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SERO- EPIDEMIOLOGICAL STUDY OF BOVINE BRUCELLOSIS IN SELECTED DAIRY FARMS OF BISHOFTU AND HOLETA TOWNS, OROMIA REGIONAL STATE, CENTRAL ETHIOPIA

MVSc Thesis

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF CLINICAL STUDIES

BY:

KEBEDE SHANKO HORDOFA

June, 2017

Bishoftu, Ethiopia

SERO EPIDEMIOLOGICAL STUDY OF BOVINE BRUCELLOSIS IN SELECTED DAIRY FARMS OF BISHOFTU AND HOLETA TOWNS, OROMIA REGIONAL STATE, CENTRAL ETHIOPIA

A Thesis submitted to School of Graduate Studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Veterinary Science in Veterinary Epidemiology.

By:

Kebede Shanko Hordofa

June, 2017

Bishoftu, Ethiopia

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Clinical Studies

As MVSc research advisers, we here certify that we have read and evaluated the Thesis prepared under our guidance by **Kebede Shanko entitled: "Sero-epidemiological study of Bovine brucellosis in selected dairy farms of Bishoftu and Holeta towns, Oromia Regional state, central Ethiopia**"; we recommend that it can be submitted as fulfilling the MVSc thesis requirement .

Dr. Fufa Abunna _____ _____

Major Advisor Signature Date

Dr.Teshale Sori _____ _____

Co- Advisor Signature Date

Dr. Getachew Tuli _____ _____

Co- Advisor Signature Date

As members of the Examining Board of the final MVSc open defense, we certify that we have read and evaluated the Thesis prepared by: **Kebede Shanko** Titled: "**Sero-epidemiological study of Bovine brucellosis in selected dairy farms of Bishoftu and Holeta towns, Oromia Regional state, central Ethiopia**" .We recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Veterinary Epidemiology.

Dr. Dinka Ayana _____ _____

Chairman Signature Date

Dr. Alemayehu Regassa _____ _____

External Examiner Signature Date

Dr. Kebede Amenu _____ _____

Internal Examiner Signature Date

DEDICATION

I dedicate this research work to my late father, ***Shanko Hordofa****, whose wise counsel has made me the person I am.*

STATEMENT OF AUTHOR

First, I declare that this thesis is my ***actual*** work and that all sources of materials 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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Name: Kebede Shanko

Signature: _____

Date of Submission: _____

Advisors' name

Main advisor - Dr. Fufa Abunna (Associate professor)

Signature: _____

Co- Advisor - Dr. Teshale Sori (DVM, MSc, PhD, Associate professor, AAU, CVMA)

Signature: _____

Co- Advisor - Dr. Getachew Tuli (DVM, MSc, NAHDIC, Sebeta)

Signature: _____

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LIST OF ABBREVIATIONS

AMOS	'Abortus-melitensis-ovis-suis'
BBAT	Buffred *Brucella* antigen tests
BCV	*Brucella*-containing vacuole
BPAT	Buffered Antigen Plate Agglutination test
BSL3	Biosafety Level 3
c-ELISA	competitive -Enzyme Linked Immunosorbent assay
CFT	Complement fixation test
CSA	Central Stastistic Agency
CVI	Cost of veterinary intervention
DALY	Disability Adjust Life Year
DNA	Deoxy riboneucleotide Acid
DS	Combinations of Doxycycline with streptomycin
EcoRV	Escherichia coli Restriction Enzyme V
ELISA	Enzyme Linked Immuno Sorbent Assay
FAO	Food and Agriculture Organization
MBM	Malachite Brucella medium
MLVA	Multiple locus variable number of tandem repeats analysis
MRT	Milk ring test
MZN	Modification of Ziehl-Neelsen
NAHDIC	National Animal Health Diagnostic and Investigation Center
OD	Optical Density
OIE	Office International des epizooties
Omp	Outer Membrane Protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RBPT	Rose Bengal plate test
RER	Rough Endoplasmic Reticulum
RFLP	Restriction fragment length polymorphism

RFM	Retained fetal membrane
SDA	Serum–Dextrose Agar
S-LPS	Smooth- Lipopolysaccharides
SNPs	Single nucleotide polymorphisms
TSA	Tryptone soya agar

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ABSTRACT

A cross-sectional seroepidemiological study of bovine brucellosis was conducted in selected dairy farms of Bishoftu and Holeta towns, central Ethiopia, from December, 2016 to May, 2017 with the aim to estimate sero-prevalence of *Brucella* infection, to identify the potential risk factors in cattle and to assess the knowledge of the farm owners toward the disease. A total of 326 dairy cattle from 30 herds were included in the study (175 animals from Bishoftu and 151 animals from Holeta).The dairy herds were stratified into small herd size (<10 animals), medium herd size (10-50) and large herd size (≥ 50). Stratified two stage sampling was used to sample both herds and individual animals.The serum samples collected were initially screened using Rose Bengal Plate test and competitive enzyme linked immunosorbent assay. Complement fixation test was used to confirm the seropositivity of the samples found positive with the above tests. The animal level prevalence was 0. 92% (95%CI: 0.19, 2.66). At herd level 3 out of 30 herds tested positive making herd level prevalence of 10.0 % (95%CI: 2.11, 26.53). Of the herds that tested seropositive, the within herd seroprevalence ranged from 2.9 % (1 out of 34) to 16.7 % (1 out of 6). Slightly higher seroprevalence was observed in highland agro-ecological area of Holeta (1.98 %; 95% CI: 0.41, 5.69) than the mid-highland area of Bishoftu 0% though the difference observed was not statistically significant (P > 0.05). At animal level only abortion history and retained fetal membranes were found to be significantly associated with the sero-positivity for *Brucella* infection using univariable analysis (P < 0.05).The odds of positivity is 38.6 (3.3,453.9) times higher in aborted cattle compared to those did not abort. Cows with history of retained fetal membrane had higher risk of infection with *Brucella* species (OR= 33.2; 2.8,387.1) than cows without history of retained fetal membrane. Up on multivariable logistic regression analysis, only abortion history was significantly associated with sero-positivity to *Brucella* infection (OR= 37.8**;** P <0.05). At herd level only reproductive disorders and herd size were found to be significantly associated with *Brucella* infection (P <0.05). Personnel in commercial dairies had better awareness (knowledge) about zoonotic brucellosis than those working in smallholder farms. Therefore, the need for implementing strong and sustainable control measures and raising public awareness focusing on the smallholder farmers .

Keywords: *Awareness, Bovine brucellosis, Central Ethiopia, Dairy cattle, seroepidemiology*

1. INTRODUCTION

Brucellosis is a contagious, zoonotic and economically important bacterial disease of animals worldwide and it is considered as one of the most widespread zoonoses in the world (Schelling *et al.*, 2003). It has been virtually eliminated from the majority of the developed countries, but it is still endemic in Africa, the Middle East, Central and Southeast Asia, Central and South America and in most of the Southern European countries. Despite being endemic in many developing countries (Donev, *et al*., 2010), brucellosis remains under diagnosed and under-reported. It is an important disease among livestock and people in sub-Saharan Africa. Furthermore, since brucellosis is an important cause of abortion especially in first calf heifers, the disease can also cause important economic losses in developing countries (Smits *et al*., 2007*).*

It is caused by *Brucella* species, is primarily affecting domestic livestock and wild animals and it is an important public health problem in many countries (Mangen *et al*., 2002; Refai, 2002). *Brucella* species are Gram-negative**,** facultative, intracellular coccobacillary organisms affecting multiple host species (OIE, 2000). Clinical manifestations of brucellosis are determined by the combination of pathogen and host species since *Brucella* species have distinct host preferences (Xavier *et al.*, 2009a, Poester *et al.*, 2013).

The disease in cattle, usually caused by *Brucella abortus* and occasionally by *Brucella melitensis* and *Brucella suis*, is characterized by late term abortion at first gestation and It is predominantly caused by biovars (mainly biotype -1) of *B. abortus* causing infertility and reduced milk production in cows (OIE, 2008), whereas bulls can develop orchitis (Carvalho Neta *et al*., 2010, Poester *et al.*, 2013). Since abortion usually occurs in the first pregnancy after infection and becomes less likely thereafter because of sustained immunity, the introduction of infected animals into immunologically naïve groups (or of unprotected replacements in infected groups) results in multiple abortions in a short period of time (“abortion storms”) (Cunningham, 1977).

Besides being a threat to human health, brucellosis often causes serious problems to the national economies. The economic loss from brucellosis in developed countries arises from the slaughter of cattle herds that are infected with and the cost of eradication and control program. In developing countries farmers suffer from the abortion of calves and the decreased milk yield, birth of weak calves that die soon after birth, retention of placenta, impaired fertility and sometimes arthritis or bursitis and the cost of tests and samples, death may occur as a result of acute metritis (Garin–Bastuju, 2003). The estimation of the financial loss caused by brucellosis depends mainly on the type of cattle farming, herd size, and loss in reproduction and reduction in meat and milk due to abortion. In Ethiopia, information on losses specifically through brucellosis in the different types of production systems is sparse, with the exception of Tariku (1994) who reported an annual loss from brucellosis that was estimated to be 88,941.96 Ethiopian Birr ($5231 equivalent) among 193 cattle, largely due to reduced milk production and abortions.

The epidemiology of the disease in livestock and humans as well as appropriate preventive measures is not well understood, and in particular such information is inadequate in sub-Saharan Africa (McDermott, 2002). There is a paucity of science-based evidence on brucellosis in sub-Saharan Africa and an appraisal of historical and contemporary epidemiology (prevalence estimates, affected host species, potential reservoirs and *Brucella* species involved) are key to implementing measures for sustainable management of this disease. The epidemiology of cattle brucellosis is complex and influenced by several factors (Crawford *et al.*, 1990).

Although in Ethiopia there is no documented information on how and when brucellosis was introduced and established, the evidences of *Brucella* infections in Ethiopian cattle have been serologically demonstrated by different authors. Indeeded, higher individual bovine brucellosis seroprevalence has been recorded in intensively managed cattle herds as compared to those in the extensive management system. Accordingly, a seroprevalence of 39% was recorded at the

Institute of Agricultural Research in Western Ethiopia (Meyer, 1980). Moreover, 22% in dairy farm in Northeastern Ethiopia (Tariku, 1994), 11 to 15% in dairy farms and ranches in Southeastern Ethiopia (Bekele *et al.,* 2000), 8.2% in Arsi area (Molla, 1989), and 8.1 % in dairy farms in and around Addis Ababa (Asfaw *et al.,* 1998) were recorded.Whereas relatively low individual animal level seroprevalence were recorded in some intensive farms in different parts of the country .for example Kassahun *et al.* (2007) documented 2.46% in Sidama Zone of Southern Ethiopia; Mussie *et al*. (2007a) reported a prevalence of 4.63% in Northwestern part of Amhara Regional State.

Even though seroprevalence of brucellosis is established in different species of animals, it is still among the known animal diseases that inflict huge problems on human health and national economies in Ethiopia. Indeed, change in demographic characterstics and agricultural practices are expected to change the occurrence of contagious diseases like brucellosis. There are many dairy farmers in the study areas who highly supplied huge amounts milk for local market as well as to national market to satisfy the highest national demand. Though there is information on the epidemiology of brucellosis in study towns in animals, there is a controversy in reporting apparent seroprevalence of bovine brucellosis in study areas. Therefore, further assessment of the status of the disease and understanding of the awareness among the community has paramount importance in order to overcome the impact of the disease. This study was undertaken in areas that are known as the milk sheds of the capital. Understanding of the awareness and knowledge of the dairy owners and community at large about the disease is also important so as to develop control strategies. Therefore, this study was carried out with the following objectives:

1.1. General Objective

- To investigate seroepidemiology of bovine brucellosis and assess the awareness of the community towards the disease in selected dairy farms of Bishoftu and Holeta towns, central Ethiopia.

1.2. Specific Objectives

- To estimate the current status of the seroprevalence of bovine brucellosis in selected dairy farms of Bishoftu and Holeta towns
- To assess the associated risk factors of bovine brucellosis, and
- To understand the knowledge of the community toward the disease in study towns.

2. REVIEW OF LITRATURE

2.1. The Etiologic Agent

Brucella species are facultative intracellular gram-negative cocco-bacilli, non-spore forming and non-capsulated. Although *Brucella* species are described as non-motile, they carry all the genes except the chemotactic system, necessary to assemble a functional flagellum. Currently ten species are recognized including the genus *Brucella* consisted of six recognized species, comprised of *B. abortus* (cattle, biovars 1-6, and 9), *B. melitensis* (goats, sheep, biovars 1-3), B. suis(pigs, reindeer and hares, biovars 1-5), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (desert wood rats). More recently, new members to the genus include *B. ceti* and *B. pinnipedialis* (dolphins/porpoises and seals respectively), *B. microti* (voles) and *B. inopinata* (reservoir undetermined) (Godfroid *et al.*, 2013). In spite of more than 94% similarity amongst the members of the genus (Verger *et al.*, 1987), bacteria of the genus *Brucella* have different host preferences.Therefore; *Brucella* species are capable of causing disease in a variety of animal species, including humans (Table 1).

Table 1. *Brucella* species and their host preferences

Species	Zoonotic importance	Host preference
Brucella melitensis	High	Sheep, Goat
Brucella abortus	Moderate	Cattle
Brucella suis	Moderate	Pig
Brucella canis	Mild	Dog
Brucella ovis	Absent	Sheep
Brucella neotomae	Absent	Deseret wood rat (*Neotome lepida*)
Brucella ceti	Mild	Ceteceans
Brucella Pinnipedials	Mild	Seals
Brucella microt	Absent	Common Voles (M*icrotus arvalis*)
Brucella inopinata	Mild	Undetermined host

Source: Godfroid *et al.* (2013)

Brucella species have a strong host preference, which is evident in their ability to establish chronic infection in individuals and maintain transmission and infection in populations of specific animal species (Godfroid *et al.*, 2013). *Brucella abortus, B. melitensis* and *B. suis* are the species that have the highest impact on domestic livestock productivity and human health (Godfroid *et al*., 2011). Although they preferentially infect cattle, small ruminants and swine, respectively, cross-infections may be significant in mixed husbandry systems or at the livestock wildlife interface (Godfroid *et al.*, 2013). *Brucella suis* have the widest host range, with established host-pathogen relationships in reindeer and hares, in addition to swine. However, almost all *Brucella* species can infect mammalian species other than their preferred host; for example, both *B melitensis* and *B. suis* are capable of colonizing bovine udders and therefore contaminating cows' milk (Refai, 2002).

As a component of their identification, *Brucella* species are also classified on the basis of the presence or absence of S-LPS; the presence of S-LPS appears to be associated with virulence. The commonly identified human pathogens *B. abortus, B. melitensis,* and *B. suis* are characterized as smooth because S-LPS is present in their outer membrane. The remaining species (*B. canis* and B. *ovis*) are characterized as rough strains, given that they express little or no S-LPS and cause less severe or no disease in humans (Ko *et al*., 2003). Molecular characterization has identified a great degree of homology among the *Brucellae* (Halling *et al.*, 2005). Common genetic fingerprinting methods such as pulsed-field gel electrophoresis and multilocus sequence typing analyses have revealed little variability among isolates of a given species.

However, multilocus sequence typing has been useful in identifying the relationship among various species and among biovars within species, and in general, the findings support the classification of *Brucella* into the 6 known species, with at least 1 new species representing the newer marine strains of *Brucella* (Whatmore *et al.,* 2007). Multiple-locus variable-number tandem repeat analysis appears more effective at discriminating between different species and strains and shows promise for differentiation of strains associated with a local outbreak or investigation. Multiple-locus variable-number tandem repeat analysis was recently used to

differentiate isolates in 2 unrelated laboratory-acquired cases of brucellosis, when the laboratory workers had been exposed to more than *1 Brucella* species isolate. Molecular genetic studies have clearly demonstrated that the genus *Brucella* forms a discrete homogenous group which clearly belongs to the Alphaproteobacteria (Garritty *et al.*, 2005).The phylogenetic tree of the *Brucella* species is presented in (Figure 1).

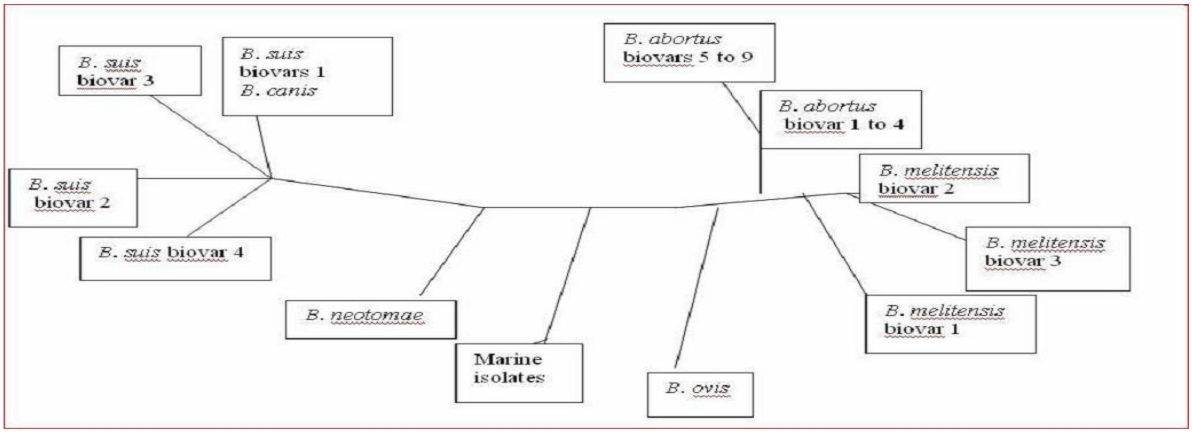

Figure 1. Phylogenetic tree of the *Brucella* species

Source: Garrity *et al.* (2005).

2.2. Morphology

Brucellae are coccobacilli or short rods 0.6 to 1.5 μm long by 0.5 to 0.7 μm in width. They are 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. *Brucella* species are non-motile. They do not form spores, flagella, or pili. True capsules are not produced. *Brucella* is Gram-negative and usually does not show bipolar staining. They are not truly acid-fast but resist decolouration by weak acids, thus stain red by the Stamp's modification of Ziehl-Neelsen method, which is sometimes used for the microscopic diagnosis of brucellosis from smears of solid or liquid specimens (Europian Commission, 2001).

2.3. Genome of *Brucella* Organism

In 1985, it was proposed that the six *Brucella* species should be grouped as biovars of a single species based on DNA-DNA hybridization studies. The genomes sequenced from genus *Brucella* are also known to be very similar in terms of both base composition and genome size. All sequenced species have a GC content of approximately 57%, and most genomes consist of approximately 3.3 Mbp divided on two chromosomes. None of the sequenced members of the *Brucella* genus have any plasmids reported. The first *Brucella* species to be sequenced was *B. melitensis* 16M (biovar 1) followed closely by *B. suis* (biovar 1) (Bohlin *et al.*, 2010). Analysis of 16S rRNA sequences places *Brucella* species as members of the alpha-2 *Proteobacteria (*Shirley *et al*., 2005). The genus *Brucella* has six recognized species, all of which exhibit distinct host preferences (Shirely *et al*., 2005).The high degree of similarity among the *Brucellae* (Gandara *et al.*,2001; Bricker,2002; Paulsen *et al*.,2002) lends support to the proposal that the classical species of *Brucella* are actually strains of *B. melitensis.* However, this view conflicts with the hypothesized evolutionary isolation of these classical species due to their intracellular existence and host preference (Shirley *et al*., 2005).

Pulsed-field gel electrophoresis (PFGE) maps of the classical *Brucella* species genomes are composed of two circular chromosomes of approximately 2.1 and 1.2 Mbp, with the exception of *B. suis* biovar 3, which has a single chromosome of 3.1 Mbp. PFGE studies revealed other differences, including a 640-kb inversion in the small chromosome of *B. abortus* and a deletion in the small chromosome of *B. ovis*. The two chromosomes of *Brucella* differ in important ways. The origin of replication of the large chromosome (Chr I) is typical of bacterial chromosomes, while that of the small chromosome (Chr II) is plasmid like. Further, most of the essential genes are located on Chr I. The GC content of the two chromosomes is nearly identical, consistent with the assertion that the assimilation and stabilization of a plasmid was an ancient event in *Brucella* (Paulsen *et al*., 2002). The genome sequences of *B. melitensis* and *B. suis* have been determined (DelVecchio *et al*., 2002). Comparative analyses revealed both that the two genomes are extremely similar and that they have many similarities to both bacterial plant and animal pathogens and symbionts (Shirley *et al*., 2005). The sequence identity for most open reading frames (ORFs) was 99% or higher. Nevertheless, unique

fragments were reported to exist between these two genomes. Prior to sequencing the *B. abortus* genome, a large number of short sequences were available in gene bank. Many of these sequences were derived from analyses of plasmids estimated to cover 20% of the genome from a random shotgun library of *B. abortus* S2308 (Bohlin *et al*., 2010).

