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ISOLATION AND SEROPREVALENCE OF *BRUCELLA*: FROM DAIRY CATTLE IN AND AROUND ASELLA AND BISHOFTU TOWNS, ETHIOPIA

MSc Thesis

By

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Bishoftu, Ethiopia

ISOLATION AND SEROPREVALENCE OF *BRUCELLA*: FROM DAIRY CATTLE IN AND AROUND ASELLA AND BISHOFTU TOWNS, ETHIOPIA

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

By

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by **Minda Asfaw** entitled: **Isolation and seroprevalence of *Brucella*: From dairy cattle in and around Asella and Bishoftu towns, Ethiopia.** And recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Veterinary Microbiology.

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STATEMENT OF AUTHOR

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LIST OF ABBREVIATIONS

AD	After the Birth of Christ
AMOS PCR	Abortus-Melitensis-Ovis-Suis PCR
AP-PCR	Arbitrarily Primed PCR
AST	Allergic skin test
BC	Before Christ
BCAP	*Brucella* Capt
BCV	*Brucella*-Containing Vacuole
BMB	*Brucella* Medium Base
BPAT	Buffered Antigen Plate Agglutination -
BSL	Biosafety Level
CFT	Complement Fixation Test
CO_2	Carbon dioxide
DC	Dendritic Cell
DNA	Deoxynucleotide Acid
DS	Combination of Doxycycline with Streptomycin
EcoRV	Escherichia coli Restriction Enzyme V
ELISA	Enzyme Linked Immuno Sorbent Assay
ERIC-PCR	Enterobacterial Intergenic Consensus Sequences PCR
ery	erythrulose-1-phosphate dehydrogenase gene
FAO	Food and Agricultural Organization
FPA	Fluorescence Polarization Assay
g/l	gram per litre
H_2S	Hydrogen sulfide
HOOF	Hyper Variable Octomeric Oligonucleotide Finger-Prints
HRM	High Resolution Melt
I g	Immunoglobulin
IS	Insertion Sequence
KAP	Knowledge, Attitude and Practices
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-of-Flight
MAT	Microscopic Agglutination Test
MBM	Malachite *Brucella* Medium

ME	Mercaptoethanol
mg/L	Milligram per Liter
MGB	Minor Groove Binding Protein
MLSA	Multilocus Sequence Assay
MLVA	Multiple Locus Variable Number of Tandem Repeats Analysis
Mm	millimeter
MR	Methly Red
MSL	Metre above Sea Level
NAD	Nicotinamide adenine dinucleotide
NAHDIC	National Animal Health Diagnostic and Investigation Center
OD	Optical Density
OIE	Office International des Epizooties
omp	Outer Membrane Protein
PAHO	Pan American Health Orgnization
PCR	Polymerase Chain Reaction
Pg	Picogram
pH	Power of Hydrogen
RBPT	Rose Bengal Plate Test
REP-PCR	Repetitive Extragenic Palindromic PCR
RER	Rough Endoplasmic Reticulum
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
rpm	revolution per minute
RSAT	Rapid Slide Agglutination Assay Test
SAT	Serum Agglutination Test
SAT	Standard Slow Agglutination Tube Test
SNPs	Single Nucleotide Polymorphism Signatures
Spp	Species
T4SS	Type IV Secretion System
TIR	Toll/interleukin-1 receptor
TLR	Toll like Receptor
TSA	Tryptone Soy Agar
UI/L	International Unit per Litre

US	United States
USDA	United States Department of Agriculture
VNTR	Variable Number of Tandem Repeat
VP	Voges Proskauer
χ^2	Pearson's Chi-square

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ABSTRACT

Brucellosis is considered by the Food and Agriculture Organisation (FAO), the World Health Organisation (WHO) and the Office International des Epizooties (OIE) as one of the most widespread zoonoses in the world. A cross-sectional study was conducted in Asella and Bishoftu towns of Oromia Region of Ethiopia to isolate and determine seroprevalence of dairy cattle brucellosis. A total of 570 dairy cattle from 35 herds were purposively included in the study based on abortion history**.** Out of 46 clinical sample cultured 6.52% (3/46) overall rate of isolation was found in the study area and all the three isolates were *B. abortus* based on biochemical test result. *B. abortus* was isolated from placental cotyledon 1/9 (11.1%), vaginal swab 2/23 (8.69%) while no isolate was obtained from milk and fetal abomasal content of dairy cattle. All serum samples collected were tested and confirmed serologically using card test, rose Bengal plate test (RBPT), Indirect enzyme linked immuno sorbent assay (iELISA) and Complement fixation test (CFT). Risk factors analysis was also conducted using Chi-square and multivariate logistic regression analysis. An overall seroprevalence was estimated 1.4% (95% CI: 0.241, 3.461) by CFT while 16 (2.81%), 15(2.63%) and 13 (2.28%) were found positive by card test, iELISA and RBPT, respectively. The higher seroprevalence of 3.23% was observed in and around Asella compared to Bishoftu (0.52%) town. A Chi-square computed statistical analysis indicated that origin ($\chi2$=6.63; P<0.05), breed type ($\chi2$= 8.49; P<0.05), abortion history ($\chi2$=92.43; P<0.001) and abortion period ($\chi2$=192.97; P<0.001) were the major risk factors for *Brucella* infection. In addition, adult age (1.82%), intensive management system **(**1.82%), large herd size (2.17%) and pluriparous (2.41%) cows were found with higher sero positivity of *Brucella*. Multivariable logistic regression statistical analysis revealed that origin and breed type were significantly associated with seropositivity (P<0.05). Conse-quently, origin was statistically identified to be the major risk factor for brucellosis to occur in relation to other factors (OR=7.56) while breed type (OR=0.19) was the second major risk factor. Therefore, more proactive measures should be taken to protect the cattle populations from *Brucella* infection to reduce its ecomonic impact to the dairy industry and the risk of zoonotic infection in exposed human population.

Key words: Asella, Bishoftu, Brucellosis, Dairy Cattle, Isolation, Risk factors, Seroprevalence

1. INTRODUCTION

Brucellosis is considered by FAO, WHO and OIE as one of the most widespread zoonoses in the world (Schelling *et al.,* 2003). According to OIE, it is the second most important zoonotic disease in the world after rabies. The disease affects cattle, swine, sheep, goats, camels and dogs. It may also infect other ruminants and marine mammals. Synonyms of Brucellosis include: undulant fever, Malta fever, Mediterranean fever, enzootic abortion, epizootic abortion, contagious abortion, Bang's disease, Gibraltar fever, Cyprus fever, Rock fever and typhomalarial fever in animal and human. It is an important zoonotic disease and causes significant reproductive losses in sexually mature animals (Forbes and Tessaro, 1996; Mantur *et al*., 2007; Wadood *et al*., 2009).

The disease is manifested by late term abortions, weak calves, still births, infertility and characterized mainly by placentitis, epididymitis and orchitis, with excretion of the organisms in uterine discharges and milk. It also causes morbidity and considerable loss of productivity (Pappas *et al*., 2006a). The disease is important from economic point of view; it is one of the most devastating trans-boundary animal diseases and also a major barrier for trade (Gul and Khan, 2007).

Brucellae are gram-negative coccibacilli, which are classified into species by various techniques such as growth patterns on media and phage susceptibility. There are six "classical" species of the genus *Brucella* (*Brucella abortus (B.abortus), Brucella melitensis (B. melitensis), Brucella suis (B. suis), Brucella ovis (B. ovis), Brucella canis (B. canis),* and *Brucella neotomae* (*B. neotomae*) (Corbel, 1997; Moreno *et al*., 2002; Godfroid *et al*., 2005; Pal, 2007; Hadush and Pal, 2013). Recently, four new *Brucella* species have been recognized and classified (*Brucella pinnipedialis* (*B. pinnipedialis), Brucella ceti (B. ceti), Brucella microti (B. microti),* and *Brucella inopinata* (*B. inopinata)* (Foster *et al*., 2007; Scholz *et al.,* 2008a; Scholz *et al*., 2008b ; Scholz *et al*., 2009).

Three of this *Brucella* spp. can be subdivided in biotypes (Bricker, 2002a; Ocampo-Sosa *et al*., 2005). Therefore, three biotypes (1-3) have been identified in *B. melitensis*; eight biotypes (1-7, 9) in *B. abortus*; and five biotypes (1-5) in *B. suis* (Whatmore, 2009). All *Brucella* spp. are considered potentially pathogenic for humans, with the exceptions of *B. neotomae, B. microti*, and *B. ovis* (Xavier *et al*., 2009a; Hadush and Pal, 2013). Bovine brucellosis is an infectious and contagious disease known for its impact on reproductive performance of cattle in Africa and is predominantly a disease of sexually mature animals (McDermott and Arimi, 2002; Rahman *et al*., 2011; 2012; Asmare *et al*., 2013).

The disease is primarily caused by *B. abortus* and occasionally by *B. melitensis* where cattle are kept together with infected sheep or goats and characteristically associated with abortion at first gestation ("abortion storm" in naïve heifers) and is mainly caused by biovars(mainly biotype -1) of *B. abortus* (OIE, 2009a; Godfroid *et al*., 2010). Chronic infection of the mammary glands due to *B.suis* has also been reported (Lopes *et al*., 2010). Clinically bovine brucellosis is characterized by impaired fertility specifically with abortion, metritis, orchitis and epididymitis (Radostits *et al*., 2007; Seleem *et al*., 2010).

Brucellosis is also classified as one of the neglected zoonoses with a serious public health importance worldwide (OIE, 2009a) and has been eradicated in many developed countries (Geering *et al*., 1995; Makita *et al*., 2008). It however remains one of the seven zoonotic diseases listed by the World Health Organization (WHO) as "neglected". The disease has a great impact on both animal and human health as well as tremendous socio-economic impact in developing countries where rural income relies largely on livestock breeding and dairy products (Roth *et al*., 2003).

The mode of transmission of the bacteria varies with the epidemiological area, the animal reservoir and the occupational exposed groups (Seleem *et al*., 2010). Sources of infection for the transmission of the bovine brucellosis are aborted fetuses, the fetal membranes after birth, and vaginal discharges and milk from infected animals (Radostits *et al*, 2000; PAHO/ WHO, 2001; Tolosa, 2004). The most common route of transmission is the gastrointestinal tract following ingestion of contaminated pasture, feed, fodder, or water, and after birth, fetuses, and newborn calves, all of

which may contain a large number of the organisms and constitute a very important source of infection (PAHO/WHO, 2001) and it can be transmitted to humans through direct contact with infected tissue via breaks in skin, ingestion of contaminated tissues or milk products, and inhalation or mucosal exposure to aerosolized bacteria (Pal and Jain, 1986; Seifert, 1996; Radostits *et al*., 2007).

In livestock, the disease results in significant economic losses due to reproductive impairment caused by abortion, stillbirth or weak calves, neonatal mortality, infertility, loss of offspring, repeat breeding, decreased milk production, culling of valuable animals, death of animals due to metritis, the cost of veterinary attendance and replacer animals (Olsen *et al.*, 2004; Radostits *et al*., 2006; Xavier *et al*., 2009a; Abernethy *et al*., 2012). In humans, *Brucella* spp. infection causes a febrile disease that may be associated with a broad spectrum of symptoms, and it may be fatal in some cases (Cutler *et al*., 2005; Pal, 2007).

Animal brucellosis is therefore of significant public health importance for livestock farmers, dairy workers, slaughterhouse personnel, veterinarians, and laboratory personnel (Rahman *et al*., 2012). Raw milk consumption, close intimacy with animals and low awareness on zoonosis facilitate transmission of the disease to men (Megersa *et al*., 2012). Public health significance includes illness, physical incapacity and loss of manpower and also results in the scarcity of animal proteins due to loss of meat. Herdsmen are the most exposed members of the population. This occupational exposure is high in countries, where herding of animals is traditional and unsafe practices which exposed to infection (Kassahun, 2003).

Brucella spp. are also a focus of interest as they are categorized as bioterrorism agents due to their high contagiousness and their impact on human and animal health. *B. suis* was among the earliest agents investigated and developed as a bioterrorism weapon in the United States offensive bioterrorism program in the 1950s. The zoonotic pathogens *B. abortus*, *B. melitensis*, and *B.suis* have been identified as category B bioterrorism agents (Rotz *et al*., 2002). These *Brucella* spp. are also designated as selected agents by the US Government (CDC, 2005).

The most frequent manifestation of brucellosis in cattle is abortion, usually during the second half of pregnancy (Acha and Zyfres, 2003; OIE, 2011). Other clinical signs include stillborn or weak calves, retained placentas, decreased milk yield (estimated 20 to 25 percent loss), decreased fertility, orchitis, epididymitis, and infertility (Acha and Zyfres, 2003; Seleem *et al.*, 2010). The outcome of infection in cattle depends on age, reproductive and immunological status, natural resistance, route of infection, infectious challenge, and virulence of the infective strain (Carvalho-Neta *et al*., 2010).

A precise diagnosis of *Brucella* spp. infection is important for the control of the disease in animals and consequently in man. Clinical diagnosis is based usually on the history of reproductive failures in livestock, but it is a presumptive diagnosis (Poester *et al*., 2010) that must be confirmed by laboratory methods (Nielsen, 2002; Poester *et al*., 2010). The "gold standard" in the diagnosis of brucellosis is bacterial isolation from specimens that requires long cultivation periods (4 to 7 days up to 40 days) (Richman *et al*., 2000). Serological tests, as serum agglutination test (SAT), RBT, CFT, Card test and ELISA are still frequently used (Pal and Jain, 1986; Pal, 2007; Gwida *et al*., 2010).

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 Friedlander, 1997; Hamdy and Amin, 2002).

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

Since the first report of brucellosis in the 1970s in Ethiopia (Domenech, 1977; Meyer, 1980) the disease has been noted as one of the important livestock diseases in the country (Asfaw *et al*., 1998; Eshetu *et al.,* 2005; Ibrahim *et al*., 2010; Kebede *et al.,* 2008). A large number of studies on bovine have been reporting individual brucellosis seroprevalence ranging from 1.1% to 22.6% in intensive management systems (Asmare *et al.,* 2007; Hailemelekot *et al.,* 2007; Tolosa *et al*., 2010; Tesfaye *et al.,* 2011) and 0.05% -15.2% in extensive management system (Berehe *et al*., 2007; Hunduma and Regassa, 2009; Asmare *et al*., 2010; Degefa *et al*., 2011; Megersa *et al*., 2011). 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 and to design *Brucella* vaccine for control of brucellosis (Bricker, 2002a; Al Dahouk *et al*., 2003). Sufficient knowledge on epidemiology, particularly on species and biotypes of *Brucella* at national and/or regional level are important to set up and implement efficient control measures against brucellosis

Even though seroprevalence of brucellosis is established in different species of animals, direct diagnosis (bacteriological and molecular methods), which are the most reliable tests for identification and isolation of *Brucella* spp. and biovars, no published report have been found in Ethiopia except the attempt of Melesse *et al.(* 2007) who isolate *B. melitensis* from small ruminants in pastoral areas. There were attempts to isolate and identify *Brucella* spp. in dairy cattle at a farm level, however, they were not yet successful .

