Brucellosis in Ethiopia: epidemiology and public health significance.

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This dissertation contains less than 65,000 words including appendices, bibliography, tables, equations and figures.

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ABSTRACT

Brucellosis is a common bacterial zoonotic disease that has important veterinary and public health concerns and economic impact in Sub-Saharan Africa including Ethiopia. A cross-sectional study was conducted in Addis Ababa Dairy farms and four selected districts of Borena Pastoral setting in Southern Ethiopia from November 2016 to February 2018 to estimate the prevalence of brucellosis and associated risk factors in cattle, sheep and goats and occupationally associated humans. A total of 2300 cattle, 882 small ruminants and 341 humans were serum sampled and serially tested for antibody against *Brucella* infection using Rose Bengal Test for screening and further confirmed by Competitive-Enzyme Linked Immunosorbent Assay. The overall individual animal level seroprevalence was 0.06 % (95% CI: 0.002 -0.4) in commercial dairy cattle in Addis Ababa, 2.4 % (CI: 1.4-3.7) in cattle, and 3.2 % (CI: 2.1-4.6) in small ruminants; both in Borena pastoral region, Southern Ethiopia. The seroprevalence recorded in humans occupationally linked to livestock production systems in Borena was 2.6 % (CI: 1.2-5). Herd size, parity, and history of abortion were risk factors associated with *Brucella* seropositivity ($p<0.05$) in cattle whereas in small ruminants the results show that district, age group, flock size, and history of abortion were found to be significantly associated risk factors with *Brucella* infection ($P<0.05$). The risk factors for *Brucella* infection in humans were assisting in calving and presence of seropositive animals at household ($p<0.05$). Existence of more than one seroreactor animal species in some villages in Borena indicates more credence to the possibility of cross-species transmission of *Brucella* infections. Human seropositivity in the households with seropositive animals provides evidence of a public health hazard. In addition, an outbreak investigation of abortion at Adami Tullu Agricultural Research centre, central Ethiopia, was made to isolate and characterise the causative agents using cultural and molecular tools. Fifteen *Brucella abortus* were isolated from various tissue samples and vaginal swabs collected during postmortem examination of seropositive animals in the centre. The *B. abortus* isolates and two *B. melitensis*

previously isolated from vaginal swab of goats with history of abortion were whole genome sequenced (WGS). Subsequent phylogenetic analysis involved whole genome SNPs,core-genome SNPs, and *in silico* analysis of Multilocus Sequence Typing and Multiple Loci Variable Number of Tandem Repeats to characterise and determine global clustering of Ethiopian isolates. The result indicated that the *B. abortus* isolates from the outbreak were distinct and there were eight genotypes comprising single, double and triple locus variants circulating in the centre. Panel 1 and Panel 2A markers in MLVA-16 typing displayed no diversity among the neotypes suggesting that these are an early branching genotypes of the same strain. The core-genome phylogeny revealed that Ethiopian *B. abortus* form a distinct African clade branching basally with isolates previously described from Mozambique and Kenya. This clade was previously represented by only a small number of isolates, and thus the placement of Ethiopian *B. abortus* from the current study within this group substantially increases the representation of these basal *B. abortus* strains in genomic databases. On the other hand, *B. melitensis* isolates fall into a distinct African cluster with most isolates from Eastern African countries such as Somalia, Kenya, Eritrea, and Tanzania. The first isolation and molecular characterisation of circulating *Brucella* species in Ethiopia is a crucial step in planning and designing intervention strategies in the country. A coordinated One Health approach involving active involvement of human and animal health efforts to enhance public health and improve livestock productivity is recommended.

Key words: *Brucellosis, cattle, sheep and goats, humans, seroprevalence, whole genome sequencing*.

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LIST OF ABBREVIATIONS

AD	Anno domini
AI	Artificial insemination
ALIPB	Aklilu Lemma Institute of Pathobiology
APHA	Animal and Plant Health Agency
ATARC	Adami Tullu Agricultural Research Centre
ATJK	Adami Tullu Jido Kombolcha
BC	Before Christ
BCCN	Brucella Culture Collection Nouzilly
BCSP	Brucella cell surface salt extractable protein
CFT	Complement fixation test
CFU	Colony forming unit
COMPELISA	Competitive Enzyme linked immunosorbent assay
CSA	Central stastical agency
CVMA	College of Veterinary Medicine and Agriculture
DALY	Disability adjusted life in years
DC	Dendritic cell
EDTA	Ethylenediamine tetraacetic acid
ENA	European Nucleotide Archive
FAO	Food and Agriculture Organization
FPA	Fluorescein Polarisation Assay
FPSR	False positive serological reactions
FRET	Fluorescence Resonance Energy Transfer
HF	Holstein Friesian
HGDI	Hunter Gaston Diversity Index
IPC	Internal positive control
KAP	Knowledge Attitude Practices
LPS	Lipopolysaccharide
MGB	Minor groove binding
NVI	National Veterinary Institute
OARI	Oromia Agricultural Research Institutes

OD	Optical density
OIE	Office International des Epizooties
OPD	*O*-Phenylenediamine dihydrochloride
QUAST	Quality Assessment Tool
RBT	Rose Bengal Test
RFM	Retained foetal membrane
SAT	Serum Agglutination Test
SDA	Serum Dextrose Agar
SNNPR	Southern Nations Nationalities and People Region
SSA	Sub-Saharan Africa
ST	Sequence types
TLR	Toll-like receptor
UK	United Kingdom
USA	Unites States of America
VNTR	Variable Number of Tandem Repeats
WGS	Whole Genome Sequence
WHO	World Health Organisation

1. CHAPTER ONE

1.1. Introduction

Brucellosis is an ancient disease that can possibly be traced back to the 5th plague of Egypt around 1600 BC. Recent examination of ancient Egyptian bones, dating to around 750 BC, showed evidence of sacroiliitis and other osteoarticular lesions, common complications of brucellosis (1). David Bruce isolated *Brucella melitensis* (*Micrococcus melitensis* at that time) in 1887 from the spleen of a British soldier who died from a febrile illness (Malta fever) common among military personnel stationed in Malta. For almost 20 years after isolation of *M. melitensis*, Malta fever remained a mystery and was thought to be a vector-borne disease until Themistocles Zammit accidentally demonstrated the zoonotic nature of the disease in 1905 by isolating *B. melitensis* from goat milk (2). It was believed that goats were not the source of infection since they did not become ill when inoculated with *Brucella* cultures. The discovery that healthy goats could be carriers of the disease has been termed one of the greatest advances ever made in the study of epidemiology (3).

In 1897, a Danish veterinarian, Bernard L.F. Bang, discovered Bang's bacillus or 'bacillus of cattle abortion' (*B. abortus*) to be the causative agent of Bang's disease (4). Alice Evans, an American scientist who did landmark work on pathogenic bacteria in dairy products, confirmed the relationship between Bang's disease and Malta fever and renamed the genus *Brucella* to honour David Bruce (5). Her work on *Brucella* was central in gaining acceptance of the pasteurization process to prevent human brucellosis in USA. The discovery of *Brucella* species in marine mammals in early 1990 has changed the concept of a land-based distribution of brucellosis (3).

The disease affects mainly cattle, swine, sheep, goats, camels and dogs but it may also infect other ruminants and marine mammals. Synonyms of Brucellosis disease include, Malta fever, Mediterranean fever, Cyprus fever, Gibraltar fever, Undulant fever, Rock fever, (6) and Bang's disease, enzootic abortion (7), in human and animal respectively.

1.2. Etiology

1.2.1. Taxonomic classification

The genus *Brucella* belongs to the family *Brucellaceae*, order *Rhizobiales*, class *Alphaproteobacteria* and phylum *Proteobacteria*. The *Proteobacteria* are the largest phylum of bacteria, harbouring a wide variety of genera of pathogens, such as *Escherichia*, *Salmonella*, *Vibrio*, *and Helicobacter* among others. Bacteria belonging to the phylum proteobacteria are Gram-negative, with an outer membrane mainly composed of lipopolysaccharides (8).

For a long period, the genus *Brucella* was considered to comprise six 'classical' species designated based on the antigenic variation and according to primary host species from where they were isolated. These classical species are *B. abortus, B. melitensis, B. suis, B. ovis, B. canis* and *B. neotomae* (9). In addition to these classical *Brucella* species, other species infecting marine mammals have been identified and classified including *B. ceti* and *B. pinnipedialis,* and a *Brucella* species isolated from the common vole (*B. microti*) (10,11). Recently, *Brucella inopinata*, was isolated from a breast implant infection in a woman with clinical signs of brucellosis (12). So far, there are 12-named *Brucella* spp. as illustrated in Table 1.1. Original citation indicates the original publication where the species was characterized (Table 1.1 is adapted from Hull and Schumaker (13).

Table 1.1: *Brucella* species by host. Zoonotic potential is classified as pathogenicity and virulence in humans.

Species	Natural host	Zoonotic potential	Original citation
B. melitensis	Sheep, goat, and camels	Yes-High	(14)
B. abortus	Cattle, elk, and bison	Yes-High	(15)
B. suis	Pigs, hare, reindeer/caribou	Yes-High	(16)
B. canis	Dogs (domestic and wild)	Yes-Moderate	(17)
B. ovis	Sheep	No reported infections	(18)
B. neotomae	Desert wood rats	Infection reported (19)	(20)
B.ceti	Cetaceans	Yes-Low	(10)
B. pinnipedialis	Pinnipeds	Yes-Low	
B. microti	Red foxes, common voles, frogs	No reported infections	(11)
B. inopinata	Unknown	Yes High	(12,21)
B. papionis	Non-human primates	No reported infections	(22,23)
B. vulpis	Red fox	No reported infections	(24,25)

1.2.2. Characteristics of Brucella

Brucella species are facultative intracellular, Gram-negative, non-spore-forming and non-capsulated, partially acid-fast coccobacilli, endospores or native plasmids. They survive freezing and thawing but most disinfectants, which are known to be active against Gram-negative bacteria, all kill *Brucella* species. Pasteurization effectively kills *Brucella* in milk (12). The bacteria are of 0.5-0.7µm in diameter and 0.6-1.5µm in length. Classical *Brucella* species are oxidase, catalase and urease positive. Although classical *Brucella* species are described as non-motile, they carry all the genes except the chemotaxis system necessary to assemble a functional flagellum (7).

The genomes of the members of *Brucella* are very similar in size and gene content (26). Each species within the genus has an average genome size of approximately 3.29 Megabases (Mb) and consists of two circular chromosomes. Chromosome I is

approximately 2.11 Mb on average and the average size of chromosome II is approximately 1.18 Mb. The G + C content of all *Brucella* genome is 57.2% for Chromosome I and 57.3% for Chromosome II (26). The *Brucella* have no classic virulence genes encoding capsules, plasmids, pili or exotoxins and compared to other bacterial pathogens relatively little is known about the factors contributing to the persistence in the host and multiplication within phagocytic cells. Also, many aspects of interaction between *Brucella* and its host remain unclear (27).

In cattle, bison and buffalo, brucellosis is mainly caused by *Brucella abortus*. Up to nine *B. abortus* biovars (1-9) have been reported, but some of these biovars differ only slightly and the status of some is under question. Other *Brucella* species occasionally associated with disease in cattle include *B. melitensis* and *B. suis* (7,28).

1.3. Pathogenesis

The ability of genus *Brucella* to replicate and persist within host cells is directly associated with its capacity to cause persistent disease and to circumvent innate and adaptive immunity (29). The mechanisms that allow host cell invasion by *Brucella* species are not completely clear, but although specific host receptors that interact with *Brucella* have not yet been identified, internalization of *Brucella* into host cells requires cytoskeletal changes (30). Interestingly, invasion through the digestive tract does not elicit any inflammatory response from the host. Therefore, *Brucella* species invade silently or unnoticed by the innate immune system of the host. In fact, *Brucella* species have mechanisms that prevent activation of the host innate immune system (31). Indeed, *Brucella* Toll/interleukin-1 receptor (TIR) domain-containing protein prevents Toll-like receptor (TLR) 2 signalling by interfering with MyD88, and also inhibits dendritic cell (DC) maturation, cytokine secretion and antigen presentation (32).

B. abortus also induces suppression of the transcription of pro-inflammatory mediators in trophoblastic cells at very early stages of infection. 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 (30,31).