2.4. Epidemiological Distribution

The epidemiology of brucellosis is complex and it changes from time to time. Wide host range and resistance of *Brucellae* to environment and host immune system facilitate its survival in the populations. Since cattle are found throughout the world, prevalence of brucellosis in cattle has been reported from a wide range of countries.

2.4.1. World distribution

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). Although in most countries brucellosis is a nationally notifiable disease and reportable to the local health authority, it is under reported and official numbers constitute only a fraction of true incidence of the disease.

Although brucellosis has been, or is close to being, eradicated from a number of developed countries, it continues to be a major public and animal health problem in many regions of the world, particularly where livestock are a major source of food and income. There are many reasons why brucellosis remains endemic. These include expansion of livestock herds and flocks, with associated uncontrolled movements; lack of veterinary support services and

vaccines; and husbandry practices. Published studies on the relative occurrence of brucellosis are largely confined to serological surveys, and are much more commonly conducted for bovine brucellosis, occasionally for shoats and rarely for pigs and Camels (McDermot and Arimi, 2002). Figure 2 depicts the world wide distribution of bovine brucellosis caused by *B. abortus* (OIE reports of 2011).

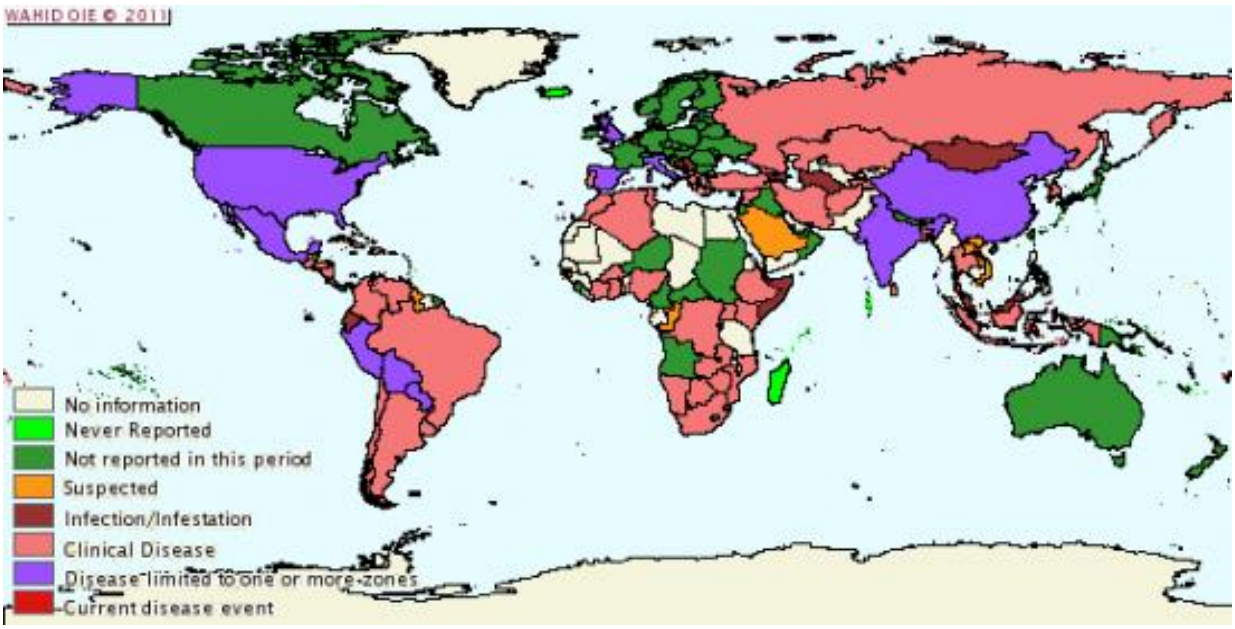

Figure 2**.** Worldwide distribution of bovine brucellosis (*B. abortus*) report
Source: OIE (2011)

2.4.2. Distribution in Africa

Brucellosis is a disease that has been known in Africa for a very long time, in both animals and humans, as evidenced by a study by (Akakpo *et al.,* 1987). It is a disease of chronic evolution, the importance of which is difficult to assess owing to the dominant livestock production systems of unrestricted grazing, transhumance and nomadism. However, official Veterinary Services are now starting to turn their attention to brucellosis, after controlling most of the major epizootics that formerly decimated livestock (including rinderpest and contagious pleuropneumonia) and in response to the intensification of dairy livestock production in many Sub-Saharan countries. It is a matter of debate whether brucellosis (sero) prevalence is higher

under extensive or intensive breeding conditions (Ducrotoy *et al*., 2014). Here is the summary of the seroprevalence of bovine brucellosis in some African countries in (Table 2).

Table 2. Seroprevalence of brucellosis in cattle on a survey of studies in some African countries

Country	No of cattle tested	Prevalence (%)	Test applied	Reference
Zambia	1245	14.1	RBPT, c-ELISA	Muma *et al*.(2007)
Kenya	393	1	c-ELISA,CFT	Kang'ethe *et al.(2007)*
Sudan	574	24.5	c-ELISA	Angara *et al*. (2010)
Ghana	444	2.9	RBPT	Folitse *et al*. (2014)
Nigeria	-	24.0	RBT+ELISA	Mai *et al*. (2012)
Uganda	-	14	RBPT	Miller *et al*. (2015)
Uganda	-	5	ELISA	Bertu *et al*. (2010)
Zimbabwe	1291	5.5	RBPT, c-ELISA	Matope *et al*. (2010)
Algeria	1032	9.7	BPAT	Aggad & Boukaa (2006)
Egypt	1966	5.4	BPAT	Samah *et al*. (2008)
South Africa	5 059	1.5	RBPT, CFT	Bishop *et al*.(1994)
Eritrea	1294	8.5	RBPT, CFT	Omer *et al*. (2000)
Ghana	183	6.6	RBPT	Kubuafor *et al*. (2000)

No: Number

2.4.3. Status of Brucellosis in Ethiopia

Both husbandry systems as well as environmental conditions greatly influence the spread of *Brucella* infection (WHO, 1997). Ethiopia has several institutionally owned commercial dairy farms, mostly situated in and around Addis Ababa and in some regional towns. These farms have been the focus of most *Brucella* surveys at country level so far. But now days, studies on the seroprevalence of bovine brucellosis have been carried out in many parts of Ethiopia by different researchers. These studies were conducted in local, pure and cross breed

of cattle. In these studies, seroprevalence of brucellosis in cattle ranging from 0.2-11.2% were reported by CFT as depicted in (Table 3).

Table 3. Seroprevalence of brucellosis in cattle based on a survey of studies published in Ethiopia since 2007-2016

Location	Prevalence rate		Reference
	RBPT	*CFT*	
Tigray Region	3.3	3.19	Berhe *et al.* (2007)
Sidama Zone	-	1.66	Asmare *et al.* (2010)
West Tigray	-	4.9	Haileselassie *et al*. (2008)
Jimma zone	-	0.77	Tolosa *et al.* (2008)
Debrebirhan and Ambo	0.7	0.2	Bashitu *et al.* (2015)
Adami Tulu	4.5	4.3	Tibesso *et al.* (2014)
Debre-Zeit, Central Ethiopia	3.3	2	Alemu *et al.* (2014)
SE. Somali and Oromia	-	0.9	Gumi *et al.* (2013)
Benishangul Gumuz	1.2	1	Adugna *et al*. (2013)
East Wollega Zone	2.96	1.97	Yohannes *et al.* (2012)
East Showa Zone	11.2	-	Hunduma and Regassa (2009)
East shoa zone	-	11.2	Dinka and Chala (2009)
Wuchale-Jida district	12.5	11.0	Kebede *et al.* (2008)
Central Oromia	4.9	2.9	Jergefa *et al*. (2009)
Arsi Negele District	2.6	-	Amenu *et al.* (2010)
Jimma zone	-	3.1	Ibrahim *et al.* (2010)
Jijjiga zone	1.84	1.38	Hailu *et al*. (2011)
Pastoral and mixed farming	-	3.5	Megersa *et al*. (2011b)
WesternTigray	-	6.1	Mekonnen *et al. (*2011)
Alage and its Surroundings	-	2.4	Asgedom *et al*. (2016)
Central Ethiopia	2.28	1.40	Geresu *et al*. (2016)

2.4.4. Reservoirs/ Carriers of Brucella species

Domestic animals

Brucellosis is a worldwide zoonotic disease caused by *Brucella* species in which domestic animals such as cattle, goats, sheep, pigs, camel, buffalo and dogs serve as a reservoir hosts (Corbel, 1997; Moreno *et al.*, 2002). Fresh milk and dairy products prepared from unpasteurized milk such as soft cheeses, yoghurts and ice creams contain high concentration of the bacteria and consumption of these is an important cause of human brucellosis (Bikas *et al.*, 2003). *Brucella* species can survive in proper environmental condition, damp soil and seawater and can be a source of infection. Notably abortion materials such as fetal parts, and fetal membranes, amniotic fluid and vaginal discharges of infected animals may contain high amounts of the bacterium and act as source of brucellosis (Henk *et al.*, 2005).

Wild animals

Brucella abortus and *B. suis* have been isolated worldwide from a great variety of wildlife species (Godfroid, 2002). Some general risk factors, which can be identified in most of the wildlife diseases, are wildlife overabundance, movements of wild and domestic animals and fomites (Gortázar *et al*., 2007). Artificial management of wild species, including fencing, feeding and translocation, can also increase the risk of transmission of infectious brucellosis (Gortázar et al., 2006). The risk of infection increases dramatically with increasing wildlife density and their exposure to *Bucella abortus* around feeding grounds (Godfroid *et al*., 2013).

Wild ruminants have been suggested as brucellosis carriers, but they are probably not true reservoirs (Gortázar *et al*., 2007; Godfroid *et al*., 2013). Other works showed that wild ruminants do not play a relevant role in the maintenance of *B. abortus* and *B. melitensis* infections since limited cases of brucellosis have been reported in wild ruminants (Godfroid *et al*., 2005; Garin-Bastuji *et al*., 2014).Only weak evidence for a direct relationship between brucellosis and size/density of the population of wild animals has been reported (Conner *et al.*, 2008) .However, a potential risk for brucellosis infection of livestock by wild animals could be associated when artificial management such as winter feeding increases aggregation (Gortázar

et al., 2007; Conner *et al.,* 2008). Thus, wild animals are often at risk as a consequence of contact with infected livestock, particularly in extensive breeding systems. With regards to elk and bison, artificial feeding management during winter results in significant congregations in the feeding grounds and increases the risk of elk being exposed to *B. abortus* (Godfroid, 2002).

2.4.5. Risk factors of Brucella infection

There are so many factors that can affect the pervasiveness of brucellosis in various species of livestock. 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).

Risk factors for human brucellosis include the handling of infected animals, ingestion of contaminated animal products such as unpasteurized milk and milk products (including cow, goat, and camel milk), meat, history of travel to endemic areas and handling of cultures of *Brucella* species in laboratories. Other risk factors include: abattoir workers, veterinarians, slaughterhouse workers and dairy workers (Corbel, 1997). The risk factors can be categorized into those associated with characteristics of host, the parasite biology and management:

2.4.5.1. Risk factors associated with the host

Susceptibility of livestock to *Brucella* infection is influenced by the age (young animals are less susceptible to *Brucella* than older animals). Age has been referred to as one of the intrinsic factors associated with brucellosis. Thus, it may also explain the higher prevalence in adult animals than in young. Brucellosis has traditionally been considered a disease of adult animals since susceptibility increases after sexual maturity and pregnancy (Nicoletti, 1980). However, variations in the age of sexual maturity among breeds could present differences between age and brucellosis positivity (Matope *et al*., 2010). *Brucella* species presented a tropism to the

reproductive tract due to the production of erythritol, a 4-carbon sugar produced in the foetal tissues of ruminants that stimulates the growth of *Brucella (*Petersen *et al*., 2013). On the other hand, a higher prevalence of brucellosis in adults has also been associated with longer contact with infected animals or with the environment.

Sex and reproductive status of individual animal (sexually mature, pregnant animals) are more susceptible to infection with the organism than sexually immature animals (Nicoletti, 1980). Female ruminants presented higher odds of brucellosis infection than male. Since brucellosis infection in males presented clinical signs such as epididymitis and orchitis, the prevalence in males be lower than females because they may be culled faster (Coelho *et al*., 2007). Placental trophoblasts produce erythritol in increasing amount during the later stage of pregnancy which coincides with the period when pregnant cattle are more susceptible to infection with *B.abortus*. The preferential utilization of erythritol rather than glucose is characteristics of pathogenic *Brucella* strains. Erythritol promotes the growth of some strains of *Brucella* and the metabolic pathway for degradation of erythritol has been described previously. However, *Brucella* has also been found in the reproductive tract of animals with no detectable levels of erythritol, the role of this sugar in the virulence of the organism has been put into question (Sangari *et al*., 2000). *B.abortus* Strain 19 is spontaneous attenuated mutant widely used to vaccinate cattle. S19 is the only *B.abortus* strain that is inhibited by erythritol (Sangari *et al*., 2000).

Other host related important risk factor for brucellosis seropositivity is herd size; being higher in large herds an increased odds ratio for seropositivity has been largely reported in cattle (Megersa *et al*., 2011a).The higher number of animals increases the likelihood of transmission of the disease by contact among them (Nicoletti, 1980). Breed and number of species also other risk factors for brucellosis seropositivity as reported by different authors like (Muma *et al*., 2007). Thus, an increase in prevalence where several species intermingle is difficult to explain but could be associated with higher chances of being *Brucella* seropositive because of multiple sources of infection. Dogs are also a potential risk in the diffusion of brucellosis, acting as

mechanical disseminators by feeding on aborted foetuses, dragging them along and spreading the bacteria (Xiang *et al* ., 2013).

B. abortus is the principal cause of brucellosis in cattle. The bacteria are shed from an infected animal at or around the time of calving or abortion. Once exposed, the likelihood of an animal becoming infected is variable, depending on age, pregnancy status, and other intrinsic factors of the animal, as well as the number of bacteria to which the animal was exposed (Radostits *et al*., 2000). The two main causes for spontaneous abortion in animals are erythritol, which can promote infections in the fetus and placenta, and the lack of anti-*Brucella* activity in the amniotic fluid. Males can also harbor the bacteria in their reproductive tracts, namely seminal vesicles, ampullae, testicles, and epididymis.

2.4.5.2. Pathogenic risk factors

Brucella is facultative intracellular organism which is capable of multiplication and survives within host phagocytes. The organisms are phagocytosed by polymorphonuclear leukocytes in which some survives and multiply. These are then transported to lymphoid tissue and foetal placenta. The inability of the leukocytes to effectively kill virulent *Brucella* at the primary site of infection is a key factor in the dissemination to regional lymphnodes, mononuclear phagocytic system, and organs such as the uterus and udder. The ability to survive within macrophages and leukocytes enables the organism to be protected from humoral and cellular bactericidal mechanisms during the periods of heamatogenous spread (Nielsen and Duncan, 1990). Naturally infected animals and those vaccinated as adults with strain 19 remain positive to the serum and other agglutination tests for long periods.

The antibody response to *Brucella* consists of an early IgA and IgM isotype response, the timing of which depends on the route of exposure, the dose of bacteria and the health status of the animal. The IgM response is followed shortly by production of IgG1 antibody and later by IgG2 (Nielsen, 2002). The total concentration of IgG2 increases with age. Most cross reacting antibody, resulting from exposure to microorganism other than *Brucella* species, consist of IgM, making serological tests which measure IgM not specific as false positive results occur,

leading to low assay specifity. In the case of *Brucella* infection, the concentration of anti-*Brucella* total IgG2 increases with the level of antigen exposure, therefore the monitoring of IgG1 and IgG2 *Brucella* antibody levels is relevant for detection of *Brucella*-infected cattle (Saegerman *et al.*, 2004).

2.4.5.3. Management risk factors

The spread of disease from one herd to another and from one area to another is most commonly due to the movement of an infected animal from an infected herd or area into a non-infected herd or area (Radostits *et al*., 2000). Whether a herd raises its own replacement animals or purchases replacement animals affects the potential for introduction into the herd (Walker, 1999; Tolosa, 2004). The unregulated movement of cattle from infected herds or areas to brucellosis-free herds or areas is the major cause of breakdowns in brucellosis eradication programs. Once the herds are infected, the time required to become free of brucellosis is increased by large herd size, by active abortion, and by loose housing (Radostits *et al*., 2000).

2.4.5.4 Other factors associated with brucellosis

It was noted that knowledge ages equal to or older than 55 years was a protective factor for brucellosis prevention *(Coelho et al., 2007; Díez et al., 2013*). This observation is difficult to explain and may be due to younger farmers' lack of experience. Older farmers have more familiarity with recognizing the clinical signs of the disease or the main route of transmission and can be more aware of the importance of preventive measures (Memish, 2001; Godfroid, 2002). Farmers who had previously experienced brucellosis in their herds had a higher probability of having greater knowledge of bovine brucellosis, which is consistent with having experience with the disease. Producer's associations, education and veterinary support have been recognized as protective factors (Blasco, 1997; Díez *et al.*, 2013). Farmer's lack of awareness about brucellosis, improper handling of aborted materials and the habit of consuming raw milk, among other factors, might contribute to further spread of brucellosis in their livestock and expose the community to a public health hazard (Megersa *et al.*, 2011a).

2.4.6. Mode of transmission

Animal brucellosis can be transmitted by both vertical and horizontal transmission. Horizontal transmission occurs through ingestion of contaminated feed, skin penetration, via conjunctiva, inhalation and udder contamination during milking. Congenital infection during parturition is frequently cleared and only few animals remained infected as adult (Radostits *et al.*, 2007). In cattle and other Bovidae, *Brucella* is usually transmitted from animal to animal by contact following an abortion. Pasture or animal barn may be contaminated and the organisms are probably most frequently acquired by ingestion and inhalation, conjunctival inoculation, skin contamination and udder inoculation from infected milking cups are other possibilities. The use of pooled colostrums for feeding newborn calves may also transmit infection. Sexual transmission usually plays little role in the epidemiology of bovine brucellosis. Bulls that remain fertile and functionally active after infection with *brucella* shed the organisms with the semen during the acute phase of the disease. During the later stage shedding may cease or become intermittent (Radostits, *et al.,* 2007).

However, artificial insemination can transmit the disease and semen must only be collected from animals known to be free of infection (Robinson *et al*., 2003).Transmission of *B. abortus* mostly occurs via the oral route because cattle tend to lick aborted fetuses and the genital discharge of an aborting cow. *Brucella* organisms can also be transmitted through the transmammary route while calves born to healthy dams are infected when fed on colostrum or milk from infected dams (*Radostits et al*., 2007).

Wildlife, birds and waterways contaminated with urine, uterine discharge, or slurry from aborting cattle) are vectors for indirect exposure to brucella organisms. Dogs carrying pieces of placenta or aborted fetuses from one place to another can disseminate the infection (Forbes, 1990). Contamination of a cowshed or pasture takes place when infected cattle abort or have full-term parturition. Although it is generally accepted that *B. abortus* is not excreted for considerable time before abortion occurs, excretion in the vaginal discharge of infected cattle may occur as early as 39 days after exposure.

A massive excretion of *Brucella* starts after abortion and may continue for 15 days. Infection due to *B. abortus* occurs less frequently in goats and may result in low prevalence (WHO, 2006) although abortion due to *B. abortus* has been documented under an experimental condition (Al-Majali *et al*., 2009).After the fetal membranes are expelled the uterine discharge diminishes and the number of *Brucella* organisms excreted decreases rapidly. The infectious material usually clears after 2-3 months from the genital tract, although some infected cattle remain carriers of *Brucella* and excrete it intermittently for many years (Nicoletti, 1989; Radiostatis, 2007). Humans acquire brucellosis mainly through ingestion of contaminated milk and unpasteurized dairy products. Contact of mucosa and skin abrasions with fluids and tissues from aborted fetuses of infected animals are also important sources of *Brucella* transmission (Hartigan, 1997; Fugier *et al*., 2007) as illustrated by (Figure 3). Survival of the organisms in the environment may also play a role in epidemiology of the disease (Abuo -Eisha, 2000).