Therefore, the objectives of this study were.

- ✓ To isolate and identify *Brucella* spp.from dairy cattle in Ethiopia with history of abortion
- ✓ To determine the seroprevalence of brucellosis in dairy cattle and associated risk factors

2. LITERATURE REVIEW

2.1 . Historical Overview of Brucellosis

Brucellosis is an ancient disease that can possibly be traced back to the 5th plague of Egypt around 1600 BC. Recent examination of the ancient Egyptian bones, dating to around 750 BC, showed evidence of sacroiliitis and other osteoarticular lesions, common complications of brucellosis (Pappas and Papadimitriou, 2007) and examination of the skeletal remains of the Roman residents of Herculaneum (Naples, Italy) killed by the catastrophic volcanic eruption of Mt.Vesuvius in the late August, 79 AD revealed vertebral bone lesions typical of brucellosis in more than 17% of the residents. Scanning electron microscopy of recovered cheese provided a likely explanation for the high incidence of the disease (Sriranganathan *et al*., 2009). The buried carbonised cheese, made from sheep's milk and found with the bones, revealed the presence of cocco-bacillary forms that were morphologically similar to *Brucella* spp. (Capasso, 2002).

Eighteen centuries later, Sir David Bruce isolated *Micrococcus melitensis* (now *B.melitensis*) from the spleen of a British soldier who died from a febrile illness (Malta fever) common among military personnel stationed on Malta, an island not far away from Herculaneum (Godfroid *et al.*, 2005). For almost 20 years, brucellosis was thought to be a vector-borne disease. The zoonotic nature of the brucellosis was accidentally demonstrated in 1905 by isolating *B. melitensis* from goat's milk used for the production of soft cheese in Malta (Nicoletti, 2002a; Godfroid *et al*., 2005; Rust, 2006). It was believed that goats were not the source of infection since they did not become ill when inoculated with *Brucella* cultures. Although raw goat's milk had been used as an essential nutritional meal for hospitalized patients suffering from Malta fever, it was decided to ban it from hospitals. The public did not follow the same recommendation and consumed infected dairy products and remained exposed to the disease (Wyatt, 2005; Sriranganathan *et al.*, 2009).

Importantly, even as late as 1955, over 200 cases of brucellosis were caused by ingestion of a special cheese from Maltese goats (Wyatt, 1999). In 1895, 1914, and 1966, *Brucella* spp were isolated from aborted bovine, swine, and canine fetuses, respectively. In 1953, *B.ovis* was identified as a cause of epididymitis in rams (Nicolleti, 2002). In the last 15 years 3 new non-classical species of *Brucella* has been identified (Ross *et al.*, 1994; Foster *et al*., 1996; Scholz *et al*., 2008a).

2.2. Taxonomy and Evolution of the Family Brucellaceae

Brucellae are facultative intracellular coccobacilli belonging to the order Rhizobiales of the α-2 subgroup of Proteobacteria. The class alpha-proteobacteria includes organisms that are either mammalian or plant pathogens or symbionts (Garrity, 2001; Ficht, 2010). Within the family Brucellaceae, *Ochrobactrum* is the closest phylogenetic neighbour of *Brucella*. Historically, Brucellae are differentiated by host tropism, pathogenicity and phenotypic traits (Al Dahouk *et al*., 2013).

Although all members of the *Brucella* genus are closely related, the genus is divided into six classical species; *B. melitensis, B.abortus, B. suis, B. ovis, B. canis* and *B. neotomae* (Osterman and Moriyon, 2006). However, it was proposed to re-classify the genus as a monospecific genus *Brucella*, i.e. combining the six species into a single species with several biotypes (Verger *et al*., 1985). In addition to the classical *Brucella* spp., the genus has expanded to include marine isolates (*B. ceti* and *B. pinnipedialis*) and a species isolated from the common vole (*B. microti*) (Foster *et al*., 2007; Scholz *et al*., 2008a). Recently, *B. inopinata*, which is the only species that has not been isolated from an animal reservoir, was isolated from a breast implant infection in a woman with clinical signs of brucellosis (Scholz *et al.*, 2010).

What led to the division of *Brucella* spp. into the different species is the difference in biochemical capabilities, susceptibilities to dyes and phages together with the difference in host preference (Cutler *et al*., 2005). Although *Brucella* spp. have a strong affiliation to specific natural hosts, they can infect heterogonous hosts (Boschiroli *et al*., 2001). The bacteria may affect a range of different mammals including cattle, sheep, goats, swine, dogs, rodents, marine animals, several wildlife species and men. The disease primarily infects the reproductive system in most host species, with concurrent loss in productivity (Cutler *et al*., 2005).

2.3. Identification of *Brucella* Species

2.3.1. Isolation

Brucella spp. are chemo-organotrophic microorganisms requiring complex media containing several amino acids, thiamin, biotin, nicotinamide and magnesium salts, while X (haemin) and V (nicotinamide adenine dinucleotide [NAD]) factors are not required (Alton *et al*., 1988; Holt *et al*., 1994). Growth is inhibited on media containing bile salts, tellurite or selenite (Alton *et al*., 1988). The growth of *Brucellae* in simple nutrient liquid medium is usually poor unless these are supplemented with blood, serum or tissue extracts (Moyer and Holocomb, 2005). *Brucella* spp. are more fastidious than other aerobes and hence their growth in liquid medium is generally poor, unless the medium is vigorously agitated to improve aeration (Alton *et al*., 1988).

Most *Brucella* spp. can be isolated in unsupplemented, enriched peptone based media, or blood agar (Alton *et al*., 1988; Quinn *et al*., 1999). Good growth is obtained on *Brucella* medium base (Oxoid), sucrose dextrose agar (Oxoid), tryptone soy agar or glycerol dextrose agar (Oxoid) supplemented with 5% bovine or horse serum (OIE, 2004; Moyer and Holocomb, 2005; Al Dahouk *et al*., 2013). Cultures of Brucellae are usually established directly on solid media as it allows colonies to be isolated and recognised clearly, but liquid media may be used either for enrichment or for voluminous specimens. A non-selective biphasic Castaneda's medium is recommended for the isolation of *Brucella* spp. from blood or other body fluids or milk where enrichment culture is advisable (OIE, 2004).

Brucella spp.are slow growing and the use of selective media is recommended for primary isolation from most clinical specimens because of the high numbers of overgrowing contaminants (Marin *et al*., 1996). Such selective media are prepared by incorporating antibiotics and bacteriostatic dyes onto basic enriched media such as *Brucella* medium base (Oxoid). An example is Farrell's medium (Oxoid), prepared by adding six antibiotics; bacitracin, vancomycin, nalidixic acid, polymixin B, nystatin and cycloheximide onto sucrose dextrose agar for the isolation of *B. abortus* from contaminated milk samples (Farrell, 1974).

Farrell's medium was found not to be an ideal medium for the isolation of *B. melitensis*, because the concentrations of nalidixic acid and vancomycin in this medium have inhibitory effects on some strains (Marin *et al.,* 1996). Therefore, the use of modified Thayer-Martin'medium supplemented with haemoglobulin (10g/l), colistin methanesulphonate, vancomycin, nitrofurantoin, nystatin and amphotericin B in tandem with Farrell's medium is believed to enhance the chances of isolating *B. melitensis* (OIE, 2004). Recently, due to its carcinogenicity, cycloheximide has been removed from the *Brucella* selective supplements used in the Farrell's medium (Anon, 2005).

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

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

For the isolation of *Brucella* spp. from milk samples, although solid media have been used successfully (Farrell, 1974), the use of enrichment media such as serum dextrose, tryptone soy or *Brucella* broth containing selective supplements of at least amphotericin B and vancomycin should be used because the microorganisms are usually present in too low numbers to be detected on solid media (OIE, 2004).

2.3.2. Growth characteristics

Brucella spp.have an aerobic type of metabolism, using the cytochrome-based electron transport system with oxygen or nitrate as terminal electron acceptor (Holt *et al*., 1994). On primary isolation, many strains would require supplementation with 5-10% CO2 (Alton *et al*., 1988; Holt *et al.*, 1994). Although the growth of *Brucella* spp. may occur between 20 ºC and 40 ºC, growth occurs optimally at 37 ºC (Holt *et al*., 1994). In static broth medium, when *Brucella* spp. are incubated at 37 ºC for seven days, smooth strains produce moderately uniform turbidity with pale powdery deposit (Alton *et al*., 1988).

Non-smooth strains may produce granule deposit, variable turbidity and pellicle formation (Alton *et al*., 1988). In semi-solid media, CO2-dependent strains produce a disc of growth few millimetres below the surface, whereas CO2- independent strains produce uniform turbidity from the surface down to a depth of a few millimetres (Alton *et al*., 1988). On solid media, growth is not apparent until about 3 to 5 days of incubation (Quinn *et al*., 1999). Growth on selective media may be delayed by several days and some strains may not produce discernible colonies until about 14 days of growth (Alton *et al*., 1988; Walker, 1999).

Brucella spp. produces colonies that are round, glistening, pin-point, 1-2 millimetres in diameter, with smooth margins (Alton *et al*., 1988; Quinn *et al.,* 1999). Later they become larger and darker but remain clear (Alton *et al*., 1988). On serum dextrose agar, or any other clear medium, when examined under low power microscope, *Brucella* colonies have a raised surface, translucent with entire margins and displaying a characteristic pale "honey drop-like" appearance (Corbel and Brinley-Morgan, 1984).

On sheep blood agar, smooth strains of *Brucella* spp. produce small, glistening, smooth and non-haemolytic colonies which become opaque with age (Quinn *et al*., 1999). Some strains of *B.suis* may over time produce large colonies which appear mucoid. The colonies of non-smooth strains are dull, yellowish, opaque and when touched with an inoculation loop are found to be brittle (Quinn *et al*., 1999). *Brucella* spp. may or may not grow on MacConkey agar depending on the fastidiousness of the strains (Alton *et al*., 1988). The more vigorous strains of *B. abortus*, *B. melitensis* and *B.suis* will grow on MacConkey agar, producing small, non lactose fermenting colonies (Corbel and Brinley-Morgan, 1984).

2.3.3. Microscopic appearance

Brucella spp. is observed as gram negative cocci, coccobacilli or short rods. They are usually arranged individually and less frequently in pairs, short chains or small groups and do not usually exhibit bipolar staining (Holt *et al*., 1994; Garritty *et al*., 2005).

2.3.4. Biochemical reactions

On the basis of biochemical tests, members of the genus *Brucella* are broadly defined as catalase positive, oxidase positive (except *B. ovis*), urease positive (except *B. ovis* and *B. neotomae*), reduce nitrates to nitrites and do not exhibit motility in semi-solid media (Alton *et al*., 1988; Quinn *et al*., 1999). *Brucella* spp., with the exception of *B. neotomae*, does not produce acid from carbohydrates in conventional peptone media (Holt *et al*., 1994). In addition, they do not produce indole, gelatinases, haemolysins, acetyl methyl carbinol (Voges Proskauer test), formic and acetic acids from glucose (Methyl red test) (Holt *et al.*, 1994). A summary of the biochemical tests used to identify *Brucella* spp. is given in Tables 1 and 2. The details of the scheme for identification of members of the genus *Brucella* and their differentiation from closely related bacteria are provided elsewhere (Barrow and Felthman, 1993; Holt *et al*., 1994; Garritty *et al*., 2005).

2.3.5. Biotyping

Definitive identification and biotyping of *Brucella* colonies are performed by using various classical microbiological tests. The most important are: the dependence on serum and CO_2 for growth, the production of H_2S, growth in the presence of some dyes, agglutination with specific antisera and lysis by specific phages (Alton *et al*., 1988; Godfroid *et al*., 2013). To conduct these tests proficiently, submitting the isolated strains to an internationally recognised laboratory for definitive typing is recommended (Godfroid *et al*., 2013).

Table 1: Differential characteristics of *Brucella* spp. from some other gram negative bacteria (Alton *et al*., 1988).

Tests	*Brucella*	*Bordetella bronchoseptica*	*Campylobater fetus*	*Moraxella* species	*Acinetobacter* species	*Yersinia enterocolitica*
Morphology	Small cocco-bacilli	Small cocco-bacilli	Coma shaped	Diplococcoid	Diplococcoid	Rods
Motility at 37 ^{o}c	-	+	+	-	-	-
Motility at 20 ^{o}c	-	-	-	-	-	+
Lactose fermentation on MacConkey agar	-	-	-	v [a]	v	-
Acid production on media containing glucose	-[b]	-	-	-	v	+
Heamolysis on blood agar	-	+	_	v	v	-
Catalase	+	+	+	v	-	-
Oxidase	+[C]	+	+	+	-	-
Urease	+d	+	-	v	v	+
Nitrate reduction	+e	+	+	v	-	+
Citrate utilization	-	+	-	-	v	-

[a] Positive and negative species within the genus; b *B. neotomae* may show some fermentation; [c] Except *B. ovis, B. neotomae* and occasional *B. abortus* strains which are negative; [d] Except *B. ovis* and occasional *B. abortus* strains which are negative; [e] Except *B. ovis* which does not reduce nitrates to nitrites; V[a], variable

Table 2: Differentiation of the species and biovars of the genus *Brucella* (OIE, 2004; Garritty *et al*., 2005).

Charectirstics	*B. melitensis* biovars			*B.abortus* biovars								*B. suis* biovars					*B. ovis*	*B. neotomae*	*B. canis*
	1	2	3	1	2	3	4	5	6[b]	7	9	1	2	3	4	5			
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+[e]	+	+	+	+	-	-	+
Urease	+	+	+	+	+	+[f]	+	+	+	+	+	+	+	+	+	+	-	+	+
CO_2 requirement	-	-	-	[+]	[+]	[+]	[+]	-	-	-	-	-	-	-	-	-	+	-	-
H_2S production	-	-	-	+	+	+	+	-	[-]	[+]	+	+	-	-	-	-	-	+	-
Growth on media containing c																			
Thionin	+	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	-[d]	+
Basic fuchsin	+	+	+	+	-	+	+	+	+	+	+	[-]	-	+	[-]	-	[-]	-	[-]
Agglutination with monospecific antisera																			
A	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+	-	-	+	-
M	+	-	+	-	-	-	+	+	-	+	+	-	-	-	+	+	-	-	-
R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+

[a]Symbols: +, positive; [+], positive for most strains, [-], negative for most strains, -, negative all strains.