Brucella species 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. However, other forms of 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 (27). 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 (31). Cyclic β-1, 2-glucans, which are also part of the outer membrane, are also required for intracellular survival of *Brucella*. *Brucella* species 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* (30,32)

Invading *Brucella* usually localize in the lymph nodes, draining the invasion site, resulting in hyperplasia of lymphoid and reticulo-endothelial tissue and the infiltration of inflammatory cells. The organism is able to survive within macrophages because it has the ability to survive phagolysosome. Survival of the first line of defence by the bacteria results in local infection and the escape of *Brucella* from the lymph nodes into the blood. During bacteraemia phase, bones, joints, eyes and brain of the host can be infected, but the bacteria are most frequently isolated from supra-mammary lymph nodes, milk, iliac lymph nodes, spleen and uterus (7). In bulls, the predilection sites for infection are also the reproductive organs and the associated lymph nodes. During the acute phase of infection, the semen contains large number of *Brucella* cells but as the infection becomes chronic, the number of *Brucella* cells excreted decreases. However, it may also continue to be excreted for years or just become intermittent (33).

If the infected animal is pregnant, *B. abortus* will colonize and replicate to high numbers in the chorionic trophoblasts of the developing foetus. Erythritol, a substance produced by the foetus and capable of stimulating the growth of *B. abortus*, occurs naturally in greatest concentration in the placental and foetal fluids and is responsible for localization of the infection in these tissues. Invasion of the gravid uterus results in a severe ulcerative endometritis of the intercotyledonary spaces. The allantochorion, foetal fluids, and placental cotyledons are invaded, and the villi are destroyed (31). The organism has a marked predilection for the ruminant placenta. In acute infections of pregnant cows, up to 85% of the bacteria are in cotyledons, placental membranes, and allantoic fluid. The resulting tissue necrosis of the foetal membranes allows transmission of the bacteria to the foetus. The net effect of chorionic and foetal colonization is abortion during the last trimester of pregnancy (30).

Sexually immature and other non-pregnant cattle can become infected but lose their humoral antibody to the organism much more quickly than cattle infected while pregnant. In the adult, non-pregnant cow, localization occurs in the udder, and the uterus, if it becomes gravid, is infected from periodic bacteraemia phases originating in the udder (7). Infected udders are clinically normal but they are important as a source of reinfection of the uterus, as a source of infection for calves or humans drinking the milk, and because they are the basis for the agglutination tests on milk and whey (32).

1.4. Clinical manifestations and post-mortem lesions

In cattle, *B. abortus* causes abortions and stillbirths; abortions usually occur during the second half of gestation. Some calves are born alive but weak and may die soon after birth. The placenta may be retained, and secondary metritis can occur. Lactation may be decreased (28). After the first abortion, subsequent pregnancies are generally normal; however, cows may shed the organism in milk and uterine discharges.

Epididymitis, seminal vesiculitis, orchitis or testicular abscesses are sometimes seen in bulls. Infertility occurs occasionally in both sexes, due to metritis or orchitis/epididymitis (30). Hygromas, particularly on the leg joints, are a common symptom in some tropical countries. Arthritis can develop in some long-term infections. Systemic signs do not usually occur in uncomplicated infections, and deaths are rare except in the foetus or newborn. Infections in non-pregnant females are usually asymptomatic (34).

In camels, bison, water buffalo, bighorn sheep and other ruminants, the symptoms are similar to cattle (35,36). Symptomatic infections have been reported in some species of carnivores. Abortions, epididymitis, polyarthritis and other symptoms occur in some *B. abortus*-infected dogs (37). In horses, *B. abortus* can cause inflammation of the supraspinous or supra-atlantal bursa; these syndromes are known, respectively, as fistulous withers and poll evil (7).

At necropsy, granulomatous inflammatory lesions may be present in the reproductive tract, udder, supramammary lymph nodes, other lymphoid tissues, and sometimes in the joints and synovial membranes. Mild to severe endometritis may be seen after an abortion. The placenta is usually thickened and oedematous and may have exudate on its surface (31). The regional lymph nodes can be enlarged, and the mammary gland may contain lesions. Some aborted foetuses appear normal; others are autolyzed or have variable amounts of subcutaneous oedema and bloodstained fluid in the body cavities. The liver may be enlarged and discoloured, and the lungs may exhibit fibrous pleuritis and pneumonia (38). In bulls, one or both sacs of the scrotum may be swollen due to orchitis, epididymitis or abscesses. Hygromas may be found at slaughter in both sexes on the knees, stifles, hock, angle of the haunch, and between the nuchal ligament and the primary thoracic spines (7).

In previously unexposed and unvaccinated cattle, *B. abortus* spreads rapidly and abortion storms are common. During these storms, the abortion prevalence varies from 30% to 80%. In herds where this organism has become endemic, only sporadic

symptoms occur and cows may abort their first pregnancies (39). Deaths are rare in adult animals of most species; however, *B. abortus* can be lethal in experimentally infected moose, and possibly in bighorn sheep (7).

In humans, *Brucella* cause systemic infections with an acute, subacute, or chronic relapsing course. Clinical presentation of human brucellosis is nonspecific and highly variable. Patients commonly have a wide range of symptoms including undulant fever, headache, chills, myalgia, and arthralgia. The disease is also associated with abortion, orchitis, acute renal failure, endocarditis, splenic abscess, spondylitis, arthritis, and encephalitis (40).

1.5. Transmission

Brucella species show different degrees of host adaptation. For cattle, infection is usually caused by *B. abortus*. However, *B. melitensis* and more rarely *B. suis* can also establish themselves in cattle and the mode of transmission is then similar to that for *B. abortus*. These infections are particularly dangerous to humans because of the high virulence of most *B. melitensis* and *B. suis* strains and of the large dose of bacilli that are excreted by these animals when infected (28).

In cattle and other Bovidae, *Brucella* is usually transmitted from animal to animal by contact following an abortion. Pasture or animal housing may be contaminated, and the organisms are probably most frequently acquired by ingestion; inhalation, conjunctival inoculation, skin contamination, and udder inoculation from infected milking cups are other possibilities of transmission (30). The use of pooled colostrum for feeding new-born calves may also transmit infection. Sexual transmission usually plays little role in the epidemiology of bovine brucellosis. However, artificial insemination can transmit the disease and semen must only be collected from animals known to be free of infection (8).

Infections in sheep and goats are highly contagious because of the pathogenicity of *B. melitensis* and because of close contact caused by the density of the flocks, the commingling of those of different owners and heavy exposure in housing. Animal-to-animal transmission occurs as a result of the large number of organisms shed in the environment (39).

The susceptibility of camels to brucellosis caused by *B. melitensis* and *B. abortus* (41,42) was observed when they were pastured together with infected sheep, goats and cattle. The result suggests that large herd size, sharing of watering points with ruminants and inadequate hygienic practices under pastoral management system all favors transmission of camel brucellosis, particularly at time of abortion or delivery, by an infected female (41).

In humans the ultimate sources of infection are infected animals. The key species are the major food-producing animals: cattle, sheep, goats, and pigs. The risk of disease and its severity is to a significant extent determined by the type of *Brucella* to which an individual is exposed. This will be influenced by the species of host animal acting as source of infection (43).

In general, 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. Contact of mucosa and skin abrasions with fluids and tissues from aborted foetuses and infected live animals are also important sources of *Brucella* transmission (44). 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 (45).

1.6. Risk factors of *Brucella* infection

The risk factors that influence the initiation, spread, maintenance, and/or control of bovine brucellosis are related to the genetic content of the animal host population, management practices, and the biology of agents (30). Risk factors for human brucellosis include the handling of infected animals, ingestion of contaminated animal products such as unpasteurized milk and milk products (including cow, goat, and camel milk), meat, history of travel to endemic areas and handling of cultures of *Brucella* species in laboratories (38).

1.6.1. 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) (46). Placental trophoblasts produce erythritol in increasing amounts 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 characteristic of pathogenic *Brucella* strains (39). Erythritol promotes the growth of some strains of *Brucella*. However, *Brucella* has also been found in the reproductive tract of animals with no detectable levels of erythritol, therefore the role of this sugar in the virulence of the organism has been put into question. *B. abortus* Strain 19 is a spontaneously attenuated mutant widely used to vaccinate cattle. S19 is the only *B. abortus* strain that is inhibited by erythritol (47).

1.6.2. Management risk factors

The risk factors associated with spread of the disease within a herd include unvaccinated animals in infected herds, herd size, population density, method of housing, and use of maternity pens. Large herd sizes are often maintained by the purchase of replacement cattle, which may be infected. It is also more difficult to

manage large herds, which may lead to managerial mistakes that allow the disease to spread (48).There is a positive association between population density (number of cattle to land area) and disease prevalence, which is attributed to increased contact between susceptible and infected animals. The use of maternity pens at calving is associated with a decrease in the prevalence of infection, presumably due to decreasing the exposure of infected animals to susceptible animals (49).

The spread of the disease from one herd to another and from one area to another is almost always due to the movement of an infected animal from an infected herd into a non-infected susceptible herd. 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 infected, the time required to become free of brucellosis is increased by increase in herd size, by active abortion and by loose housing (30).

1.6.3. Pathogen risk factors

The bacterium possesses an unconventional non-endotoxic lipopolysaccharide, which confers resistance to antimicrobial attacks and modulates the host immune response. These properties make lipopolysaccharide an important virulence factor for *Brucella* survival and replication in the host (50). The organism can survive on grass for variable periods depending on environmental conditions. In temperate climates, infectivity may persist for 100 days in winter and 30 days in summer. The organism is susceptible to heat, sunlight, and standard disinfectants but freezing permits almost indefinite survival (7).

1.7. Public health significance

Brucellosis is the world's most widespread zoonosis, but ranks as one of the seven most neglected diseases, according to the World Health Organization (WHO) (13,51). It is a potential cause for chronic, debilitating maladies, due to its non-descript clinical presentation in human populations. This leads to major economic ramifications due

to the loss of normal daily activities (39).

Seven out of twelve known *Brucella* species can infect humans (Table 1). The most pathogenic and invasive species for humans are, *B. melitensis, B. suis and B. abortus,* and are on selected agents' list by the contents for disease control and prevention program in USA (29). This is due to the highly infectious nature of these three species, as they can be aerosolized. Moreover an outbreak of brucellosis would be difficult to detect because the initial symptoms are easily confused with those of influenza (39).

There are approximately 500,000 reported incident cases of human brucellosis annually; however, the true incidence is estimated at 5,000,000 to 12,500,000 cases annually (52).The most affected countries includes Syria, which has been reported to have the highest incidence (1,603.4 cases per 1,000,000 individuals) followed by Mongolia (3910), Iraq (268.8), Tajikistan (211.9), Saudi Arabia (149.5),and Iran (141.6) based on statistics they report to WHO (13,43,53,54). Turkey and Kyrgyzstan have had incidence above 200 in the past decade, however, have since decreased dramatically to 49.5 and 88.0, respectively (13,55). The population tend to be affected in these endemic areas include animal husbandry workers, shepherds, slaughterhouse workers, and most rural communities living in close contact with animals and animal by products (56–58). It is to be noted that there are many countries known to be endemic with human brucellosis, which are reported to be have no data. This is because of the lack of surveillance and reporting to the WHO as well as the lack of peer-reviewed publications elucidating the incidence of the disease (13,43).

Human brucellosis is endemic in sub-Saharan Africa with fragmented serological evidence in time and place (49,59). Besides, diagnosis of human brucellosis in sub-Saharan Africa is often challenging to clinicians due to the wide spectrum of clinical manifestations and lack of reliable diagnostic tests. This frequently results in misdiagnosis as other febrile diseases. Thus brucellosis remains severely underreported (49). Despite the high burden of the disease in many low-income countries, the disease does not attract the appropriate attention of health systems (39,40).In Ethiopia, information on human brucellosis is scarce. However, a few

studies conducted involving occupationally risk groups such as slaughterhouse and farm workers indicated an average prevalence 2.7% (60–62).

The prevalence of infection in animal reservoirs provides a key of its occurrence in humans (29,39). As a result, human infection is through contact with infected animals or byproducts and ingestion of contaminated animal products such as milk, meat, or carcasses (39,63). Brucellosis is, therefore, remains an occupational hazard to veterinarians, slaughterhouse workers, farmers, animal attendants and laboratory personnel (45,64,65). Brucellosis is recognized as the world's most common laboratory acquired infection (66). This is attributed to the low infectious dose, estimated between 10–100 bacterial cells by aerosol or subcutaneous route (67,68). Few cases of human-to-human transmission have been reported (69).