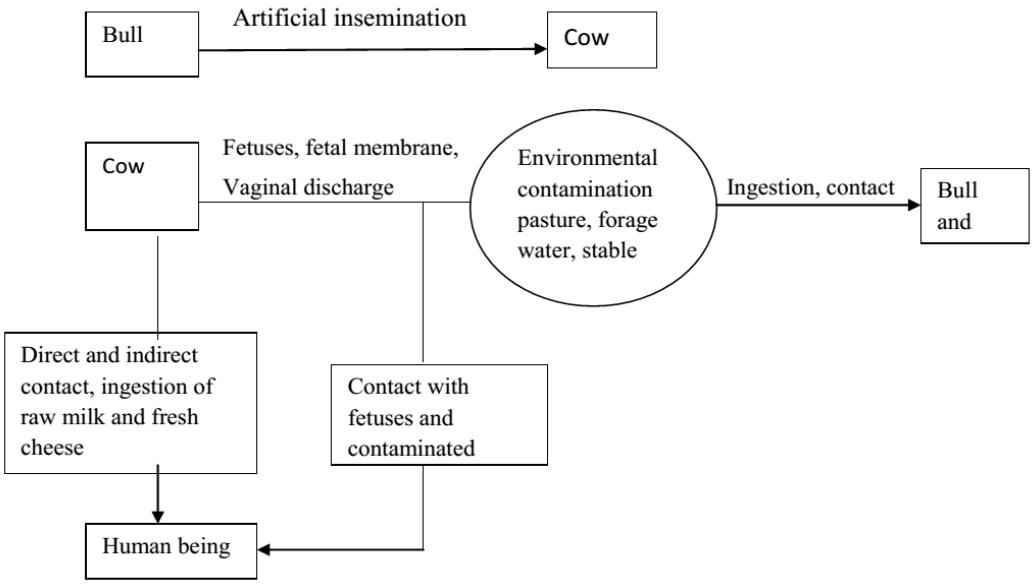

Figure 3. Mode of Transmission of Bovine Brucellosis

Source: Acha and Szyfres (2001)

2.5. Pathogenesis and Pathology

The ability of *Brucella* species to cause disease requires a few critical steps during infection. *Brucella* specie can invade epithelial cells of the host, allowing infection through mucosal surfaces: M cells in the intestine have been identified as a portal of entry for *Brucella* species (Kohler *et al*., 2002).Once *Brucella* species has invaded, usually through the digestive or respiratory tract, they are capable of surviving intracellularly within phagocytic or non-phagocytic host cells (Pizarro-Cerdá *et al*., 2000). *Brucella* has the ability to interfere with intracellular trafficking, preventing fusion of the *Brucella*-containing vacuole (BCV) with lysosome markers, and directing the vacuole towards a compartment that has rough endoplasmic reticulum (RER), which is highly permissive to intracellular replication of *Brucella* (Pizarro-Cerdá *et al*., 2000).

The pathogenesity of *Brucella* is due to its ability to adapt to the environmental conditions encountered in its intracellular replicative niche including low levels of nutrients and oxygen, acidic pH and reactive oxygen intermediates (Kohler *et al*., 2002). It seems that the initiation of *Brucella* infection depends on exposure dose, virulence of the organism and natural resistance of the animal to *Brucella* (Radostits *et al*., 2007). Resistance to infection is based on the host's ability to prevent the establishment of a mucosal infection by the destruction of the invading organism. Invading *Brucella* usually localize in the lymph nodes, draining the invasion site, resulting in hyperplasia of lymphoid and reticulo-endothelial tissue and the infiltration of inflammatory cells. Survival of the first-line of defense by the bacteria results in local infection and the escape of *Brucella* from the lymph nodes into the blood. Smooth *Brucella* inhibit host cell apoptosis, favoring bacterial intracellular survival by escaping host immune surveillance, while rough *Brucella* mutants (*Brucella canis* and *Brucella ovis* are two exceptions) induce necrosis in macrophage (Pei *et al*., 2006).

However, the mechanisms and virulence factors that mediate macrophage cell death have not been identified. In contrast to other pathogenic bacteria, no classical virulence factors, such as exotoxins, cytolysins, capsules, fimbria, plasmids, lysogenic phages, drug resistant forms,

antigenic variation, endotoxic, lipopolysaccaride (LPS) have been described in *Brucella* (Moreno and Moriyon, 2002). *Brucella* uses a number of mechanisms for avoiding or suppressing bactericidal responses inside macrophages. 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* (Lapaque *et al*., 2005).

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).

Research suggests that the smooth, non-endotoxic lipopolysaccharides help block the development of innate and specific immunity during the early stage of infection, and protect the pathogen from the microbicidal activities of the immune system (Porte *et al*., 2003). *Brucella melitensis* LPS does not stimulate production of tumor necrosis factor-α or nitric oxide (Tumurkhuu *et al.,* 2006). *Brucella* LPS plays a role in protecting against bactericidal cationic peptides (defensin NP-2, lactoferrin, cecropines, lysozyme, bactenecin-derived peptides, and the defensin-like antibiotic polymyxin B, and the crude lysosomal extracts from polymorphonuclear leukocytes) (Lapaque *et al*., 2005).

Smooth *Brucella suis* interact with lipid-rafts through an unknown receptor on the surface of macrophages and enter cells via a pathway allowing it to avoid fusion with lysosomes (Lapaque *et al*., 2005). In contrast, the rough strain (lacking O-side chain) seems not to enter by lipid-rafts, and fuses rapidly with lysosomes. Although naturally rough strains of *Brucella* (*B. ovis and B. canis*) lack O-side chain in their LPS, they are pathogenic for rams and dogs,

respectively, and induce long-lasting infections with high levels of splenic colonization in laboratory animals (Caro-Hernandezet *et al*., 2007).

Brucellae display strong tissue tropism and replicate within vacuoles of macrophages, dendritic cells (DCs), and placental trophoblasts. However, the pathogen has the ability to replicate in a wide variety of mammalian cell types, including microglia, fibroblasts, epithelial cells, and endothelial cells. The intracellular lifestyle of *Brucella* limits exposure to the host innate and adaptive immune responses, (Martirosyan and Gorvel, 2013) sequesters the organism from the effects of some antibiotics, and drives the unique features of pathology in infected hosts, which is typically divided into three distinct phases: the incubation phase before clinical symptoms are evident, the acute phase during which time the pathogen invades and disseminates in host tissue, and the chronic phase that can eventually result in severe organ damage and death of the host organism. Nonspecific influenza-like symptoms observed in humans include pyrexia, diaphoresis, fatigue, anorexia, myalgia, and arthralgia. Furthermore, increasing evidence from endemic regions suggests that an elevated risk of human abortion is associated with exposure. Chronic infection results from the ability of the organism to persist in the cells of the host in which *Brucellae* are distributed by way of the lymphoreticular system to eventually cause cardiovascular, hepatic, lymphoreticular, neurologic, and osteoarticular disease (Baud and Greub, 2011).

The tropism of *Brucella* to the male or female reproductive tract was thought to be by erythritol, which stimulates the growth of the organism, but *Brucella* has also been found in the reproductive tract of animals with no detectable levels of erythritol. In the acute stage of infection, abortion occurs at four or five months into pregnancy and cattle usually abort only once. Excretion of *Brucella* after parturition may persist for months or years and may re-occur after any consecutive normal parturition. Infected cattle excrete *Brucella* in the colostrum or milk although it cannot always be detected (Quinn *et al.,* 2002).

Abortion and expulsion of the fetus was thought to be the results of placentitis caused by *Brucella*. Proliferation of *brucella* in the uterus induces necrosis and destruction of the fetal

and maternal placental membranes resulting in death and then expulsion of the fetus. The pathologic changes in the caruncules and cotyledons prevent normal separation and expulsion of the placenta. Although placentitis impairs the normal function of the placenta, *Brucella* endotoxins may also play a role in inducing abortion (Anderson *et al*., 1986). *Brucella abortus* may induce production of high concentration of cortisol that decreases progesterone production and increases estrogen production. Decreases in progesterone level and increases in estrogen levels induce a premature parturition (Enright *et al.,* 1984).

2.6. Intracellular Survival of *Brucell*a and Immune Responses

Intracellular survival of *Brucella*: *Brucellae* are facultative intracellular bacteria and can become secluded within the endoplasmic reticulum of cells and thereby avoid lysosome fusion. By controlling the maturation of the brucellosome (*Brucella*-containing vacuole) at the onset of infection, unopsonized *Brucella* can enter, survive and replicate in a variety of cells, including dendritic cells and macrophages (Pappas *et al*., 2005) to evade the host innate immune response before activation of anti- *Brucella* mechanisms by adaptive immunity. To restrict long-term protective immunity, the organism first avoids the innate immune response by stealthy entry into host cells. From there, the organism controls aspects of protein secretion, intracellular trafficking, and bacterial replication, (De Jong *et al.,* 2013), ultimately altering the course of the innate and adaptive immune responses (Xavier *et al.,* 2013).

The two-component BvrR/BvrS gene sensing system that also acts through a cascade of protein phosphorylation to modulate bacterial gene expression is thought to be one of the key factors involved in the modulation of cell binding and penetration. In *Brucella,* VirB is thought to be essential for intracellular survival; however, the transported effectors substrate in *Brucella* has not yet been identified and it is very unlikely that the transported molecule is a classic virulence factor. In these brucellosome, *Brucella* organisms are able to produce virulence genes (*VirB*) which promote multiplication of the organisms in such environments (Kohler *et al.,* 2003).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).

The absence of the BvrR/BvrS sensory-regulatory system results in major changes in the bacterial outer membrane that alters cellular uptake of the organism. The BvrR/BvrS regulators also include carbon and nitrogen metabolic functions and the expression of additional transcriptional regulators among 127 differentially regulated genes. The absence of VirB alone may explain the attenuated virulence of bvrR/bvrS mutants. In the absence of a functioning BvrR/BvrS, the organism fails to replicate intracellularly and is avirulent in the mouse model (Viadas *et al*., 2010).

Experimental results suggest that the early phagosome is very acidic and poor in nutrients, resulting in induction of VirB and genes encoding stress proteins. VirB participates in the creation of the 'brucellosome' characterized by: (1) absence of fusion with lysosomes, (2) neutral pH, and absence of certain nutrient components (Figure 4).

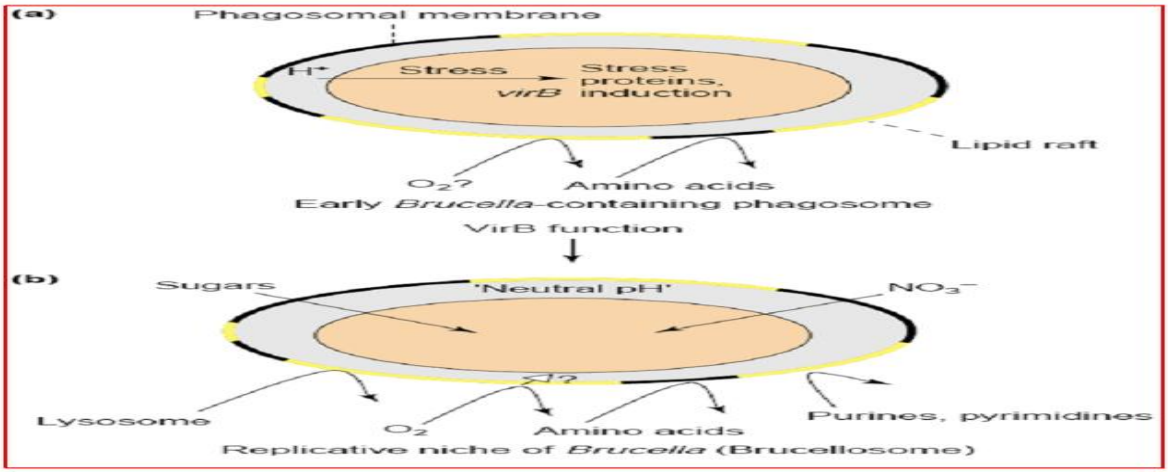

Figure 4. Characteristic properties of (a) an early vacuole and (b) the replicative niche of *Brucella* species.

Source**:** Kohler *et al. (*2003).

Immune responses: Naturally infected and vaccinated animals can be serological reactors. After infection, the level of immunoglobulin IgM, IgG and IgA will significantly increase in serum (Radostits *et al.*, 2007). IgM antibodies, which appear initially after infection and low levels of IgG, will cause complement-mediated lysis of *Brucella.* Secretory IgA is tending to be abundant in milk, where as IgG is high in serum (Walker, 1999). The O-chain of the smooth lipo-polysaccharide complex of the bacterial cell envelope together with the outer proteins is potent immunogens. On the other hand, the immunogenicity of the non-smooth cell variant is relatively low (Corbel *et al*., 1980). The O-chain specific antibodies play a major role in protective immunity, but do not eliminate the bacteria as they are protected by their intracellular niche (WHO, 1997). This highlights the lack of correlation between protection and high antibody level (Walker, 1999).Interestingly, cellular and humoral immune responses against similar *Brucella* strains vary significantly among susceptible hosts. This confounding aspect of *Brucella* immunobiology has presented important challenges in the identification of reliable correlates of immune protection in tractable model animal systems.

Cytokines are key players in protection against brucellosis, mediating both innate and adaptive immune responses. IL-12 produced by B cells and macrophages leads to a Th1 response and induction of interferon- gamma, which activates macrophages. The activity of interferon gamma is maximized by tumor necrosis factor- alpha produced by macrophages and natural killer cells. Reports also indicate that IL-1 dependent induction of colony-stimulating factor increases neutrophil and macrophage infiltration into the spleen (Doyle *et al.,* 1992).

2.7. Clinical Manifestations

The most common clinical signs of cattle infected with Brucellosis are high incidences of abortions, arthritic joints, and retained placenta (Radostits *et al*., 2000) .The incubation period varies between 14 and 120 days (Seifert, 1996; Tolosa, 2004). Primary clinical manifestations of brucellosis are related to the reproductive tract. In highly susceptible non-vaccinated pregnant cattle, abortion after the 5^{th} month of pregnancy is cardinal feature of the disease

(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, reduced milk yield and metritis (Walker, 1999; Acha and Szyfres, 2003; Shareef, 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). Orchitis and epididymitis are typical signs in males, and hygroma (Figure 5) is usually common during chronic infection.

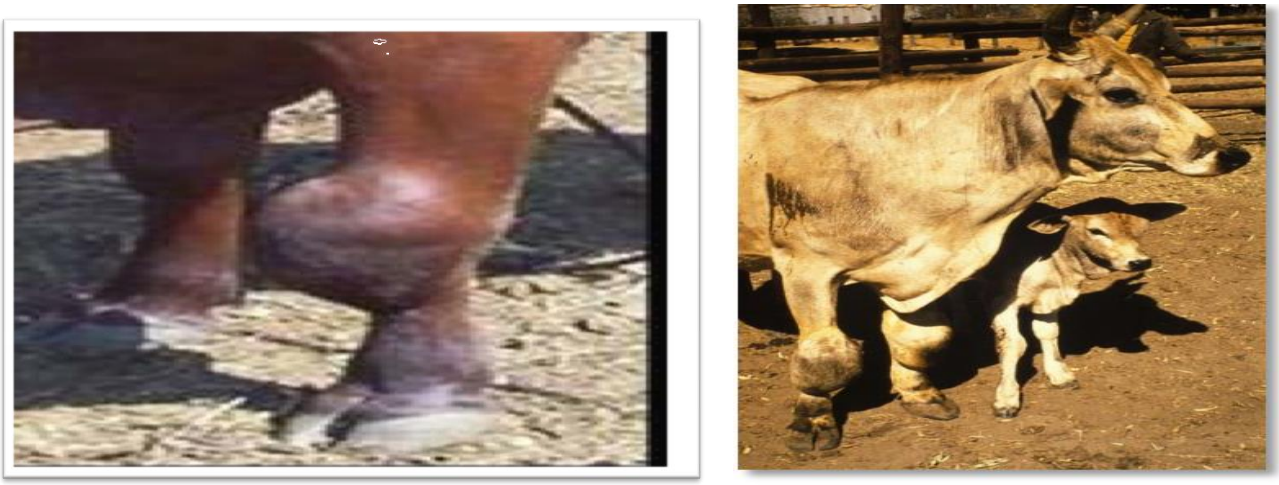

Figure 5. Unilateral and bilateral *Brucella abortus*-induced hygroma in cattle
Source**:** Matope *et al*. (2010)

2.8. Diagnostic Methods

Brucellosis diagnostics is based on bacteriological and molecular methods (direct tests), and serological in vitro and allergic in vivo methods (indirect tests) (Poester *et al*., 2010; Smirnova *et al., 2013*). Since, brucellosis signs are non-pathognomonic in livestock, and definitive diagnosis depends on laboratory testing. 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). *Brucella* species are highly monomorphic, with minimal genetic variation among species (Tiller *et al*., 2009) and maintain a close taxonomic relationship and can only be distinguished by rigorous metabolic, immunologic, and biochemical analyses. The similarities among the *Brucella* species extend to the genetic level at which all species share greater than 90 % DNA homology (Hoyer and McCullough, 1968 (a). Species of *Brucella* were differentiated in the laboratory by colonial morphology, growth requirement, various biochemical tests and lysis by bacteriophage (Christina, 1998). The identification of one or more infected animals is sufficient evidence that infection is present in the herd, and that other serologically negative animals may be incubating the disease and present a risk. Diagnostic tests fall into two categories: those that demonstrate the presence of the organisms and those that detect an immune response to its antigens (Corbel, 2006).

2.8.1. Direct methods for diagnosis of brucellosis

2.8.1.1. Bacteriological methods

The "gold standard" of the brucellosis diagnosis is the direct bacteriological testing: cultivation of *Brucella*, isolated from body fluids (blood, cerebrospinal fluid, urine and others) or tissues (Yagupsky, 1999; Smirnova *et al.,* 2013).The isolation and identification of *Brucella* offers a definitive diagnosis of brucellosis and may be useful for epidemiological purposes and to monitor the progress of a vaccination programme. It should be noted that all infected materials present a serious hazard, and they must be handled with adequate precautions during collection, transport and processing (Corbel, 2006). Isolation of the organism is considered the

gold standard diagnostic method for brucellosis since it is specific and allows biotyping of the isolate, which is relevant under an epidemiological point of view (Bricker, 2002a; Al Dahouk *et al*., 2003). However, in spite of its high specificity, culture of *Brucella* species is challenging. *Brucella* species 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 are not available in most developing countries (Hadush and Pal, 2013). However, the isolation and cultivation of bacteria are also necessary preliminary steps for staining and biotyping of *Brucella* species.

Staining: Stamp staining is still often used, even though this technique is not specific: other abortive agents such as *Chlamydophila abortus* (formerly *Chlamydia psittaci)* or *Coxiella burnetii* are also stained red *(*Corbel, 2006). It provides valuable information for the analysis of aborted material (Alton *et al*., 1988). They are not truly acid fast; however, they are resistant to decolonization by weak acids, and stain red against a blue background with the Stamp's modification of the Ziehl-Neelsen method. *Brucella* species is a coccobacillus measuring 0.6-1.5 µm long and 0.5-0.7 µm wide. They generally occur singly and are observed in clusters of two or more. Smears of placental cotyledon, vaginal discharge or fetal stomach contents may be stained using modified Ziehl-Neelsen (Stamp) or Kosters' methods. The presence of large aggregates of intracellular, weakly acid-fast organisms with *Brucella* morphology is presumptive evidence of brucellosis.

Culture: Bacterial isolation is always required for the biotyping of strains. For the definitive diagnosis of brucellosis, the choice of samples depends on the clinical signs observed. In the case of clinical brucellosis, valid samples include aborted fetuses (stomach, spleen, and lung), fetal membranes, vaginal secretions, colostrum, milk, sperm, and fluid collected from arthritis or hygroma. At slaughter, in order to confirm suspected cases of acute or chronic brucellosis, the preferred tissues are the genital and oropharyngeal lymph nodes, the spleen, and the mammary gland and associated lymph nodes. For the isolation of *Brucella* species, the most commonly used medium is the Farrell medium, which contains antibiotics able to inhibit the

growth of other bacteria present in clinical samples. Some *Brucella* species, like *B. abortus* wild type (biovars 1-4), need CO 2 for growth, while others, like *B. abortus* wild type (biovars 5, 6, 9), *B. abortus* S19 vaccine strain, *B. melitensis*, and *B. suis*, do not (Poster *et al*., 2010). For liquid samples (milk or blood), sensitivity is increased by the use of a biphasic medium like the Castaneda medium, originally described for use with human blood cultures. Growth may appear after 2-3 days, but cultures are usually considered negative after 2-3 weeks of incubation (Alton *et al*., 1988). The identification of *Brucella* species is based on morphology, staining and metabolic profile (catalase, oxidase, and urease) (Alton *et al*., 1988; Corbel *et al*., 2005).