[b]For more certain differentiation of biovar 3 and 6, thionine at 1:25, 000 (w/v) is used; biovar 3 gives a positive growth response, biovar 6 is negative.,[c]Dye concentration, 1:50, 000 (w/v).

[d]Growth will occur in the presence of thionine at a concentration of 1:150, 000 (w/v).

[e]Rapid reaction, most strains of *B. suis* test positive within 5 minutes

[f]Some field strains of *B. abortus* may be negative

2.3.6. Molecular typing

The AMOS PCR (AMOS = abortus, melitensis, ovis, suis; PCR = Polymerase chain reaction) has been used to type *Brucella* spp. (Bricker, *et al*., 2003a). This test allows discrimination among *Brucella* spp. and between vaccine and wild-type strains but does not allow differentiation between the biovars of a given *Brucella* spp. However, not all the biovars of *B. abortus* (biovars 3, 5, 6, 7, 9) and *B.suis* (biovars 2, 3, 4, 5), some of which are epidemiologically important in Europe, can be identified in this assay. A newly developed multiplex PCR assay (named Bruce-ladder) can, for the first time, identify and differentiate between all the *Brucella* spp. and the vaccine strains in the same test (López-Goñi *et al*., 2011).

New techniques appear promising, such as single nucleotide polymorphism signatures (SNPs), aimed at detecting DNA sequence variations that occur when a single nucleotide in the genome differs between members of a species; MLSA, aimed at directly measuring the DNA sequence variations in a set of housekeeping genes and characterising strains by their unique allelic profiles; and MLVA, which analyses the variability of loci presenting repeated sequences (Le Flèche *et al*., 2006; Whatmore, 2009).

Molecular typing of brucellae has been attempted using DNA-DNA or DNA-RNA hybridisation methods, PCR based methods such as the repetitive extragenic palindromic PCR (REP-PCR) and the enterobacterial intergenic consensus sequences PCR (ERIC-PCR) (Mercier *et al.*, 1996), the arbitrarily primed PCR (AP-PCR) (Fekete *et al*., 1992) and the restriction fragment length polymorphism PCR (RFLP-PCR) (Cloeckaert *et al*., 2001).

These PCR based methods have been reviewed in detail by Bricker (2002a). Recently, using the variable number of tandem repeats (VNTR) analysis(Bricker *et al.*, 2003b) found the technique to be the most discriminatory for *Brucella* spp. However, using this technique, *Brucella* spp.have been found to be highly homogenous. Other studies of the genome of *Brucella* spp. have demonstrated the existence of more than 90% homology (Clavareau *et al*., 1998; Cloeckaert *et al*., 2001) and based on DNA-DNA hybridisation, a single species of *B. melitensis* has been proposed, with the other species being biovars (e.g. *B. melitensis* biovar *abortus*) (Verger *et al*., 1985).

This genomic similarity makes the differentiation of *Brucella* spp. difficult, and often a study of biological and physiological characteristics is required (Alton *et al*., 1988). The debate of whether the Brucellae should comprise a single genospecies or multiple species has been a source of much controversy (Cutler *et al*., 2005). But the recent reappraisal of the *Brucella* spp. by review of their population structure and analysis of their genetic diversity by methods other than DNA-DNA hybridisation (Moreno *et al*., 2002) has reasserted the return to the pre-1986 taxonomy where the multiple species and biovars concept is used (Osterman and Moriyon, 2003; Foster *et al*., 2007). The recent discovery of new *Brucella* spp. from marine mammals has given further support for use of this nomenclature (Cloeckaert *et al*., 2001).

2.4. Pathogenesis of Brucellosis

Brucellae have a predilection for macrophages, dendritic cells (DCs) and trophoblasts (Billard *et al*., 2005) and the bacteria can enter, survive, and replicate within these cells and cause disease (Delrue *et al.*, 2004). *Brucella* gain access to the host through inhalation, conjunctiva, skin abrasions and ingestion (Vassalos *et al*., 2009). The virulence factors of *Brucella* spp. are unknown, although there have been preliminary reports of involvement of toxins, fimbriae and plasmids, none of these has been confirmed, and like other gram-negative bacteria, lipopolysaccharides (LPS) are presumed to play an important role (Ficht, 2003; Delrue *et al*., 2004).

The ability of *Brucella* spp. to cause disease requires a few critical steps during infection. *Brucella* spp. 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* spp. (Ackermann *et al.,* 1988). Once *Brucella* spp.have invaded, usually through the digestive or respiratory tract, they are capable of surviving intracellularly within phagocytic or non-phagocytic host cells (Carvalho-Neta *et al*., 2010). *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* (Anderson *et al*., 1986; Pizarro-Cerdá *et al*., 1998; 2000).

The mechanisms that allow host cell invasion by *Brucella* spp. are not completely clear, but although specific host receptors that interact with *Brucella* have not yet been identified, internalisation of *Brucella* into host cells requires cytoskeletal changes (Pizarro-Cerda *et al*., 1999; Guzmán-Verri *et al*., 2001). Interestingly, invasion through the digestive tract does not elicit any inflammatory response from the host (Paixão *et al*., 2009). Therefore, *Brucella* spp. invades silently or unnoticed by the innate immune system of the host. In fact, *Brucella* spp.have mechanisms that prevent activation of the host innate immune system (Barquero-Calvo *et al.,* 2007). Indeed, *Brucella* Toll/interleukin-1 receptor (TIR) domain-containing protein prevents Toll-like receptor (TLR) 2 signalling by interfering with MyD88, and also inhibits DC maturation, cytokine secretion and antigen presentation (Cirl *et al.,* 2008; Salcedo *et al.,* 2008).

B.abortus also induces suppression of the transcription of pro-inflammatory mediators in trophoblastic cells at very early stages of infection (Carvalho-Neta *et al.,* 2008). Trophoblasts are placental cells that are targeted during infection of pregnant cows. After an initial suppression of pro-inflammatory transcripts, *B. abortus* induces expression of pro-inflammatory chemokines by cultured trophoblastic cells, which correlates with the profile of expression observed *in vivo* in the placenta of infected cows (Carvalho-Neta *et al*., 2008; Poester *et al.,* 2013).

Brucella spp. lack classical bacterial virulence factors such as exotoxins, cytolysins, a capsule, fimbriae, flagella, plasmids, lysogenic phages, endotoxic lipopolysaccharide (LPS), and inducers of host cell apoptosis (Seleem *et al*, 2008). However, LPS plays an important role in *Brucella* virulence because it prevents complement-mediated bacterial killing and provides resistance against antimicrobial peptides such as defensins and lactoferrin (Allen *et al.*, 1998; Lapaque *et al*., 2005). Another important virulence mechanism of *Brucella* is the BvrR/BvrS two-component regulatory system, which is required for modulation of the host cell cytoskeleton upon *Brucella* invasion, and for regulation of the expression of outer membrane proteins, some of which are required for full virulence (López-Goñi *et al*., 2002). Cyclic β-1, 2-glucans, which are also part of the outer membrane, is also required for intracellular survival of *Brucella* (Briones *et al*., 2001).

Brucella spp. express a type IV secretion system (T4SS), encoded by the components of the *virB* operon, that is crucial for intracellular survival in host cells and virulence *in vivo* (Hong *et al.,* 2000; O'Callaghan *et al.,* 1999). Unlike other type IV systems, which are expressed extracellularly, transcription of the *virB* operon is induced specifically within macrophages, and phagosome acidification is a key intracellular signal inducing *virB* expression (Boschiroli *et al.,* 2002).

The *Brucella* T4SS is required for persistence in mice and induction of the host immune response (Rolán and Tsolis, 2007; Roux *et al*., 2007). It is also essential for elicitation of inflammatory and immune responses during *Brucella* infection in mice (Roux *et al*., 2007), and it is required for microgranuloma formation during *Brucella* infection (Rolán *et al*., 2009). The T4SS is required for *Brucella* to reach its intracellular replication niche (Celli *et al*., 2003), and its expression is regulated by the BvrR/BvrS two-component regulatory system (Martínez-Núñez *et al*., 2010). The T4SS delivers *Brucella* effector proteins into the host cell cytosol, but the identities and roles of these effectors have only recently begun to emerge (Marchesini *et al*., 2011).

A novel strategy used for screening proteins of unknown function that are involved in protein-protein interactions was used to identify *Brucella* effectors. Using this approach, four putative proteins (BPE043, BPE005, BPE275 and BPE123), which were translocated into mouse macrophages by *B. abortus*, are hypothesised to be candidates for modulation of host cell functions (De Jong and Tsolis, 2012).

2.5. Epidemiology

2.5.1 .Prevalence of infection

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 (Mantur *et al.*, 2007). Brucellosis is the most common zoonotic infection worldwide (Corbel, 1997). It is endemic in the Mediterranean region, the Middle East, Latin America and parts of Asia and Africa, but the epidemiology is changing over the last decades due to socioeconomic changes, improved disease recognition and eradication programmes (Pappas *et al.*, 2005a).

2.5.2. Transmission

Brucella spp. is commonly transmitted to other animals by indirect or direct contact with infected animals or their discharges (OIE, 2009a). Transmission in cattle occurs mainly by ingestion of contaminated feed and water by organisms, which are present in large numbers in aborted fetuses, fetal membranes and uterine discharge (FAO, 1986; Acha and Szyfres, 2001). However, infection through injured/intact skin, the mucosa at the respiratory system and conjunctiva frequently occurs (Seifert, 1996; Degefa *et al*., 2011).

Brucellosis is transmitted to human through contaminated and unpasteurized milk and milk products or by direct contact with infected animals or animal carcasses. Abortion materials, uterine exudates, and colostrum are highly infectious (Earhart *et al*., 2009). Primary routes of infection include penetration of the oral or gastric mucosa through ingestion of unpasteurized or contaminated dairy products, inhalation and penetration of the ocular mucosa, or through direct inoculation into the bloodstream through abrasions in the skin or vaccination. Occupational exposure to animals or animal products is the most common risk factor for brucellosis. Abattoir workers, farm or dairy workers, veterinarians and veterinary assistants, as well as healthcare and laboratory workers are well-recognized risk groups (Hartigan, 1997; Memish and Mah, 2001).

Humans acquire brucellosis mainly through ingestion of contaminated milk and unpasteurized dairy products. Contact of mucosas 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).

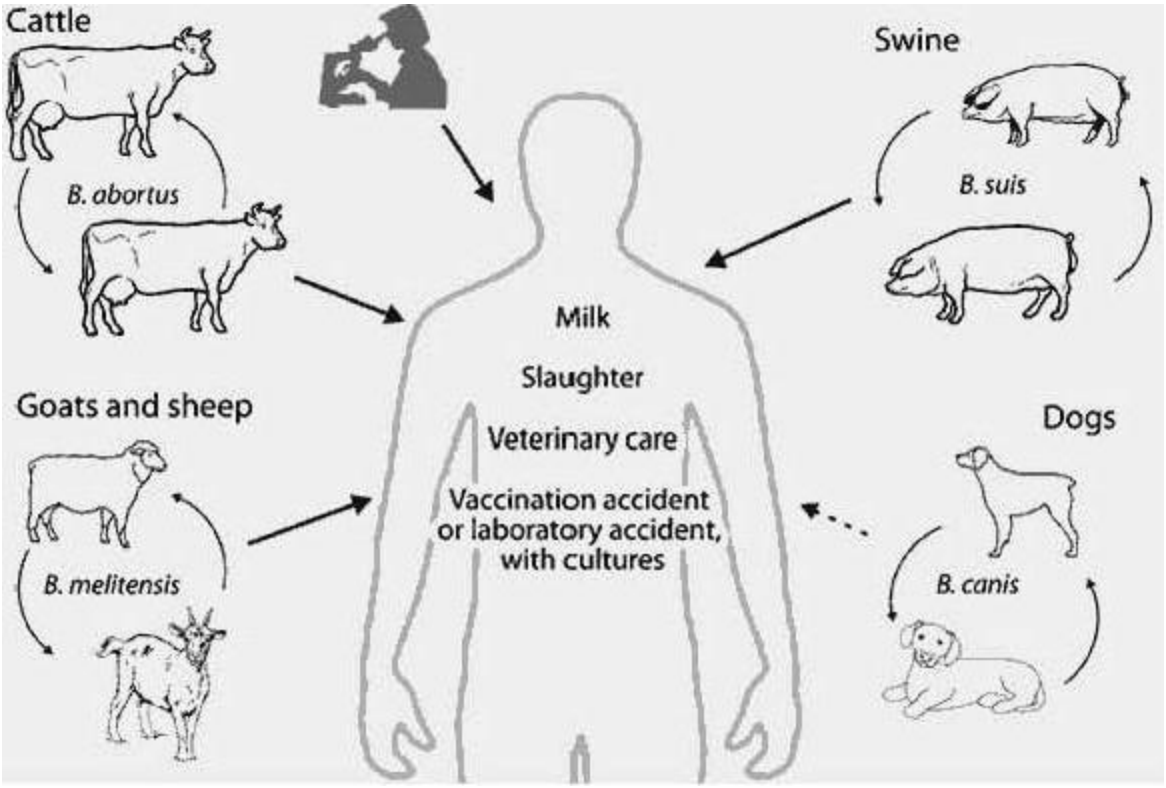

Figure 1: Transmission of *Brucella* to humans (Gadaga, 2013)

2.5.3. Reservoirs of Brucella species

Brucellosis is a worldwide zoonotic disease caused by *Brucella spp.* 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* spp can survive in proper environmental condition, damp soil and seawater and can be a source of infection. Notably abortion materials such as placenta, fetal parts, 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).

2.5.4. 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* spp. in laboratories. Other risk factors include: abattoir workers, veterinarians, slaughterhouse workers and dairy workers (Corbel, 1997).

2.5.4.1. Host risk factors

Susceptibility of livestock to *Brucella* infection is influenced by the age (young animals are less susceptible to *Brucella* than older animals), 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).

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).

2.5.4.2. Agent 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* spp., 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).

2.5.4.3. 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*., 1994; 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).

2.6. Clinical Manifestations

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 and metritis (Walker, 1999; Acha and Szyfres, 2003; Shareef *et al.,* 2006). The infected cows usually abort only once after which a degree of immunity develops and the animals remain infected. At subsequent calvings, the previously infected cows excrete huge numbers of *Brucella* in the fetal fluids (Silva *et al*., 2000). Brucellosis does not usually result gross organic lesions (Schlafer and Miler, 2007), but sometimes a mild interstitial inflammatory reaction in the mammary gland may be observed ,which is associated with elimination of bacteria in the milk (Xavier *et al*., 2009a).