Asymptomatic infections can occur in humans. In clinical cases, the manifestations of the disease are extremely variable and non-specific. Brucellosis usually begins as an acute febrile illness with nonspecific flu-like syndromes such as fever, headache, malaise, back pain, myalgia and generalized aches (28). Drenching sweats can occur, predominantly at night. The complications observed often include arthritis, spondylitis, chronic fatigue, and epididymo-orchitis. Neurologic signs (including, meningitis, uveitis, optic neuritis and personality changes), anaemia, internal abscesses, nephritis, endocarditis and dermatitis can also occur. Other internal organs and tissues can also be affected, resulting in a wide variety of syndromes (30,70).

Economic losses caused by the disease in humans are a consequence of the cost of hospital treatment, cost of drugs, patient out-of-pocket treatment expenses, and loss of work or income due to illness. A study by Felix *et al* (71) indicated that a total of 49, 027 disability-adjusted life years (DALYs) were averted by mass vaccination of animals, where 52% reduction of brucellosis transmission was achieved. Broader human DALY burdens for brucellosis are yet to be estimated globally. This reflects the fact that human brucellosis is even more under-reported and underestimated than animal brucellosis. It usually presents as an acute febrile illness, often mistaken for malaria or typhoid (72).

1.8. Diagnosis

A precise diagnosis of *Brucella* species infection is important for the control of the disease in animals and consequently in man. Clinical diagnosis of brucellosis is not reliable and is usually based on the history of reproductive failures in livestock, but it is a presumptive diagnosis that must be confirmed by laboratory methods (73). Laboratory testing is therefore very important for the detection and confirmation in animals and a correct identification of the disease in humans. Definitive diagnosis is normally done by isolation and identification of the causative agent, which are able to identify the species and biovars of *Brucella* in samples (73,74). Definitive, isolation is time-consuming and must be performed by highly skilled personnel, as it is hazardous. For these reasons, serological tests are normally preferred. Brucellosis serology has advanced considerably in the last two decades with very sensitive and specific new tests available(75). Modern genetic characterization of *Brucella* using molecular DNA technology has been developed. Several PCR-based assays have been proposed, from the rapid recognition of genus to differential identification of species and strains (28,37).

1.8.1. Direct methods for diagnosis of brucellosis

Bacteriological diagnosis

Isolation of the organism is considered the gold standard diagnostic method for brucellosis since it is specific and allows bio typing of the isolate, which is relevant from an epidemiological point of view (50). However, in spite of its high specificity, culturing of *Brucella* species is challenging. *Brucella* species is a fastidious bacterium and require rich media for primary cultures. Furthermore, its isolation requires a large number of viable bacteria in clinical samples, proper incubation and quick delivery to the diagnostic laboratory (28,70). Furthermore, handling of live cultures poses risk of laboratory exposure and infection. As a result, molecular typing has been used as a replacement to biochemical typing for rapid identification of and characterisation of

Brucella species(76–78). The low sensitivity of microbiological culture compared to PCR has been reported by various studies (50,79,80).

For the cultural diagnosis of brucellosis, the organism may be recovered from a variety of materials, which usually depends on the presenting clinical signs. In animals, the placenta is the most infective and contains the greatest concentration of bacteria; this is followed by the lymph nodes and milk; and from blood in humans (73). Furthermore, other materials rich in the organism include stomach contents, spleen and lungs from aborted foetuses, vaginal swabs, semen, and arthritis or hygroma fluids from adult animals(50). Vaginal secretions should be sampled after abortion or parturition, preferably using a swab with transporter medium, allowing sampling and isolation of the organism up to six weeks post parturition or abortion (73). Milk samples should be a pool from all four mammary glands. Non-pasteurized dairy products can also be sampled for isolation of *Brucella* (70,73).

From animal carcasses, the preferred tissues for culture are the mammary gland, the supramammary, medial and internal iliac, retropharyngeal, parotid and prescapular lymph nodes, and the spleen. When sampling for diagnosis from animal tissue, all specimens must be packed separately, cooled and transported immediately to the laboratory in leak proof containers (50,81). For humans, blood for culture is the material of choice, but specimens need to be obtained early in the course of the disease. The samples should be kept in cold chain or frozen until required for culture (70).

Direct culture and isolation of *Brucella* are usually performed on solid media. This is generally the most satisfactory method as it enables the developing colonies to be isolated and recognised clearly. Such media also limit the establishment of non-smooth mutants and excessive development of contaminants. However, the use of liquid media may be recommended for voluminous samples or for the purpose of enrichment of a primary culture (70). A wide range of commercial dehydrated basal media is available, e.g. *Brucella* medium base, tryptose (or trypticase)–soy agar (TSA).

The addition of 2–5% bovine or equine serum is necessary for the growth of strains such as *B. abortus* bv.2, and many laboratories systematically add serum to basal media, such as blood agar base or Columbia agar, with excellent results. Other satisfactory media, such as serum–dextrose agar (SDA) or glycerol–dextrose agar, can be used (82). SDA is usually preferred for observation of colonial morphology. A non-selective, biphasic medium, known as Castañeda's medium, is recommended for the isolation of *Brucella* from blood and other body fluids or milk, where enrichment culture is advised. Castañeda's medium is used because brucellae tend to dissociate in broth medium, and this interferes with biotyping by conventional bacteriological techniques (83).

Frequently, field samples are contaminated with other bacteria; thus, selective media should be used to avoid overgrowth by fast growing agents. All the basal media mentioned above can be used for the preparation of selective media. Appropriate antibiotics are added to suppress the growth of organisms other than *Brucella*. The most widely used selective medium is the modified Farrell's medium (FM) (84), added to 1litre of agar: polymyxin B sulphate (5000 units = 5mg); bacitracin (25,000 units = 25mg); natamycin (50mg); nalidixic acid (5mg); nystatin (100,000 units); vancomycin (20mg). A corresponding freeze-dried antibiotic supplement is available commercially. However, nalidixic acid and bacitracin, at the concentration used in FM, have inhibitory effects on some *B. abortus, B. melitensis* and *B. suis* strains (70). Accordingly, the simultaneous use of FM and the less selective Thayer–Martin's modified (mTM) culture media has been considered the strategy of choice for *Brucella* primary isolation from field veterinary samples. However, the mTM is not translucent because of the haemoglobin contained as a basal component, being thus unsuitable for the direct observation of colonial morphology, probably the most practical procedure for the presumptive identification of *Brucella* (82).

A new selective and translucent culture medium (named CITA) has recently been formulated. For its preparation, blood agar base is used as a basal component, supplemented with 5% sterile calf serum and containing vancomycin (20mg/litre),

colistin methanesulfonate (7.5mg/litre), nitrofurantoin (10mg/litre), nystatin (100,000 International Units (IU)/litre), and amphotericin B (4mg/litre) (85). This new CITA medium inhibits most contaminant microorganisms but allows simultaneously the growth of all *Brucella* species and it is more sensitive than both mTM and Farrell's media for isolating all smooth *Brucella* species from field samples, being thus the selective medium of choice for overall *Brucella* isolation, although the maximal diagnostic sensitivity is obtained using both FM and CITA simultaneously (83,85). Contrary to the situation with several *B. abortus* biovars as well as *B. ovis*, the growth of *B. melitensis* or *B. suis* is not dependent on an incubating atmosphere containing 5–10% CO_2, but such a CO_2 enriched-atmosphere is optimal for the culture of all *Brucella* (83).

After 48-72h of incubation at 37°C, *Brucella* colonies are usually 0.5 to 1.0 mm in diameter with a convex and circular outline. Smooth strains are transparent and pale yellow, resembling droplets of honey with a shiny surface when observed in transmitted light. Rough colonies are opaquer with a granular surface. The 'smooth' or 'rough' colony morphology is exhibited depending on LPS structure (86). The 'smooth' phenotype is due to the presence of a complete LPS which is composed of lipid A, a core oligosaccharide and an O-side chains. Most *Brucella* species are considered 'smooth' although 'rough' mutants can occur especially following repeated laboratory subculture. *B. ovis* and *B. canis* are naturally occurring 'rough' species (87,88).

Dissociation of *Brucella* can be detected by the emulsification of a colony in 0.1% w/v aqueous acriflavine (89). Smooth colonies produce a yellow uniform suspension whereas rough colonies produce granular agglutinates. Colonial variation can also be detected by examining the plates under oblique light after staining the colonies with crystal violet (90). Smooth colonies appear translucent and pale yellow and rough colonies are stained with red, purple or blue with opaque and granular appearance. Colonial morphology, staining, slide agglutination with anti-*Brucella* serum (smooth or rough), urease, catalase and oxidase tests are the basis for a culture to be identified

as belonging to the genus *Brucella* (73). Once a culture has been identified as *Brucella*, it is important to classify the species and the biovars. This further classification should be done in specialized or reference laboratories. These tests are cumbersome and include CO_2 requirement, production of hydrogen sulphide (H_2S), dye sensitivity (thionin and basic fuchsin), phage lysis, and agglutination with A, M or R specific antisera, and in some cases it is necessary to use the oxidative metabolic method (50,73).

Molecular diagnosis

Molecular techniques are important tools for diagnosis and epidemiologic studies, providing relevant information for identification of species and biotypes of *Brucella* species, allowing differentiation between virulent and vaccine strains (37). Molecular detection of *Brucella* species 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 (91).

The use of the Polymerase Chain Reaction (PCR) to identify *Brucella* DNA at genus, species and even biovar levels has become preferred diagnostic test and a diversity of methods has been developed. Applications for PCR methods range from the diagnosis of the disease to characterization of field isolates for epidemiological purposes including taxonomic studies (73). PCR and its variants, based on amplification of specific genomic sequences of the genus, species or even biotypes of *Brucella* species, are the most broadly used molecular technique for brucellosis diagnosis (37,50,70).

The standard PCR assays include one pair of primers which is used to amplify the target genomic sequence of *Brucella* spp. Pairs used include the primers for sequences encoding 16S rRNA (92,93), outer membrane proteins *(omp2a*, *omp2b* and *omp31*) (94,95), 31 kDa immunogenic *Brucella abortus* protein (BCSP 31 B4/B5) (96–98), 16S-23S ribosomal DNA interspace region (ITS66/ITS279) (99,100), and insertion sequence *IS711* (101,102).

The first species-specific multiplex PCR was called AMOS-PCR assay which is used to identify and differentiate *B. abortus* biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1, based on the polymorphism arising from species-specific localization of the insertion sequence *IS711* in the *Brucella* chromosome (50). An improvement of this technique was introduced by incorporating additional strain specific primers into the primer mixture for identification of the vaccine strains *Brucella abortus* S19 and RB51. A further modification of the assay called BaSS-PCR (*Brucella abortus* Strain Specific PCR assay) was developed to identify and distinguish field strains of *B. abortus* biovars 1, 2 and 4 and to distinguish these from vaccine strains and other *Brucella* species from cattle (73,91). A new primer was developed, which together with the *IS711* AMOS primer produced a PCR to identify the isolates of biovars 3, 5, 6 and 9 of *B. abortus* (91).

In addition to the commonly used PCR assays, a new Multiplex-PCR assay was developed that specifically identified *B. neotomae*, *B. pinnipedialis*, *B. ceti*, and *B. microti*. Furthermore, it differentiated *B. abortus* biovars 1, 2, 4 from biovars 3, 5, 6, 9, as well as between *B. suis* biovar 1, biovars 3, 4, and biovars 2 and 5 (103). A *Bruce-ladder* multiplex PCR assay was also developed for identification and differentiation of the classical *Brucella* sp. including those from marine mammals and vaccine strains in a single reaction (104). Further evaluation of this assay, however, revealed that it was unable to distinguish between some *B. canis* strains from *B. suis* (105). More work to advance the Bruceladder Multiplex PCR assay allowed the differentiation of the nine recognised *Brucella* species including *B. micoti, B. ceti, B. inopinata,* and *B. pinnipedialis* (106). Moreover, the previously specious identification of some *B. canis* strains as *B. suis* was resolved by replacing the primers BMEI1436f/BMEI1435r in the original primer mix with the new primers BMEI1426/1427 (78,103,107).

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 recently been described in order to test *Brucella* culture cells (108) and for presence

in urine (109), blood, and paraffin-embedded tissues (110).

Three separate real-time PCR assays 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 PCR assays derived from the insertion element *IS711* whereas the reverse primer and Fluorescence Resonance Energy Transfer (FRET) probes are selected from unique species or biovar-specific chromosomal loci. Sensitivity of the *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 (108).

