12 and ≤ 4 years old. Several previous studies have indicated that higher seropre-valence of brucellosis in adult age group of cattle (Zewold *et al*., 2012; Bekele *et al*., 2011a). 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 (Radostits *et al*. 2007).

The present study pointed out that the overall seroprevalence of bovine brucellosis was 2.4%. This finding is consistent with the earlier reports of Fekadu *et al*. (2014) who reported 2.0% in Eastern Showa, Asmare *et al* (2007) who reported 2.46% and Kassahun (2004) who reported 2.5% in Sidama zone of southern Ethiopia, Jegerfa *et al*. (2009) who reported 2.9% in Central Oromia, Nuraddis *et al*. 2010 who reported 3.1% in Jimma Zone.

The seroprevalence of bovine brucellosis in the current study indicated to be 2.4% which was found to be relatively higher than the previous reports of Degefu *et al*. (2011) who found out 0.5% in Arsi zone of Oromia Regional State, Tolosa *et al.* (2011a) who reported 1.1% in north Gondar zone, North western Ethiopia. Furthermore, Tolosa *et al.* (2010b) reported 0.5% in Jimma area, Hailu *et al. (*2011) who reported 1.38% in Jijiga Zone and Tesfaye *et al.* (2011) who reported 1.5% in Addis Ababa.

There were reports on the contrary with a relatively higher seroprevalence of bovine brucellosis in other parts of the country. Hunduma and Regassa (2009) reported 11.2%; Ibrahim *et al*., (2010) reported 3.1%; Hailesillasie *et al.,* (2011) reported 4.9%; Megersa *et al*. (2012) reported 8.0% and Tibesso *et al*. (2014) reported 4.3%. Moreover, Berhe *et al*. (2007) reported 42.31% and Kebede *et al*. (2008) reported 45.9% in Ethiopia. Aggad and

Boukraa (2006) reported 31.5% in Algeria and Ahmad *et al*. (2009) reported 25.8% in Jordan.

The differences in seroprevalence of the current finding from others could be mainly due to the fact that, the current study included animals that had history of recent abortion which indicated 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 variations, and sample size.

There was also statistically insignificant difference (P>0.05) in seroprevalence of the present study of cattle brucellosis among sex, age, body condition, parity status, in Fisher`s exact analysis. Even though age was not significantly associated with *Brucella* seropositivity (P> 0.05), a seroprevalence of 4.62% was found among adult age group unlike young age group of cattle with no *Brucella* seropositivity in the study sites. Several previous reports similarly indicated that higher seroprevalence of brucellosis was observed in adult age group of cattle (Magona *et al.*, 2009; Megersa *et al.*, 2011).

Sex was not significantly associated with *Brucella* seropositivity (P> 0.05). A seroprevalen ce of 1.5% was found among female animals whereas no Brucella seropositivity was observed in male animals. Female animals were found to be more likely seropositive than male animals. The reason was explained by Kebede *et al*. (2008) that males are kept for relatively shorter time duration in breeding herd than females and thus the chance of exposure is lower for males.

The disease was found to be higher in animals in 3-4 years of age and greater than 4 years, respectively than in animals in 1-2 years of age. A significantly higher seroprevalence in older than younger animals can be attributed to the practice of leaving youngers around home premises, when adult cattle were taken to graze communally, lessening the risk of youngers to acquire the infection via contact from common grazing and watering grounds (Kiputa *et al*., 2007). This is common phenomenon in Amibara pastoral areas, in Afar.

In addition, this phenomenon could be explained by sexual maturity and pregnancy due to the influence of sex hormones and placenta erythritol play a role on the pathogenesis of brucellosis (Radostits *et al*., 2007). Sexually mature animals are more susceptible to

Brucella infection than sexually immature animals of either sex (Bekele *et al*., 2011). This may be due to the fact that sex hormones and erythritol, which stimulate the growth and multiplication of *Brucella* organisms, tend to increase in concentration with age and sexual maturity (Radostits *et al*. 2007; Alan, 2013).