Bulls can be infected but they do not readily spread the disease. *B. abortus* is a common cause of orchitis that is often associated with a vesiculitis and epididymitis. Infection in males may result in either temporary or permanent infertility, depending on the intensity of the lesions (Eaglesome and Garcia, 1992; Megid *et al*., 2010). Orchitis is occasionally manifested, and when it occurs it is usually unilateral, but both testicles may be affected. Scattered foci of necrosis coalesce to produce total testicular necrosis (Foster and Ladds, 2007).

2.7. Diagnosis

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

2.7.1. Direct methods for diagnosis of brucellosis

2.7.1.1. Bacteriological diagnosis

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* spp. is challenging. *Brucella* spp. is a fastidious bacterium and requires rich media for primary cultures. Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper storage and quick delivery to the diagnostic laboratory and it requires BSL3 facilities which is not available in most developing countries (Refai, 2003; Seleem *et al*., 2010; Hadush and Pal, 2013).

Contamination of clinical samples is a complicating factor for *Brucella* spp. isolation. Therefore, the use of nutrient-rich media supplemented with antibiotics (Polymixin B 5,000 UI/L; bacitracin 25,000 UI/L; cyclohexamide 100 mg/L; nalidixic acid 5 mg/L; nystatin 100,000 UI/L and vancomycin 20 mg/L) is used to inhibit growth of contaminants that may prevent isolation of *Brucella* spp. (De Miguel *et al*., 2011).

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

Vaginal secretions should be sampled after abortion or parturition, preferably using a swab with transporter medium, allowing isolation of the organism up to six weeks post parturition or abortion (Poester *et al*., 2010). Milk samples should be a pool from all four mammary glands. Non pasteurized dairy products can also be sampled for isolation (Lage *et al.*, 2008; Poester *et al*., 2010). Samples of choice in slaughterhouses include mammary, iliac, pharyngeal, parotids and cervical lymph nodes, and spleen. Samples must be immediately sent to the laboratory, preferentially frozen at -20 ºC (+4 ºC for milk sample), and they must be identified as suspect of *Brucella* spp. infection (Poester *et al*., 2010). Vaginal swabs, semen and seminal fluid have low numbers of viable organisms, and therefore isolation is more difficult, often resulting in false negative results. Enrichment media containing selected antibiotics can improve the sensitivity in these cases (De Miguel *et al*., 2011).

Brucella spp. colonies are elevated, transparent, convex, with intact borders, smooth, and a brilliant surface. The colonies have a honey 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. Some *Brucella* spp. requires CO_2 for growth. Typical colonies appears after 2 to 30 days of incubation, but a culture can only be considered negative when there are no colonies after 2 to 3 weeks of incubation. False negative results should be considered in the absence of bacterial growth since the sensitivity of culture is low (Poster *et al*., 2010).

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).

2.7.1.2. Immunohistochemistry

Immunohistochemistry is an alternative technique for direct diagnosis of *Brucella* spp. 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).

2.7.1.3. Molecular methods for *Brucella* species genotyping

Molecular techniques are important tools for diagnosis and epidemiologic studies, providing relevant information for identification of species and biotypes of *Brucella* spp., allowing differentiation between virulent and vaccine strains (Ocampo-Sosa *et al*., 2005; Le Flèche *et al*., 2006; López-Goñi *et al*., 2008). Molecular detection of *Brucella* spp. can be done directly on clinical samples without previous isolation of the organism. In addition, these techniques can be used to complement results obtained from phenotypic tests (Bricker *et al*., 2002b).

PCR and /its variants, based on amplification of specific genomic sequences of the genus, species or even biotypes of *Brucella* spp., are the most broadly used molecular technique for brucellosis diagnosis (Bricker, 2002a).The technique is chosen based on the type of biological sample and the goal, i.e. diagnosis or molecular characterization or epidemiological survey. Most of the molecular diagnostic methods for brucellosis have sensitivity ranging from 50% to 100% and specificity between 60% and 98%. The DNA extraction protocol, type of clinical sample, and detection limits of each protocol, are factors that can influence the efficiency of the technique (Mitka *et al*., 2007).

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).

PCR DNA-based methods such as gene probes and PCR utilize primers derived from different polymorphic regions in the genomes of *Brucella* spp. Different PCR methods for the detection of *Brucella* spp. that utilize primers derived from different polymorphic regions in the genomes of *Brucella* spp. as i.e. (1) a gene encoding a 31-kDa *B. abortus* antigen which is conserved in all *Brucella* spp. (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-k-Da (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.

Multiplex PCR typing: 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. The first multiplex PCR, called AMOS PCR assay 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. Identity was determined by the size of the product amplified from primers hybridizing at various distances from the element. This method could identify three biovars (1, 2, and 4) of *B. abortus*, all three biovars of *B. melitensis*, all *B. ovis* biovars and biovar 1 of *B. suis*. An abbreviated multiplex AMOS PCR assay based on three additional primers was developed to differentiate *B. abortus* vaccine strains S19 and RB51 from field strains (Ewalt and Bricker, 2000). In 2005, the finding of a deletion next to one of the *IS711* copies in *B. abortus* biovars 5, 6, 9 and in some field strains of biovars 3 of *B. abortus* has allowed to design and add a specific primer to the eight primer mixtures of AMOS PCR, allowing to enhance the discrimination power of this assay (Sosa *et al*., 2005).

A random amplified polymorphic DNA (RAPD-PCR) was used in order to develop a multiplex PCR that uses the AMOS primers, additional specific loci of the insertion element *IS711*, and other unique insertions and deletions. This novel PCR assay differentiates between all presently recognized *Brucella* species, including the recently described species *B. ceti* (formerly named '*Brucella maris*' or '*Brucella cetaceae*'), *B. pinnipedialis* (formerly named '*Brucella maris'* or '*Brucella pinnipediae*'), and *B. microti*, including some more recently described strains of the latter species (Scholz *et al.,* 2008a; 2008b;2009), and also allows accurate differentiation of certain biovars of *B. abortus* and *B. suis* (Huber *et al*., 2009).

A new generation of multiplex PCR assays has been developed on the basis of the knowledge arisen from the recent availability of genome data. Garcia Yoldi *et al*. (2006) described a multiplex PCR assay for the identification of all six classical species, *Brucella* isolates from marine mammals, the vaccine strains *B. abortus* RB51 and S19 and *B. melitensis* Rev 1. The eight species-specific primer pairs amplified fragments of different sizes that showed a unique profile for each species following agarose gel electrophoresis. However, this multiplex PCR was unable to differentiate

B. microti from *B. suis* and *B. ceti* from *B. pinnipedialis*. A similar multiplex approach based on species-specific differences was recently described as being able to distinguish the six classical species but with some problems with *B. canis* and *B.suis* differentiation (Hinic *et al*., 2008). In addition some single target PCRs have proven particularly useful e.g. the presence of an insertion sequence, *IS711*, downstream of the bp26 gene, a feature specific to the marine mammal *Brucella* strains (Cloeckaert *et al*., 2000a).

An advancement of the Garcia Yoldi protocol for the differentiation of all currently `described *Brucella* species was published by Mayer-Scholl *et al*. (2010). The primer pair identifying *B. microti* (Scholz *et al*., 2008a) was included in the multiplex PCR described by Garcia Yoldi *et al*. (2006), and the assay was set up on the DNA of *Brucella* reference strains and field isolates. The assay allowed the identification of all currently known *Brucella*, distinguishing also between the marine species *B. ceti* and *B. pinnipedialis* and identifying the recently described species *B. microti* and *B. inopinata*.

Real-time PCR: Real-time PCR 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. 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, and paraffin-embedded tissues (Kattar *et al*., 2007).

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).

High resolution melt: The development of a molecular technique which utilizes real-time PCR followed by high-resolution melt (HRM) curve analysis to reliably type members of this genus have been described by Winchell *et al*. (2010). The assay targeted discriminating loci within the genomes of *Brucella* spp. and through the dissociation curve analysis allowing the accurately identification of *Brucella* isolates at the species level and of unusual *Brucella* isolates such as BO1 and BO2. This assay also proved successful for discriminating *B. suis* from *B. canis*, but was unable to accurately differentiate a *B. suis* biovar 4 from *B. canis*. However, this particular *B. suis* biovar has previously been reported to exhibit a genotypic pattern identical to *B. canis*, and it is still debated as to whether this is truly a unique biovar of *B. suis* (Whatmore *et al*., 2007; Huynh *et al*., 2008).

Restriction fragment length polymorphism based approaches: PCR-restriction fragment length polymorphism (PCR-RFLP) is a common approach for typing of *Brucella* spp., providing a good tool for taxonomic, epidemiological, evolutionary and diagnostic studies. The method has especially been utilized in studies of various outer membrane protein (omp) genes (Al Dahouk *et al*., 2005).

Recently, PCR-RFLP has provided evidence of polymorphism in a number of genes including the outer membrane protein 2 (omp2), the heat shock protein, dnaK, htr, and the erythrulose-1-phosphate dehydrogenase gene (ery). Particularly, the DNA polymorphism in omp2a, omp2b, omp25 and omp31 has been found to be useful for the differentiation between the *Brucella* species and their biovars, including the marine mammal *Brucella* isolates (Bricker, 2002b; Al Dahouk *et al*., 2005). Results of PCR-RFLP allowed to identify in omp25 a marker for *B. melitensis* in the form of absence of an EcoRV site though *B. suis* biovars 3 and 4 and *B. canis* could still not be distinguished. Other omp genes examined include omp31, known to be deleted in *B. abortus* (Vizcaino *et al*., 1997), but which has markers for *B. canis, B. suis* biovar 2 and *B. ovis* (Garcia Yoldi *et al*., 2005).

Single nucleotide polymorphisms typing: Single nucleotide polymorphisms (SNPs) represent powerful markers that allow accurately describing the phylogenetic framework of a species, particularly in a genetically conserved group as *Brucella*. The approach is based on a series of discrimination assays interrogating SNPs that shown to be specific to a particular *Brucella* spp. Scott *et al*. (2007) described the use of SNPs in order to develop a multiplex SNP detection assay, based on primer extension technology that can rapidly and unambiguously identify an isolate as a member of one of the six classical *Brucella* spp. or as a member of the recently identified marine mammal group. An alternative approach based on minor groove binding protein (MGB) probes applied on a real-time PCR platform was described (Foster *et al*., 2008; Gopaul *et al*., 2008).

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Bacterial identification based on peptidic spectra obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was proposed 30 years ago. This method represents a new diagnostic tool in established microbiological laboratories (Seng *et al*., 2009). Databases have been developed that include the main pathogenic microorganisms, thus allowing the use of this method in routine bacterial identification from plate culture. Recently, to identify *Brucella* species a reference library was constructed using 12 *Brucella* strains. With this '*Brucella library'* discrimination was not possible to the species level (Ferreria *et al*., 2010).

Tandem repeat based typing: In the last years the availability of microbial genome sequences has facilitated the development of multilocus sequence-based typing approaches such as multiple locus variable number of tandem repeats (VNTR) analysis (MLVA). The VNTR, allelic hyper variability related to variation in the number of tandemly repeated sequences observed at several genomic loci in the *Brucella* genomes, were used for the discrimination of bacterial species that display very little genomic diversity. The first application of VNTR based typing to *Brucella* was the HOOF-Prints scheme (Hyper variable Octomeric Oligonucleotide Finger-Prints) published by Bricker *et al*. (2003b).

2.7.2. Indirect methods for diagnosis of brucellosis

2.7.2.1. Serological tests

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

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 (Nielsen, 2002b; Poester *et al*., 2010).

Standard slow agglutination tube test: Standard slow agglutination tube test (SAT), which was the first developed serological test for diagnosis of brucellosis, is based on bacterial antigen agglutination, particularly by IgM under neutral pH. This test has low specificity, and therefore it is not recommended (OIE, 2009a; Poester *et al*., 2010).

Milk ring test (MRT): The milk ring test is based on agglutination of antibodies secreted into the milk. This test allows screening of large number of cattle by using milk samples from tanks or pools from several cows. 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. 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).

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. 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). 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).

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

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

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

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

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

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

The indirect ELISA (iELISA) 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). O ELISAi has been usually used for smooth LPS *Brucella* spp., 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 ELISAi varies from 96 to 100%, and its specificity from 93.8% and 100% (Gall *et al*., 2001; Gall and Nielsen, 2004). The competitive ELISA (ELISAc) with smooth *Brucella* LPS as antigen is used for detection of anti-*Brucella* in serum samples from cattle, sheep, goats, and pigs. This test is capable of differentiating vaccine antibody response from actual infections, and its sensitivity varies from 92 to 100%, whereas the specificity ranges from 90 and 99% (Godfroid *et al*., 2010; Perrett *et al*., 2010).

Fluorescence polarization assay: The fluorescence polarization assay (FPA) was initially developed for testing serum. However, the technology has been extended to testing whole blood and milk samples from individual animals.The FPA is based on the rotational differences between a small soluble antigen molecule in solution and the antigen molecule complex with its antibody. It measures the size of a fluorescent tagged molecule such as an antigen. The utilization of the O-side chain of LPS from *Brucella* spp. has shown encouraging results. The test is a valuable alternative to conventional serological tests. Sensitivity of FPA is 96% for culture-confirmed human brucellosis and specificity is about 98% (Nielsen and Gall, 2001; Supriya *et al*., 2010).

The fluorescence polarization assay has been used for the diagnosis of *Brucella* spp. infection in man (Lucero *et al*., 2003) and several animal species, using serum, milk or whole blood in EDTA (Gall *et al*., 2001). This test can be performed under field conditions (Nielsen, 2002b). Sensitivity of the fluorescence polarization assay varies from 87.5 and 100%, and specificity from 84 to 100% (Godfroid *et al*., 2010), which is similar to the levels obtained with ELISAc (Gall *et al*., 2001).

Agar gel immunodiffusion test: The agar gel immunodiffusion test is based on precipitation of the antigen-antibody complex. This method is often used for the diagnosis of *B. ovis* infection. This test has a low cost, it is easily performed and it has sensitivity levels that are comparable to complement fixation. However, it has some disadvantages such as a marked decrease in sensitivity in chronic infections and high variability of the quality of commercially available antigens. Therefore, it is highly advisable to perform complementary diagnostic techniques such as PCR (Costa *et al*., 2012). Sensitivity of the agar gel immunodiffusion test varies from 50 to 92.7% and the specificity from 94.3 and 100% (Estein *et al*., 2002; Gall and Nielsen, 2004).

Coombs test: This is the most suitable and sensitive test for confirmation of relapsing patients with persistent disease (Supriya *et al*., 2010). It is an extension of the SAT test i.e. if the SAT test yields negative results due to the presence of blocking antibodies, Coombs test may be used instead. Agglutination can be determined visually, as for SAT, by using an agglutinoscope or a drop on a slide examined under the microscope. Coombs test is used for detection of incomplete, blocking or non-agglutinating IgG. It is time consuming, technically difficult, requires skilled personnel and not routinely performed in clinical laboratories. It is good for complicated and chronic cases but misses about 7% of cases compared with ELISA (Sarguzel *et al*., 2011).