The predominance of real-time PCR in terms of sensitivity and specificity is well documented in various studies. Queipo-Ortuno *et al*., (109) performed real-time PCR with SYBR Light Cycler Green I in blood cultures of serum samples and whole blood of patients with brucellosis using primers B4 and B5 (targeting *bcsp31* gene) and compared their results with PCR-enzyme linked immunosorbent assay (110). Real-time PCR in serum samples had better sensitivity. Surucuoglu *et al.* (111) used the TaqMan real time PCR technique which targeted the *IS711*, *bcsp31* and *per* genes in patients with various clinical forms of brucellosis and compared the results of their method with other conventional methods using serum samples. The *IS711*-based assay was the most sensitive, specific, efficient and reproducible method to detect *Brucella* species. Further reports have documented the specificity, sensitivity and rapid results of different real-time PCR assays (112,113).

Single nucleotide polymorphisms (SNPs) typing single nucleotide polymorphisms 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* species. Scott *et al*. (114) described the use of SNPs in order to develop a multiplex SNP detection assay, based on primer extension technology that can rapidly and clearly 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 (115). An alternative approach for rapid, simple and unambiguous

characterisation of *Brucella* to the species level and differentiate vaccine strains based on minor groove binding protein (MGB) probes applied on a real-time PCR platform was also described (77,115).

Multilocus Sequence Typing (MLST) and Multiple Loci Variable Number of Tandem Repeats (VNTR) Analysis (MLVA): these protocols were reported to be highly discriminatory and provide a clustering of strains in accordance with the currently recognised Brucella species and biovars. They have also been described in identifying subtypes within each species or biovar on geographic origin or host specificity (116). A number of studies have indicated that MLVA typing has been useful in outbreak and epidemiological investigations (117–120). The MLVA and MLST were reported to be insensitive in detecting new mutations from new clades (121,122). Whole genome sequencing has been described as a robust and unbiased method to resolve within species relationships for closely related species such as *Brucella* species (123,124).

1.8.2. Indirect methods for diagnosis of brucellosis

Serological tests

Serological tests are crucial for laboratory diagnosis of brucellosis since most of control and eradication programmes rely on these methods. Inactivated whole bacteria or purified fractions (*i.e*. lipopolysaccharides or membrane proteins) are used as antigens for detecting antibodies generated by the host during the infection. Antibodies against smooth *Brucella* species such as *B. abortus, B. melitensis,* and *B. suis* cross react with antigen preparations from *B. abortus*, whereas antibodies against rough *Brucella* species (e.g. *B. ovis* and *B. canis*) cross react only with each other (70,74).

There are various serologic tests that are based on the detection of either whole-cell antigen or the sLPS (74). Overall, serologic tests are an ideal first line test. One major drawback are organisms that share the sLPS (that may include *Yersinia enterocolitica, Vibrio cholerae, Ochrobactrum anthropi, Salmonella enterica serotype Urbana,*

Francisella tularensis, and *Escherichia coli O157:H7*) and cross-react on these tests and produce False Positive Serological Reactions (FPSR) (125,126). False negative results of varying etiology can also occur (delayed seroconversion, blocking antibodies and prozone phenomenon) (127).

In order to validate serological tests, results should be analysed according to the true infectious status of an animal. The presence of anti-*Brucella* antibodies suggests exposure to *Brucella* spp., but it does not indicate which *Brucella* species induced production of those antibodies. Moreover, seropositivity does not necessarily mean that the animals have current or active infection at the time of sampling (128). In fact, studies of experimental and natural infections indicate that nearly all animal species vulnerable to *Brucella* infection can lose their antibody titres. This means that the actual prevalence of brucellosis may be higher than that indicated by antibody screening. Therefore, the "gold standard" in brucellosis remains the isolation of *Brucella* spp. by culturing. If brucellosis is suspected in livestock or in wildlife because of positive serological results, attempts to isolate the organism are considered mandatory and should always be performed (129).

It has been reported, however, that culture of human clinical samples has 100 % specificity, but usually poor sensitivity, particularly in chronic cases (130). Cultures from patients with the acute disease can have sensitivities of 50% to 80%; while the chronic form is less likely (less than 5%) to produce culture(13,131,132). A study in the US indicated that only 30-50% of seropositive animals are culturable, which leaves the question of the disposition of 50-70% of seropositive animals that are culture negative (13,133,134). Antibodies against *Brucella* also varies depending on the stage of infection. A specific IgM antibodies dominate during the acute phase of the disease while anti-*Brucella* IgG antibodies are present in the serum of patients at later stages of the illness and in the serum of relapsing patients(135,136). ELISA has been reported to discriminate between the presence of specific IgM and IgG antibodies and to roughly assess the stage of illness(137,138).

Although several serological tests are currently available, these tests can be classified as screening tests (*e.g.*, Rose Bengal Plate Test - RBT), 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 (73,74).

Slow Agglutination Test or Slow Agglutination of Wright (SAT or SAW): The principle of this test is to detect agglutinin antibodies mainly of the IgM isotype directed against *Brucella* species at an optimum concentration of antigen and antibodies, large antigen-antibody complexes form and precipitate at the bottom of the test tube. This reaction is slow because, in contrast to the rapid agglutination tests, it requires an overnight incubation at 37°C. This technique can also be practiced in micro method (micro agglutination test) in a reaction volume of 100 µL, without a change in performance. Reading the result is facilitated by the addition of a dye that stains the cells (74). The specificity of the test is increased by treating the serum with a chelating agent such as EDTA, which reduces cross-reactions due to IgM. Although this test is no longer recommended by the OIE for bovine brucellosis diagnosis, it is still widely used in human brucellosis diagnosis (70,129).

Buffered *Brucella* antigen tests: The Rose Bengal (RB) and buffered plate agglutination (BA) tests are the well-known buffered *Brucella* antigen tests. These tests are rapid agglutination tests lasting 4 minutes and performed on a glass plate with the help of an acidic-buffered antigen (pH 3.65 ± 0.05). These tests have been introduced in many countries as the standard screening test because it is very simple and thought to be more sensitive than the SAT. The OIE considers these tests "prescribed tests for trade" (50,83).

Complement fixation test: The Complement Fixation Test (CFT) allows the detection of anti-*Brucella* antibodies that are able to activate complement. Cattle immunoglobulins (Ig) that can activate bovine complement are the IgG and the IgM.

According to some literature this test is not highly sensitive but shows an excellent specificity. Because the test is difficult to standardize, it is progressively being replaced by ELISA tests (129). This test is a “prescribed test for trade” by the OIE (70).

ELISA tests: ELISA tests are divided into two categories, the indirect ELISA (iELISA) and the competitive ELISA (cELISA). Most iELISA use purified smooth LPS as antigen but a good deal of variation exists in the anti-bovine Ig conjugate used (129). Most iELISA detect mainly IgGs or IgG sub-classes. Their main quality is their high sensitivity, but they are also more vulnerable to non-specific reactions, notably those due to *Yersinia enterocolitica* O:9 infections. These cross-reactions seen in iELISA motivated the development of cELISA. The O-chain of the smooth LPS of *Brucella* contains specific epitopes that are not shared with the LPS of YO9. Therefore, by using monoclonal antibodies directed against specific epitopes of the LPS of *Brucella*, the development of more specific cELISA has been possible. These tests are more specific, but less sensitive, than iELISA (73,74). The OIE considers these tests “prescribed tests for trade” (83).

Fluorescence Polarization Assay: The fluorescence polarization assay (FPA) is based on a physical principle: how quickly a molecule spins in a liquid medium correlates with its mass. Molecules of small size spin faster and depolarize a polarized light beam more, while bigger molecules spin more slowly and, consequently, depolarize light less. FPA measures the degree of depolarization in milli-polarization units (mP)(139). During the test, serum samples are incubated with a specific antigen of *B. abortus* labelled with fluorescein isothiocyanate. In the presence of antibodies against *Brucella* species, large fluorescent complexes are formed. In negative samples, the antigen remains not complexed. These smaller molecules spin more quickly and therefore cause greater depolarization of the light than do the samples positive for *Brucella* species. This test can be easily automated and is very quick, since after mixing the labelled antigen and serum the reading is almost instantaneous (129).

Milk tests

Milk Ring Test: The test consists of mixing coloured *Brucella* whole-cell antigen with fresh bulk/tank milk. In the presence of anti-*Brucella* antibodies, antigen-antibody complexes form and migrate to the cream layer, forming a purple ring on the surface. In the absence of antigen-antibody complexes, the cream remains colourless. This test is not considered sensitive, but this lack of sensitivity is compensated by the fact that the test can be repeated, usually monthly, due to its very low cost. This test is prescribed by the OIE for use only with cow milk (73,83).

ELISAs and Fluorescence Polarization Assay: The two tests, discussed above in the context of serum samples, can also be applied to milk samples to detect infected animals. These tests are less sensitive when applied to milk than to serum samples. Indeed, before they can be used on tank milk, which may come from hundreds of cows, their sensitivity must first be checked on pools of samples (50,73,74). Increasing the testing frequency can often compensate this lower sensitivity in the case of tank milk. These tests are prescribed by the OIE for testing the milk of cattle and small ruminants (70).

Skin test: The skin test detects the specific cellular immune response induced by *Brucella* species infection. The injection of brucellergen, a protein extract of a rough strain of *Brucella* species *(Brucella melitensis strain B115)*, 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 (129). 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 (50,74). This test is prescribed as an alternative test by the OIE (70).

1.9. Epidemiology

The global distribution of brucellosis is constantly changing, with new foci emerging or re-emerging. The epidemiology of human brucellosis has considerably been changing over time because of various sanitary, socioeconomic, and political reasons, together with increased international travel. New foci of human brucellosis have continued to emerge, particularly in central Asia (28). The disease in animals occurs worldwide, except in those countries where brucellosis has been eliminated (defined as the absence of any reported cases for at least five years). These countries include Australia, Canada, Cyprus, Denmark, Finland, The Netherlands, New Zealand, Norway, Sweden, and the United Kingdom. The Mediterranean Countries of Europe, northern and eastern Africa, Near East countries, India, Central Asia, Mexico and Central and South America are still not brucellosis free. While *B. melitensis* has never been detected in some countries, there are no reliable reports that it has ever been eradicated from small ruminants in any country besides the ones given above(140).

In sub-Saharan Africa (SSA), the introduction of exotic animals with better productivity is partly constrained by infectious diseases, of which bovine brucellosis is one of them (49,141). Serological evidence of brucellosis is abundant throughout SSA. Evidence of brucellosis and its impact in cattle can be found in a range of reports from countries including Ethiopia, Egypt, Kenya, Uganda, Zambia, and Zimbabwe (142–148). However, it is scattered in time and space and, in addition, good quality data that can be interpreted in strict epidemiological terms are rather scarce (49,141,149). Over a decade ago, McDermott and Arimi (49) noted that the figures reported had to be interpreted with caution because of uncertainties in test implementation and validation. Despite these diagnostic uncertainties, a number of studies in Africa show that individual brucellosis seroprevalence may correlate with the number of abortions (49,150,151).

Whether brucellosis sero-prevalence is higher under extensive or intensive breeding conditions has been debated (149). In sub-Saharan African countries, McDermott and Arimi (49) reviewed individual animal level data in cattle as varying from 7.5 to 40%

for pastoralists in arid and semiarid areas, 0.3–25.4% for cash/subsistence crops with livestock in sub-humid areas, 1.5–16.2% for crop-livestock in tropical highlands. The wide range in values indicates that, based on early evidence, it is not possible to draw general conclusions on the significance of these production systems on the individual prevalence, as already observed by Mangen *et al*. (141).

In small ruminants, there is limited information on brucellosis in SSA. It is considered that brucellosis in these livestock species is caused mostly by *B. melitensis* but, as there are very limited bacteriological studies, different epidemiological scenarios are plausible. (49,149). The few recent studies generally indicate a low individual prevalence that could reflect a common existence of chronically infected flocks and herds. However, flock or herd prevalences have been rarely if ever reported, and these are necessary for a complete picture and an assessment of the problems that the disease may pose if breeding conditions and epidemiological circumstances change (152).

In Sahel and East African arid and semi-arid areas, camels often react in serological tests for brucellosis, suggesting usually low individual seroprevalence (42,49,142,149,153–156). The herd seroprevalence in camels were found to be relatively high, 15% in Borena, Southern Ethiopia (154) and 24% in Afar, Eastern Ethiopia (157). However, the tests used in these surveys have not been validated in these animals (156).

1.9.1. Bovine brucellosis in Ethiopia

In Ethiopia, brucellosis in cattle has been reported from different regions of the country, in both intensive and extensive management systems. A 38% seroprevalence of bovine brucellosis in western Ethiopia has been reported (158), while most of the studies suggested a low seroprevalence of below 5% in cattle, as summarized in Table 1.2.

Table 1.2: Individual animal level seroprevalence of bovine brucellosis from different geographical areas of Ethiopia.