Brucella species colonies are elevated, transparent, and convex, with intact borders, smooth, and a brilliant surface. The colonies have a honey color 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. Usually, solid media such as dextrose agar, tryptose agar, and trypticase soy agar, 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 Castañeda's medium is recommended for improving sensitivity (Poester *et al*., 2010).

2.8.1.2. Immunohistochemistry

Immunohistochemistry is an alternative technique for direct diagnosis of *Brucella* species infection. It has been extensively used in studies of pathogenesis and diagnosis of brucellosis, allowing *in situ* localization of the organisms within *Brucella* induced lesions (Xavier *et al*., 2009b). An advantage of this technique is that it does not require viable bacteria and allow retrospective studies. Although immunohistochemistry is simple; several factors may affect the result, including the fixation protocol and selection of the primary antibody (Santos *et al*., 1998; Geresu and Kassa, 20016).

2.8.1.3. Molecular methods

In order to avoid difficulties of bacteriological testing the molecular biological techniques, often based on the polymerase chain reaction (PCR) amplification, are successfully used for *Brucella* identification and typing (Yu and Nielsen, 2010; Smirnova *et al., 2013*). 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* species, allowing differentiation between virulent and vaccine strains (Le Flèche *et al*., 2006; López-Goñi *et al*., 2008). Initially, PCR based identification has been developed for the determination of bacterial isolates (Ouahrani-Bettach et *al*., 1996) but now these methods are also used for detection of *Brucella* species in clinical samples of human and animals without previous isolation of the organism(Baddour and Alkhalifa, 2008; Smirnova *et al., 2013*). In addition, these techniques can be used to complement results obtained from phenotypic tests (Bricker *et al*., 2002b).

Since the routine identification and differentiation of brucellosis suspected specimens, based on culture isolation and phenotypic characterization, requires Biosafety level-3 (BSL3) protocols for the high risk of laboratory-acquired infections (Boschiroli *et al*., 2001), molecular methods have been explored in order to overcome these difficulties. Furthermore, the polymerase chain reaction (PCR)-based assays have shown a higher sensitivity with respect to the standard microbiological assay for the diagnosis of brucellosis (Hoover and Freidlander, 1997).The PCR, including the real-time format, provides an additional means of detection and identification of *Brucella* species (Bricker, 2002). Despite the high degree of DNA homology within the genus *Brucella,* several molecular methods, including PCR, PCR restriction fragment length polymorphism (RFLP), Single nucleotide polymorphisms (SNPs), multiple locus variable number of tandem repeats (VNTR) analysis (MLVA) and Southern blot, have been developed that allow, to a certain extent, differentiation between Brucella species and some of their biovars (Moreno *et al.,* 2002). Pulse-field gel electrophoresis has been developed that allows the differentiation of several *Brucella* species.*Brucella* biotyping and distinguishing

vaccine strains by PCR can be accomplished satisfactorily but there has been limited validation of the PCR for primary diagnosis.

PCR DNA-based methods such as gene probes and PCR utilize primers derived from different polymorphic regions in the genomes of *Brucella* species. Different PCR methods for the detection of *Brucella* species that utilize primers derived from different polymorphic regions in the genomes of *Brucella* species as i.e. (1) a gene encoding a 31 - kDa *B. abortus* antigen which is conserved in all *Brucella* species (primers B4/B5) (Baily *et al*., 1992), (2) a sequence +16S rRNA of *B. abortus* (primers F4/R2) (Romero *et al.,* 1995), (3). a gene encoding an outer membrane protein of 26-kDa (omp-2) (primers JPF/JPR and primers P1/P2) (Bardenstein *et al.,* 2002), (4) outer membrane proteins (omp 2b, omp2a and omp31) (Imoaka *et al*., 2007), (5) proteins of the omp25/omp31 family of *Brucella* spp. (Vizcaino *et al.,* 2004), the entire bp26 gene of *B. melitensis* 16M, encoding the BP26 protein (omp 28) (primers 26A/26B) (Cloeckaert *et al.,* 2000b) were described.

Real-time PCR assays have been recently described in order to test *Brucella* cells (Redkar *et al*., 2001), urine (Queipo-Ortuno *et al.,* 2005), blood, paraffin-embedded tissues (Kattar *et al.,* 2007), serum, and other tissues (Smirnova *et al.,* 2013). It is more rapid and more sensitive than conventional PCR. It does not require post amplification handling of PCR products, thereby reducing the risk of laboratory contamination and false-positive results. Three separate real-time PCRs were developed to specifically identify seven biovars of *B. abortus*, three biovars of *B. melitensis* and biovar one of *B. suis* using fluorescence resonance energy transfer. The upstream primers used in these real-time PCRs derived from the insertion element, IS711 whereas the reverse primer and FRET probes are selected from unique species or biovar-specific chromosomal loci. Sensitivity of *B. abortus*-specific assay was as low as 0.25 pg DNA corresponding to 16-25 genome copies and similar detection levels were also observed *for B. melitensis* and *B. suis*-specific assays (Redkar *et al.,* 2001).

There still remains the problem of false negative results, because the PCR is inhibited in the presence of some admixtures, such as EDTA, RNAases, DNAases, gems, heparin,

phenols,urea, and many others, from the clinical samples or DNA isolation and purification procedures. False positive results may also occur as a result of sample contamination. It is further necessary to develop the positive and negative controls and standardize the conditions for PCR reactions with clinical samples (Yu and Nielsen, 2010; Smirnova *et al*., 2013). The advantages of PCR are numerous. Independent of the disease stage, it is more sensitive than blood cultures and more specific than serological tests (Wei and Klaus, 2010). Real time PCR using the IS711 -based insertion element assay has been shown to be the most sensitive, specific, efficient, and reproducible method to detect *Brucella* species (Wei and Klaus, 2010).

Multiplex PCR typing is more effective method of diagnosis and identification of *Brucella*. Several multiplex PCRs which identify the genus *Brucella* at the species level and partly at the biovar level using different primer combinations have been reported. It provides identification of all known *Brucella* species, including pathogens of marine mammals, at the species or even biovars level by using certain combinations of primer pairs. The first multiplex PCR based test for *Brucella* detection was developed in 1994 (Bricker and Halling, 1994; Smirnova *et al*., 2013), it is also called AMOS PCR assay. It allowed identification of the four *Brucella* species (*Brucella abortus, Brucella melitensis, Brucella ovis* and *Brucella suis*) and was named AMOS PCR (AMOS is an acronym from ‘‘abortus-melitensis-ovis-suis’’) for the first letters of species names. It comprised five oligonucleotide primers for the identification of selected biovars of four species of *Brucella*.The assay exploited the polymorphism arising from species-specific localization of the genetic element IS711 in the *Brucella* chromosome.

2.8.2. Indirect methods for diagnosis of brucellosis

2.8.2.1. Classical serological methods

The indirect methods of brucellosis diagnostics are based on the detection of the immune response to a bacterial infection. Most of these methods have been initially developed for testing of cattle and then were used to test the domestic goats and sheep (except for the analysis of milk), and later were adapted for the monitoring of certain species of wild animals (Godfroid *et al*., 2010; Smirnova *et al.,* 2013). Serology is the mainstay of diagnosis for

brucellosis because the diagnostic material is relatively easily accessible, and the tests are relatively cheap, available and sensitive. Therefore, 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. lipopolysaccaride or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Antibodies against smooth *Brucella* species (e.g. *B. abortus*, *B. melitensis*, and *B. suis*) cross react with antigen preparations from *B. abortus*, whereas antibodies against rough *Brucella* species (e.g. *B. ovis* and *B. canis*) cross react with antigen preparations from *B. ovis* (Nielsen, 2002).

Although several serological methods are currently available, these tests can be classified as screening tests (e.g. buffered antigen plate agglutination - BPAT), monitoring or epidemiological surveillance tests (e.g. milk ring test), and complementary or confirmatory tests (e.g. 2-mercaptoethanol, complement fixation, ELISAs, and fluorescence polarization assay). Selection of a given test should take into account the species affected as well as local regulations (Poester *et al*., 2010). No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals (Poester *et al*., 2010). Consideration should be given to all factors that impact on the relevance of the test method and test results to a specific diagnostic interpretation or application.

In epidemiological units where vaccination with smooth *Brucella* is practised, false-positive reactions may be expected among the vaccinated animals because of antibodies cross-reacting with wild strain infection. The presence of anti-*Brucella* antibodies suggests exposure to *Brucella* species, but it does not indicate which *Brucella* species induced production of those antibodies. Moreover, seropositivity does not necessarily mean that the animals have current or active infection at the time of sampling. In fact, studies of experimental and natural infections indicate that nearly all animal species vulnerable to *Brucella* infection can lose their antibody titers (Godfroid *et al*., 2010).

Buffred Brucella antigen tests (BBAT)

The Rose Bengal (RB) and buffred antigen plate agglutination (BPAT) tests are the well-known buffered *Brucella* antigen tests. The RBT/RBPT is one of a group of tests known as the buffered *Brucella* antigen tests which rely on the principle that the ability of IgM antibodies to bind to antigen is markedly reduced at a low pH. The RBT is a simple spot agglutination test where drops of stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction. The test is an excellent screening test but may be oversensitive for diagnosis in individual animals, particularly vaccinated ones (Corbel, 2006). 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 RBPT 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).

RPBT is an agglutination test that is based on reactivity of antibodies against smooth lipopolysaccaride (LPS). As sensitivity is high, false negative results are rarely encountered. One of the approaches to remove none specific reactivity is the precipitation of high molecular weight serum glycoproteins. It is commonly done by addition of rivanol to serum followed by removal of the precipitate by centrifugation and either a rapid plate type agglutination test with undiluted serum or a tube test using serum dilutions starting at 1:25 Poester *et al*., 2010; 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).

Enzyme linked immune sorbent assays test (ELISA)

ELISAs are divided into two categories, the indirect ELISA (iELISA) and the competitive ELISA (c-ELISAs) (Saegerman *et al.,* 2004). 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. Competitive ELISA (c-ELISA) and Indirect ELISA (iELISA) tests can be used as supplementary tests to CFT (McGiven *et al.,* 2013). 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). 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).

The indirect ELISA (i-ELISA)

The method is based on the specific binding of antibodies present in the test sample with immobilized antigen. The binding event is visualized using chemically or enzymatically derived fluorescent, luminescent or colorimetric reaction. Many iELISA tests are available on the market (Poester *et al*., 2010). It has been used for serologic diagnosis of brucellosis in sheep, goats and pigs. It has also been used for diagnosis using serum or milk from cattle (Gall *et al*., 2003; Di Febo *et al*., 2012). i-ELISA has been usually used for smooth LPS *Brucella* species, and it is sensitive and specific for *B. abortus* or *B. melitensis*, but it is not capable of differentiating antibodies induced by the vaccine strains S19 or Rev1 (Eoh *et al*., 2010; Lim *et al*., 2012). Sensitivity of i-ELISA varies from 96 to 100%, and its specificity from 93.8% and 100% (Gall *et al*., 2001; Gall and Nielsen, 2004).

The competitive ELISA (c-ELISA)

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). It can also be used both for screening and confirmatory tests (FAO, 2003). Antibodies against smooth LPS are used in all the above mentioned tests. They have a common significant disadvantage: O-polysaccharides of *Brucella* are similar to that of *Yersinia enterocolitica* and other bacteria. It leads to the false positive results and thus reduces the specificity of the test (Nielsen *et al.*, 2004). Partly this problem is solved in the competitive ELISA (cELISA), where the specific epitopes of *Brucella* O-polysaccharides are used as antigens, but the sensitivity of c-ELISA is significantly lower than the iELISA (Nielsen *et al*., 1995). By selecting a monoclonal antibody with slightly higher affinity for the antigen than most of the vaccinal/cross-reacting antibody but with lower affinity than most antibodies arising from infection, reactivity by vaccinal antibody could be eliminated in the majority of cases.

Fluorescence polarization assay (FPA)

The FPA is based on the fact that, when polarized light excites fluorescent molecules, they will emit polarized light. In solution, the polarization of the emitted light is inversely proportional to the molecule's rotational speed, which is influenced by the solution's viscosity, absolute temperature, molecular volume and the gas constant (Poiester *et al.,* 2010). In brucellosis serology; small molecular weight subunit of OPS is labelled with fluorescein isothiocyanate and used as the antigen. When testing serum, blood or milk, if antibody to the OPS is present, the rate of rotation of the labelled antigen will be reduced at a rate which is proportional to the amount of antibody present (Muma *et al.,* 2007). The FPA is very accurate, and the sensitivity/specificity can be manipulated by altering the cutoff value between positive and negative reactions to provide a highly sensitive screening test as well as a highly specific

confirmatory test. The FPA can distinguish vaccinal antibody in most vaccinated animals, and it can as well eliminate reactivity by some cross-reacting antibodies (Yu and Nielsen, 2010).

Complement fixation test (CFT)

This test is regarded throughout the world as being the confirmatory test for the serological detection of infected animals. The sensitivity and specificity of the CFT is good, but it is a complex method to perform requiring good laboratory facilities and trained staff. If these are available and the test is carried out regularly with good attention to quality assurance, then it can be very satisfactory. It is essential to titrate each serum sample because of the occurrence of the prozone phenomenon whereby low dilutions of some sera from infected animals do not fix complement. This is due to the presence of high levels of non-complement fixing antibody isotypes competing for binding to the antigen. At higher dilutions these are diluted out and complement is fixed. Such positive samples will be missed if they are only screened at a single dilution. In other cases, contaminating bacteria or other factors in serum samples fix or destroy complement causing a positive reaction in the test, even in the absence of antigen. Such "anti-complementary" reactions make the test void and a CFT result cannot be obtained (Corbel, 2006).

Due to its high accuracy, complement fixation is used as confirmatory test for *B. abortus*, *B. melitensis*, and *B. suis* infections (Chin *et al.*, 1991), and it is the reference test recommended by the OIE for international transit of animals (OIE, 2009a, b). The (CFT) using a hot saline extracted antigen preparation has been used for the diagnosis of *B. ovis* infection in sheep (Searson, 1982). The CFT detects mainly the IgG1isotype antibody, as the IgM isotypes are partially destroyed during the inactivation process. Since antibodies of the IgG1 type usually appear after antibodies of the IgM type, control and surveillance of this disease is best done with CFT (WHO/MZCP, 1998).

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). It is thus so used as a confirmatory test (FAO, 2003). The test distinguishes reaction caused by

other factors like other bacterial infections: *Escherichia coli* O157, *Yersinia enterocolitica O:9, Vibrio cholerae, Pseudomonas mallophilia and Salmonella* serotypes which share common chain of lipopolysaccharide (LPS) antigen with smooth *Brucella strains* and therefore cross react. 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).

2-Mercaptoethanol (2-ME)

The 2-mercaptoethanol is a confirmatory test that allows selective quantification of IgG anti-*Brucella* due to inactivation of IgM in the test sample. Production of IgG is usually associated with chronic infection, and therefore, a positive result with this test is a strong indicator of brucellosis. Dithiothreitol is preferable because of the toxicity of 2-mercaptoethanol. The test measures mainly IgG, because the disulphide bridges of IgM are broken, reducing it to monometric molecules, and therefore, unable to agglutinate. However, IgG can also be reduced in the process, giving false negative results; though in general, reduction of IgM increases specificity (Poiester *et al.,* 2010).

The test does not eliminate vaccine generated antibodies, therefore is not recommended for international trade. However, this test has some drawbacks including the toxicity of mercaptoethanol, which requires a fume hood for its manipulation, and the possibility of IgG degradation caused by the 2-mercaptoethanol, which may result in false negative results (Poester *et al*., 2010). The agglutinating ability of IgM and IgA is destroyed by 2-ME; therefore agglutination in this test is indicative of presence of IgG and likelihood of persisting infection. Sensitivity of the 2-mercaptoethanol test varies from 88.4 and 99.6%, and its specificity from 91.5 and 99.8% (Nielsen *et al*., 2004).

Milk ring test (MRT)

The agglutination test has been adapted to test milk for antibody to *Brucella* species. The format of the milk ring test (MRT) is a little different in that haematoxylin stained *Brucella*

cells are mixed with whole milk or whole milk with cream added (Sutra *et al*., 1986). This test allows screening of large number of cattle by using milk samples from tanks or pools from several cows. Immunoglobulins present in the milk will in part be attached to fat globules via the Fc portion of the molecule. If antibody to *Brucella* species is present, antigen will attach to it, resulting in a purple band in the cream layer. If no antibody is present, the fat layer will remain a buff colour and the purple antigen will be evenly distributed throughout the milk. This test is useful for monitoring cattle herds or areas free of brucellosis so it is classified as surveillance or monitoring test (OIE, 2009a).

Importantly, the number of false positive results is proportional to the number of cows secreting acidic milk due to colostrums or mastitis. The immunoglobulins detected by MRT are IgM and IgA. A positive result indicates the presence of infected cattle in the herd so the test should be followed by individual serological test in the entire herd (OIE, 2009a).When a positive test result is obtained, all cows contributing milk should be blood tested. The milk I-ELISA is a sensitive and specific test, and is particularly valuable for testing large herds. The milk ring test (MRT) is a suitable alternative if the ELISA is not available. Still, in spite of its problems, it may be used as an inexpensive screening test in conjunction with other tests.

2.8.2.2. Brucellin allergic skin test

The skin test is an allergic test that detects the specific cellular immune response induced by *Brucella* species infection. The injection of brucellergene, a protein extract of a rough strain of *Brucella* species, is followed by a local inflammatory response in a sensitized animal. This delayed type hypersensitivity reaction is measured by the increase in skin thickness at the site of inoculation. A strong positive reaction is easily recognised by local swelling and indurations. However, borderline reactions require careful interpretation. Skin thickening of 1.5–2 mm would be considered as a positive reaction. This test is highly efficient in discriminating between true brucellosis cases and false positive serological reactions. The skin test is highly specific but its weak sensitivity makes it a good test for herds but not for individual certification. It cannot discriminate between infection and vaccination (Saegerman, 1999). Pouillot and his colleagues (1997) made an assessment of the diagnostic value of the

Brucellin allergic skin test (AST) in a brucellosis false positive serological reaction and reported that allergenic skin test is to be more specific than RBT and CFT. Therefore, this test could be used as a confirmatory test on cattle non-vaccinated against brucellosis. This test is prescribed as an alternative test by the OIE (2009a).

2.8.2.3. Interferon gamma test

As the prevalence of brucellosis decreases, accuracy of serological tests becomes more important. False positive reactions result in trace-backs and epidemiological investigations that are expensive and time consuming. Therefore, assays that eliminate FPSR will become more and more useful. In general, the interferon gamma test involves stimulation of lymphocytes in whole blood with a suitable antigen, in this case, *Brucellin* has been shown to work well and then measuring the resulting gamma interferon production by a capture ELISA (Weynants *et al*., 1998). This test could be useful in the discrimination of FPSR but more specific antigens are needed and the protocol needs to be standardized.

7.8.3. Recent advances in proteomic methods of Brucella diagnostics

Both classical microbiological and serological methods of brucellosis diagnosis, as well as PCR based methods have some significant disadvantages despite their intensive development. Moreover, recently the results of *Brucella* study using system biology methods of genomics and proteomics were published. Through the sequencing of the complete genomes of 8 bacterial isolates from five *Brucella* species (*Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis* and *Brucella canis*) (Chain *et al.,* 2005), it became possible to create a library of the predicted protein-coding open reading frames (ORFs) in *Brucella* genome. Such an ORFeome library was made for *B. melitensis,* but since the high degree of homology between the genomes of *Brucella* species (Chain *et al.,* 2005); *B. melitensis* ORFeome library can be used to analyze any type of *Brucella*. The protein coding DNA fragments were amplified using PCR and cloned into the DNA vector, which has been transformed into *E. coli* cells. The resulting library consisted of 3091 clones, each containing a single ORF. It was 96.7 percent of the total number of found ORF. *B. melitensis.* ORFeome library was immobilized on

DNA microarray and used for the analysis of the expression of *Brucella abortus* genes (Viadas *et al*., 2009).