Dipstick assay: The IgM dipstick assay is one of the tests that have been adapted to detect IgM antibodies to the smooth LPS. The assay has shown high sensitivity for patients with disease lasting less than 3 months (Clavijo *et al*., 2003; Lim and Richman, 2004; Taleski, 2010). IgM dipstick assay offers higher sensitivity and easier manipulation than IgM ELISA to detect IgM antibodies to *Brucella* species and improves the interpretation of results thus establishing cut-off points. IgM dipstick

assay could be used as a rapid and simple alternative to the ELISA IgM for the serodiagnosis of patients with acute brucellosis. The combined results of SAT and IgM dipstick assays can provide an indication of the stage of disease for those patients in whom the onset of clinical manifestations is not known (Clavijo *et al*., 2003).

Immunocapture agglutination test; *Brucella Capt*: Recently, new immunocapture agglutination for anti-*Brucella* (BCAP) assay has been developed to detect agglutinating and non-agglutinating antibodies with high sensitivity (Ozdemir *et al*., 2011). It is based on the sandwich ELISA system, where a microwell is covered with Coombs antibodies against human origin IgG, IgA and IgM antibodies. This *Brucella* agglutination test occurs in a microwell and is performed with Coombs antibodies and determines the 3 antibodies that form against brucellosis. It has been suggested as a possible substitute for Coombs test and a better marker for disease activity (Ozdemir *et al*., 2011; Mol *et al*., 2012). Compared to Coombs test, it has similar sensitivity and specificity but both can remain positive for long time after treatment in cured patients. BCAP determines blocking antibodies at diagnosis and during follow up for patients having brucellosis (Ozdemir *et al*., 2011). It is easier to carry out in 24 hours without a second step necessary as in Coombs test (Casao *et al*., 2004).

In comparison with other tests: it is more complex, expensive and slow. It can hardly replace rapid screening tests such as RBT and dipstick as a screening or first diagnostic test. However, it could help to diagnose disease in patients with longstanding evolution of brucellosis that is not detected by SAT. So, like Coombs test, *Brucella Capt* which is based on the immunocapture-agglutination of the total anti-*Brucella* antibodies, could be a second level serological test (Supriya *et al*., 2010; Taleski, 2010).

Lateral flow assay: An immunochromatographic *Brucella* IgM / IgG lateral flow assay is a simplified version of the ELISA test and has a great potential as a rapid point-of-care assay. The test has high sensitivity and specificity for *Brucella* IgM and IgG. It uses a drop of blood obtained by finger prick. It can be done as a bedside procedure. So it is a rapid and a simple diagnostic test that is also easy to interpret (Supriya *et al*., 2010).

Rapid slide agglutination test: Since routine brucellosis diagnosis does not include *B. canis* investigation, infection with this species may be more widespread than is currently suspected. The rapid slide agglutination assay test (RSAT) could be a suitable screening test for the diagnosis of human brucellosis and a supplementary technique, such as ELISA, performed on all positive RSAT samples that were negative by *B. abortus* antigen could ensure diagnostic specificity and confirm the diagnosis. It is recommended to use MAT and 2-ME/RSAT to check sera of all patients, who have symptoms of brucellosis but are negative for brucellosis using a smooth *Brucella* antigen (Lucero *et al*., 2005; Sayan *et al.*, 2011).

2.7.2.2. Brucellin allergic skin test

The skin test is an allergic test that detects the specific cellular immune response induced by *Brucella* spp. infection. The injection of brucellergene, a protein extract of a rough strain of *Brucella* spp., 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. 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. Treatment, Prevention and Control

Brucellosis is one of the drug-neglected diseases and treatment of brucellosis in domestic animals is not indicated (Kassahun, 2003). 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 treatment failure and relapse rates are high and depend on the drug combination and patient compliance (Seleem *et al*., 2008).

The optimal treatment for brucellosis is a combination regimen using two antibiotics since monotherapies with single antibiotics have been associated with high relapse rates (Solera *et al*., 1997; Pappas *et al*., 2005b; 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 (Alp *et al*., 2006; Ersoy *et al*., 2005; Falagas and Bliziotis, 2006; Seleem *et al*., 2009).

In the developed world, control of animal brucellosis has been successfully achieved through the combination of vaccination and test-and-slaughter programs and human brucellosis through milk pasteurization (Dwight,1999; McDermott and Arimi, 2002; Pappas *et al*., 2006a)coupled with effective disease surveillance and animal movement control(Godfroid *et al*., 2011). In developing countries, however, control by test-and- slaughter is hardly achievable because of limited resources to indemnify farmers whose animals are slaughtered during such screening programs (McDermott and Arimi, 2002). 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).

Human brucellosis is usually prevented by controlling the infection in animals. Pasteurization of dairy products is an important safety measure where this disease is endemic. Unpasteurized dairy products and raw or undercooked animal products (including bone marrow) should not be consumed. Good hygiene and protective clothing/equipment are very important in preventing occupational exposure. Precautions should be taken to avoid contamination of the skin, as well as inhalation or accidental ingestion of organisms when assisting at a birth, performing a necropsy, or butchering an animal for consumption (CFSPH, 2007).

In regions with high prevalence of the disease, the only way of controlling and eradicating brucellosis is by vaccination of all susceptible hosts and elimination of infected animals (Briones *et al*., 2001). The most commonly used vaccines against bovine brucellosis are *B. abortus* strain 19 and the recently USDA approved strain RB51; the latter unlike strain 19 does not interfere with serological diagnoses .The use of *B. abortus* strain 19 vaccine leads to the production of antibodies whose persistence depends mainly on the age of the animals at the time of vaccination. Based on test and slaughter coupled with control by vaccination, for a policy of eradication to be successful, there must be rigid control of the age at which strain 19 vaccinations is allowed (Seleem *et al*., 2010).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in two purposively selected sites in central Ethiopia, Bishoftu, East Shewa zone and Assela, East Arsi zone. These study areas were selected based on the abundance of dairy farms that constituted the known milk sheds (Land O'Lakes Inc., 2010).

Bishoftu is located at 47kms south east of Addis Ababa. The area is located at 9°N latitude and 40°E longitudes at an altitude of 1850 meters above sea level in the central high land of Ethiopia. It has an annual rainfall of 866mm of which 84% is in the long rainy season (June to September). The dry season extends from October to February. The mean annual maximum and minimum temperatures are 26°C and 14°C respectively, with mean relatively humidity of 61.3% (ADARDO, 2007). Farmers in the vicinity of Bishoftu town use a mixed crop and live stock farming system. Moreover Bishoftu and its surrounding have variable and yet representative agro-ecologies of the country. These agro-climatic zones are inhabited with different plant and animal species (Conway and McKenzie, 2007).

The second study area was Asella, which is located at 175 km south east of Addis Ababa and the altitude and annual rainfall of the area ranges from 502-4130 MSL and 200-400mm respectively with mean annual temperature of 22.5 ^{o}C. It is one of the highly populated area in Ethiopia with estimated human population of 2,521,349 and live stock population of cattle-82,190, sheep- 51,292, goat-8, 11, 479, poultry- 5, 62, 915, equine- 22,055 (Deselegn *et al*., 2011).

3.2. Definitions

In Ethiopia dairy cattle production systems are classified into rural smallholder (mixed crop-livestock) production, pastoral and agro-pastoral production, urban and peri-urban smallholder dairy production, and commercial dairy production systems (Land O'Lakes Inc., 2010; Asmare *et al*., 2013). This study focuses on the latter two production systems.

Urban and peri-urban dairy is one of the four dairy production systems in Ethiopia producing milk either as a full time or a part time business in urban and peri-urban areas. These smallholder dairy farms predominantly keep a small number (≤10 animals) of cross-bred cows in a zero grazing system to produce milk for both home use and sale. Commercial dairy farms are also farms located in urban and peri-urban areas mainly in and around the major cities and produce milk exclusively for sale. The farms were classified according to herd size and level of production into smallholder farm (<10 animals), medium farms (10 to 50 animals) and large farms with more than 50 animals (Megersa *et al*., 2011).

3.3. Risk Factors Assessment

In this study covariates (hypothesized explanatory variables) were assessed at both individual and farm level. Information was extracted from herd records where possible, if this information was not available owners were interviewed using semi-structured questionnaires (Annex 14).

The presence of abortion /still birth history in the farm, separate parturition maternity pen, separation of cows during parturition, breed of dairy cattle (Holestein-Friesian, cross or local) , awareness about brucellosis (knowledge), brucellosis test in the farm, frequent contact between animals with other herds were categorized as yes or no variables. Breeding was characterized by service types (artificial insemination (AI), bull or both). The method of after birth disposal (placenta, aborted material and dead fetus) was also categorized into burying/burning, or thrown to the environment (open dump).

The culling criterion of animals from the farm was categorized as reproductive problems, non-reproductive problems or both variables. The method of cleaning of calving pen after parturition was categorized as flushing with water, disinfecting with detergents or both variables.

The replacement stock of each farm was defined as buy in, raise own replacement or both. Culling, test and slaughter or both were considered as the measures taken against the known *Brucella* infected animals. Individual animals were categorized as young (≤ 36 months) and adult (>36 months), origin of each individual animal was defined as either Asella or Bishoftu while the location of the farm was classified as urban or peri-urban. Parity of the animals in the farm were categorized as primiparous, pluriparous or not applicable variables where as the abortion stage was classified as first trimester, second trimester or third trimester.

3.4. Study Design and Methods

The target populations were cattle in urban and peri-urban dairy (both smallholder and commercial) farms of Asella and Bishoftu towns which are composed of Holstein-Friesian, their crosses and local breeds established in the major milk sheds of the study sites.

A cross-sectional study design was conducted to determine the prevalence of *Brucella* infection in dairy cattle in the two selected towns and to identify the potential risk factors associated with the seropositivity and to isolate *Brucella* spp. by tracing back seropositivity and abortion history of the animals. Dairy cattle above six months of age were selected for this study. Relevant individual animal biodata and farm level information were collected using a semi-structured questionnaire.

The sampling was performed using a two level approach, selecting first individual farms with abortion history and then randomly selecting individual animals systematically inside each farm, while all animals in each farm with the history of abortion were sampled purposively for bacteriological culture and isolation. About 57% of the sampled cattle were from smallholder farm (small herd size) while the remaining 43 % were from medium and large herd size around urban and peri-urban including the commercial dairy farms. A list of dairy farms was prepared for each of the two study areas in collaboration with the respective district livestock health departments.

The sample size for cattle in Asella was calculated on the basis of previous report of 14.14% seroprevalence of bovine brucellosis in Arsi Zone (Deselegn *et al.*, 2011). Therefore to determine the sample size of dairy cattle in this area, 14.14% was used as P_{exp} and 95% confidence interval and 5% required precision (Thrusfield, 1995).

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

$$n = \frac{3.84 \times 0.1414 \times (1-0.1414)}{(0.05)^2} = 186$$

In Bishoftu, since there was no previous study done in the area, by considering 50% expected prevalence, 95% confidence interval and 5% required precision, 384 cattle were selected for this study (Thrusfield, 1995).

$$n = \frac{1.96^2 \times P_{exp} \times (1-P_{exp})}{d^2} \quad \text{(Thrusfield, 1995)}$$

Where n= required sample size, d= desired absolute precision, P_{exp}= expected prevalence (50%). Hence, a total of 570 dairy cattle (186 from Asella and 384 from Bishoftu) were considered for this study from 35 farms in the study areas.

3.5. Sample Collection and Laboratory Tests

3.5.1. Serological blood sample collection

Blood samples (5ml) were collected from the jugular vein of each animal, using sterile needles and plain vacutainer tubes. The blood samples were allowed to stand overnight at room temperature and centrifuged at 1500 × g for 10 min. to obtain the serum. Sera were decanted into cryovials, identified and transported to the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebeta, Ethiopia in ice packs and stored at −20 °C until been screened for antibodies against natural *Brucella* exposure using serological analysis.

3.5.1.1. Serological laboratoratory techniques

Rose Bengal plate test (RBPT): All sera samples collected were initially screened by RBPT using RBPT antigen (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) according to OIE (2004) and Alton *et al.* (1975) procedures (Annex 11). Briefly, sera and antigen were taken from refrigerator and left at room temperature for half an hour before the test to maintain to room temperature and processed following the recommended procedures.

Indirect enzyme linked immunosorbent assay (iELISA): For further laboratory analysis iELISA was performed using a commercial iELISA kit (BRUCELISA(160+400), (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom) to detect circulating antibodies to *Brucella* in cattle serum sample, and the protocol provided by the developers was followed precisely (Annex 12).

The test sera were analyzed at a final dilution of 1/200. The positive and negative controls were used at a dilution of 1/40 as has been indicated by the manufacturer. Following the addition of the conjugate and substrate - chromogen mixture at a recommended strength, the plate was incubated and examined for the intensity of reaction with automated ELISA reader at 405nm. Color development within a well indicates that the tested serum has antibodies to *Brucella.* A positive/ negative cut-off was calculated as 10 % of the mean of the optical density (OD) of the eight positive control wells. Any test serum with an OD value equal to or above this value was considered positive.

Card test: The brucellosis card test is a macroscopic agglutination procedure utilizing disposable materials, a stained buffered whole cell antigen suspension of *B. abortus* strain119-3 and contained in compact kits of minimal size. The card test for brucellosis is a rapid, sensitive and reliable procedure for detecting serologic evidence of *Brucella* infection. This test was performed according to instructions of the manufacturer (United States Department of Agriculture (USDA), APHIS, Veterinary Services, USA). A positive serum showed characteristic agglutination, moderate to

large clumps where as a negative one showed a pattern of dispersed particles without characteristic clumps and those showed no clumping (Annex 13).

Complement fixation test (CFT): Sera that tested positive to card test, iELISA and RBPT were further tested using CFT for confirmation using standard *B. abortus* antigen S99 (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, United Kingdom). Preparation of the reagent was evaluated by titration and performed according to protocols recommended by World Organisation for Animal Health (OIE, 2009b) (Annex 13). 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.

3.5.2. Bacteriological sample collection

Foetal and placental cotyledon: Aborted cattle foetuses (aspirate of stomach content) and placental cotyledon were collected during the visits to the farms after the report of bovine abortion cases and transported to NAHDIC in ice packs and stored at −20 ˚C until processed.

Milk: Initially the cattle in the dairy farm were screened serologically using RBPTand positive animal's milk samples were collected. Samples of milk were collected cleanly after washing and drying the whole udder and disinfecting the teats. The samples were containing milk from all quarters, and 10 ml of milk was taken from each teat. The first streams were discarded and the sample is milked directly into a sterile vessel and transported to NAHDIC in ice packs and stored at +4 ˚C until processed.