Study area	Nº tested	Prevalence (%)	95% CI	Type of test	Production system	Author/s
Central Oromia	1,238	2.9	2.0-4.0	RBT, CFT	Extensive & Intensive	(159)
Tigray	816	3.19	2.0-4.6	RBT, CFT	Extensive	(160)
Jimma Zone	1,595	3.1	2.2-4.0	RBT, CFT	Extensive & Intensive	(161)
Jigjiga Zone	435	1.38	0.5-3.0	RBT, CFT	Agro-pastorals	(162)
West Tigray	1354	4.9	3.7-6.2	RBT, CFT	Extensive & Intensive	(163)
Southern & Eastern Ethiopia	1,623	3.5	2.6-4.4	RBT, CFT	Extensive and pastoral	(150)
Addis Ababa	1,202	1.5	0.8-2.4	RBT, CFT	Intensive	(164)
Borena	575	8.0	5.9-10.5	RBT, CFT	Pastoral	(154)
East Wollega	406	1.97	0.8-3.8	RBT, CFT	Extensive	(165)
Western Ethiopia	1,152	1.0	0.5-1.8	RBT, CFT	Extensive	(166)
Northern Ethiopia	441	0.4	0.05-1.6	RBT, CFT	Intensive	(143)
Southern Ethiopia	719	3.2	2.0-4.7	RBT, CFT	Intensive	(143)
Central Ethiopia	567	1.6	0.7-2.9	RBT, CFT	Intensive	(143)
Somali & Borena	862	1.4	0.7-2.4	RBT, ELISA	Pastoral	(167)
Arsi	417	1.7	0.6-3.4	RBT, i-ELISA	Intensive	(168)
East Shewa	300	2.0	0.7-4.3	RBT, CFT	Intensive	(169)
Alage	804	2.4	1.4-3.6	RBT,c-ELISA	Extensive and intensive	(170)
Asella & Bishoftu	570	1.4	0.6-2.7	RBT, CFT	Intensive	(171)
Harar, Dire Dawa	967	1.3	0.7-2.2	RBT, CFT	Intensive	(172)
Nechsar park	268	9.7	7.0-14.7	RBT/i-ELISA, CFT	Extensive	(173)
Addis Ababa	1550	0.06	0.002-0.3	RBT, C-ELISA	Intensive	(174)

1.9.2. Small ruminant brucellosis in Ethiopia

Brucellosis in small ruminants has been reported by various studies in Ethiopia. Most studies conducted in small ruminants were from pastoral livestock production systems where mainly sheep and goats are reared together. The prevalence of brucellosis in small ruminants ranged from 0.4% in mixed crop livestock production system in Bahir Dar (175) to 13.7% in Afar pastoral region (176). Seroprevalence recorded of small ruminant brucellosis in Ethiopia from different geographical areas is indicated in Table 1.3.

Table 1.3: Individual animal seroprevalence of small ruminant brucellosis from different geographical areas of Ethiopia.

Study	No Tested	Prevalence (%)	95% CI	Type of test	Production	Authors
Afar and Somali	2000	9.7	8.4-11.1	RBT, i-ELISA	Pastoral	(177)
Afar Region	1568	4.8	3.8-6.0	RBT, CFT	Pastoral	(178)
South Omo	384	4.2	2.4-6.6	RBT, CFT	Pastoral	(179)
Bahir Dar	500	0.4	0.04-1.4	RBT, CFT	Extensive	(175)
Jigjiga	730	1.51	0.7-2.6	RBT, CFT	Pastoral	(180)
Dire Dawa	384	9.1	6.4-12.4	RBT, CFT	Pastoral	(181)
Yabello, Oromia	384	1.56	0.5-3.3	RBT, CFT	Pastoral	(182)
Southern Tigray	985	3.5	2.4-4.7	CFT	Extensive	(183)
Afar region	1050	13	11.0-15.2	RBT, CFT	Pastoral	(157)
Southern & Central	3315	1.9	1.4-2.4	RBT, CFT	Extensive and	(184)
Somali and Oromia	420	3.6	2.0-5.8	RBT, CFT	Pastoral	(185)
Afar Region	414	13.7	10.5-17.4	RBT, CFT	Pastoral	(176)
Mojo abattoir	853	1.76	1.0-2.8	RBT, CFT	Pastoral	(186)
Arsi and East Shewa	840	4.6	3.3-6.3	RBT, i-ELISA	Extensive	(187)
Afar Region	1190	12.4	10.5-14.4	RBR, CFT	Extensive	(188)
Somali region	291	1.37	0.4-3.4	RBT, CFT	Pastoral	(189)

1.9.3. Camel brucellosis in Ethiopia

In Ethiopia, camels are a subset of large livestock resource with a population of 1.1 million (190). Among the pastoral and agropastoral communities of Ethiopia, camels are the most important livestock species uniquely adapted to live in hot and arid environments that are inhospitable to other domestic animals (191). Camels are traditionally raised by these communities primarily for milk production (192). Despite the presence of large populations of camels in the pastoral areas of Ethiopia, camel brucellosis in Ethiopia is largely unstudied. Initial data published on camel brucellosis (193) in the provinces of Sidamo, Harar and Tigray reported a an individual animal seroprevalence of 4.4% (n=977). In addition, Teshome *et al.* (194) reported a seroprevalence of 4.2 % by testing 1,442 camels in arid and semi-arid camel- rearing regions (Afar, Somali and Borena) of Ethiopia. In Borena lowland, Megersa *et al*. (195) and Megersa *et al*. (196), detected anti-*Brucella* antibodies in 1.8% (58/3,218) and 2.2% (17/756), respectively, of camels tested. In camels of southeast lowland areas of the Somali Region, Tilahun *et al.,* (191) reported an individual animal and herd seroprevalence of 2.43% (n=822) and 10.3% (n=185), respectively.

1.9.4. Human brucellosis in Ethiopia

As compared to studies of animal brucellosis, studies of human brucellosis in Ethiopia are sparse with even less information on risk factors for human infection. The literature survey indicated that studies conducted on human brucellosis mainly focused on hospitals or health clinics, involving patients with febrile illnesses. However, there were few studies involving occupationally linked groups. Table 1.4 shows prevalence/incidence of human brucellosis in various geographic locations in Ethiopia.

Table 1.4: Seroprevalence of human Brucellosis from different geographic areas of Ethiopia.

Study area	No tested	Prevalence/ Incidence (%)	95% CI	Type of test	Sampled population	Authors
West Gojjam	653	2.6	1.8-4.7	SAT	Febrile patients	(197)
Yabello	88	34	24.3-45.0	IgM/IgG lateral flow assay	Febrile patients	(198)
Matama	100	3	0.6-8.5	IgM/IgG lateral flow assay	Febrile patients	(198)
Jimma	56	3.6	0.4-12.3	RBT/CFT	Febrile patients	(199)
Afar Region	200	15	10.3-20.7	RBT/CFT	Febrile patients	(200)
Addis Ababa	336	4.8	2.7-7.6	2-Mercapto Ethanol Test	Occupationally linked	(60)
Western Tigray	246	2.1	0.3-3.5	RBT/CFT	Occupationally linked	(201)
Adami Tulu	93	2.1	0.3-7.5	RBT/CFT	Occupationally linked	(61)
Modjo	149	1.3	0.2-4.7	RBT/CFT	Abattoir workers	(62)
Arba Minch	254	10.6	7.1-15.0	Standard Tube titration	Blood donors	(202)

1.10. Economic impact of brucellosis

Estimates of the economic impact of the disease include direct and indirect economic losses attributable to brucellosis and the wide breadth of consequences that brucellosis has on the livelihood of livestock stakeholders in low-resource communities. Direct effects include those that are visible or directly evident to the stakeholder, and those that are invisible or that include forgone production potential (203). Visible losses include livestock abortion, reduced milk production, lost draught power, reduced weight gain from chronic infections and ill-thrift, premature death or culling of unproductive stock, veterinary costs associated with diagnostics and vaccination, and diminished animal welfare. In endemic areas, *Brucella* spp. can cause a significant reduction in herd productivity that compromises food security and the livelihood of farmers who depend on the sale or trade of surplus meat, dairy, and offspring from their animals (204).

The invisible direct losses include reduced herd fertility and the costs associated with changing the herd structure to compensate for the overall reduction in productivity and fertility. Infertility due to brucellosis in cattle is caused by post-abortion metritis and retained placentas (33). Additional invisible losses are associated with spill-over of *Brucella* infection from livestock to wildlife or feral animals that can then be a source of spill back of the disease to surrounding herds (204). Moreover, forgone revenue related to brucellosis includes trade restrictions from areas endemic with *B. melitensis*, *B. abortus*, and *B. suis*. These *Brucella* spp. are listed as notifiable diseases by the OIE – a list made in compliance with the Sanitary and Phytosanitary Agreement of the World Trade Organization that helps to guide policy development concerning the international trade of products contaminated with specific biological agents (83).

In endemic areas, economic impact of brucellosis in humans includes direct costs related to healthcare, or medical expenditures for the diagnosis, treatment and management of clinically-ill patients, and non-healthcare costs, or those that provide a patient with access to care (205). Non-healthcare costs include expenses related to a patient's access to care and the prevention of disease. Transport to and from

medical facilities, housing accommodations while care is being received, and a loss of workdays or leisure time when seeking medical attention can amount to significant expenses that the patient or their families are responsible for paying out of-pocket. It should also be noted that while individuals in low-resource settings have an increased prevalence of health complications and shorter life expectancy, access to health services in these areas is significantly lacking (206). Indirect costs include those associated with the morbidity and mortality of a disease that specifically affect the patient and society in which the patient lives (205).

1.11. Treatment

Brucellosis is one of the neglected diseases and treatment of brucellosis in domestic animals is not indicated. In humans, due to intracellular localization of *Brucella* and its ability to adapt to the environmental conditions encountered in its replicative niche, *e.g.* macrophages, treatment failure and relapse rates are high, being influenced by the drug combination and patient compliance (27). There is strong evidence that the tetracyclines (especially doxycycline and minocycline) are the most effective drugs for brucellosis treatment (207). The choice of regimen and duration of antimicrobial therapy should be based on whether focal disease is present or there are underlying conditions that contra-indicate certain antibiotics (*e.g*. pregnant patients or young children) (208).

Most individuals with acute brucellosis respond well to a combination of doxycycline plus aminoglycosides or rifampicin for 6 weeks. Monotherapy with doxycycline or minocycline, or a combination of doxycycline with trimethoprim–sulfamethoxazole (TMP–SMZ), a quinolone and rifampicin may be an alternative. Patients with focal disease, such as spondylitis or endocarditis, may require longer courses of antibiotics, depending on clinical evolution (39). Patients with persistent symptoms following extended antibiotic therapy, for whom focal disease or relapse have been ruled out, pose a difficult clinical management problem. This disabling syndrome, sometimes called chronic brucellosis, is similar to chronic fatigue syndrome and must be treated symptomatically (209).

1.12. Control and prevention

In the developed world, for more than four decades, control and eradication programmes of brucellosis in livestock have been implemented by national veterinary services. Classically after a first phase in which the infection is controlled by compulsory vaccination, then vaccination is gradually restricted and eventually prohibited with a “test and slaughter” policy implemented in order to eradicate the infection once incidence is sufficiently low to make this practical. More than a decade is usually needed to complete the brucellosis eradication programme by a “test-and-slaughter” policy and key for success is a sufficient financial compensation scheme for farmers for their culled livestock (210). In the European Union (EU), such national programmes are co-financed by the EU and the Member States (MSs). This policy has been successfully implemented for bovine as well as ovine and caprine brucellosis in Northern MSs, whereas eradication programmes, particularly ovine and caprine brucellosis eradication programmes are not yet completed in some Southern European MSs (52).

In resource poor countries, the implementation of an efficient eradication policy is very challenging and thus innovative approaches taking into account the scarcity of financial resources as well as the perceptions and attitudes of communities have to be defined where human brucellosis is documented to be a public health problem (211). One example of such an innovative approach has been studied in Mongolia where the economic benefit, cost-effectiveness, and distribution of benefit of improving human health through the control of brucellosis by mass vaccination of livestock has been estimated (71,212). In Tajikistan biannual conjunctival vaccination of small ruminants with Rev 1 reduced the seroprevalence by 80 per cent in 5 years and the prevalence of households with evidence of infection in their animals dropped from 25.1 to 7.5 percent (213).