The expansion of genomics, next-generation sequencing, and omics technologies has enabled in-depth analysis of the pathogenesis of brucellosis. Large-scale simultaneous *Brucella* and host global expression data sets can now be combined with proteomics and metabolomics data from in vitro and in vivo experiments in target species and nonhuman primates to generate cellular pathway and gene regulatory networks that enable full-scale systems biology analysis and improved whole system understanding of *Brucella* pathogenesis (Gomez-Cabrero *et al*., 2014). System biology methods also provide possibility to identify the antibodies produced by the organism in response to *Brucella infection* and also to predict the serodiagnostic properties of bacterial antigens. These studies provide provocative examples in support of using systems biology to more effectively integrate and exploit data for model development, for causal discovery, for the prediction of biological activities, for improving the design of in vitro and in vivo experiments, for finding biomarkers for enhanced brucellosis diagnosis, and for druggable targets for more effective treatment of brucellosis (Gomez *et al*., 2013).

2.9. Public Health Importance of Brucellosis

Brucellosis in humans is known as "undulant fever" or "Mediterranean fever", "Malta fever" or "Bangs disease" (Corbel, 2006).It is recognized as an important zoonotic disease worldwide posing serious health hazards. In endemic regions without brucellosis eradication programmes, zoonotic risk still represents an important public health threat (Moreno, 2014). Infection happens due to contact with infected animals or consumption of their products, mostly unpasteurized milk and milk products of sheep, goats, cattle and Camels (Tabak *et al*., 2008). In particular, immunocompromised persons, including the elderly, pregnant women, infants and young children, are at the highest risk of contracting brucellosis (CID *et al*., 2014). Human brucellosis is caused by four species of *Brucella*, namely, *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* , with the majority of cases of the disease in humans being attributed to *B. melitensis*, although there is a possibility that human infection by the other three species is

underappreciated (Lucero *et al.,* 2010; Guerrier *et al*., 2011). In humans, brucellosis is described as a chronic febrile debilitating disease with an incubation period of 2 to 24 weeks, which leads to significant socioeconomic losses owing to long-term treatment and inability of the affected individuals to provide for their families (Atluri *et al*., 2011).

It presents special importance in those regions where trading of raw milk and raw milk products is a common practice among farmers (Lindahl *et al*., 2014). The survival of *Brucella* in milk and dairy products is related with curing methods, humidity, temperature and/or changes in pH. For milk, *Brucella* survival is inversely proportional to the pH (El-Daher *et al*., 1990). *Brucella* can be responsible for milk-borne diseases, particularly since the appearance and taste of the milk are rarely affected by the presence of the bacteria (Dhanashekar *et al*., 2012). In addition, brucellosis vaccines such as Rev-1 and RB51 are live dried living vaccines. Thus, needle stick accidents during their preparation or administration could also be a risk factor for human infection. Close contact with animals may occur when farmers or veterinarians assist animals during parturition or abortion or handling of stillbirth. In some parts of the world it is also common practice for farmers to separate the placenta manually, thereby increasing their exposition to tissues infected with Brucella (Lindahl *et al.,* 2014).

Dairy farmers who milk with bare hands have a greater chance of becoming infected from Brucella infected animals (Lachapelle, 2012) as do farmers or slaughterhouse workers who have skin lesions which provide an entry point for the bacteria (Islam *et al*., 2013). Also, inhalation of *Brucella* has been previously reported in slaughterhouse workers where the concentration of *Brucella* can be high due to aerosol generation (Sammartino *et al*., 2006).Zoonotic brucellosis from marine mammal includes individuals in traditional communities where products from whales and seals are still an important part of their diet (Tryland *et al.,* 2014). In addition, occupational acquired infection in people handling stranded marine mammals, whale and seal hunters, marine researchers and other people handling raw products from the ocean could be exposed (Waltzek *et al*., 2012). Also, it is suggested that marine avian species may harbour *Brucella* by eating infected fish and thus become vectors of zoonotic infections (Thakur *et al*., 2012). Tourists who swim and interact closely with captive

dolphins can be at risk when *Brucella* species could be circulating in these colonies (Muñoz *et al*., 2006).

Brucellosis in humans is a multisystemic, acute to chronic, disease characterized by fever, headache, joint pains, musculo-skeletal pains, sweating, malaise and body wasting. Because of these rather non-specific signs, this has caused tremendous problems with the clinical diagnosis of brucellosis in sub-Saharan Africa, where it is constantly mis-diagnosed as malaria, which is very prevalent. Animal brucellosis constitutes significant public health importance for a pastoral community where close intimacy with animals, raw milk consumption and low awareness 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 consumes it, is also a source of infection with milk-borne zoonoses such as brucellosis (Scherlling *et al*., 2003).

Additionally, brucellosis is considered one of the most common laboratory-acquired infections worldwide because Brucella can be easily transmitted via aerosol, a fact that has also led this pathogen to be developed as a potential biological weapon in the past (Xavier *et al*.,2010). Importantly, both vertical and horizontal human-to-human transmission are rare (Aydin *et al*., 2013), which leads to the conclusion that infected humans are accidental hosts and should be considered a dead end as far as *Brucella* species transmission is concerned. At present, there is no available vaccine to prevent Brucell*a* infection in humans. The treatment of choice for human brucellosis consists of combination antibiotic therapy for long periods of at least 6 weeks; however, significant treatment failures and relapses of infection have been reported (Rubach *et al.*, 2013). Therefore, a better understanding of the disease pathogenesis in both animal and human hosts is crucial for the development of more efficient tools for the prevention and treatment of *Brucella* infection.

Boiling or heating of milk at 80–85 ºC (176–185 ºF) for several minutes (approximately 10 minutes) will destroy the bacteria. Bacteria cannot survive if the cheese is cured longer than 3 months (Nicoletti, 1989). In acidified soft cheeses and dry cheese, their survival is greater. Thus, European legislation requires that all cheeses made from raw milk be submitted to a cure

period of not less than 60 days Regulation (EC) (2004). Survival time in meat is lower, except in frozen meat where the microorganism can survive for several years (Pessegueiro *et al*., 2003). Although zoonotic brucellosis is mainly associated with farmers in high prevalence areas, even in low prevalence countries brucellosis represents an important threat as a work-acquired infection among dairy farmers, butchers, veterinary practitioners, meat inspectors, slaughter house personnel or artificial inseminators who do not take adequate Biosafety precautions while performing their jobs (Luce *et al*., 2012; Ali *et al.*, 2013; Traxler *et al.*, 2013). The global health burden caused by brucellosis has been recently estimated to be >250,000 Disability Adjusted Life Years (DALYs) (Kirk *et al.*, 2015).

In places where brucellosis is endemic, humans can get infected via contact with infected animals or consumption of their products, mostly milk and milk products especially cheese made from unpasteurized milk of sheep and goats and rennet from infected lambs and kids. Some specific occupational groups including farm workers, veterinarians, ranchers, and meat-packing employees are considered at higher risk (Tabak *et al*., 2008). *B. abortus* and *B. suis* infections usually affect occupational groups, while *B. melitensis* infections occur more frequently than the other *Brucella* species in the general population. Because person-to-person transmission rarely occurs, infected persons do not pose a threat to their surroundings (Sriranganathan *et al*., 2010). Although in general the number of human cases should follow the incidence in animals and be higher in the classical risk groups (McDermott and Arimi, 2002; Ducrotoy *et al.*, 2014), the situation can fluctuate depending on the proximity of contact between humans and livestock, the existence of different Brucella species and biovars, the alimentary and cultural habits of the population and possibly other factors. Yet human brucellosis remains the commonest zoonotic disease worldwide with more than 500 000 new cases annually (Pappas *et al*., 2005). Figure 6 depicts the incidence of human brucellosis worldwide status since 2000.

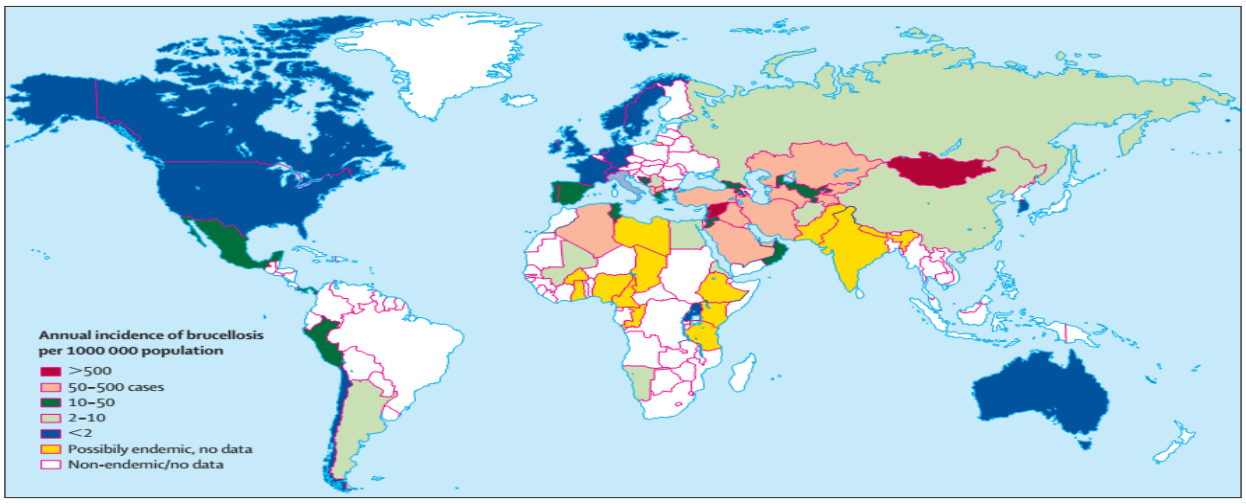

Figure 6. Worldwide incidence of human brucellosis

Source: Pappas *et al*. (2006)

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).Thus the true incidence of human brucellosis is unknown and the estimated burden of the disease varies widely, from <0.03 to >160 per 100,000 population (Pappas *et al*., 2006).Seven republics of the former Soviet Union (Kyrgyzstan, Tajikistan, Kazakhstan, Azerbaijan, Turkmenistan, Armenia and Uzbekistan) are included in the 25 countries with the highest incidence of the disease worldwide, while another country of this region, Mongolia, is ranked the second. Syria has the highest annual incidence of human brucellosis worldwide (Pappas *et al.*, 2006).

2.10. Economic Impact

Food and Agriculture Organization of the United Nations (FAO) and the Organization of Animal Health (OIE) consider brucellosis as has not only direct public health implications, it also poses a barrier to trade of animals and animal products (Fitcht, 2003) and has a wide

socioeconomic impacts especially in countries where people in rural areas rely to a large extent on livestock breeding and dairy products as a source of income (Zinsstag *et al*., 2005). Brucellosis is consistently ranked among the most economically important zoonoses globally. It is a 'multiple burdens' disease with economic impacts attributable to human, livestock and wildlife disease. The epidemiology and economic impact of brucellosis vary by geography and livestock system. In many high-income countries, brucellosis has been successfully controlled or eliminated in livestock populations. Where it persists, wildlife populations have become the main reservoirs (for example, bison and elk in North America). In emerging middle-income countries, the brucellosis picture is much more variable. Middle-income countries tend to report the greatest number of outbreaks and animal losses (WHO, 2009; ILRI, 2012).

Despite the existence of effective vaccines for cattle (S19 and RB 51) and goats (Rev 1), control efforts in economically poor endemic areas have failed as a result of the absence of vaccine provision, inconsistent infrastructure and lack of funding. Notably, most cases of human brucellosis in non-endemic developed countries result from dairy products imported from endemic area or from patients who import the disease. Data on the yearly economic impact of brucellosis in the developing world associated with disease in livestock have generally been hard to assess, especially in Africa (Smits *et al*., 2007).

In countries such as Argentina and Mexico, which depend heavily on the sale of livestock products for both domestic and international markets, these annual costs for control are estimated to be US$60 million and $200 million, respectively. Studies done in developing countries by the United Nations highlight the need for effective control programmes which have an obvious benefit to the health of both human beings and livestock. If the costs of control programmes are shared between the public and private sectors and include international aid, they are likely to be profitable and cost effective (Roth *et al*., 2003; Smits *et al*., 2007). The economic impact in terms of human disease has been even harder to gauge; not only must the cost of treatment and diagnosis be considered, but also the cost in terms of disability-adjusted life years. Regardless of the measures used, the economic burden of human

brucellosis in endemic areas is high and justifies widespread and sustained control efforts (Roth *et al*., 2003; Smits *et al*., 2007).

A recent study by analyzing the economic costs and benefits of brucellosis in Mongolia provides an excellent framework for brucellosis control decision-making. Their analysis combines both economic impacts in animals, DALY losses in humans and costs to the health system and then uses this information to evaluate the benefits of different vaccination strategies under varying efficacy assumptions. This methodology, adapted to local circumstances, would provide the key information required for animal health policy and decision makers in countries and areas where brucellosis is prevalent (Roth *et al*., 2003). The economic losses due to bovine brucellosis include: losses of calves due to abortion, reduced milk yield, culling and condemnation of valuable cows because of breeding failure, endangering animal export trading of a nation, loss of man power, medical costs and government cost for research and eradication programs (Chukwu, 1987) . In pregnant females, abortion occurs during the second half of the pregnancy, often with retention of the placenta and resultant metritis, which may cause permanent infertility. It is estimated that the infection causes a 20% to 25% loss in milk production as a result of interrupted lactation due to abortion and delayed conception. For instance, calves produced from Rose Bengal test positive and negative cows in a transhuman system in southern Sudan had, approximately 10% less calves than negative. Abortion rates were 22% in positive versus 11% in negatives (Mc Dermott and Arimi, 2002).

In Africa, very few countries tackled this chapter on estimating economic losses, no doubt owing to lack of data on funding brucellosis control and on assessing direct economic losses and loss of earnings. Algeria, Gabon, Mauritania, Morocco, the Democratic Republic of Congo, Tanzania, Tunisia and Swaziland gave a few indications regarding the annual cost of brucellosis control. The countries receive public or private financing (livestock producers). Public financing amounts to 19 459.13 EUR in Swaziland, 20 890 EUR in Tanzania and 1 897 288 EUR in Algeria. In Swaziland, economic losses arising from abortion total 2900023 EUR, while milk losses are assessed at 1272 210 EUR and export losses at 47384 EUR. Tunisia and the Democratic Republic of Congo report economic losses from abortion reduced agricultural

manpower and lower milk yields, although they provide no financial evaluation of the losses (Ayayi *et al.*, 2009).

2.11. Treatment, Prevention and Control

2.11.1. Treatment

Brucellosis is one of the drug-neglected diseases and treatment of brucellosis in domestic animals is not indicated (Kassahun, 2003). The essential administration of effective antibiotics for an adequate length of time. This element in the treatment of all forms of human brucellosis should be within the context of general medical supervision and, for severely ill patients, is best carried out in hospital if circumstances permit. In human, 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), treatment failure and relapse rates are high and depend on the drug combination and patient compliance. 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). 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*.

Although the DS regimen is considered as the gold standard treatment, it is less practical because the streptomycin must be administered parenterally for 3 weeks. 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).

2.11.2. Prevention and control

Brucellosis is an infectious disease which has been controlled and eradicated in some countries in the world (Godfroid *et al*., 2005). In sub-Saharan Africa, animal health services delivered by

the public sector have greatly decreased over the last 20 years due to various factors such as decreasing government budgets, particularly for operational costs of disease control. Thus, programs that require coordinated surveillance, information exchange and application of control measure are not implemented in many sub-Saharan countries (McDermott and Arimi 2002; Smits and Cutler, 2004). The prevention of brucellosis is mainly by control of infection in domestic livestock by mass vaccination. The use *B. abortus* strain S19 in cattle and *B. melitensis* strain Rev-1 in goat and sheep has drastically reduced its incidence in many endemic areas. Vaccination of livestock is relatively cheap and will increase the value and productivity of their animals. It is not only important to improve the health of their animals but also is an important step to reduce the risk of severe illness and disability for themselves and their family members and also reduce the transmission to the human population.

It is nearly always more economical and practical to prevent diseases than to attempt to control or eliminate them. For brucellosis, the measures of prevention include: Careful selection of replacement animals. These, whether 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 replacements animals 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. Since animals are not often kept as business enterprises, the off takes are often low (Muma *et al*., 2009). Thus animals tend to live long resulting in emotional attachment of the farmers to their animals. It has been suggested that any disease control strategies need to take into account the need and perceptions of the communities (Marcotty *et al*., 2009).

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 fetuses. Disinfection of contaminated areas should be performed thoroughly. Cooperation with public health authorities to investigate human cases. Animal brucellosis, especially when caused by *B. melitensis*, can often be identified through investigations of cases in humans (Corbel, 2006).

Ensuring safe food is paramount for the protection of human health and for enhancement of the quality of life. Risk based programmes should aim at preventing or decreasing the transmission of zoonoses, including brucellosis, through adequate policy frameworks, prevention and control measures, and education, to prevent and control the spread of all zoonoses and foodborne diseases. Pasteurization of milk and sanitary or herd surveillance, with animal vaccination and culling techniques for infected animals and herds of cattle and other animals for brucellosis have considerably reduced the incidence of outbreaks in many developed countries (New Zeland, Sweeden, the U.K. and France have earned the designation brucellosis free). Agricultural efforts and investments in brucellosis control and eradication programmes are worth many millions of dollars (Russo *et al.*, 2009).

3. MATERIALS AND METHODS

3.1. Description of Study Areas

The study was conducted in two purposively selected towns of Bishoftu and Holeta that are known for their well developed dairy production constituting the major milk sheds of the capital (Land O'Lakes Inc., 2010). The two towns are far apart from each other approximately by about 76 Km and the study areas were sketched in (Figure 7) below.

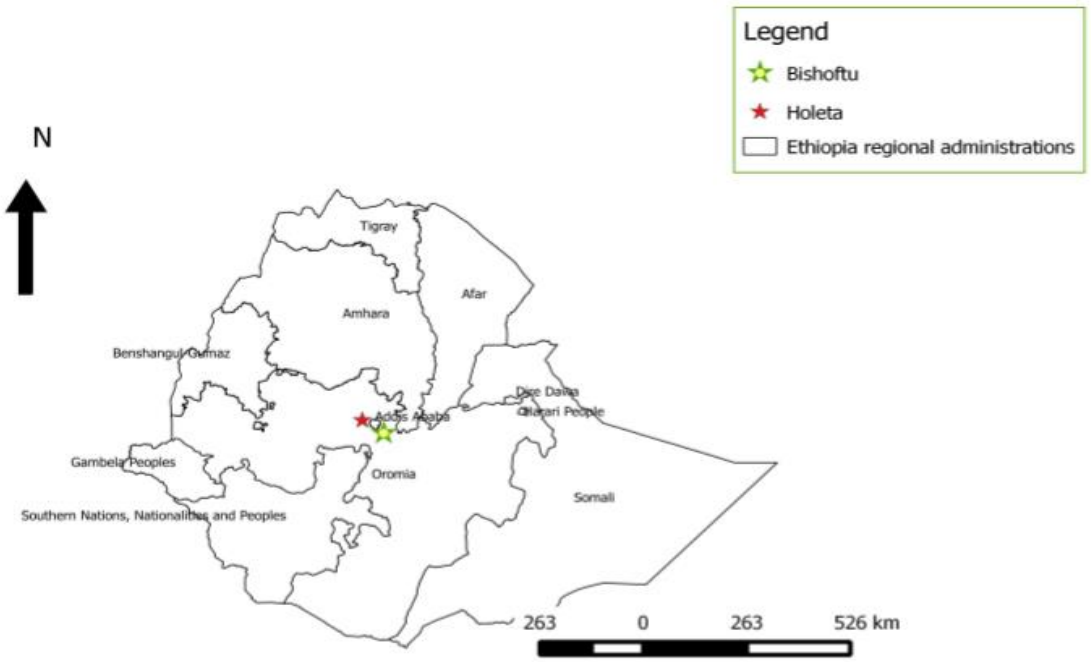

Figure 7. Map of Ethiopia showing the study areas

Bishoftu is a town located in the East Shoa zone at 47 kilometers south of the capital, Addis Ababa, on the main international road to Djibouti. The 2007 national census reported a total population for Bishoftu of 99,928, of whom 47,860 were men and 52,068 were women. The absolute location of Bishoftu is geographically located at 8°45′N latitude and 38°59′E longitude. Topographically the town is located in tepid to cool sub-moist mid highland at an altitude of about 1920 meters above sea level (m.a.s.l). The mean annual maximum and

minimum temperatures are 26°C and 14°C, respectively; with mean relatively humidity of 61.3% .It has an annual rainfall of 866 mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. Farmers in the vicinity of Bishoftu town use a mixed crop- live stock farming system. The area has diverse climatic zones, which are inhibited with different plant and animal species.