There was statistically significant association ($P<0.05$) between abortion period and sero-positivity of brucellosis in the present study. This could be explained by the presence of higher seropositivity in cows in the last trimester may be due to the preferential localization of *Brucella* in the uterus in which allantoic fluid factors such as erythritol could stimulate the growth of *Brucella* and elevated in the placenta and fetal fluid from about the 5^{th} month of gestation (Coetzer and Tustin, 2004; Radostits *et al*., 2007).

Age, of status remained significant in multivariable logistic regression analysis. Multivaria ble logistic regression analysis revealed that seropositivity for *Brucella* infection increases approximately 12 times in $\geq$5-12 years old animals compared to >12 and $\leq$ 4 years old. Several previous studies have indicated that higher seroprevalence of brucellosis in adult age group of cattle (Fekadu *et al*., 2014; 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 (Radostits *et al*. 2007

6. CONCLUSIONS AND RECOMMENDATIONS

In this study, brucellosis in camels and cattle that had a recent history of abortion was found to be still prevalent in the study area. In the present study, *B. abortus* was isolated in Amibara district from seropositive cattle with history of abortion. The organisms were isolated from vaginal swab (three isolates) and synovial fluid (two isolates). Moreover, the seroprevalence recorded in the present study revealed that brucellosis is an established disease in Amibara district of Afar regional state. Higher seroprevalence was observed in cattle than camels compared to both species. In conclusion, the bacteriological isolation and identification of *B. abortus* from cattle combined with the prevailing *Brucella* seropositivity indicate the importance of brucellosis in livestock production system of the areas and potential public health implication for human population in the study areas.

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

- Isolation of *Brucella abortus* from clinical abortion case of cattle shows the species is the main causative agent in the area and further confirmation of the bio variant should be carried out using molecular techniques.
- Since the majority of isolation of the agent were from vaginal sawab, contact of the pastoralist community without propoer protection might expose for zoonotic brucellosis hence, 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.
- Comprehensive study in various animal species in different areas of the region should be carried out to determine the general status of brucellosis.
- Detailed diagnostic tools including molecular methods must be used for identification of *Brucell*a species circulating in the area.

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8. ANNEXES

Annex 1**:**The Rose Bengal Plate Test

Procedure

1. 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 30µl serum was mixed with 30ul 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 2: Complement Fixation Test

Procedure

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

2. Using standard 96-well U-bottom microtitre plates, 25µl volumes of diluted test serum are placed in the wells of the first and second rows, and 25µl volumes of veronal buffered saline are added to all wells except those of the first row.
3. Serial doubling dilutions are then made by transferring 25µl volumes of serum from the second row onwards continuing for at least four dilutions.
4. Repeat steps ii and iii above for each serum to act as anticomplementary serum controls (see below).
5. Volumes (25µl) of complement at 1.25 MHD, are added to each well and 25µl of antigen, diluted to working strength, are added to all wells excluding those of the anticomplementary controls. These latter wells receive 25µl of veronal buffered saline instead.
6. Control wells containing: diluent only, negative serum + complement + diluent, antigen + complement + diluent, and complement + diluent, are set up to contain 75µl total volume in each case.
7. The plates are incubated at 37°C for 30 minutes with agitation at least for the initial 10 minutes, or at 4°C for 14- 18 hours.
8. Volumes (25µl) of sensitised SRBC suspension are added to each well, and the plates are reincubated at 37°C for 30 minutes with agitation at least for the first 10 minutes.
9. The results are read after the plates have been left to stand at 4°C for up to 1 hour to allow unlysed cells to settle.

Interpretation

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

Annex 3: Competitive ELISA procedure

1. All reagents should equilibrate to room temperature 18-25 °c before use.
2. Add samples

   Add 45µl of Sample Dilution Buffer into each well that will be used for serum sample, serum controls and conjugate controls

A. Add 5µl of positive, weak positive and negative serum controls, into each of the appropriate wells, respectively. For conformation purpose it is recommended to run the control sera in duplicates.

B. Add 5µl of Sample Dilution Buffer into two appropriate wells(designated as conjugate controls, Cc)

C. Add 5µl of test sample to each of the appropriate wells. The samples can be tested in singlicates or duplicates. However for conformation purpose it is recommended to run the samples in duplicates.

3. Add 50µl of mAb- Solution into all wells used for controls and samples. Note: The time difference between controls/ samples and mAb- Solution addition must not exceed 10 minutes.