The re-emerging of *B. melitensis* infection in sheep and goats and its public health impacts in many South Eastern European (SEE) and Mediterranean countries has worsened for over a decade. Brucellosis, from low prevalence became an important

endemic disease, especially in the Balkans, with high prevalence in sheep and goats, as well as in humans (214,215). In part, this has been the result of political changes, conflicts and wars in the new countries established in early 1990s after breaking away from the former Yugoslavia (43,216). In addition, shortages of funds reduced vigilance or ceased implementation of the recommended control programmes and, together with more intensive international human travelling, cross-border movement of animals, and insufficiently controlled trade in livestock and agricultural products, have resulted in a resurgence and worsening of the prevalence of brucellosis in the SEE countries (217).

In Sub Saharan African, it has been reported that vaccination was rarely conducted outside of southern Africa and that, if done, it was mostly on an *ad hoc* basis, rather than being part of a systematic campaign. For this region, the disease was reported to be notifiable in several countries, and surveillance, movement control, and stamping out or vaccinations were implemented in Botswana, Namibia, Lesotho and South Africa (49). In Zimbabwe control programmes were targeted to specific cattle production systems. A test-and-slaughter programme was partially implemented in goats in KwaZulu-Natal, which apparently managed to reduce prevalence but not to eradicate the disease. Problems reported in the implementation of this policy were not different from those observed elsewhere (i.e. time and financial constraints and stockowner disillusionments with the compensations) (218).

Despite the huge efforts invested on the control of animal brucellosis, results have not always matched the expectations. This situation may be the consequence of the combined effect of several factors, including those inherent to the disease regardless of the etiological agent/infected host and limited sensitivity of some diagnostic tests in certain epidemiological situations and also other factors associated with the etiological agent and the host (29,39). Still, three major strategies have been demonstrated as effective tools to control brucellosis in domestic animals when used in combination: strict biosecurity at the farm level, test-and-slaughter programmes and immunization of the susceptible population (49).

The sole implementation of one of these measures is however much less effective since optimal results are obtained when at least two of them are applied jointly. Still, the best strategy will depend on the epidemiological situation in a given setting, the availability of resources, etc. Moreover, in addition to these "classical" strategies, other complementary tools should be considered to ensure the success of the programme (animal identification, animal movement control, economic compensations, etc.) (219).

1.12.1. Management and biosecurity

Management and hygienic measures against *Brucella* infection must be focused on diminishing the possibility of contact with viable *Brucella*, including both infected animals and contaminated environments. The most frequent route of entry of *Brucella* onto a free farm are the following: purchase of infected animals that can shed the bacteria to the environment, therefore exposing susceptible individuals, contact with infected material, pastures, etc. (220).

The use of appropriate biosecurity measures is of critical importance to prevent the entrance of the disease in a naïve epidemiological unit. These strategies include the implementation of quarantine before the introduction of new animals, the separation of animals with an unknown/uncertain status, the control of animal movements, the adequate management of replacement, the isolation of pregnant females before parturition, and the strict quality/sanitary control of semen (219). It is also important to avoid or limit the contact between livestock containing bulls for artificial insemination and wildlife in environments where wild animals have been seen to be a source of infection (49).

In infected settings, in addition to the biosecurity recommendations cited above, hygienic measures are essential to limit and control the bacterial load in the environment, decreasing the possibility of contact with viable *Brucella* species, and should be systematically implemented. Removal of abortion products, full cleaning

and disinfection of premises, elimination of infected manure, and incineration of infected material are some examples of measures to attain this objective (219). In human, the most rational approach for preventing human brucellosis is control and eradication of the infection in animal reservoirs. In addition, there is a need to educate the farmers to take care in handling and disposing of aborted foetus, foetal membrane and discharges. Education of the general populace, in the dangers of unpasteurized milk and dairy products and abattoir workers in transmission of infection especially via skin abrasion has paramount importance (3,39,43).

1.12.2. Test and slaughter programmes

The main aim of this approach is the early detection and removal of possible sources of infection, thus avoiding circulation of *Brucella*. Despite the effectiveness of the diagnostic strategy used, there is always a certain risk of having infected animals that may remain as silent carriers maintaining the pathogen in the flock and, if there is a drop in the immunity of the herd, it may lead to an abortion storm (39). This strategy is most useful in low-prevalence settings where economic resources and veterinary expertise are available for its support (48). Test-and-slaughter strategies may also be useful for the management of outbreaks, particularly when numbers of animals make the implementation of stamping-out measures unfeasible. In some cases, the only measure that achieves complete elimination of the bacteria on the flock is the stamping out followed by a thorough cleaning and disinfection of barn and replacement with *Brucella*-free animals (29,39).

1.12.3. Immunization

As a general rule, vaccination efforts are often focused in the most susceptible individuals in order to stimulate a protective immune response against the pathogen of interest. However, in the case of animal brucellosis, vaccination campaigns target a less susceptible population, while vaccination of the most susceptible subset is avoided due to the side effects of vaccination of pregnant animals (219).

The characteristics of the "ideal vaccine against brucellosis" include the following (221):

(i) to induce a solid and long-lasting protection against the infection by different *Brucella* species without the need of re-vaccinations,
(ii) to be innocuous regardless of the reproductive stage of the animal,
(iii) to have no or very low residual virulence for human and be susceptible to the antibiotics typically used to treat human brucellosis,
(iv) to avoid the induction of cross-reacting antibodies in the serological techniques traditionally used in control/eradication programmes,
(v) to be affordable, and
(vi) to possess stability at different environmental temperatures

Even though we still do not have an ideal vaccine, vaccination with available vaccine strains remains the most successful method for the prevention and control of brucellosis in cattle, being a critical component of most brucellosis control and eradication programmes throughout the world (222). Numerous countries have adopted control measures against bovine brucellosis in order to reduce the prevalence or eradicate the disease from domestic livestock, in an effort to prevent transmission to humans and mitigate economic losses (223). Vaccination of female calves is the central point of any brucellosis control programme, since it has performed well in the reduction of disease prevalence, and is therefore useful at the disease control stage (221). Considering that vaccination alone is not enough to control and eradicate the disease, it should be associated with continuous elimination of infected animals, as they are the source of new infections. Thus, besides vaccination, most bovine brucellosis eradication programmes also include test–and–slaughter policies, surveillance and hygiene measures (223).

Only a few vaccines have been widely used in cattle immunization against *B. abortus* including S19, RB51, 45/20 and SR82 with S19 and RB51 being the most widely used

(83) . However, many *B. abortus* vaccine candidates have been developed, including DNA, subunit, recombinant *B. abortus* and recombinant vector vaccines. All of them are evaluated principally in experimental mouse models, and with a few exceptions, the majority of these new vaccines have not been tested in cattle or were not protective in cattle, the target species (222).

The smooth S19 strain is the most widely employed vaccine in the case of bovine brucellosis, and even though it is currently considered the reference strain, it presents similar limitations as *B. melitensis* strain Rev.1 vaccine despite its demonstrated efficacy (70). For these reasons, the rough strain RB51 is increasingly used in some regions of the world as an alternative for vaccination against bovine brucellosis since its usage does not induce the production of cross-reacting antibodies, even though its efficacy is still under discussion in certain epidemiological situations (223).

It has been reported that intravenous administration of full doses (1-3.4x10^{10} colony-forming units [CFU]) of *B. abortus* strain RB51 were found to induce severe placentitis and placental infection in most vaccinated cattle, as a result there is excretion of the vaccine strain in milk in a relevant number of vaccinated animals. Experiences from field also indicates that it can induce abortion and increased perinatal mortality if applied to pregnant cattle(83). When the dose of the vaccine is reduced (1×10^{9} CFU), no abortions or placentitis lesions have been reported, but a significant proportion of vaccinated animals can shed the vaccine strain. However, this reduced dose does not protect against *B. abortus* when used as a vaccine in calves, and the protection against *B. abortus* is only moderate when used as an adult vaccine (223).

Brucella melitensis Rev.1 is the most widely used vaccine for the prevention of brucellosis in sheep and goats, and, despite its drawbacks, remains the reference vaccine with which any other vaccines should be compared. By contrast, the rough *B. abortus* RB51 vaccine is not effective against *B. melitensis* infection in sheep(224). The Rev.1 vaccine is used as a freeze-dried suspension of live *B. melitensis* Rev.1 strain for the immunisation of sheep and goats. It should be given to lambs and kids aged between three and five months as a single subcutaneous or conjunctival inoculation,

five months being the upper time limit to minimise the antibody response to make this vaccination compatible with further serological testing. No matter the inoculation route, the standard dose must be between 0.5×10^9 and 2.0×10^9 viable organisms(70). The reduced doses confer a significantly lower protection than the standard doses and should not be recommended for vaccinating sheep and goats. The subcutaneous vaccination induces long lasting serological responses, causing strong interferences in serological tests and should not be recommended for use in combined eradication programmes. However, when this vaccine is administered conjunctivally at the standard dose, it produces a similar protection without inducing a persistent antibody response, thus facilitating the application of eradication programmes combined with vaccination (83).

Care must be taken when using *B. melitensis* Rev.1 vaccine to avoid the risk of contaminating the environment or causing human infection. In many developing countries and endemic areas, vaccination of the whole population has to be considered as the best option for the control of the disease (225). However, the Rev.1 vaccine is known to often cause abortion and excretion in milk when animals are vaccinated during pregnancy, either with a full or reduced dose (225). These side effects are considerably reduced when adult animals are vaccinated conjunctivally (full dose) during lambing/kidding, lactation or before mating. Therefore, when mass vaccination is the only means of controlling the disease, a vaccination campaign should be recommended using the standard dose of Rev.1 administered by the conjunctival route when the animals are not pregnant or during the late lambing/kidding and pre-breeding season (225).

In general, the inability to vaccinate pregnant animals is a major disadvantage in animal brucellosis vaccination since it complicates achieving a quick increase in the proportion of the resistant subset of the population, by reducing the transmission between animals by means of mass vaccination (221). The success of the use of live vaccines for immunization against animal brucellosis is based on a balance between an adequate colonization of the host, triggering a solid protection against infection

with other *Brucella* field strains, and a limited replication that minimizes the residual virulence of these vaccine strains (223). Although immunological mechanisms induced by live vaccines have not been completely elucidated, they should stimulate the innate immunity, activate CD8+ and CD4+ cells, and generate an adequate population of memory cells, among other mechanisms, to induce a solid protection (219).

Most of the drawbacks associated with the use of live vaccines could be overcome with the use of killed bacteria or subunit vaccines; however, the ability of these inactivated vaccines to provide a solid and long-lasting immune response against *Brucella* has traditionally been considered lower than that triggered by live attenuated vaccines. New approaches are being investigated to elude the main drawbacks of inactivated vaccines to induce protective immunity in domestic livestock (221,223).

1.13. Thesis objectives

Ethiopia is believed to have the largest livestock population in Africa (226) with an estimated population of 54 million cattle, 25.5 million sheep, and 24.06 million goats. From the total cattle population 98.95% are local breeds and the remaining are crossbred and exotic breeds. Ninety nine per cent of the sheep and nearly 100% of the goat population in the country are local breeds (226).

Livestock production systems in Ethiopia have been classified into pastoral, agro-pastoral, mixed crop-livestock farming, urban and peri-urban dairy farming and specialized intensive dairy farming systems based on integration of livestock with crop production , level of input and intensity of production, agro-ecology and market orientation(227–229). There are also breeding farms, which are owned by the government and with the primary objective of breed improvement through crossing of indigenous breeds with exotic breeds (Holstein-Friesian and Jersey). These farms supply pregnant cross breed heifers or bulls to rural small holder dairy farms (143). In this PhD project a cross-sectional study design was employed to investigate the epidemiology of brucellosis in urban, peri-urban and commercial dairy farms in Addis Ababa, in cattle, sheep and goats and in pastoral communities who had a frequent

close contact with livestock in Borena pastoral zone and an outbreak investigation and molecular characterisation of the causative agents at Adami Tullu Agricultural Research Centre (ATARC) dairy breeding unit, central Ethiopia. In cross-section studies a cross section of target population was systematically sampled, tested for anti-*Brucella* antibody using serological tests and potential risk factors precipitating the disease in a study population were determined. Outbreak investigation involved serological testing, postmortem examination and molecular characterisation of the causative agents.

The dairy industry has been growing to meet an ever-increasing demand for milk and milk products in the country. Crossbreeding indigenous cattle with high yielding exotic cattle is the main policy established by the Ethiopian government to bridge the gap between supply and demand for dairy products. Since the first report of brucellosis in Ethiopia in 1970s (230), the disease has been noted as one of the important livestock diseases in the country (161,231). As brucellosis is known to hamper livestock productivity through abortion and infertility, establishing current and reliable scientific data on such a disease is of paramount importance.