Holeta is a town of Welmera district and situated in Oromia special zone surrounding Finfinne. The town is located 29 kilometers west of Addis Ababa at 9°30' N and 38°30' E with altitude ranging from 2300-3800m above sea level which is actually parts of central highlands of Ethiopia.The average annual minimum and maximum temperatures were 6° and 22°C, respectively. The area is also characterized by occasional frost that occurs in the months of October to December, where temperatures below zero for few days during these months. The annual rainfall ranges from 900-1100 mm. According to the population and housing census of 2007 the population of the town is 23,296 (male=11512, female=11,784). The major livestock production systems in the area include: mixed crop-livestock farming surrounding the towns, where animals are managed under extensive traditional grazing systems; market oriented, peri-urban dairy production and urban dairy production systems.

3.2. Target Population and Characterizations

The target populations were dairy cattle in urban and peri-urban (both smallholder and commercial) dairy farms of Bishoftu and Holeta towns. Since dairy farms use more Artificial insemination than natural mating for breeding purpose, male animals were excluded from study. The dairy cattle under study comprised of breeds of Pure Holstein Frisian and indigenous crossbreed with Holstein Frisian. Cattle breeds established in the major milk sheds of the study sites with no history of vaccination against brucellosis. Dairy cattle older than six months were included into the study as the disease wasn't common in the cattle less than 6 months of age due to maternal antibody. The animals were classified into two age groups (6-17 months and $\geq$ 17months) as young and adult based on Asmare *et al*. (2013).

3.3. Study Design and Methods

A cross-sectional seroepidemiological study of bovine brucellosis was conducted from December, 2016 to May, 2017. The towns were purposively selected based on the abundance of dairy farms that constituting the known milk sheds to the capital (Land O' Lakes Inc., 2010) and agroecological differences. The sampling frame was prepared and a sampling unit was divided into groups, or strata based on herd size and level of production into three groups: smallholder (small herd size), medium herd size and large herd size. Stratified random sampling was employed to sample the herds and the individual animal from each stratum sequentially by using lottery method. The sampling was performed using a two level approach, selecting first individual herds from each stratum and then randomly selecting individual animals inside each stratum proportionally (stratified two stage sampling). Then, relevant individual animal bio data and farm / herd level information were collected using structured format.

3.4. Sample Size Determination and Sampling Methodology

The sample size of each town was calculated based on the reports of previous sero- prevalence of bovine brucellosis in study areas:

$$\mathbf{n= \frac{1.96^2 * P_{exp}(1-P_{exp})}{d^2}}$$ **.....** Thrusfield (2005)

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

Thus, the sample size for Bishoftu and Holeta towns were calculated based on a previous report of 3.48% expected sero prevalence of bovine brucellosis by Jergefa *et al*. (2009). Thus,

$n= \frac{1.96^2 * P_{exp}(1-P_{exp})}{d^2} = \frac{1.96^2 * 0.348(1-0.348)}{(0.05)^2}$ = 53 dairy cattle and when multiplied by two, 106 dairy cattle from the two study areas were supposed to be included into the study. However, approximately by more than three folds sample size was increased so as to increase

precision and reduce standard error. Accordingly, in the present study a total of 326 dairy cattle (175 and 151 dairy cattle from Bishoftu and Holeta, respectively) were selected to investigate *Brucella* infection for this study purpose.

Before sample collection, members of the target population were identified by constructing a list (the sampling frame). Thus, a list of 95 dairy farms (40 and 55 dairy farms from Bishoftu and Holeta, respectively) was generated with the help of the respective district livestock health departments of the two study areas.Then, the study population was divided into strata based on different ranges of herd (herd size) and level of production (Thrusfield, 2005). Three strata were formed such as: small herd size (<10), medium herd size (10-50) and large herd size (≥50) based on (Geresu *et al.,* 2016). Accordingly, a total of 30 dairy farms (14 dairy farms from Bishoftu and 16 from Holeta study areas) were proportionally selected randomly from the strata based on the herd number in each stratum. That means, 15(111 dairy cattle), 11(320 dairy cattle) and 4(250 dairy cattle) dairy farms were selected as small herd size, medium herd size and large herd size, from the sampling frame, respectively.Of caurse the minimum and maximum herd size were 4 and 94 dairy cattle,respectively. Then, individual dairy cattle were also proportionally selected randomly by lottery methods from each substratum of the selected dairy herds. Accordingly, about 64,161 and 101 dairy cattle from small herd size, medium herd size and large herd size were included into the study based on stratiefied two stage sampling, respectively.

3.5. Blood Sample and Data Collection

3.5.1. Collection of blood samples

Blood samples were collected aseptically from jugular vein of individual animals for serological analysis. Approximately 10 mL of blood was aseptically collected from each cow using plain vacutainer tubes and then the collected blood samples were kept overnight to allow clotting at room temperature. Then, the separated sera were carefully collected/ harvested into cryovial without mixing with the clotted blood. The harvested sera were transported to National Animal Health Diagnostic and Investigation center (NAHDIC) serology laboratory in

icebox with the aid of ice pack. In the laboratory, the sera were stored at -20°C until processed. Information regarding to the attributes of individual animal such as breed, age, abortion history, herd size, etc., was recorded during blood sample collection (Annex 1).

3.5.2. Questionnaire survey

Verbal consent was obtained from the respondents and the objective of the survey explained to them before start of the interview. Then, parallel to collection of blood samples, pretested structured questionnaire (Annex 2) was used to collect data from 30 dairy farms owners, on possible factors that could contribute to transmission of brucellosis in livestock, through interviews. A number of Socio-demographic character such as age, education level and gender of the respondents, and herd-level predictor variables such as herd size, categorized as small (<10 animals), medium (10-50) and large (50 and above), source of replacement stock (from own, market and both), the presence of a separate parturition maternity pen, separate cows during parturition, reproductive disorders, selling of dairy breeding cattle, visitors and stray animals control were recorded. The hygienic status of the farm was also categorized as good, fair and poor based on manure disposal, drainage, physical appearance of the animal and ventilation status. The predominant selling reasons for dairy cattle were coded either as diseases, infertility or shortage of money. The culling criteria were also coded as reproductive problems, non-reproductive problems or both. The method of placenta, aborted material and dead animal disposal was also categorized into burying, open dump or feed to dogs. Calving pen after parturition treated as flushing with water, disinfecting with detergents or both. Furthermore, key questions on zoonoses including brucellosis were included into the questionnaire to assess the knowledge (awareness) of the respondents at the study areas.

3.6. Serological Diagnostic Tests

Rose Bengal Plate Test (RBPT): The sera samples were first screened using Rose Bengal Plate Test (RBPT) as described by Nielsen (2002). RBPT *Brucella* antigen from (Veterinary Laboratories Agency, Addlestone, United Kingdom), together with positive and negative control sera were used. Briefly, 30 µl of sera samples were dispensed on to the plate, and 30 µl of RBPT antigen was dropped alongside the sera. Using an applicator stick, the antigen and the

sera were mixed and rocked for about 4 minutes, and examined for agglutination. Of course, antigen for the Rose Bengal Plate Test was prepared from *B. abortus* strain 99 stained with Rose Bengal dye and suspended in acid buffer pH 3.65. Positive and negative controls were employed for interpretation of the results. Results of (RBPT were interpreted as 0, +, ++, and +++ as has been described by Nielsen (2002).

Competitive –ELISA (c- ELISA) Kit (SVANOVIR® (Svanova Biotech, Uppsala, Sweden), was used according to the manufacturer's instructions. In this procedure, the samples together with a mouse monoclonal antibody (mAb) specific for an epitope on the O-polysaccharide portion of the S-LPS antigen are exposed to *Brucella abortus* smooth lipopolysaccaride coated wells on microtitre plates. If anti- *Brucella* antibodies are present in the test sample they will bind to the antigens in the well and block these antigenic sites. If anti-*Brucella* antibodies are absent in the sample, these sites will remain free and the mAb which was added after the sample will bind to these free antigenic sites. After an incubation period the unbound materials are removed by rinsing and a goat anti-mouse horseradish peroxidase (HRP) conjugated IgG is added to plate. The HRP conjugate will bind with specific mAb in absence of anti-*Brucella* antibodies in the sample. Unbound materials are removed by rising prior to the addition of the substrate. Subsequently a blue colour develops which is due to the conversion of the substrate by the conjugate. A negative result is indicated by the development of a blue color. The reaction is stopped by addition of stop solution; the colour changes to yellow. The result can be read by a micro plate photometer, where the optical density (OD) is measured at 450 nm. Sera from strain 19 vaccinated cattle do not compete with the mAb because of their specificity and lower affinity, leading to negative reaction. Samples with PI values of ≥30% are considered to brucella infection where as those with PI value <30% are considered as negative.

Complement Fixation Test (CFT): RBPT and c-ELISA positive sera were further tested using complement fixation test (CFT) as described by MacMillan (1990) for confirmation using standard *B. abortus* antigen for CFT (Veterinary Laboratories Agency, Addlestone, United Kingdom). The use of RBT/CFT combinations, the most widely used serial scheme, is generally recommended to maximize specificity of the test result by ruling out false positive results due to serological cross-reactions (Dohoo *et al.*, 2003). An animal was considered

positive if it gives positive results on RBPT, c-ELISA and CFT. Interpretation of the result is based on the degree of fixation of the complement and considered strong reaction (+3) when more than 75% fixation of the complement occurred at a dilution of 1: 5 where as the reaction is considered as weak positive (+2) when 50% fixation of complement was fixed at a dilution of 1: 10 and above. *Brucella* antigen for the complement fixation test was prepared from *B. abortus* S99. Brucella antigen and positive control for complement fixation test were obtained from AH-VLA (Animal Health Veterinary Laboratory Agency, UK). Hemolytic serum and guinea pig complement was obtained from ID VET (Innovative Veterinary Diagnostic) Company.

3.7. Data Management and Analysis

Putative biological and environmental factors believed to be associated with the occurrence of brucellosis were recorded in a Microsoft Excel® Spread Sheet. Data for serological and questionnaire were entered into Microsoft Excel spreadsheet and analysis was done using STATA version13.0 for windows (Stata Corp, College Station, TX). Prevalence was calculated by dividing the number of RBPT, c-ELISA and CFT-positive animals by the total number of animals tested and reported for each test result. Herd prevalence was calculated by dividing the number of herds with at least one reactor in RBPT, c-ELISA and CFT by the number of all herds tested. The within-herd prevalence was calculated by dividing the number of RBPT, c-ELISA and CFT reactors within a herd by the number of serum samples tested in the herd. Asssociations of risk factors with *Brucella* seropositivity were used Fisher's exact test for both animal and herd levels. But for the none zero outcome of individual animal risk factors, logistic regression were used to measure association of potential risk factors. Odds ratio (OR) was utilized to measure the degree of association between risk factors such as management system, breed types, abortion history and RFM with animal level seroprevalence. All risk factors that had none zero outcome value in their category in the univariate logistic regression analysis were subjected to multivariable logistic regression analysis except RFM due to multicollinearity effect with abortion history. Likelihood ratio test was used to simplify the multivariable logistic regression analysis. P value less than 0.05 was considered statistically significant in all analysis.

3.8. Ethical Considerations

Ethical clearance certificate with Ref.no. VM/ERC/26/06/09/2016 was obtained from the animal research ethical review committee of the College of Veterinary Medicine and Agriculture (CVMA) evidencing that humble handling of the sampled animals was done to minimize unnecessary suffering during sample collection (Annex 8).

4. RESULTS

4.1. Results of Seroprevalence of Bovine Brucellosis and Associated Risk Factors

4.1.1. Prevalence of anti- Brucella antibodies

The sero-prevalence distribution of *Brucella* infection at individual animal and herd level in the study areas is presented in Figure 8. Among the 326 dairy cattle tested for bovine brucellosis, 7 (2.14%) and 4 (1.23%) were found to be positive by RBPT and c-ELISA, respectively. When tested further serially with CFT 3 (42.9% and 75% of RBPT and c-ELISA positive sera, respectively) were confirmed to be positive. The animal level sero-prevalence of bovine brucellosis of the present study was 0.92% (95% CI: 0.19%, 2.66%).The herd level sero-prevalence of bovine brucellosis was 16.7% and 13.3% by RBPT and c-ELISA, respectively. Results of CFT showed that a herd prevalence of bovine brucellosis was 10% (95% CI: 2.11%, 26.53%). The within-herd prevalence varied between nil to 16.67 % (1/6) based on CFT result. Likewise, among herds that tested seropositive, the within-herd seroprevalence ranged from 2.94 % to 16.67%. The mean apparent prevalence among animals in the seropositive herds was 4.6% (n = 65; 95% CI: 0.9%, 12.9%) (Figure 8).

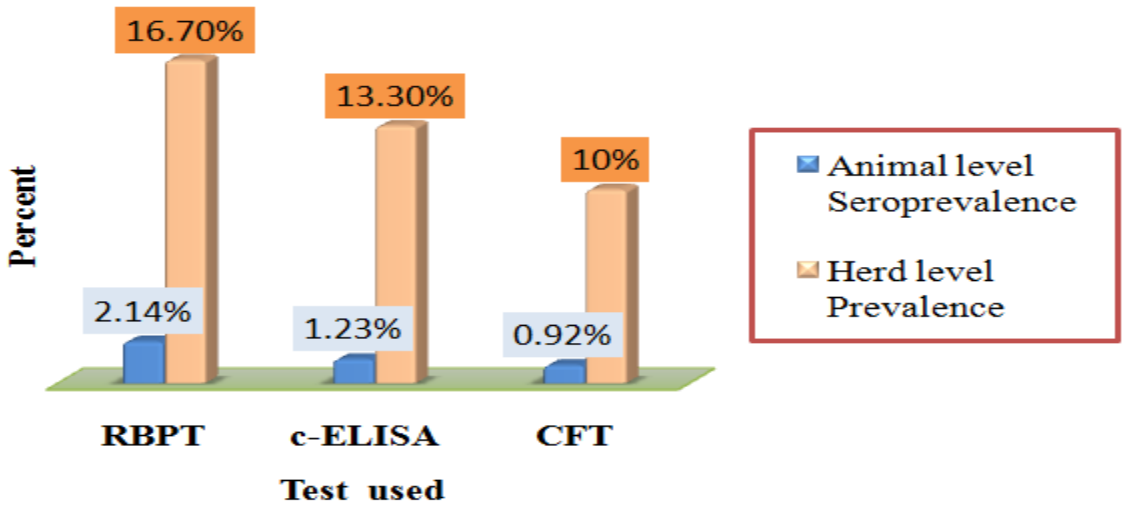

Figure 8. Graphical representation of apparent seroprevalence of bovine brucellosis at animal and herd levels using RBPT, c-ELISA and CFT

4.1.2. Putative variables for infection with Brucella species at animals level

Table 4 present results of animal level *Brucella* seropositivity and their association with exposure variables using fisher's exact test. The result respectively showed 0.0% and 1.09 % seroprevalence were young and adult animals. However, the difference in the prevalence observed was not statistically significant (P >0.05). The prevalence was not significantly associated with other factors such as agro-ecology, herd size and parity as well (P > 0.05).

Table 4. Association of bovine *Brucella* sero-positivity with Animal-level risk factors

Variable	Level	No of animals studied	Prevalence (%) (95% CI)	P-value♦
Age	Adult	274	1.09 (0.22, 3.16)	0.593
	Young	52	0.0(-)	
Agro-ecology	Highland	151	1.98 (0.41,5.69)	0.098
	Mid-highland	175	0.0(-)	
Herd size	Small	64	1.6 (0.39,8.40)	0.128
	Large	101	1.9 (0.24,6.97)	
	Medium	161	0.0(-)	
Parity	Non pariturated	67	1.49(0.04, 8.04)	
	Primiparus	71	0.0 (-)	
	Pluriparus	188	1.06 (0.13, 3.79)	0.782

CI: Confidence interval

♦Based on Fisher's exact test

The following Table 5 present results of animal level univariable analysis showing the magnitude and association of the putative variables with *Brucella* sero-positivity. Seroprevalence of bovine brucellosis did not show significant variations among breed types and management system (P > 0.05) using univariate logistic regeration analysis. Other risk factors like the history of abortion and retained fetal membrane were also analyzed and for analysis of these factors; the age group of up to two years was removed from the data to avoid bias as there are no chances of abortion and RFM in animals below two years of age. Of the

remaining 267 animals (of more than two years of age), 15 animals (5.61%) and 17 animals (6.36%) were found to have a history of abortion and RFM, respectively. The seroprevalence of brucellosis was found to be significantly higher (P<0.05) in animals with a history of abortion (13.3%) and RFM (11.7%) than in those without such a history (0.39%) and (0.4%), respectively. The animals with history of abortion were 38.6 times more likely to be seropositive than those without such history. Similarly, Animals that had suffered from retained fetal membranes were 33.2 times at risk of being positive to *Brucella* infection than those without such history (Table 5).

Table 5. Risk factors of infection with *Brucella* in Cattle in the study areas as shown by logistic regression analysis

Variable	Level	No of animals studied	Prevalence (%) (95% CI)	P-value	OR(95% CI)
Breed type	Pure HF	64	1.6 (0.04,8.40)	0.557	2.1(0.2,23.1)
	HF cross	262	0.8 (0.09,2.73)		1.0
Mgt system	Intensive	171	1.3 (0.15,4.58)	0.516	0.45(0.1, 5.0)
	Semi-intensive	155	0.6 (0.01, 3.21)		1.0
History of abortion	Yes	15	13.3 (1.65,40.46)	0.004**	38.6(3.3,453.9)
	No	252	0.39 (0.01,2.19)		1.0
RFM	Yes	17	11.7(1.46,36.4)	0.005**	33.2(2.8,387.1)
	No	250	0.4 (0.01,2.21)		1.0

HF: Holstein-Friesian; OR: Odds ratio; Yes: means the presence of the factor; No: means the absence of the factor**,** Mgt: Management; CI: Confidence interval; *: statistically significant

4.1.3. Multivariable analysis of animal level risk factors with Brucella sero-positivity

Except variables that had zero outcomes in their category among individual animal risk factors, the variables from univariable analysis were included in the final multivariable logistic model.. One variable, retained fetal membrane that showed multicollinearity with abortion history was not included in the multivariable logistic regression model. A risk linked abortion history was observed in final model of animal level analysis. Thus, in the multivariable analysis, only abortion remained to be independently associated with brucellosis seropositivity (Table 6).

Table 6. Multivariable model for risk factors of bovine *Brucella* seropositivity at animal level

Variable category	Prevalence (%) (95% CI)	P value	OR (95%CI)
Breed types (HF)	1.6 (0.04,8.40)	0.932	1.13 (0.06, 21.16)
HF-cross	0.8 (0.09,2.73)		1.0
Mgt system (Intensive)	1.3 (0.15,4.58)	0.953	0.91(0.05, 16.57)
Semi-intensive	0.6 (0.01, 3.21)		1.0
Abortion history (Yes)	13.3 (1.65,40.46)	0.004**	37.8 (3.17, 451.3)
No	0.39 (0.01,2.19)		

CI: Confidence Interval; OR: Odds ratio; *: statistically significant

4.2. Results of Questionnaire Survey

4.2.1. Socio-demographic charactrestics of the respondents

Of the 30 farm owners' interviewed during field survey, the youngest was 24 while the oldest was 74 years. The majority of the respondents' ages categorized between 30-50 years which accounts for about 70% of the respondents who were participated in the study. As far as the education level of respondents is concerned, the data indicated that a majority of respondents, 70% had primary and secondary school while 30% of the respondents were being vocational and college level. However, these sociodemographic factors included into the present study were not showed significant association with herd seroprevalence of bovine brucellosis ($\rho > 0.05$) as shown in (Table 7).