4. Seal the plate and mix the reagents thoroughly for 5 minutes, either by using a plate shaker or by tapping the sides of the plate.

5. Incubate the plate at room temperature 18-25°c for 30 minutes.

6. Rinse the plates/strips 4 times with PBS-Tweeen Buffer: fill up the wells at each rinse, empty the plate and tap hard to remove all remains of fluid.

7. Add 100µl of Conjugate Solution into each wells. Seal the plate and incubate at room temperature 18-25°c for 30 minutes.

8. Repeat step #6.

9. Add 100µl Substrate solutions to each well and incubate for 10 minutes at room temperature 18-25°c for 30 minutes. Begin timing after the first well is filled.

10. Stop the reaction by adding 50µl of Stop solution to each well and mix thoroughly. Add the Stop solution in the same order as the Substrate solution was added in step #9.

11. Measure the Optical density (OD) of the controls and samples at 450 nm in microplate photometer (use air as blank).Measure the OD within 15 minutes after the addition of Stop solution to prevent fluctuation in OD values.

Calculations

1.Calculate the mean OD (Optical density) values for each of the controls and samples.

2.Calculate the percent Inhibition (PI) values for controls as well as samples, using the following formula :

$$PI = 100 - \frac{(OD_{\text{samples or controls}})}{(OD_{\text{Conjugate controls Cc}})} * 100$$

Interpretation

The status of a test sample is determined as

PI	Status
<30%	Negative
≥ 30%	Positive

Annex 4: 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 (FD161) was added and the media is then poured in to sterile Glass petridishes.

Annex 5: Procedure of staining

a)Stamps Modified Ziehl-Neelsen Staining procedure for Brucella

Procedure

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

Interpretation

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

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 6: 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% H_20_2 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_20_2 is not necessary

Tube methods:

Directly add 1.0ml of H_20_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 colour change after 15-30 seconds (kovac's reagent)

Indirect paper procedure

Place a 6cm2 pieces of whatman no. 1 filter paper in petridish

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 colour reaction occur within 5-10 seconds

Interpretation

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

Oxidase negative: no colour 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^{o}c for 18-24 hrs.

Interpretation

Organisms that hydrolyse 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 H_2S production

When a sensitive technique for detecting H_2S production is required, the lead acetate paper test is recommended. For this, you have to:

Inoculate a tube or bottle of sterile trypicase 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^{o}C, and examine daily for a blackening of the lower part of the strip.

Expected Results

Blackening ——————————— Positive test H_2S produced

No blackening ———————————- Negative test No H_2S produced

Annex 7: 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^{0}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 45-560C 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 petridish.
Annex 8: Agglutination with positive and negative *Brucella* sera

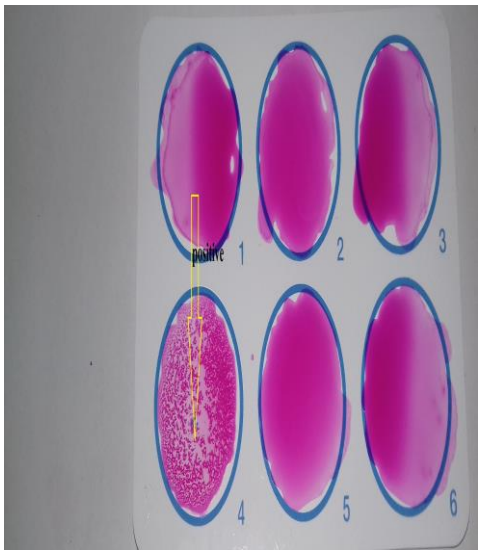
For antigen production, the seed culture is used to inoculate a number of potato-infusion agar slopes that are then incubated at 37°C for 48 hours. SDA and TSA, to which 5% equine or new born calf serum and/or 0.1% yeast extract may be added, are satisfactory solid media provided a suitable seed is used as recommended above. The growth is checked for purity, resuspended 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.