Vaginal discharge: A vaginal swab was taken after abortion or parturition in Stuart medium and transported to NAHDIC in ice packs and stored at -20 ˚C until processed.

3.5.2.1. Bacteriological isolation of *Brucella*

Brucella isolation from foetal and placental contyledon were performed according to Farrell method (Farrell, 1974). Approximately 1ml of foetal abomasal contents and placental cotyledon collected were rubbed on to *Brucella* medium base supplemented with 5% horse serum (Oxoid, CM 0169) and onto Farrell's medium (Farrell, 1974), selective medium, which is prepared by the addition of *Brucella* selective supplement (Oxoid,SR0083A) (containing Polymyxin B(as SO_4) = 2,500IU, Bacitracin = 12,500IU, cyclohexamide = 50.0mg, nalidixic acid = 2.5 mg, nyastatin =50,000 IU, vancomycin(as HCL) = 10.0 mg), 5% horse serum , 50% methanol and 50% dextrose on both *Brucella* medium base and tyrptic soy agar.

Milk samples for isolation of *Brucella* were processed according to Alton (1988). The milk samples were centrifuged at 3000 rpm for 10 minutes to obtain the sediment-cream mixture which then was cultured on both basal media (*Brucella* medium base supplemented with 5% horse serum) and Farrell's medium (*Brucella* selective medium). The plates were incubated in presence of 10% CO_2 and in normal air condition at 37 °C for 2 weeks.Vaginal swabs were streaked on to solid media similar to milk and placental cotyledon samples.

The inoculated plates from different clinical specimen were incubated at 37ºC both in the absence and presence of 10% CO_2 for up to 2 weeks. After the incubation, the suspected colonies were examined for *Brucella* spp growth. *Brucella*-suspected colonies were characterised by their typical round, glistening, pinpoint and honey drop-like appearance , positive Gram stain, modified Ziehl-Nelsen stain, motility(at both 37^{o}c and 20^{o}C), oxidase, catalase, urease production while negative for methyl red,voges proskuaer test, acid production on media containing glucose, citrate utilization, indole test, and no growth on Mac Conkey agar and non-hemolytic on blood agar .

3.6. Data Analysis

Data generated from questionnaire survey and laboratory investigations were recorded and coded using Microsoft Excel spreadsheet (Microsoft Corporation) and analyzed using STATA version 11.0 for Windows (Stata Corp. College Station, TX, USA). The seroprevalence was calculated as the number of seropositive samples divided by the total number of samples tested. To identify association of seropositivity with the potential risk factors (origin, age, management system, breed type, herd size, separate parturition, abortion history, abortion period and parity) were computed by Pearson's Chi-square test and multivariable logistic regression. The agreement between card test, RBPT, iELISA with CFT, considering as gold-standard test, were done using kappa test and interpreted according the recommendations of Dohoo *et al.* (2003) who states Kappa values as: <0.2: slight agreement, 02–0.4: fair agreement, 0.4–0.6: moderate agreement, 0.6–0.8: substantial agreement and >0.8: almost perfect agreement. The 95% confidence interval and a significance level of $\alpha = 0.05$ were used.

4. RESULTS

4.1. *Brucella* Isolation

Of 46 clinical sample cultured in the present study 6.52% (3/46) overall rate of isolation was found and all the three isolates were *B. abortus* based on biochemical tests result. *B. abortus* was isolated from placental cotyledon 1/9 (11.1%), vaginal swab 2/23 (8.69%) while no isolate was obtained from milk and fetal abomasal contents (abomasal aspirate) of dairy cattle (Table 3). Three colonies of *B. abortus* were observed on *Brucella* selective media (Farrell's medium) after 4 days of incubation, as pinpoint, round, convex with smooth margin, translucent and pale honey in colour. All the colonies were grown in 10% carbon dioxide supplied incubator and agglutinated with positive control serum for *B. abortus* while negative with the negative control serum (slide agglutination test with an anti-*Brucella* polyclonal serum). The culture smear showed Gram negative coccobacilli in Gram's staining and red stained coccobacilli in modified Ziehl-Neelsen staining. The isolated colonies were not grown on MacConkey agar (non-lactose fermenter) and non-heamolytic on blood agar. Growth was noticed in plate with basic fuchsin. The detailed result of basic biochemical and metabolic profiles of field *B.abortus* isolated from the study area were depicted in Table 4.

Table 3: *Brucella* isolates recovered from clinical samples of seropositive animals

Sample type	No. of examined samples	Positive culture isolation	%
Foetal abomasal content	1	0	0
Placental cotyledon	9	1	11.11
Milk	13	0	0
Vaginal swab	23	2	8.69
Total	46	3	6.52

Table 4: Basic biochemical and metabolic profiles of field *B. abortus* isolated from dairy cattle in the study area.

Brucella isolate	Biochemical properties										Growth on dyes
	Cat	Oxi	[a]Ure	Mot	Ind	MZN	Cit	MR	VP	Glu	BF
Placental cotyledon	+	+	+	-	-	+	-	-	-	-	+
Vaginal swab 1	+	+	+	-	-	+	-	-	-	-	+
Vaginal swab 2	+	+	+	-	-	+	-	-	-	-	+

Cat,Catalase; Oxi,Oxidase; Ure, Urea hydrolysis; Mot,Motility test(+ , motile, - , non-motile; Ind, Indole production ; MZN, Modified Zeihl Neelsen stain; Cit,Citrate Utilization; MR, Methyl Red; VP, Voges Proskauer ; Glu,Glucose; BF, Basic Fuchsin; TSA, Tryptic Soy Agar; + , Positive test result; - , Negative test result

[a]Urea hydrolysis= All isolate positive within 2 hours of culture.

4.2. Knowledge, Attitudes and Practices of the Farm Owner about Brucellosis

From 35 farms studied, 80%, 55.56% and 100% of the farm owners in small, medium and large herd sizes responded as they were aware of brucellosis, respectively. It was also found out that all farm owners of the study area were dependent on culling of the known *Brucella* infected animals while most of farm owners dispose after birth to open dump in small and medium herd size farms (Table 5).

Table 5: Knowledge, attitudes and practices (KAP) of farm owner's about *Brucella* infection in small, medium and large herd size in the study areas.

Variables	Proportion of respondents (n)		
	Herd size		
	Small(n=20) n (%)	Medium(n=9) n (%)	Large(n=6) n (%)
Awareness about brucellosis			
No	4(20)	0)	0(0)
Yes	16(80)	5(55.56)	6(100)
Brucella infected animal			
Test and slaughter	0(0)	0(0)	0(0)
Culling	20(100)	9(100)	6(100)
Both	0(0)	0(0)	0(0)
After birth disposal			
Burrying/ Burning	1(5)	1(11.1)	5(83.33)
open dump	19(95)	8(88.89)	1(16.67)

n= number

4.3. Farm Characteristics

Of the 35 farms assessed by questionnaire survey, it was found that all of the large herd size farms had bulls on their farms whereas only 5 (25%) of small farms have bulls. The study revealed that all farms in the study area had no frequent contact with other herds. Majority (95%) of small farms and all of the large farms were using AI for breeding purpose. The practices of provision of separate parturition pens were 83.3 % in large farms whereas only 5% for small farms (Table 6).

Table 6: Summary of the proportion of variables in the three herd (farm) size

Variables category	Herd size		
	Small (n=20) Frequency (%)	Medium (n=9) Frequency (%)	Large (n=6) Frequency (%)
Bull			
No	15 (75)	2 (22.2)	0
Yes	5 (25)	7 (77.8)	6 (100)
Frequent contact with other herd			
No	20 (100)	9 (100)	6 (100)
Yes	0 (0)	0 (0)	0 (0)
Service type			
AI	19 (95)	7 (77.8)	6 (100)
Bull	0 (0)	0 (0)	0 (0)
Both	1 (5)	2 (22.2)	0 (0)
Parturition pen			
No	19 (95)	5 (55.6)	1 (16.7)
Yes	1 (5)	4 (44.4)	5 (83.3)
Cleaning of calving pen			
Flushing with water	15 (75)	6 (66.7)	1 (16.7)
Both*	4 (20)	2 (22.2)	5(83.3)
Replacement stock			
Buy in	0 (0)	0 (0)	0 (0)
Raise own stock	19 (95)	7 (77.8)	5 (83.3)
Both	1 (5)	2 (22.2)	1 (16.7)

Both* = Flushing with water and disinfection with detergent

4. 4. Seroprevalence of Brucellosis

In the present study, an overall seroprevalence was estimated 1.4% (95% CI: 0.241, 3.461) by CFT. Among 570 tested samples, 13(2.28%), 15 (2.63%) and 16 (2.81%) were found positive by RBPT, iELISA, and card test, respectively. The higher seroprevalence, 3.23% (95% CI: 3.0, 7.4) was observed in and around Asella town compared to Bishoftu (0.52%) (Table 7).

Table 7: Results of Card test, RBT, iELISA and CFT of brucellosis by origin

Origin	N	Card test	RBT	iELISA	CFT
		Number positive (%)	Number positive (%)	Number positive (%)	Number positive (%)
Bishoftu	384	4(1.04)	3(0.78)	3(0.78)	2(0.52)
Asella	186	12(6.45)	10(5.38)	12(6.45)	6(3.23)
Total	570	16(2.81)	13(2.28)	15(2.63)	8(1.40)

N= number of animal tested

4.5. Comparison of Serological Test Agreement

The kappa statistics showed that there was substantial agreement between card test, RBT with CFT as gold-standard test while almost perfect agreement was observed between iELISA and CFT (Table 8).

Table 8: Kappa test for agreement between card test, RBPT, i-ELISA and CFT

	CFT		Kappa Value	Kappa value interpretation	P-value
	-	+			
Card Test			0.660		0.001
Negative	554	0		Substantial agreement	
Positive	8	8			
Rose Bengal Test			0.758		0.001
Negative	557	0		Substantial agreement	
Positive	5	8			
i-ELISA			0.839		0.001
Negative	559	0		Almost perfect agreement	
Positive	3	8			

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

4.6. Chi-square Analysis of Association of the Putative Risk Factors with *Brucella* Seropositivity

A Chi-square analysis revealed origin, breed, abortion history and abortion period were significantly associated ($P< 0.05$) with sero positivity of bovine brucellosis than among other factors considered during the study (Table 9.)

Table 9: Association of risk factors with *Brucella* sero positivity

Variables	Number tested	Number positive	χ^2 (*P*-value)
Origin			6.63(0.010*)
Bishoftu	384	2(0.52%)	
Asella	186	6(3.23%)	
Age			2.42(0.120)
Young	131	0(0)	
Adult	439	8(1.82%)	
Management			1.51(0.469
Extensive	8	0(0)	
Semi-intensive	178	1(0.56%)	
Intensive	384	7(1.82%)	
Herd size			3.73(0.155)
Medium	163	0(0)	
Small	85	1(1.18%)	
Large	322	7(2.17%)	
Breed type			8.49(0.014*)
HF	94	0(0)	
Cross	468	7(1.50%)	
Local	8	1(12.5%)	
Abortion history			92.43(0.001**)
No	524	0(0)	
Yes	46	8(17.39%)	
Separate parturition			3.17 (0.075)
No	160	0(0)	
Yes	410	8(1.95%)	

Abortion period			192.97(0.001**)
First trimester	19	0(0)	
Second trimester	5	0(0)	
Not applicable	523	0(0)	
Third trimester	23	8(34.78%)	
Location			1.73(0.189)
Urban	100	0(0)	
Peri-urban	470	8(1.70%)	
Parity			4.66(0.097)
Not applicable	160	0(0)	
Primiparous	119	1(0.84%)	
Pluriparous	291	7(2.41%)	

*statistically significant; **, statistically highly significant

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

The logistic regression analysis of the putative risk factors indicated that cattle originated from Asella were more likely to be infected (OR= 6.4, 95 % CI: 1.27 - 31.85) with *Brucella* than cattle from Bishoftu (Table 10).

Table 10: Multivariable logistic regression analysis of putitive risk factors with *Brucella* sero positivity

Variables	No.of animal tested	Prevalence (%)	Crude OR(95%CI)	Adjusted OR (95% CI)
Origin				
Bishoftu	384	0.52	1	1
Asella	186	3.23	6.37(1.27,31.85)	7.56(1.48,38.61)
Breed type				
HF	94	0	1	1
Cross	468	1.50	-	-
Local	8	12.50	0.11(1.02,86.99)	0.19(0.53,52.45)

OR, Odds ratio; CI, Confidence interval; 1, Reference

5. DISCUSSION

5.1. *Brucella* Isolation

In the present study, isolation of *B. abortus* from seropositive cattle with history of abortion was carried out in Ethiopia for the first time. This confirmatory isolation of *B. abortus* were from clinically aborted cattle placental cotyledon (11.1%) and vaginal swab (8.69%) while no isolate was obtained from milk and foetal abomasal content. The low isolation rate (6.52%) of *B. abortus* obtained in the present study from seropositive animals with history of abortion was in agreement with previous report of 6.4% (Celebi and Otlu, 2011). This might be because of the slow growing and fastidious nature of the pathogen (Seleem *et al.*, 2010).

In contrast to this result, a higher rate of isolation of *B. abortus* was reported by Gülhan *et al.* (2011) (26.7%), Ali *et al. (*2014) (40%), Ünver *et al. (*2006) (55.6%) from aborted cattle fetuses. This difference may be related to the usage of more than one selective culture media in their study while in the present study only Farrell's medium was used. Isolation of *B.abortus* can be improved if more than one selective culture medium is used (Ali *et al.*, 2014).

In the present study, bacteriological cultural, morphological and biochemical tests confirmed that all the three isolates obtained from the cases of placental cotyledon and vaginal swabs of aborted cattle were *B. abortus*. Similar to the earlier reports all the *Brucella* isolates found in this study were positive for catalase, oxidase and urea hydrolysis and negative for indole production, citrate utilization, methyl red, and voges-proskauer tests revealing them to be *Brucella* spp. (Koneman *et al*., 1997). Other reports have also indicated that on the basis of cultural, morphological, and biochemical characteristics, it possible to identify *Brucella* spp. (Alton *et al*., 1988; Koneman *et al*., 1997).