With the second largest human population in Africa (232–235), Ethiopia is particularly vulnerable to the effect of zoonotic diseases because the economy is largely dependent on agriculture (236,237) and roughly 80% of households have direct contact with domestic animals, creating an important risk for infection and spread of disease (238,239). Ethiopia also ranks very high in the health burden of zoonotic diseases and in having a large population of poor livestock keepers (240).

Brucellosis has been listed as a priority animal disease of socioeconomic and trade significance and the third priority zoonotic disease next to rabies and anthrax in Ethiopia calling for multi-sectoral collaboration to develop feasible control strategy(241). However, most serological evidences generated so far have limited geographic coverage. Comprehensive studies on brucellosis in different domestic animal species, zoonotic significance in occupationally linked humans and which *Brucella* species are circulating and responsible for natural *Brucella* infection in the country are scarce.

So far, apart from the study by Tekle *et al.* (242) there was no any attempt to isolate and characterize of *Brucella* spp. in Ethiopia and all available reports are based on serological evidence. Isolation and characterisation of circulating *Brucella* species among livestock systems will play a crucial role in future planning and designing feasible control strategies. Knowledge of genetic diversity of *Brucella* would also help to better understand the epidemiology and transmission dynamics across different species and geographic regions. Hence, to collect potential samples for *Brucella* culture, it is important that a clinical case of brucellosis such as abortion cases during last trimester of gestation period in cattle and sheep and goats should be recruited. The poor livestock disease reporting system in Ethiopia means that most outbreak cases of abortion in villages and private farms go unnoticed and therefore underreported. Establishing a surveillance network involving regional veterinary laboratories, dairy cattle cross breeding centres, dairy cooperative unions and regional veterinarians will help to identify infected foci to make further investigation.

The need for a better understanding of occurrence of brucellosis in emerging dairy systems in central Ethiopia, cattle and small ruminants in traditional livestock production system sharing common eco-zone and management under Borena pastoral setting and parallel study to determine risk of zoonosis in occupationally linked livestock keepers and herders, and isolation and molecular characterisation of circulating *Brucella* species lead to the inception of this PhD project.

The general objectives of this research project are to investigate the epidemiology of brucellosis in urban and peri-urban dairy production systems in Addis Ababa, Central Ethiopia, in domestic ruminants kept under traditional livestock production systems among pastoralists in Southern Ethiopia, which *Brucella* species are circulating in the study area, and to explore its public health significance in these settings. Better understanding of the epidemiology of the disease and its zoonotic risk would be a vital input for policy makers in public and livestock health departments of the country to help them shape future intervention programmes. With serological surveillance in these two different livestock productions systems and outbreak investigation of

brucellosis, this work set out to achieve the following specific objectives:

1. Estimate the prevalence of *Brucella* infection in urban and peri-urban dairy production systems of Addis Ababa using serological tests.
2. Estimate the prevalence of *Brucella* infection in cattle, small ruminants and occupationally associated animal attendants and shepherds in Borena pastoral zone using serological tests.
3. Assess the knowledge, attitude and practices in occupationally associated farm/herd owners and animal attendants both Addis Ababa and Borena.
4. Identify risk factors precipitating brucellosis infection in cattle, shoats and occupationally associated animal owners, animal attendants and shepherds
5. Isolation and molecular characterization of the causative agent of brucellosis circulating in the study areas.
6. Investigate the genetic diversity of Ethiopian *B. abortus* and *B. melitensis* isolates, and their phylogenetic placement relative to the global diversity of these species.

2. CHAPTER TWO

All the fieldwork in Addis Ababa including sample collection, testing and data analysis was conducted by the PI (Bedaso Mammo Edao) of this PhD project. Technical assistants from Addis Ababa University, Aklilu Lemma Institute of Pathobiology and farm workers assisted during animal restraining and sample collection.

The work in this chapter has been published in BMC Veterinary Research Journal.

Bedaso Mammo Edao, Gizachew Hailegebreal, Stefan Berg, Aboma Zewude, Yemiserach Zeleke, Teshale Sori, Gizat Almaw, Adrian M. Whatmore, Gobena Ameni and James L. N. Wood. (2018). Brucellosis in Addis Ababa dairy cattle: the myths and the realities. *BMC Veterinary Research* **14**:396.

2.1. Background

Ethiopia has one of the largest cattle populations in Africa (243) despite gaining minimum return from this resource as a result of various technical and non-technical factors, including infectious diseases. Bovine brucellosis is one of the infectious diseases hampering productivity of cattle and has been reported from several parts of the country (161). Bovine brucellosis is a zoonotic disease with economic and public health impact, particularly for human and animal populations in developing countries that rely mainly on livestock production (155). The disease can cause significant loss of productivity through abortion, stillbirth, low herd fertility and low milk production (48).

Market-oriented new dairy industries are emerging in Ethiopia to contribute towards filling the gap between an increasing national demand and supply of milk and milk products. The dairy development roadmap of the country is aimed at increasing the productivity of indigenous cattle through improvements in genetics, health and feeding to satisfy demand and to facilitate export of milk and milk products. Hence, public and private dairy industries and cooperatives require up to date and consistent scientific and surveillance data on bovine brucellosis, which can also assist in establishing nationwide bovine brucellosis intervention policies aimed at controlling

and eradicating the disease. This study was designed to estimate the seroprevalence of brucellosis in dairy cattle in Addis Ababa, identify potential risk factors and to assess Knowledge Attitude and Practices (KAP) of the farm workers towards the disease.

2.2. Methods

2.2.1. Description of the study area

The study was conducted in Addis Ababa dairy farms. Addis Ababa, the capital city of Ethiopia, lies at an elevation of 2,300 meters (7,500 ft) above sea level and is featured by a grassland biome. It is geographically located at 9°1'48"N latitude and 38°44'24"E longitude. It has a typical highland climate with temperature ranging from 11°C - 24°C. Addis Ababa has a mean annual rainfall of 1,300 mm with bimodal distribution. The city is divided into 10 boroughs (Figure 2.1), called sub cities, and 99 wards (kebeles) (244).

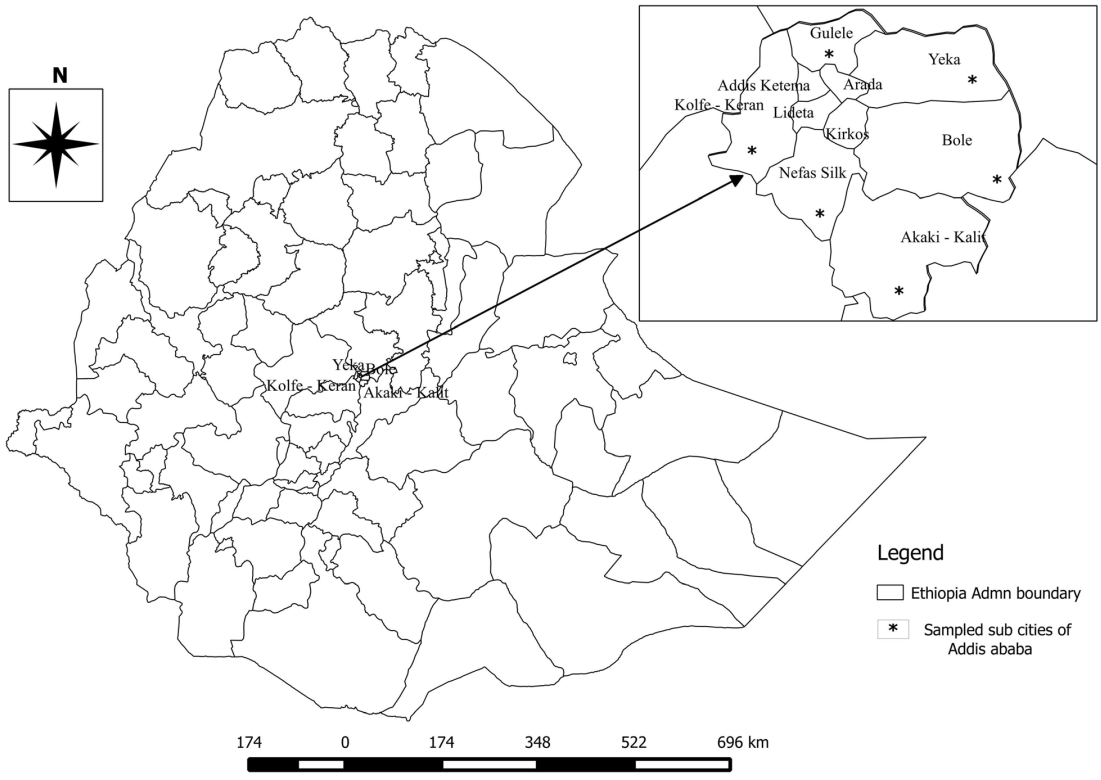

Figure 2.1: Map of Ethiopia and Addis Ababa. Legend: The map is developed from Ethiopian shape files using QGIS Software,2013. The polygons in the map of Addis Ababa represents studied boroughs/sub-cities.

Dairy cattle production systems in Ethiopia are classified into commercial dairy systems, urban and peri-urban smallholder dairy, rural smallholder (mixed crop and livestock production), and pastoral and agro pastoral (143). The dairy systems hold mainly exotic breeds or crosses with the local zebu breeds, while the rural husbandry systems stock mainly zebus. This study focused on urban and peri-urban smallholder farms, which produce milk for home use and sale, and commercial dairy systems, which are producing milk as a full-time business. These dairies constitute the main dairy source for the capital and produce milk for sale (171). Within these systems dairy farms were classified based on size of the herd and herd management into large scale farms, with more than 50 animals, medium scale farms (20 to 50 animals) and smallholder farms (<20 animals).

2.2.2. Variables collected

Explanatory variables hypothesized to be risk factors for the disease were assessed at both individual animal and farm level. Information related to herd structure was extracted either from individual herd records or from farm owners or managers interviewed using pretested structured questionnaires. Herd level parameters included herd size, the presence of reproductive problems such as abortion, retained foetal membranes and still birth in the farm, separate calving pen, brucellosis testing in the farm, frequent contact with animals in other herds and species, which were categorized as yes or no variables. The major reasons for culling were coded either as reproductive problems, old age or logistics. Breeding strategy was classed as artificial insemination, bull or both. The afterbirth (aborted foetuses and foetal membranes) disposal method was categorized into burying, open dump or feeding to dogs. The feeding and water supply strategies were classified into communal and own (Appendix 3).

Individual animals were categorized as young (6-17 months old) or adult (≥17 months old), breed as Holstein-Friesian (HF), cross, or as local Zebu. The origin of each individual animal was defined as either own stock or purchased. The clinical reproductive disorder history in the past twelve months, including abortion, stillbirth

and retained foetal membrane, was categorized as either yes or no variables. Parity number and frequency of some of the aforementioned disorders were also recorded. Physiological status of an animal was categorized as pregnant, non-pregnant, lactating and lactating pregnant. Repeat breeding was also assessed based on the animal owner's general observation. Accordingly, cows that needed three or more services per pregnancy were categorized as a repeat breeder otherwise they were categorized as a non-repeat breeder (Appendix 3).

2.2.3. Study population

The target study populations were dairy cattle above six months of age, consisting of breeding females, replacement heifers, and available bulls. The breeds of these animals were crosses of local Zebu breeds with HF. For the KAP study, occupationally associated farm workers, willing to be interviewed, were included.

2.2.4. Study design and sample size

A cross-sectional study design was employed from November 2016 to May 2017. According to Addis Ababa city urban agriculture bureau there are about 880 registered smallholder, medium scale and large commercial dairy farms in Addis Ababa. An individual farm was considered as a herd and the primary sampling frame. The sample size for dairy farms was calculated considering a 9.1% herd level seroprevalence of bovine brucellosis (143), 95% confidence interval (CI) ,5% required precision and 80 % power using the following formula previously described (245).

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

Where n = required sample size; P_{ex} = expected prevalence; d = desired absolute precision.

Hence, a total of 127 dairy farms were considered for this study and proportional allocation was made for each sub city based on the number of farms. In each sub city, herd-sampling frame was established in collaboration with sub city veterinary

department and farms were selected randomly using computer generated random numbers. Before data collection, consent was made with the identified farms owners requesting their farms to be included in the study. Farms where the owners were not willing to participate in the study were replaced by other farms. All cattle above six months of age in the selected dairy farms were sampled and a total of 1550 animals were sampled for serological screening. For the KAP study, farm workers from sero surveyed farms, who agreed to be interviewed, were included. Hence, 130 farm workers from 59 farms participated in the study.