Table 7. Socio-demographic characteristics of the respondents and their association with *Brucella* herd seropositivity using Fisher's Exact Test

Description	Proportion of respondents (n=30) (%)	Number of positive herds (%)	P value♦
Education level			
Primary school	12 (40)	1(8.3)	0.172
Secondary school	9 (30)	0.0(0)	
Vocational	4 (13.3)	0.0(0)	
College/ University	5(16.7)	2(40)	
Age			
< 30	5(16.7)	0.0(0)	0.548
[30-40)	10(33.3)	1(10)	
[40-50)	11(36.7)	2(18)	
≥ 50	4(13.3)	0.0(0)	
Gender			
Male	23(76.7)	2(8.6)	0.654
Female	7 (23.3)	1(14.3)	

♦ Based on Fisher's exact test

4.2.2. Association of herd level risk factors with bovine seropositivity to Brucella species

Among the 30 interviewed respondents 90% (n= 27) reared crossbreed (indigenous- Holstein Frisian) dairy cattle where as 10% (n= 3) of them reared pure Holstein Frisian. Nevertheless, the herd level seropositivity to brucellosis did not show statistically significant variation between the breeds (P > 0.05). According to the respondents, 56.6% (n= 17) of farms raised their stock from their own farms; 3.3% (n= 1) of the used to replace the stock from the market and 40% (n= 12) employed both options. About 76.6 % (n=23) of the farms were sold the female breeding cattle whereas 23.4% (n=7) of them did not. According to the respondents the main reasons for the selling of female breeding cattle was infertility 60.8%(n=14) followed by diseases problem, and shortage of money as shown in (Table 8 and 9).Most of the farms' hygiene was fair, and a few were good and poor based on manure disposal, drainage, and

physical appearance of the animal and ventilation status of the farms. None of these risk factors were associated with herd seroprevalence to brucellosis in present study (Table 8).

The presence of abortion history in dairy herds were responded by 26.7% (n= 8) of the respondents and about 30% (n= 9) of the respondents were reported the occurrence of retained fetal membrane during abortion or parturition in their animals. The presence of abortion history and RFM were significantly associated with herd-level seropositivity to brucellosis ($P < 0.05$). Moreover, results of Fisher's exact test also showed that herd size ($P < 0.05$) and stillbirths ($P<0.05$) were significantly associated with herd level *Brucella* seropositivity, respectively. However, agro ecological areas, the absence or presence of stray animals control, visitors control, parturition maternity pen, selling of dairy breeding cattle and the way of cleaning calving pen after parturition were not associated with herd-level seropositivity to brucellosis ($P > 0.05$) as illustrated in (Table 8).

Table 8. Association of herd level risk factors with bovine brucellosis by using Fisher's Exact Test

Type of risk factor	Serum investigated by CFT at herd level		P-value♦
	Number of herd tested (n=30) (%)	Number of test positive herd (%)	
General characteristics of farms:			
Herd size			
Small	15(50)	1(6.7)	
Medium	11(36.7)	0.0(0)	
Large	4(13.3)	2(50)	0.014*
Agro- ecology			
Highland	15(50)	3(20)	0.224
Mid-highland	15(50)	0.0(0)	
Breed types			
Pure HF	3 (10)	1(33.3)	0.280
HF cross	27 (90)	2(7.4)	
Introduction of infection to farms:			
Source of replacement stock			
From market	1 (3.3)	0.0(0)	
Raise from own farm	17 (56.6)	1(5.9)	
Both	12 (40)	2(16.7)	0.598
Selling of dairy breeding cattle			
Yes	23 (76.6)	2(8.7)	0.564
No	7 (23.4)	1(14.3)	
Management systems of the farms:			
Farm Hygiene			
Poor	4(13.3)	2(50)	0.635
Fair	21(70)	1(4.7)	
Good	5(16.7)	0.0(0)	
Exposure and maintenance of disease:			
Stray animals control			
Yes	22 (73.3)	3(13.6)	0.545
No	8 (26.7)	0.0(0)	
Visitors control			
Yes	20 (66.7)	3(15)	0.532
No	10 (33.3)	0.0(0)	
Parturition maternity pen			

Yes	3 (10)	1(33.3)	0.280
NO	27 (90)	2(7.4)	
Occurrence of abortion			
Yes	8(26.7)	2(25)	0.014*
No	22(73.3)	1(4.5)	
RFM			
Yes	9(30)	2(22.2)	0.021*
No	21(70)	1(4.7)	
History of still birth			
Yes	10(53.3)	3 (30)	0.030*
No	20(66.7)	0.0(0)	
Calving pen after parturition			
Flushing with water	18(60)	3(16.7)	0.329
Disinfect with detergent	6(20)	0.0(0)	
Both	6(20)	0.0(0)	

Both: Water with detergents; RFM: Retained fetal membrane

♦ Based on Fisher's exact test; *: statistically significant

4.2.3. Awareness and practices of the respondents on herd health management and Brucellosis

The questionnaire survey provided information regarding the knowledge (awareness) and practices of farm owners about zoonotic diseases, particularly brucellosis. Though the owners seemed to be aware of the risk of zoonotic diseases transmission through dirking raw milk and contact/ handling of fetal and aborted materials, still a high number of respondents had no detailed and accurate knowledge about zoonotic brucellosis. That is, in this study about 33.3%, 54.5% and 100% respondents owning small, medium and large herd sizes, respectively were aware of the existence of brucellosis within the community. There was a low level of awareness among the owners of small herd sized farms on brucellosis.

It was also found that the majority of the farm owners 70 %(n=21) were dependent on burying of the fetal and aborted materials while some of the farm owners dispose after birth or abortion to open dump, and still few of them feed to dogs especially those of small and medium herd size farms (Table 9). About 53.3% (n=16) of the respondents were culled prominently dairy cattle due to reproductive problems whereas the other culled for both reproductive and non reproductive

problems. Even though some of the practices that have been done in those farms seemed attractive, further attentions on the awareness issue of the disease were needed.

Table 9. Knowledge and practices of respondents on heard health and brucellosis.

Variables	Frequency of respondent(n= 30)			
	Herd size			
	Small(n= 15) n(%)	Medium(n= 11) n (%)	Large(n=4) n (%)	Total (n=30) n (%)
Awareness about zoonotic diseases transmission through drinking raw milk				
Yes	10(66.7)	9(81.8)	4(100)	23(76.7)
No	5(33.3)	2(18.2)	0.0(0)	7 (23.3)
Awareness about diseases transmitted during handling of fetal and aborted materials				
Yes	6(40)	8(72.7)	4(100)	20(66.7)
No	9 (60)	3(27.3)	0.0(0)	10(33.3)
Awareness about zoonotic brucellosis				
Yes	5(33.3)	6(54.5)	4(100)	15(50)
No	10(66.7)	5(45.5)	0.0(0)	15(50)
Handling of fetal and aborted material				
Burying	10(66.7)	7(63.7)	4(100)	21(70)
Open dump	2(13.3)	2(18.2)	0.0(0)	4(13.3)
Feed to dogs	3(20)	2(18.2)	0.0(0)	5(16.7)
Reasons for selling dairy cattle (n=23)				
Infertility	5(45.5)	7(77.8)	2(66.7)	14(60.8)
Diseases	1(9)	1(11.1)	1 (33.3)	4(17.4)
Shortage of money	5(45.5)	1(11.1)	0.0 (0)	5(21.8)
Reasons for culling dairy cattle				
Reproductive problem	8(53.3)	6(54.5)	2(50)	16(53.3)
Non reproductive problem	2(13.4)	3(27.3)	1 (25)	6(20)
Both	5(33.3	2(18.2)	1 (25)	8(26.7)

n= number of participants; Both: represents reproductive and non- reproductive problems

4.3. Comparison of Serological Test Agreement

When compared to c-ELISA, Rose Bengal Plate test diagnosed 1 false negative and 4 false positives .Then; there was a substantial test agreement between RBPT and c- ELISA. Similarly, a almost perfect agreement between CFT and c-ELISA and moderate agreement between CFT and RBPT was observed according to Kappa by using CFT as a confirmatory test as depicted in (Table 10).

Table 10. Kappa test for agreement between RBPT, c-ELISA and CFT for diagnosis of bovine brucellosis

		c-ELISA		Kappa value	Kappa value interpretation
		Positive	Negative		
RBPT	Positive	3	4	0.69	substantial agree
	Negative	1	318		
		CFT			
RBPT	Positive	3	4	0.54	Moderate agree
	Negative	0	319		
c-ELISA	Positive	3	1	0.855	Almost perfect agree
	Negative	0	322		

Source**:** (Everitt, 1989**)** K = 0: no agreement; K = 0 – 0.20: slight. K = 0.21 – 0.40: fair; K = 0.41 – 0.60: moderate; K = 0.61 ‑ 0.80: substantial agree; K > 0.81: almost perfect agree; K = 1: perfect agreement

5. DISCUSSION

It is considered that dairy animals are subjected to more stress conditions on farms, leading to a higher susceptibility to brucellosis infection (Kataria and Gahlot, 2010). However, the prevalence of bovine brucellosis recorded in this study at animal level is low (0.92%, 95% CI: 0.19%, 2.66%).That is, even though its publich health impact remains standing, its occurrence in dairy cattle in the study areas is minimal. The finding is comparable with the earlier reports of Tolosa *et al*. (2008) (0.77 %); Hailu *et al*. (2011) (1.38%); Gumi *et al. (*2013) (0.9%); Adugna *et al*. (2013) (1.0%); Asmare *et al*. (2013) (1.9%); Geresu *et al. (*2016) (1.4%)) from Ethiopia and Kang'ethe *et al.* (2007) (1.0%) from Kenya. On the other hand, there were reports with a relatively higher seroprevalence of bovine brucellosis in other parts of the country Berhe *et al.*(2007) (3.19%); Hailesillasie *et al*. (2008)(4.9%); Kebede *et al. (*2008) (11.0%); Jergefa *et al.* (2009) (2.9%*);* Ibrahim *et al*. (2010) (3.1%); Megersa *et al.(* 2011b) (3.5%); Mekonnen *et al.(* 2011)(6.1%); Tibesso *et al*.(2014) (4.3%). Similarly, relatively higher seroprevalence were reported in other African countries by other authors: 24.5% Angara *et al*. (2010) from Sudan; 24.0% Mai *et al*. (2012) from Nigeria, 5.5% Matope *et al*. (2010) from Zimbabwe; 8.5% Omer *et al*. (2000) from Eritrea were some of the reports.

The difference observed in prevalence could be due to variation in production systems and animal management. Because most of the previous authors who reported higher prevalence studied extensively managed herds, where cattle from several owners mingle at grazing or watering points. Hence, the reasons for the low prevalence of bovine brucellosis in this study areas could possibly be explained by better hygienic practices, separation of cows during parturition, cleaning and disinfection activities, culling of infected animals and depending on own herds for replacing stock in these intensive farms, and the prevailing management differences between the intensive, semi-intensive and extensive production systems (McDermott and Arimi, 2002 and Matope *et al*., 2010). This is also reflected the relatively good hygienic status of the farms and practices in disposing aborted materials to ward off contact with animals. The use of different tests and laboratories also could have affected the variation in the proportion

of animals found positive.

There were also reports with a relatively lower seroprevalence of bovine brucellosis than the present finding in other parts of the country by (Bashitu et *al*., 2015) 0.2 %, Ambo and Debreberhan, and Alem and Solomon (2002) and Belihu (2002) failed to find any seroreactive cattle in Eastern and Western Showa zones of central Ethiopia and in intensive dairy farms in Addis Ababa area, respectively. Similarly, Belihu (2002) could not find positive reactors in intensive dairy farms in Addis Ababa area (n=747). Variation in the seroprevalence findings might be seen to due to relatively more farm managements in these studies than the present study.

In addition to estimation of seroprevalence, this study was also carried out to assess the risk factors associated with disease occurrence. Even though the present study revealed that agroecology of the dairy cattle was not significantly associated with brucellosis in dairy cattle, the results showed higher individual animal level seroprevalence at high land agroecological area of Holeta (1.98%) than mid-high land area of Bishoftu (0.0%). Absence of seropositive animal in mid highland (Bishoftu) may be due to a high awareness of the farmers coupled with low prevalence of brucellosis. This finding is in accord with those of the previous findings such as that of Jergefa *et al.* (2009), Geresu *et al*. (2016) in which the agroecology was not significantly associated with seropositivity. The reasons for similarity in brucellosis seroprevalence between the agro ecological areas might be overwhelmed due to similarity in management practice performed in the two study sites. But, the observation was not in concordance with the findings of Berhe *et al.* (2007) in which agroclimate was significantly associated with brucellosis seropositivity in which low seroprevalence was reported in low land areas. Similarly, literature witnessed that the seroprevalence of brucellosis is lower in low land agro-climate which is unsuitable for survival of *Brucella* organisms than highlands (Radostits *et al.*, 1994).

Though the present study revealed that the herd size of dairy cattle was not significantly associated with animal level seroprevalence of brucellosis in dairy cattle, the results showed

higher individual animal seroprevalence in large (2%) and small (1.6%) compared to medium ones in which no positive animal was observed (Table 4). This could be due to similar herd management between the farms irrespective of the herd size. The result was in line with the findings of Kebede *et al. (* 2008), Jergefa *et al*.(2009), Asmare *et al*. (2013) who reported that the risk of seropositivity were independent of herd size. However, herd size has been identified consistently in previous and more recent studies by different reseachers to be a risk factor of brucellosis (Akakpo, 1987; Asfaw *et al*., 1998; Berhe *et al*., 2007; Muma *et al*., 2007a, b; Mekonnen *et al.,* 2010; Megersa *et al*., 2011b, b; Sanogo *et al.,* 2012; Geresu *et al*., 2016). This can be explained by the fact that an increase in herd size is usually accompanied by an increase in stocking density, one of the determinants for exposure to *Brucella* infection especially following abortion or calving (Crawford *et al.,* 1990). Similarly, the increased odd of herd seropositivity with increasing herd size has also been reported in Zimbabwe (Matop *et al*., 2010).

In the present study unlike most reports, there was no statistically significant association between the age groups at animal level seropositivity to brucellosis. But a seroprevalence of 1.09% was found among adult age group whereas no *Brucella* seropositivity was observed in young age group of dairy cattle in the study sites (Table 4).The similarity of the present finding between age groups might be due to similar proper management across the herds irrespective of the ages in the study areas. Though it was not statistically significant, the aged animals have a greater chance of becoming infected and of coming into contact with other animals. Similar findings were also reported by (Jergefa *et al*., 2009; Amenu *et al.,* 2010; Geresu *et al.,* 2016) where age was not significantly associated with *Brucella* seropositivity. Moreover, statistically significance difference of a higher seroprevalence in the adult age groups has been reported by many researchers (Kebede *et al.,* 2008; Hailesillasie *et al.,* 2010; Megersa *et al*., 2011b; Asmare *et al*., 2013). It is possible that the higher prevalence of brucellosis among older cows may be related to their advanced age as the organism may remain latent or chronic for an unspecified period before manifesting as clinical disease. The other justification also possible as age is one of the intrinsic factors which influence the susceptibility to *Brucella* infection. Brucellosis appears to be more associated with sexual maturity (Radostits *et al*., 2000). It is essentially a disease of sexually mature animals and susceptibility increases with sexual maturity and

pregnancy due to the influence of sex hormones and placenta erythritol on the pathogenesis of brucellosis (Radostits *et al.,* 2007).On the other hand, younger animals tend to be more resistant to infection and frequently clear infections, although latent infections could occur (Walker, 1999).

There is still a controversy among different researchers on the issue of breed susceptibility to brucellosis. The present study revealed that *Brucella* infection did not show significant variation between breeds (Table 5). The similarity in herd management and variation in number of animals sampled per group might be accountable for the absence of significant variation of *Brucella* infection between the breeds. The present finding agrees with the previous reports of (Kebede *et al*., 2008; Amenu *et al.,* 2010; Asmare *et al*., 2013; Geresu *et al.,* 2016) who reported seropositivity of *Brucella* infection was independent of the breeds. However, Jergefa *et al*. (2009) found that breed of cattle has significant effect on the seroprevalence of brucellosis and he reported higher seroprevalence of brucellosis in cross-bred.

The finding of this study revealed that relatively higher seroprevalence of brucellosis was observed in intensive (1.3%) than semi-intensive (0.6%) production systems though the finding was not statistically significant. This similarity could be explained by the fact that both production systems manage their dairy production relatively in a similar way. However, this finding is not in line with the previous report of Jergefa *et al. (*2009) and Asmare *et al*. (2010) who have indicated a significant higher seroprevalence of *Brucella* among dairy cattle in intensive production systems of Ethiopia.

Parity was not found to be significantly associated with *Brucella* seropositivity in the univariable analysis (Table 5). In present study, 2 of the seropositive cows were in their pluriparus parity status whereas 1 from animals not yet gives birth. Furthermore, of parity status 57.7 % (n=188) of the female animals were pluriparus whereas about 42.3% were non pariturated (n=67) and primiparus (n=71). Hence, the present finding is in line with reports of (Tolosa *et al*., 2008; Adugna *et al*., 2013; Asmare *et al*., 2013) where parity status were not associated with animal level *Brucella* seropositivity. The reason of the similarity of *Brucella* infection among the parity

could be due to low sero-epidemiological distribution of bovine brucellosis in the present study.

In present study, a previous history of abortion and retained fetal membrane were significantly associated with animal level *Brucella* seropositivity (Table 5). This could be explained by the fact that abortions or /and retained placenta are typical outcomes of brucellosis (Swell *et al., 1990;* Radostits *et al*., 1994*)*. Aborted foetuses and discharges contain large number of infectious organisms and transmit the disease within and between herds. Other studies have also shown a significant association between seropositivity and history of abortion and retained fetal membrane (Berhe *et al*., 2007; Tolosa *et al*., 2008; Ibrahim *et al*., 2010; Megersa *et al.,* 2011a; Adugna *et al.,* 2013; Alemu *et al*., 2014). *S*imilarly, a number of studies in different African countries also show that individual animal *Brucella* seropositivity is associated with the number of abortions (Akakpo, 1987; Kubuafor *et al*., 2000; McDermott and Arimi, 2002; Schelling *et al*., 2003; Muma *et al*., 2012). However, the present finding is not in corcondance with the reports of (Kebede *et al.,* 2008; Asmare *et al*., 2013) where *Brucella i*nfections were not associated with reproductive disorders. This might be due to the presence of non-specific causes of abortion and/or RFM, or possibly to information bias from a lack of record-keeping by the herd owners.

The overall herd seroprevalence of bovine brucellosis was 10% (95% CI: 2.11%, 26.53%), and the results are briefly presented on (Figure 6). The prevalence of bovine brucellosis was low to moderate at herd level. The result was comparable with the previous finding of Asmare *et al*. (2013) 10.6% from Ethiopia .In addition, relatively similar herd seroprevalences (6.5–10.2%) were found in traditional cattle reared areas by other authors such as (Muma *et al*., 2007a, b; Yohannes *et al.,* 2013, and even in urban and periurban (Mugizi *et al*., 2015a). Nevertheless, higher herd level seroprevalences also have been recorded by other authors: 62 % from Zambia (Samui *et al*., 2007), 55% from Uganda (Faye *et al*., 2005), and 26.1 % (Megersa *et al*., 2011b), 13.6% (Jergefa *et al.,* 2009), 24.1 % (Mekonnen *et al.,* 2010) and 42.2% (Berhe *et al.,* 2007) from Ethiopia. The within-herd prevalence varied between nil to 16.67 % (1/6) based on CFT test. Similarly, when the 3 seropositive herds alone were considered, the within-herd apparent seroprevalence ranged from 2.94 % to 16.67%.The variation in seroprevalence within the herds

may indicate clustering and that more transmission occurs within herds than between herds. This indicates that the spread of bovine brucellosis between herds can be controlled through the elimination of seroconverters in the infected herds.

In the present study, risk factors related to respondents' age, sex and education level were studied to see their effect on herd prevalence of brucellosis. In fact, it was also noted that knowledge ages equal to or older than 55 years was a protective factor for brucellosis prevention *(*Coelho *et al.,* 2007*;* Díez *et al.,* 2013) although in the present study the overall effect of age groups of respondents were not significantly associated ($p>0.05$) with the herd seroprevalence of brucellosis . Even though Producer's associations, education level and veterinary support have been recognized as protective factors (Díez *et al.,* 2013*),* in present study herd prevalence was not showed significant variation across level of education of the respondents ($P> 0.05$).