Shedding of *Brucella* in the milk of infected animals is an important source of transmission of disease to humans if the raw milk is consumed (Tantillo *et al.* 2003). In contrary to this fact, there was no recovery of *Brucella* spp. from 13 milk samples of RBPT positive cows in the present study. This result might be due to the secretion of an organism in the milk a few days (2 to 5 days) after abortion, small number of sample cultured and use of only Farrell's medium in the present study. The isolation of *Brucella* from milk samples may be improved if more than one culture medium is used (Ali *et al*., 2014). Hence, the result should not underestimate the risk of consuming milk as source of *Brucella* infection in the study areas. The fact that *Brucella* spp were isolated from milk of cattle from different studies with rate of 3.2% (Ali *et al.,* 2014) in Pakistan and 4.4% (Celebi and Otlu, 2011) in Turkey shows the need for further investigation in Ethiopia.

In the present study, based on the questionnaire survey, most of the respondents were well aware about brucellosis and practice culling of the known *Brucella* infected animals in their farm. Among the prevention of brucellosis transmission, culling is the most known measures against animal brucellosis (Radostits *et al*., 2000). In addition, most of the respondents in this study with the small herd size (95%) did not bury afterbirth (aborted fetus, still birth and retained foetal membrane) rather left them on open dump. Moreover, all the respondents did not use protective glove while handling parturating or aborting animals. These factors combined with the poor cleaning practice by the owners could pose a great risk of spread of the disease to unaffected animals (Tolosa, 2004).

5. 2. Brucellosis Seroprevalence

The present study revealed that the overall seroprevalence of bovine brucellosis was 1.40%. This finding is consistent with the earlier reports of Degefu *et al*. (2011) (1.38%) in agro pastoral areas of Jijjiga zone of Somali Regional State. Comparable to this finding Asmare *et al.* (2007) reported (1.92%) in Sidama zone, southern Ethiopia, (1.6%), Tolosa *et al.*(2012) in Jimma area (1.97%) and Tesfaye *et al.* (2011)(1.5%) in Addis Ababa.

On the other hand, there were reports with a relatively higher seroprevalence of bovine brucellosis in other parts of the country Hunduma and Regassa, (2009) (11.2%); Ibrahim *et al*., 2010 (3.1%); Hailesillasie *et al*., 2011(4.9%); Megersa *et al*., 2012 (8.0%) and Tibesso *et al*., 2014 (4.3%). However, most of these reports were from the extensively managed herds, where cattle from several owners mingle at grazing or watering points. Hence, the low seroprevalence observed in this study could possibly be explained by the developed awareness and instituted informal culling practice and proper disposal of afterbirths as it has been also suggested by Tesfaye *et al*. (2011) and/or the prevailing management differences between the intensive, semi-intensive and extensive production systems (McDermott and Arimi, 2002; Matope *et al*., 2011).

The present study revealed that origin of dairy cattle was significantly associated with brucellosis in dairy cattle ($P<0.05$) and the results showed higher individual animal seroprevalence in Asella (3.23%) compared to Bishoftu (0.52%). The reasons for the variations in brucellosis seroprevalence among the study areas might be related to difference in management practice performed in the two study sites. At the onset of the dairy schemes in Asella, farm owners purchased *Bos taurus* cattle from commercial farms, but the screening of these for brucellosis was not done due to limited availability of veterinary services, while the practice of screening for brucellosis before purchasing cattle in most of Bishoftu dairy farms. According to the report of different studies, purchasing of cattle from commercial farms without screening for brucellosis increases the chances of contact with infected herds (Omer *et al.,* 2000; Reviriego *et al*., 2000; Muma *et al*.,2007b).

In addition, different studies revealed that the seroprevalence of brucellosis is lower in low land agro-climate which is unsuitable for survival of *Brucella* organisms than highland (Radostits *et al.,* 1994).Therefore, the practice of purchasing cattle from commercial farms without screening for brucellosis together with other agro-ecological factors could partly explain the observed higher sero prevalence of dairy cattle brucellosis in Asella as compared to Bishoftu.

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In the present study, the higher seroprevalence of dairy cattle brucellosis was observed in large herd size in the study sites. This study finding was inline with that of Asfaw *et al*. (1998) in which he found significant association between *Brucella* seropositivity and large herd size. However, in contrary to this Kebede *et al*. (2008) reported that the risk of seropositivity was independent of herd size in small holder farms from Wuchale Jida district of East Wollega zone of Ethiopia. Higher sero positivity in large herd size 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).

Even though age was not significantly associated with *Brucella* seropositivity (P> 0.05) a seroprevalence of 1.82% was found among adult age group whereas no *Brucella* seorpositivity was observed in young age group of dairy cattle in the study sites. Several previous reports have indicated that higher seroprevalence of brucellosis in adult age group of cattle (Magona *et al.,* 2009; Megersa *et al.,* 2011) similar to the findings of this study. This could be explained by sexual maturity and pregnancy due to the influence of sex hormones and placenta erythritol on the pathogenesis of brucellosis (Radostits *et al*., 2007).

The finding of this study revealed that higher seroprevalence of brucellosis was observed in intensive production system. Similar to this findings, previous reports have indicated that higher seroprevalence of *Brucella* from highland areas of Ethiopia among dairy cattle in intensive production systems (Jergefa *et al.,* 2009; Asmare *et al*., 2010). The higher seroprevalence of brucellosis in intensive production systems particularly in Asella could be explained by the fact that there is a greater chance of contact between infected and healthy animals in these systems, or between healthy animals and infectious materials, since most of farm owners' do not follow hygienic practices which was in agreement with the report of Jergefa *et al. (*2009).

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The present study revealed that a history of previous abortions was significantly associated (P<0.001) with brucellosis sero positivity. A seroprevalence of 17.39% was recorded for the occurrence of previous abortion in these study areas based on questionnaire survey. This finding was consistent with Tolosa (2004) who reported 17.6% in selected sites of Jimma Zones. This could be explained by the fact that abortions or stillbirths and retained placenta are typical outcomes of brucellosis (Swell and Brocklesby, 1990; Radostits *et al*., 1994). In addition, in highly susceptible non-vaccinated pregnant cattle, abortion after the 5th month of pregnancy is cardinal feature of the disease (Radostits *et al*., 2000). In contrary to this findings, a relatively lower seroprevalence were reported by Tesfaye (1996) (6.1%) in Mekele dairy cattle and Yayeh (2003) (6.7%) in North Gondar, Ethiopia.

There is still a controversy among different researchers on the issue of breed susceptibility to brucellosis. This study revealed that significant difference between breed type and *Brucella* seropositivity in dairy cattle. This might be due to the origin of the animal from the previously infected or exposed herds (Deselegn *et al*., 2011). Inspite of small sample size of local breed in the present study as potential limitaion, a higher seroprevalence of 12.50% was found in local bred cattle in the study sites. This finding was not inline to Yohannes *et al*. (2012) who reported 1.7% in local bred cattle in Asella, Oromia Regional State, Ethiopia. In contrary to the present study, Jergefa *et al*. (2009) found that breed of cattle has significant effect on the serological prevalence of brucellosis and he reported higher seroprevalence of brucellosis in cross-bred than in indigenous (local) ones.

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

On the basis of parity, the difference observed in seroprevalence was statistically insignificant. Similar observations were made by Berhe *et al*. (2007). Though there is insignificant association between parity and brucellosis seropositivity, the higher seroprevalence was observed in pluriparous (2.41%) than primiparous cattle (0.84%) in the study areas. The higher seroprevalenceof brucellosis in the pluriarous cattle of this study was in line with Asmare *et al. (*2013) who reported 2.5% in pluriparous dairy cattle and breeding farms with special emphasis on cross and exotic bred.

With regard to serological test comparision, almost perfect agreement with significant association was observed between iELISA and CFT (K=0.839) whereas substantial agreement was found between card test (K=0.66) and RBPT (K=0.76) with CFT. This finding is inconsistent with Asfaw *et al*., (1998) who reported a moderate agreement (K=0.44) between RBT and CFT. On the other hand, almost perfect agreement (K=0.98) was reported between RBPT and CFT by Abay (1999).

6. CONCLUSION AND RECOMMENDATIONS

Bovine brucellosis caused by *B. abortus* has a major impact on human health, besides causing significant economical losses in dairy industry. In Ethiopia, inspite of a number of research reports on seroprevalence of brucellosis in cattle and widespread occurrence of brucellosis in different production system. In the present study, *B. abortus* were isolated for the first time in Ethiopia from seropositive dairy cattle with history of abortion. The oraganisms were isolated from placental cotyledon (one isolate) and vaginal swab (two isolates). Moreover, the seroprevalence recorded in the present study revealed that brucellosis is an established disease in Asella and Bishoftu dairy farms. Higher seroprevalence was observed in Asella dairy farms compared to Bishoftu. In addition, local breeds, cattle with history of abortion, and the third trimester abortion period were significantly associated with *Brucella* seropositivity. In conclusion, the bacteriological isolation and identification of *B. abortus* from dairy cattle combined with the prevailing *Brucella* seropositivity in most of the dairy farms indicate the importance of brucellosis in dairy cattle industry of the areas and potential public health implication for human population in the study areas.

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

- ✓ More proactive measures should be taken to protect the cattle populations from *Brucella* infection to reduce its ecomonic impact to the dairy industry and the risk of zoonotic infection in exposed human population.
- ✓ In order to design and utilize effective *Brucella* vaccines in Ethiopia, further indepth investigation on isolation and molecular characterization of *Brucella* species and biovars circulating in the country should be undertaken.
- ✓ Since the routine identification and differentiation of *Brucella* species based on culture isolation and phenotypic characterization requires, biosafety level 3 protocols , establishement of biosafety level 3 laboratory facilitites at National and Regional laboratories should be encouraged.
- ✓ Further research on the isolation and characterization of circulating *Brucella* species in other livestock (small ruminants, camel, equine and dog) of Ethiopia should be initiated.

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8. ANNEXES

Annex 1: The Gram Stain

Procedure

From a liquid culture, take a loop full of bacteria emulsify it in a small drop of water or saline on the slide. This should be a thin, not milky, suspension or it will not stain properly. Air dries the slide. This is done automatically in the virtual module.

To begin:

1. Heat fixes the slide: click on the Bunsen burner; pass the slide gently two or three times (1-2 seconds) through the flame. Do not overheat - this will cause distortion of the cells.
2. Flood the slide with crystal violet for 1 minute
3. Rinse with H_20
4. Flood the slide with iodine for 1 minute
5. Rinse with H_20
6. Decolorize with alcohol for 5-10 seconds
7. Rinse with H_20
8. Flood the slide with safranin for 1 minute
9. Rinse with H_20
10. View slide under the microscope

Interpretation

Gram positive bacteria appear blue

Gram negative bacteria stained red

Annex 2: Modified Zeihl Neelsen Stain (*Brucella* Staining)

Procedure

1. Fix a smear by heat
2. Overlay the slide completely with dilute carbolfuchsin for 15 minutes
3. Differentiate the smear for 15 seconds in 0.05% sulphuric acid/0.5%acetic acid and wash it with tap water

4. Counter stain with 3% malachite green solution or methylene blue for 2minutes, wash again with water and dry it.

Interpretation

Brucella species appeared red

Other bacteria and the background appear green

Annex 3: Catalase Test

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

Procedure:

Slide method

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

Add a drop of 3% H_20_2 over organisms on slide

Test cannot be applied if blood agar is used

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

Do not mix with inoculating needle or loop.

Mixing of culture and H_20_2 is not necessary

Tube methods

Directly add 1.0ml of H_20_2 to an 18 to 24 hrs heavily inoculated pure agar slant culture.

Observe for immediate bubbling and record the result.

Interpretation

Positive result: immediate bubbling, easily observed

Negative: no bubbling

Annex 4: Oxidase test

The oxidase test is based on the bacterial production of an oxidase enzyme. The oxidase reaction is due to the presence of a cytochrome oxidase system, which activates the oxidation of reduced cytochrome by molecular oxygen. This in turn as an electron acceptor in terminal stage of electron transfers system. The cytochrome is an iron containing hemo proteins that acts as the last link in the chain of anaerobic

respiration by trasfering electron (hydrogen) to oxygen with the formation of the water or H_20_2.

Oxidase reagents

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

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

Procedure:

Direct plate procedure

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

Indirect paper procedure

Place a $6cm^2$ pieces of whatman no. 1 filter paper in petri dish

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

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

A positive color reaction occur within 5-10 seconds

Interpretation

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

Oxidase negative: no color formation

Annex 5: Urease Test

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

Procedure

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

Interpretation

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

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

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

No urea hydrolysis – Medium used original yellow colour.

Annex 6: Indole test

The test is used to determine the ablity of the organisms to split indole from tryptophan molecules.

Procedure

Inoculate the indole media with the test organisms and incubate at 37^{o}c for 18- 24hrs.

Add 5 drops of Kovac's reagent directly to incubated tube and shake the tube gently. Keep it for a minute and see colour change at surface of the media.

Interpretation

Positive – pink to red colour at the interface of the reagent and the broth

Negative- no colour change (remains bright yellow)

Annex 7: Methyl Red Test

Methyl red is a quantitative test for acid production (mainly used in the identification of enterobactericeae) requiring positive organisms to produce strong acid (lactic, acetic and formic) from glucose through the mixed acid formation pathway. Since many species of enteriobacteriaceae may produce sufficient quantities of strong acid that can be detected methyl red indicator during the initial phase of incubation, only organisms that maintain this low PH after prolonged incubation (48-72 hrs) overcoming the PH buffer system of the medium can be called methyl red positive.

Interpretation

The developments of stable red color on the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Since other organisms may produce lesser quantities of acid from the test substrate intermediate orange color between yellow and red color may developed this does not indicate a positive test.

Annex 8: **Voges Proskauer (VP)**

To detect acetyl methyl carbinol formed by bacterial metabolism, a product of the butylenes glycol.

Principle

Pyruvic acid the pivotal compound formed in the fermentative degradation of glucose is further metabolized through a number of metabolic pathways depending on the enzyeme system possessed by different bacteria. One such pathway results in the production of the acetoin (acetyl methyl carbinol) a neutral reacting end product. Some organisms produce acetoin as the chief end product of glucose metabolism and form fewer quantities of mixed acid. In the presence of atmospheric oxygen and 40% potassium hydroxide acetoin is converted to diacetyl and alpha naphtol serves as a catalyst to bring out a red color.

Procedure

Incubate a tube of MR/VP broth with a pure culture of the test organisms. Incubate for 24hrs at 35^0c at the end of this time add 0.6ml of 5% alpha -naphtol, followed by 0.2ml of 40% KOH. It is essential that the reagent added in this order. Shake the tube gently to expose the medium to the atmospheric oxygen and allow the tube to remain undisturbed for 10-15 minutes.

Interpretation

A positive test is represented by the developments of the red color 15 minute or more after addition of the reagents, indicating the presence of the diacetyl, the oxidation product of acetoin. The test should not be read after standing for over one hours. Because negative VP culture may produce a copper like colour potentially resulting in a false positive reaction.