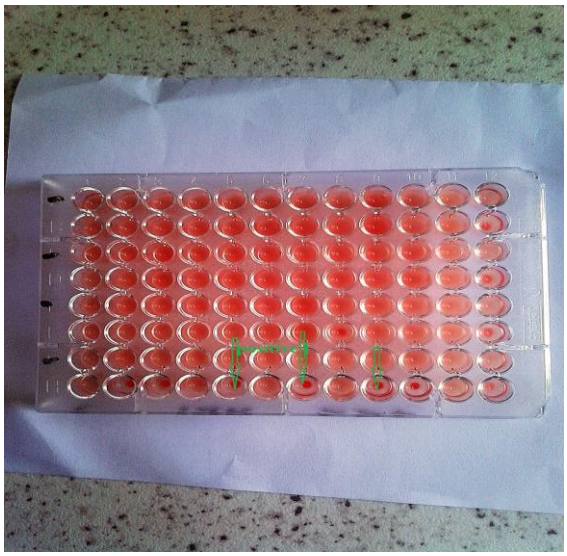
Procedure and Interpretation

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.

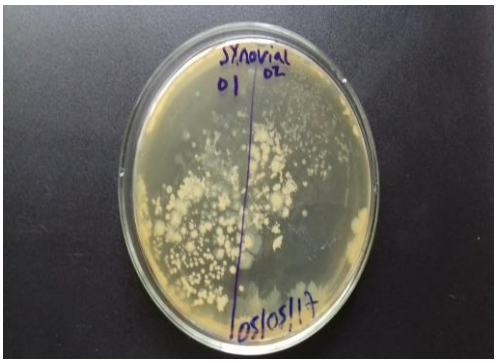
Equation 9: Pictures of serological and bacteriological test results



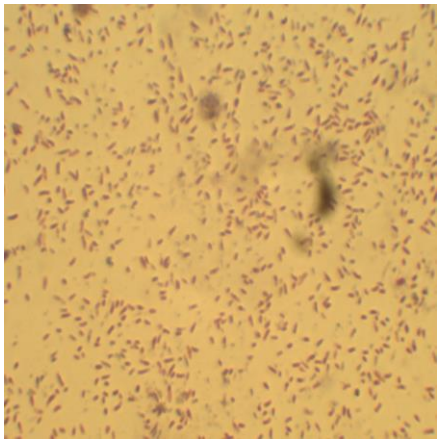
a) RBPT test, Arrows indicate positive Test result positive test result.

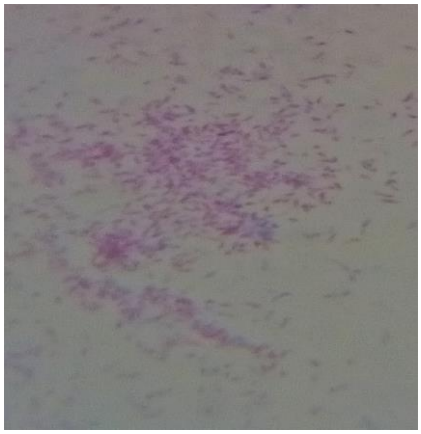


b) CFT test result Arrows indicate Positive test result



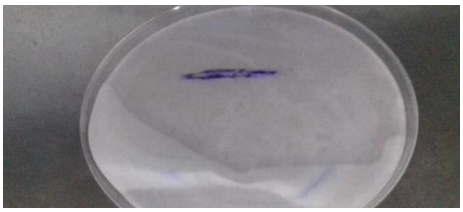
a) *Brucella* Species Colony Cultured on Brucella Selective agar after (96 hrs) 4 day's incubation



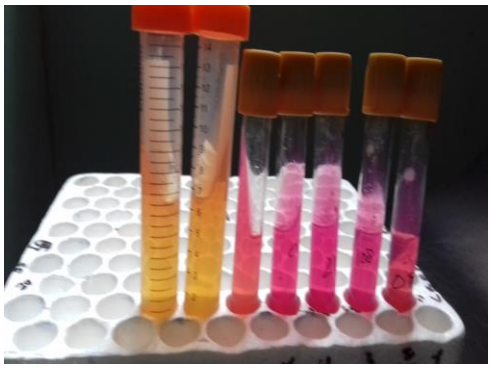


b) Photo taken from Microscope, MZN staining (Left) and Gram's stain (Right) of *Brucella*
(red, short coccobacilli)

c) Catalase positive test Result



d) Oxidase positive test Result



e) Urease Test Result positive (Right) negative (left)