The highest herd seroprevalence (50%) was observed in a group of herds with herd of size ≥ 50(large) animals whereas no reactor was observed in herds of size between 10-50 (medium) animals (Table 8). Increased in herd prevalence with increased herd size was observed with highly significant association with occurrence of bovine brucellosis ($p<0.05$) even if totally seronegative result was record in medium herd size. Such finding is in accordance with the earlier reports of (Tun *et al*., 2008) who reported significantly higher risk when the herd size is greater than 50 animals. Likewise, other researchers found herd size to be a significant factor in relation to herd seropositivity (Asfaw *et al.,* 1998; Haileselassie *et al.,* 2010; Ibrahim *et al*., 2010; Matope *et al.,* 2010; Shafee *et al*., 2011; Calistri *et al*., 2013; Adugna *et al.,* 2013; Patel *et al.,* 2014). On contrary, non-significant association between herd size and herd prevalence of brucellosis were also reported by (Kebede *et al.,* 2008; Tolosa *et al*., 2008; Jergefa *et al*., 2009; Chand and Chhabra, 2013).

Out of 30 herds, 90% (n=27) were indigenous - Holstein Frisian cross breeds whereas only 10% (n= 3) herds had pure Holstein Frisian cattle. But, the herd seroprevalence of bovine brucellosis was not significantly associated between the breeds.This could be due to the difference in numbers of breeds included in category (breeds were not proportionally included into the study).

The present finding is in line with the findings of (Kebede *et al*., 2008; Chand and Chhabra, 2013) who have reported seropositivity independent to breed and species, respectively. In contrary to the present findings, (Swai *et al*., 2005) reported significant association of breed with herd prevalence of brucellosis.

Different risk factors were also studied in the present study through questionnaire such as sources of replacement stock and selling of dairy cattle. None of these risk factors showed significant association with occurrence of bovine brucellosis. The present finding is in line with the findings of (Jergefa *et al*., 2009) who observed that sources of replacement stock had no effect on the herd prevalence of bovine brucellosis. However, unrestricted movement of animals (Gwida *et al*., 2010) and purchase of animals as replacement or breeding (Dias *et al*., 2009; Oliveira *et al*., 2013) and removal of sero-positive reactors after testing (Evangelista and Goncalves, 2009) are considered to be important risk factors for introduction of infection. Similarly, whether a herd raises its own replacement animals or purchases replacement animals affects the potential for introduction into the herd (Walker, 1999; Tolosa, 2004).

Farm hygiene was observed based on manure disposal, drainage, and physical appearance of the animal and ventilation status of the farms. Accordingly, most of the farms' hygiene were fair, and a few were good and poor (Table 8). This risk factor had no any statistical significant association with occurrence of brucellosis. Though, keeping good hygiene at dairy farm (Mugizi, 2009) are considered as a protective factor for brucellosis, unhygienic practices were identified as factors that will facilitate the spread of *Brucella* infections (Adesokan *et al*., 2013).The risk of infection among the level of factors might be overwhelmed by low sero prevalence bovine brucellosis found at study areas.

The distribution of different risk factors on exposure and maintenance of disease and their association with herd sero- prevalence of bovine brucellosis is presented in (Table 8). In almost all of the herds, there were no parturition maternity pen 90% (n=27) at all. Only about 20% (n=6) of the farms were disinfect with detergent the calving box (areas) after parturition whereas majority of the farms flushed only with water. None of these findings was statistical significance

with herd seropositivity to brucellosis. Even though statistically significant difference was not shown between these factors with *Brucella* seropositivity, lack of calving pen enhances risk of exposure and maintence of *Brucella* infection within the farm. Majority of herds were having control over visitors 66.7% (n=20) and stray animals 73.3% (n=22). However, none of these risk factors showed significant association with occurrence of bovine brucellosis at herd level. For instance, dogs carrying pieces of placenta or aborted fetuses from one place to another can disseminate the infection from one herd to another (Forbes and Tessaro, 1990). Indeed, it is a known fact that restriction over visitors and stray animals is helpful in reducing spread of infection which is further supported by an observation of (Tun *et al.,* 2008*)* who found significant increased prevalence with poor bio-security measures like control of visitors and stray animals.

The herd seroprevalence of brucellosis was higher in herds that had a history of abortion (25%) and retained fetal membrane (22.2%), compared with herds with no history of abortion (4.5%) or RFM (4.7%), respectively in (Table 8). These reproductive disorders (abortion history, RFM and still birth) showed significant association with occurrence of bovine brucellosis at herd level. The result was in accordance with the findings of researchers who had reported significant association with reproductive disorders like abortion, retention of placenta and still birth (Rahman *et al.*, 2011; Sikder *et al*., 2012; Chand and Chhabra, 2013) with herd level seropositivity. Of utmost importance is the separation of animals during calving and in the next weeks that follow, plus proper disposal of aborted foetuses and placentas, the removal of replacements born to infected mothers, and other well-known general hygiene measures (Corbel *et al*., 2006). It is a fact that the shedding of large numbers of organisms occurs during 10 days after abortion or calving of infected cows is the most significant feature of bovine brucellosis epidemiology and therefore, provision of calving box to down cowers is definitely helpful to reduce the chance of spread of infection if any FAO (2003). Still the result contrary to the finding of (Kebede *et al*., 2008) where reproductive disorders were not showed significant association with occurrence of bovine brucellosis at herd level.

Knowledge of diseases is a crucial step in the development of prevention and control measures (WHO, 2004).Thus, based on information gathered from the respondents, there was a huge gap

in knowledge on brucellosis (Table 9). Most of the respondents from small holder dairy farms relatively did not understand the root of zoonotic disease transmission including brucellosis. Farmer's lack of awareness about brucellosis, improper handling of aborted materials and the habit of consuming raw milk, among other factors, might contribute to further spread of brucellosis in their livestock and expose the community to a public health hazard (Megersa *et al*., 2011b). This low awareness is a limiting factor if control strategies are to be implemented. Lack of knowledge on the causative agent, mode of transmission and preventative measures against brucellosis can be detrimental. It is therefore important to establish an educational campaign in the study areas to enlighten the communities on the disease, risk factors as well as control strategies particularly in both livestock and humans.The respondents were practiced culling of dairy cattle when they faced reproductive problems than other problems.This could be best practice due to its protective effects but test and segregation is more preferred for further protection of the herds.

The serological test comparison was done in (Table 10) accordingly, almost perfect agreement with significant association was observed between c-ELISA and CFT (K=0.855) whereas moderate agreement RBPT (K=0.540) with CFT. The substantial agreement was also seen between RBPT and c-ELISA (K=0.69).This finding is consistent with Asfaw *et al*., (1998) who reported a moderate agreement (K=0.44) between RBT and CFT. On the other hand, it was conflicting with the finding of Abay (1999) (K=0.98) and Geresu *et al*. (2016) (K= 0.758) which were almost perfect agreement and substantial agreement between RBPT and CFT, respectively. Overall, a kappa statistic was used to measure the agreement between the various serological tests. In general, the kappa statistics were quite low, suggesting that various serological tests may detect different antibody isotypes. Thus, no single test is capable of giving conclusive diagnosis in detecting all positive cases. It is advisable to combine at least two serological test methods to screen and confirm brucellosis on herd level combine i.e. RBT and CFT.

6. CONCLUSION AND RECOMMENDATIONS

The present study revealed that the overall sero-prevalence of bovine brucellosis at animal level was 0.92% in selected dairy cattle of Bishoftu and Holeta towns, central Ethiopia. The herd level sero-prevalence of bovine brucellosis was also recorded 10%. Thus, it can be concluded that the overall prevalence of bovine brucellosis was relatively low at individual animal and low to moderate prevalent at herd level in study areas. Only history of abortion and retained fetal membranes are important risk factors associated with occurrence of bovine brucellosis at animal level. Moreover, in the present study the infection is strongly associated with large herds and is manifested by reproduction disorders at herd level. Lack of calving pen and low awareness of the communities are important gaps observed during the study. Above all, it is necessary to carry out careful herd management and implementing basic biosecurity measures in the study areas. Therefore, based on the present findings the following recommendations are worth mentioning:

- Strict pre-purchase tests policy has to be advocated among the farmers to prevent the entry of the diseases to naïve population.
- Proper farm sanitation should be practiced to reduce risk of exposure to *Brucella* species.
- Public education and awareness should be given among the community on the seriousness of the causes and modes of transmission of the diseases.
- Further isolation and characterization of the causative agents, the need to implement a transparency policy and effective control measures in Ethiopia.
- **F**urther intensive epidemiological study has to be conducted to establish the disease situation in other domestic animals and humans in the study areas.

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8. ANNEXES

Annex 1. Format used for individual Animal sampling

No	**Farm names**	**Breed types**	**Agro-ecology**	**Age**	**Parity status**	**Herd size**	**Abortion History**	**History of RFM**	**Management System**
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									

Annex 2. Questionnaire Format Used for Interview Purpose

I. Sociodemographic characteristics of the respondents

1. Name of owner (Respondent): _____

2. Gender of the respondent: ☐ Male (1) ☐ Female (0)

3. Age (years): _____

4. Marital status Single (1) ☐ Married (2) ☐ Widowed (3) ☐ Divorced (4) ☐

5. Respondent's position in the farms (with respect to the head):

☐ Farm manager (1) ☐ 2) husband ☐ wife (3) ☐ Relative living in a house (4) ☐

Farm laborer ☐ (5) other ☐ (6) Please specify_____

6. What is the highest level of education attained by the head of the respondent?

☐ Primary school (1) ☐ Secondary school (2) ☐ Vocational school (3)

☐ College / University (4) ☐ No formal Education (5)

II. General characteristics of the farms, farm management, ways of diseases exposure and introduction to the farms.

7. Herd size ----------

8. What types of breed of cattle you reared at your farm? a. Local ☐ b. Exotic breed ☐ c. Cross breed ☐

9. Where do you get the replacement stock? a. Market ☐ b. Raise own replacement ☐ c. Both ☐

10. What is the level of your herd/farm hygiene? a. Good ☐ b. Fair ☐ c.Poor ☐

11. What types of housing system of the farm? a. Loose ☐. Tying ☐ c. Both ☐

12. What types of watering system you use? a. Tap ☐ b. Under ground water ☐ c. Surface water ☐

13. Have you ever sold breeding females? a. Yes ☐ b. No ☐

14. If your answer is yes what was the reason of selling? a. Disease ☐ b. Infertility ☐ c. Shortage of money ☐

15. Do you control stray animals from your herd? a.Yes ☐ b. No ☐

16. Do you control visitors? a. Yes ☐ b. No ☐

17. Are there separate parturition maternity pen? a. Yes ☐ b. No ☐

III. Knowledge and practices on herd health management and brucellosis

18. What are your culling criteria? a. Reproductive problems ☐ b. Non-reproductive ☐ problems c. Both ☐

19. If reproductive problems what are they? a. Old age ☐ b. Poor production performance ☐ c. Both ☐

20. Do you know any disease transmitted from animal to human through handling of infected animals and its products? a. Yes ☐ b. No ☐

21. Do you know any zoonotic diseases that transmit through milk consumption a. Yes ☐ b. No ☐

22. Do you know any diseases that transmit during handling of delivery or abortion? a. Yes ☐ b. No ☐

23. Have you had cases of abortions in your herd in the past years? a. Yes ☐ No ☐

24. What do think that cause abortion in cows? a. Infectious disease ☐ Non infectious ☐

25. Have you ever heard of brucellosis? a. Yes ☐ b. No ☐

26. Have the farm/ herd been tested for brucellosis? a. Yes ☐ No ☐

27. What do you do with the known *Brucella* infected animlas? a. Culling ☐ b. Test and slaughter ☐ c. Both ☐

28. How do you handle aborted material?

a. Burying ☐ b. open dump ☐ c. feed to dog ☐ d. others_____

29. Is there any still birth in your herd so far? a. Yes ☐ b. No ☐

30. Do you separate cows during parturition? a. Yes ☐ b. No ☐

31. What do you do to the calving pen after parturition? a. Flushing with water ☐ b. disinfecting with detergents ☐ c. Both ☐

Annex 3. Rose Bengal Plate Test Procedures

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

1. 30 µl serum is placed and mixed with an equal volume of antigen on a white enamel plate to produce a zone approximately 2 cm in diameter.
2. The antigen and serum are mixed thoroughly using an applicator stick (a stick being used only once)
3. Plate is rocked by hand for about 4 minutes
4. It is examined for agglutination in a good light

Interpretation

0 = no agglutination

+ = barely perceptible

++ = fine agglutination, some clearing +++ = coarse clumping, definite clearing 97.

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

Annex 4. Competitive ELISA Procedure

1. All reagents should equilibrate to room temperature 18-25 °c before use.
2. Samples are added
    a. 45 µl of sample Dilution Buffer is added into each well that will be used for serum sample, serum controls and conjugate controls
    b. 5 µl of positive, weak positive and negative serum controls are added, into each of the appropriate wells, respectively. For conformation purpose it is recommended to run the control sera in duplicates.

c. 5 µl of Sample Dilution Buffer is added into two appropriate wells(designated as conjugate controls, Cc)
d. 5 µl of test sample is added 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. 50 µl of mAb- Solution is added 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. The plate are sealed and mixed the reagents thoroughly for 5 minutes, either by using a plate shaker or by tapping the sides of the plate.
5. The plate is incubated at room temperature 18-25°c for 30 minutes.
6. The plates/strips are rinsed 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. 100 µl of Conjugate Solution are added into each wells. The plate sealed and incubated at room temperature 18-25°c for 30 minutes.
8. Repeat step #6.
9. 100 µl Substrate solutions are added 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. The reaction is stopped by adding 50 µl 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. The Optical density (OD) of the controls and samples are measured at 450 nm in microplate photometer (use air as blank). The OD is measured 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 :

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

Annex 5. Complement Fixation Test procedure

Principle

The complement system consists of series of proteins that, if triggered by an antigen - antibody complex react in a sequential manner to cause cell lysis. The test has two steps, in the first step antigen, test serum and complement are mixed and incubated and in the second step an indicator system which consists of sheep red blood cells (SRBC) and Amboceptor (that sensitizes RBC to the action of complement) is added. If the test serum contains antibodies to *Brucella* an antigen- antibody complex is formed (positive reactions) and the complement is used up, so it cannot react in the hemolytic system. Therefore, no lysis of SRBC will occur and SRBC will remain intact. If the test serum does not contain *Brucella* antibodies (negative reaction) complement will not be fixed and lysis of RBC will occur .In indicator system SRBC and Amboceptor forms immune complex. In the positive test sample SRBC-amboceptor complex remains unaffected resulting in sedimentation of the RBC. The negative test sample has unbound complement that will bind to RBC-amboceptor immune complex this binding to the erythrocyte surface will disrupt the erythrocytes and hemolysis will occur.

Reagents

1. Complement
2. Hemolytic serum(Amboceptor)
3. Hemolytic system
4. *Brucella* antigen
5. Sheep Red Blood cells(SRBC)
6. *Brucella* abortus positive control serum
7. Negative reference serum
8. CFT buffer- veronal calcium magnesium(VCM)

9. Alsever's solution

Procedure

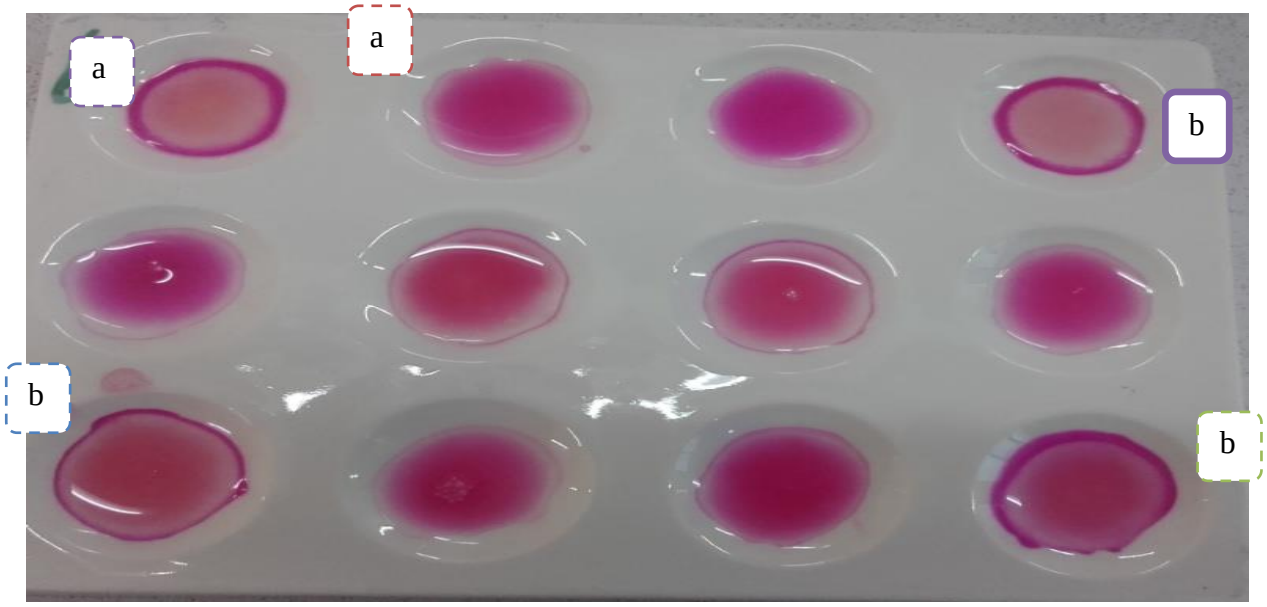
1. Test sera and appropriate working standards are diluted with an equal volume of veronal buffered saline in small tubes and incubated at 58°C (+/-2°C) for 30 minutes in order to inactivate the native complement.
2. A U-shaped 96-well microtitre plate are prepared
3. Column 1 through 12 is used for test serum
4. Each well of row "A" is used for anti-complementary control
5. 25 µL of VCM is dispense by using a hand held 12 channel micropipette into wells of rows A-H
6. Using standard 96-well U-bottom microtitre plates, 25 µL volumes of diluted test serum are placed in the wells of the A, B and C rows and homogenize wells of row C (column 1-12) of the test plate and pick up 25 µL from row C of the test plate and deliver to the wells of row D (column 1-12) .The serial dilution is continued to row H (column 1-12) from which after homogenization 25 µL is picked up and discarded.
   a. 25 µL of diluted antigen is added into all the wells of rows B-H and wells antigen and positive and negative control wells.
   b. 25 µL of diluted complement is added into the wells of rows A-H and all controls wells except hemolytic system.
   c. The plate is covered with sealer and incubated at +37°C under constant agitation on incubator shaker for 30 minutes.
   d. 25 µL of hemolytic system is added to all wells including control wells.
   e. The plate is covered with sealer and incubated at +37°C under constant agitation on incubator shaker for 30 minutes.
7. Control wells containing: Positive serum +diluents +Complement +Ag +HS, negative serum + complement + diluents+Ag +HS, antigen + complement + diluents +HS, HS + diluents and complement + diluents +HS, are set up to contain 75 µl total volumes in each case.

8. 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 6. Pictures of Serological Test Result



a*. Positive and negative control, respectively b*. Positive samples by RBPT

Annex 7. Pictures during sample collection and process

a. Picture during sample collection

b. Picture during sample processing

Annex 8. Ethical clearance certificate

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የ እንሳት ሕክምናና
ግብርና ኮሌጅ
ቢሾፍቱ/ደብረ ዘይት

ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu/Debre Zeit

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/26/06/09/2017

Name of Applicant: Kebede Shanko **(DVM,** MSc fellow)

Address: College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: Seroepidemiolgical study of bovine brucellosis in selected dairy cattle of Bishoftu and Holota towns, Oromia Regional State, Central Ethiopia

Date of application: **02/06//2017**
Nature of the project: **Non-invasive**
Target animal species: **Cattle**
Number of animals involved: **326**
Study area: Bishoftu and Holeta, **Ethiopia**

Minutes No. and date of review: **VM/ERC/06/09/017, 06/06/2017**

The above indicated research project is acceptable from ethical perspective, relevance, originality and technical competence points of view. Hence the project is allowed to be executed provided that:

1. All procedures and conditions stipulated in the proposal are respected and any deviation or changes be reported to the committee
2. The project activities be open for occasional supervision by the committee whenever this is deemed necessary

Dr Getachew Terefe
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Dr. Dinka Ayana
Dean
College of Veterinary Medicine and Agriculture

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ፋክስ} Fax 251-11-4339933
ስልክ } Tel. +251 114338450
P.o.x. Box}34
ቢሾፍቱ/ደብረዘይት፣ ኢትዮጵያ
Bishoftu/Debre Zeit, Ethiopia