Annex 9: Citrate Utilization

To determine the utilization of the citrate as a sole source of the carbon

Sodium citrate is a salt of citric acid, a simple organic compound found as one of the metabolites in the tri carboxylic acid cycle (Krebs cycle). Some bacteria can obtain energy in the manner other than the fermentation of the carbohydrates by utilizing citrate as the sole of the carbon sources. The measurement of this characteristic is important in the identification of the many members of the enterobacteriaceae.

Principle

The utilization of the citrate by a test bacterium produce an alkaline by products. The medium includes sodium citrate an anion as the sole carbon and ammonium phosphate as the source of the nitrogen. Bacteria that can utilize citrate can also extract nitrogen from the ammonium salt with the production of the ammonia NH^+

leading to alkalinization of the medium from conversion of the NH_3^{2+} to ammonium hydroxide (NH_4OH), pH 7.6 is the indicator.

Procedure

All well isolated colony is picked from the surface of the primary isolation medium and inoculated as a single streak on the slant surface of the citrate agar tube. The tube is incubated at 35^0c for 24-48hrs.

Interpretation

A positive test is represented by the development of a deep blue color within 24-48 hrs indicating the test organisms has been able to utilize the citrate containing in the medium with the production of the alkaline products. A positive test may also be read without a blue color of there is a visible colonial growth along the inoculation streak line. This is possible only if carbon and nitrogen have assimilated. A positive interpretation from reading the streak line can be confirmed by incubating the tube for additional 24 hrs when a blue color usually develops. Negative and uninoculated control citrate media is green in color.

Annex 10: Rose Bengal Plate Test

Procedure

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

1.30 µl serum was mixed with an equal volume of antigen on a white tile or enamel plate to produce a zone approximately 2 cm in diameter.

2. The antigen and serum were mixed thoroughly using an applicator stick (a stick being used only once)

3. Rock plate by hand for about 4 minutes

4. Examine for agglutination in a good light

5. Use magnifying glass when micro agglutination suspected

Interpretation

0 = no agglutination

+ = barely perceptible

++ = fine agglutination, some clearing

+++ = coarse clumping, definite clearing

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

+++, ++++ were recorded as positive.

Annex 11: Indirect Enzyme Linked Immuno Sorbent Assay (iELISA)

Procedure

1. Make a primary dilution of 1/40 of all test and control sera by adding 25µl of serum to 1ml of diluting buffer
2. Prepare the plate by adding 80 µl of diluting buffer to all wells.
3. Add 20µl of each of the primary diluted samples to all prepared wells. This gives a final dilution of 1/200. Leave columns 11 and 12 for the serum controls.
4. Add 20µl of the primary diluted positive control to each of the wells in column 11.
5. Add 20µl of the primary diluted negative control to each of the wells in column 12 except well H12 which should have no sample added as it is to be used to blank the plate. Cover the plates with lid.
6. Incubate the plate at room temperature for 30 minutes on a rotary shaker(or at 37^{o}c for 1 hour if no shaker available)
7. Shake out the contents of the plate and rinse 5 times with washing solution and then thoroughly dry by tapping the plate on absorbent paper towel
8. Prepare the conjugate solution. Dilute to working strength with diluting buffer as instructed on the ampoule label. This solution cannot be stored
9. Add 100µl of the conjugate solution to all wells. Cover the plate with a lid.
10. As step 6
11. Wash as in step 7.
12. Switch on microplate reader and allow unit to stabilize for 10 minutes
13. Immediately before use prepare the substrate solution by adding 300µl of ABTS chromogen to 12ml of substrate buffer plus 60µl of substrate (hydrogen peroxide). Mix well and add 100µl to all wells .This solution cannot be stored
14. Leave the plate at room temperature for a minimum of 10 minutes and a maximum of 15 minutes.
15. Slow the reaction by adding 100µl of stopping solution to all wells

16. Remove condensation from the bottom of the plate with a absorbent paper towel. Read the plate at 405nm blanked on well H12

Interpretation

Green fluorescent …positive

No green fluorescent….Negative

Annex 12: Complement Fixation Test

Procedure

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

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

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

4. Repeat steps ii and iii above for each serum to act as anticomplementary serum controls (see below).

5. Volumes (25 µl) of complement at 1.25 MHD, are added to each well and 25 µl of antigen, diluted to working strength, are added to all wells excluding those of the anticomplementary controls. These latter wells receive 25 µl of veronal buffered saline instead.

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

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

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

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

Interpretation

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

Annex 13: Card Test

Procedure

1. With a capillary tube attached a rubber bulb (micro pippete), 30µl of serum was delivered to the test area of card.
2. Two drops (0.015ml in each drop) of card- test antigen were placed adjacent to the drop of serum.
3. The serum and antigen were mixed with stirrer over the surface of the "tear-drop" area.
4. The card was rocked for 4 min with a rocking machine at approximately 10 to 12 rocks per minute

Interpretation

Positive serum showed moderate to large clumps

Negative serum showed dispersed particles without characteristic clumps or showed no clumping.

Annex 14: Questionnaire Format

I. General Information of Questionnaire Survey

1. Name----------Sex----------Age----------

2. Occupation a. Government employee----------

b. Non-government employee (NGO) ----------

c. Self-business----------

3. Address a. Asella----------

b. Bishoftu

4. Are you practicing dairy as the only way of life?

a. Yes----------

b. No----------

5. Location a. Urban----------

b. Peri-urban----------

II. Information on herd

6. Herd size----------

7. Is there frequent contact between your animals with other herds?

a. Yes b. No

8. Breed of the dairy cattle

a. Local b. Fresian c. Cross

9. Number of milking cows----------

10. Number of first lactation heifers----------

III. Information on brucellosis

11. What are your culling criteria?

a. Reproductive problems b. Non-reproductive problems C. Both

12. If reproductive problems what are they?

a. Old age b. Poor production c. Both

13. What type of service do you have for your animals?

a. AI b. Bull c. Both

14. Do you know brucellosis?

a. Yes b. No

15. Are there separate parturition maternity pen?

a. Yes b. No

16. Do you separate cows during parturition?

a. Yes b. No

17. What do you do to the calving pen after parturition?

a. Flushing with water b. Disinfecting with detergents c. Both

18. How do you dispose of the after birth?

a. Burning b. Burying c. Both d. Thrown to the environment (open dump)

19. Where do you get the replacement stock?

a. Buy in b. Raise own replacement c. Both

20. What do you do with the known *Brucella* infected animlas?

a. Culling b. Test and slaughter c. Both

21. Have you observed abortion/still birth in your farm?

a. Yes b. No

22. How many abortions/still births or retained after birth have you encountered during the last three years? ----------

a. Number of abortion---------- b. Number of still birth----------c. Number of retained foetal membrane----------

23. Parity of the animals

a. Primiparus b. Pluriparus c. Not applicable

24. When did abortion occur?

a. First trimester b. Second trimester c. Third trimester

25. Did the farm been tested for brucellosis?

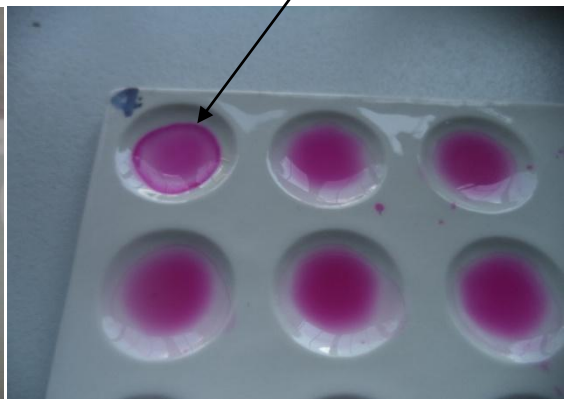
a. Yes b. No

26. Did vaccination for brucellosis carried out since inception?

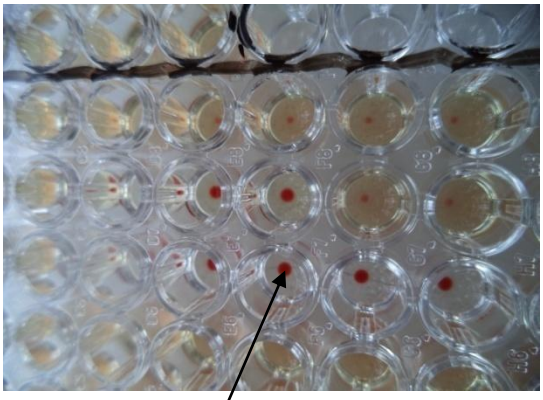
a. Yes b. No

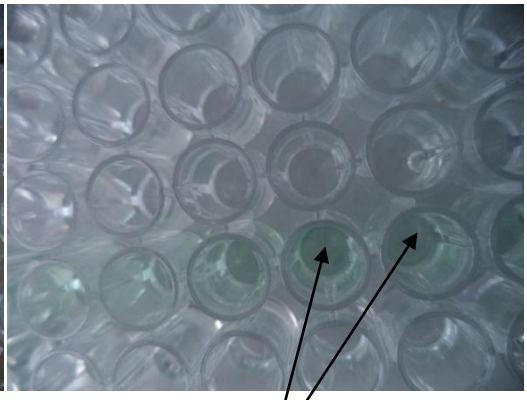
Annex 15: Serological and bacteriological result pictures

1.Serological test result



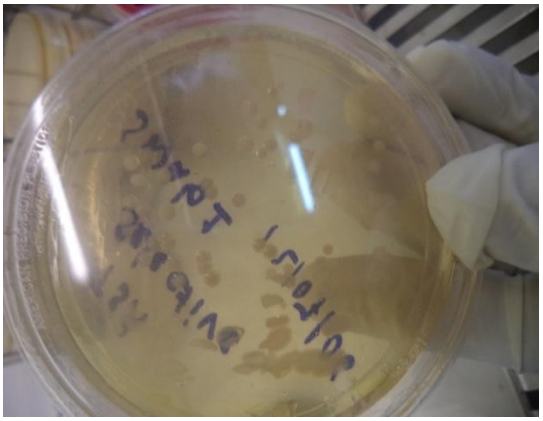
A.Card test B.RBPT

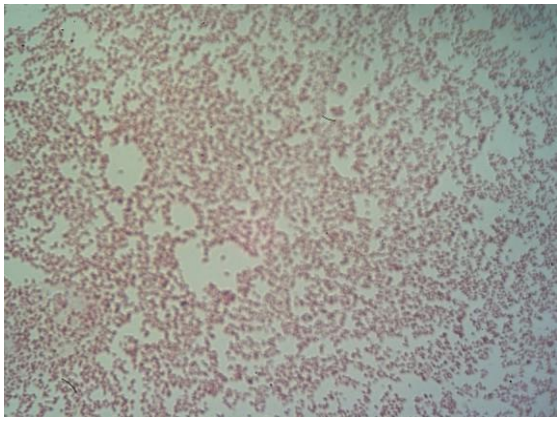




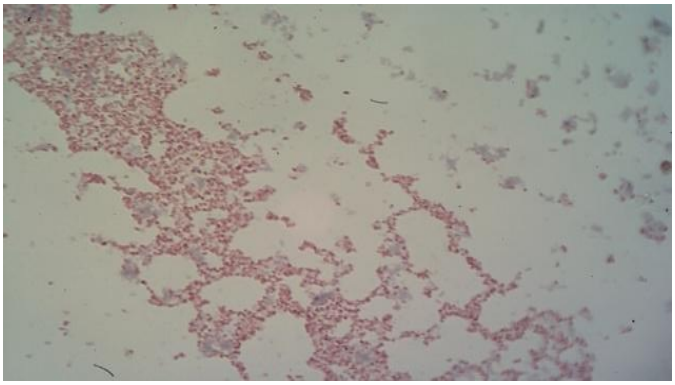
C. CFT D.iELISA

2. Bacteriological culture result





A.Growth on Farrell's medium B. Gram's stain (coccobacilli)

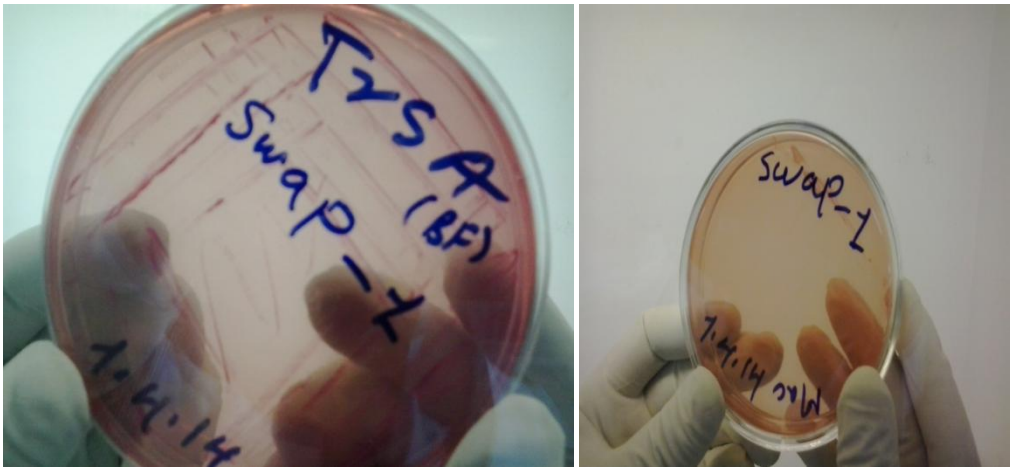


C. Modified Zeihl Neelsen (Red coccobacilli against blue back ground)

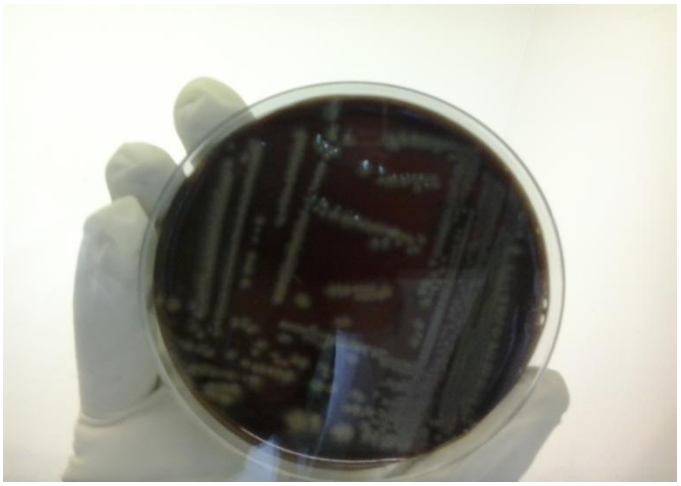
D. Urease positive within one and half an hour

E. Agglutination on positive control sera of *B.abortus*

F. No agglutination on negative control sera of *B.abortus*



G. Growth on media containing basic fuchsin dye H.No growth on MacConkey agar



I.Non-heamolytic on blood agar