2.2.5. Data collection

Data concerning farm workers KAPs towards the disease were collected by interviewing individuals using a pre-tested structured questionnaire. Verbal consent was obtained from the respondents and the objective of the survey explained to them before start of the interview. The interviews were conducted in local languages (Afaan Oromo or Amharic). The questionnaire focused on demographic characteristic of the interviewee, knowledge-attitude about the disease, handling and afterbirth/aborted fetus disposal practices, habit of raw animal product consumption and animal feeding and housing practices.

Blood samples (10 ml) from the jugular vein of each animal were collected, using sterile needles and plain vacutainer tubes labelled with individual animal identification number. The blood samples were centrifuged at 3000 × g for 10 min to obtain the serum within 12 hours of collection. Sera were decanted into cryo-vials, identified and stored at -20°C until screened for antibodies against natural *Brucella* exposure using serological analysis.

Rose Bengal plate test (RBT): All sera samples collected were initially screened by RBT using RBT antigen (Animal and Plant Health Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) according to (70) procedures. Sera and antigen were taken from refrigerator and left at room temperature for half an hour before the test

to reach room temperature. Briefly, RBT antigen (30 µl) was added onto a glass slide next to an equal amount of cattle sera. The antigen and test serum were mixed thoroughly in a plastic applicator, shaken for 4 min, and agglutination was read immediately. Any observed agglutination by the naked eye was considered to be a positive reaction.

Competitive ELISA: All RBT positive sera were further tested using COMPELISA 160 or 400, a competitive ELISA kit for the detection of antibodies against *Brucella* in serum samples (Animal and Plant Health Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom) at Addis Ababa University, Aklilu Lemma Institute of Pathobiology (AAU-ALIPB). The test was performed according to the manufacturer's instructions. The test was conducted in 96-well polystyrene plates that are pre-coated with *Brucella* species lipopolysaccharide (LPS) antigen. 20 µl of each test serum was added to each well followed by 100 µl of prepared conjugate solution. The plates were then shaken vigorously for two minutes and incubated at room temperature for 30 minutes on rotary shaker, at 160 revs/min. Plates were washed 5 times and dried. Hydrogen peroxidase substrate and chromogen solution was developed for 10 min. 100 µl of *o*-Phenylenediamine dihydrochloride (OPD) solution was added to all wells and the plates were incubated at room temperature for 10 to 20 minutes. Micro plate reader was switched on and the units allowed to stabilize for 10 minutes. The reaction was then stopped using stopping solution. Optical density (OD) was read at 450 nm using micro plate reader. The lack of color development indicated that the sample tested was positive. A positive/negative cut-off was calculated as 60% of the mean of the OD of the 4 conjugate control wells. Any test sample giving an OD equal to or below this value was regarded as being positive. An animal was considered positive if it tested seropositive on both RBT and c-ELISA in serial interpretation.

Complement Fixation Test (CFT): All samples that were RBT-positive were further subjected to complement fixation test as a confirmatory test at the National Veterinary Institute (NVI), Bishoftu, Ethiopia. The *Brucella* antigen and control sera (positive and negative) used during the test were produced by Animal and Plant Health

Agency, UK. The standardization of the antigen was made at 1:20 working dilution (strength). The *Brucella* antigen complement, and 3% sensitized sheep red blood cells were added after the test sera were serially diluted (1:5, 1:10, 1:20, and 1:40) in microtitre plates. Then the plates were incubated at 37°C for 30 min. The test was considered positive when the reading was as partial fixation (50% haemolysis) or complete fixation (no haemolysis) at 1:10 dilution. The test was considered valid when there was complete haemolysis in negative control serum and the positive control shows inhibition of haemolysis.

2.2.6. Case definition

Animals were considered positive to brucellosis when they tested positive on either RBT/CFT or RBT/c-ELISA tests in parallel interpretation. Similarly, a herd or farm was considered seropositive when at least one animal in a herd or farm tested positive. Since there is no history of vaccination against brucellosis in Ethiopia, seropositivity observed in this study was considered to be due to natural infection.

2.2.7. Ethics approval and consent to participate

Ethical clearance for this study was obtained from University of Cambridge, Department of Veterinary Medicine, Addis Ababa University, Aklilu Lema Institute of Pathobiology, Addis Ababa University, College of Veterinary Medicine and Agriculture (AAU-CVMA), Minutes of Animal Research Ethics and Review committee. Before conducting the research, participants were informed with the objectives of the study. A written and signed consent was obtained from the farm owners or managers of the dairy farms to take samples from their cattle and verbal consent was obtained from farm workers to be included in the KAP survey.

2.2.8. Data Analysis

Data generated from the questionnaire survey and laboratory investigations were recorded and coded using a Microsoft Excel spreadsheet (Microsoft Corporation) and analysed using STATA version 13.0 for Windows (Stata Corp. College Station, TX, USA). Descriptive statistics were used for demographic and farm characteristics as well as KAPs relating to bovine brucellosis. The seroprevalence was calculated as the number of seropositive animals divided by the total number of animals tested.

2.3. Results

2.3.1. Knowledge-Attitude and Practices (KAP) of the farm workers about brucellosis

Demographic characteristics

Out of 127 surveyed farms, 130 farm workers in 59 farms were willing to participate in the KAP study. Most workers in the remaining farms refused to participate. Some reported that they were employed in the farm recently and had less contact with animals in the farm, and others preferred not to mention. Of the 130 farm workers responsible for the management of the farm, the majority (88%) was male, and more than 50% were between ages 25 to 60 years. Half of the participants were not married (51.5%) and most of them had family size below 10 people. Eighty percent of the respondents had attended only primary school and about seven percent were illiterate (Table 2.1).

Table 2.1: Demographic characteristics of farm workers in the study area (n = 130).

Demographic characteristics	Category	N	%
Gender	Female	16	12.3
	Male	114	87.7
Age	12-24	54	41.5
	25-60	72	55.4
	>60	4	3.1
Marital status	Single	67	51.5
	Married	63	48.5
Level of education	Illiterate	9	6.9
	Primary	104	80
	Secondary	13	10
	Technical/Diploma	3	2.3
	Degree	1	0.8
No of people in the household *	1-5	61	46.9
	6-10	67	51.5
	>10	2	1.6

* Household defined as family members regularly living together and sharing meals, n = number

Knowledge-attitudes and practices

Most of the study participants reported several risk factors for acquiring bovine brucellosis. The majority of participants, 96% of farm workers in small scale, 100% in medium size and 92.6% in large herd sized farms were not aware of bovine brucellosis. Most respondents, 83.6% in small scale, 60% in medium size and 81.5% in large farms disposed of dead fetus/after birth on an open dump in the environment and approximately seven percent of participants in small herd sized farms fed aborted materials to dogs. Almost all participants in small scale and medium sized farms and 77.8% in large-scale farms practiced assisted parturition without wearing protective

gloves or masks. At least 60% and 80% of farm workers in all farm sizes consume raw milk and meat, respectively (Table 2.2).

Table 2.2: Knowledge-attitudes and practices of farm workers about *Brucella* infection in the study area.

Variables	Proportion of respondents (n)		
	Herd size		
	Small (n=73) n (%)	Medium (n=30) n (%)	Large (n=27) n (%)
Awareness about brucellosis			
Yes	3 (4)	0 (0)	2 (7.4)
No	70 (96)	30(100)	25 (92.6)
Dead fetus/After birth disposal			
Burning/Burying	7 (9.6)	12 (40)	5 (18.5)
Open dump	61 (83.6)	18 (60)	22 (81.5)
Give to dog	5 (6.8)	0 (0)	0 (0)
Assist parturition			
Yes	72 (98.6)	30(100)	21 (77.8)
No	1 (1.4)	0 (0)	6 (22.2)
Consume raw milk			
Yes	50 (68.5)	18 (60)	20 (74)
No	23 (31.5)	12 (40)	7 (26)
Consume raw meat			
Yes	60 (82.2)	25(83.3)	24 (88.9)
No	13 (17.8)	5 (16.7)	3 (11.1.)

n= number

2.3.2. Farm characteristics

Of the 127 farms, 103, 17 and 7 were small, medium and large herd sized farms, respectively. Of the farms assessed by a questionnaire survey, more than 70% of medium and large farms, as well as 43% of small sized farms, had reproductive problems (abortion, stillbirth, retained foetal membrane and repeat breeding) on their farms. The majority of farms were using artificial insemination (AI) for breeding purposes and 71.4% of large sized farms raised their own replacement animals whereas most of small and medium sized farms used both (raised their own stock and purchase). The practices of provision of separate pens for parturition and aborted animals were 28.6% and 14.3% in large sized farms, respectively, whereas there was no such practice in small and medium sized farms and almost all farms used flushing with tap water to clean pens after parturition. The majority (85.7%) of the large herd sized farms, and more than 40% of medium and small-scale farms, used separate feed and water supply for each animal. Reproductive problems and age were prominent culling criteria in all farms and the majority of farms (>70%) in the study area did not report frequent contact of dairy animals with other species (sheep and goat) (Table 2.3).

Table 2.3: Summary of characteristics of dairy farms in the study area.

Variables	**Herd size**		
	Small (n=103)	Medium (n=17)	Large (n=7)
	Frequency (%)	Frequency (%)	Frequency (%)
Reproductive problems			
Yes	44 (42.7)	12 (70.6)	5 (71.4)
No	59 (57.3)	5 (29.4)	2 (28.6)
Service type			
AI	71 (69)	9 (53)	5 (71.4)
Bull	9 (8.7)	4 (23.5)	0 (0)
Both	23 (22.3)	4 (23.5)	2 (28.6)
Replacement stock			
Raised own	31(30.1)	8 (47)	5 (71.4)
Purchased	5 (4.9)	1 (6)	0 (0)
Both	67 (65)	8 (47)	2 (28.6)
Parturition pen			
Yes	0 (0)	0 (0)	2 (28.6)
No	103 (100)	17 (100)	5 (71.4)
Cleaning pen after parturition			
Flushed with tap water	101 (97)	17 (100)	7 (100)
Flushed with water and disinfect	2 (3)	0(0)	0 (0)
Separate pen for aborted cow			
Yes	1 (1)	0 (0)	1 (14.3)
No	102 (99)	17 (100)	6 (85.7)
Feed and water supply			
Own	50 (48.5)	7 (41.2)	6 (85.7)
Communal	5 (4.9)	4 (23.5)	0 (0)
Both	48 (46.6)	6 (35.3)	1 (14.3)
Culling criteria			
Reproductive problem	57 (55.3)	6 (35.3)	4 (57.1)
Logistics	7 (6.8)	3 (17.7)	0 (0)
Age	39 (37.9)	8 (47)	3 (42.9)
Contact with other spp. *			
Yes	12 (11.6)	4 (23.5)	2 (28.6)
No	91 (88.4)	13 (76.5)	5 (71.4)

Contact with other spp. * = Sheep and goat

2.3.3. Seroprevalence of Bovine brucellosis in dairy cattle

A total of 1550 dairy cattle were tested with RBT and 43 (2.77 %) of them were positive in this test. The RBT positive sera samples were further tested using c-ELISA and CFT. Only one animal was confirmed seropositive for bovine brucellosis in the study area based on c-ELISA and no sero reactor animal was found by CFT. In addition to RBT positive sera samples, equal number of randomly selected RBT negative sera were shipped to APHA, Weybridge, UK and further tested using RBT and C-ELISA. However, the result was the same. The seroprevalence of bovine brucellosis in Addis Ababa dairy farms was thus 0.06% (1/1550) based on c-ELISA test. The overall herd level seroprevalence of brucellosis based on c-ELISA was 0.8% (1/127). When stratified on herd size, the prevalence of antibodies to *Brucella* spp. in small, medium and large sized cattle farms was 0%, 5.8% (1/17) and 0%, respectively (Table 2.4).

Table 2.4: Individual animal level and herd level prevalence of bovine brucellosis in dairy farms of Addis Ababa.

Farm type	Individual farm				Herd level			
	No tested	RBT Positive n* (%)	C-ELISA Positive n* (%)	95% CI**	No Tested	RBT Positive n* (%)	C-ELISA Positive n* (%)	95% CI**
Small	821	5(0.6)	0(0)	-	103	5(4.8)	0(0)	-
Medium	363	9(2.5)	1(0.27)	0.01-1.5	17	6(35.3)	1(5.8)	0.15-28.6
Large	366	29(7.9)	0(0)	-	7	3(42.9)	0(0)	-
Total	**1550**	**43(2.77)**	**1(0.06)**	**0.002-0.4**	**127**	**14(11)**	**1(0.8)**	**0.02-4.3**

n*= number positive

CI**= Confidence interval.

2.4. Discussion

Improvement of knowledge-attitudes and practices among urban dairy farm workers could have a significant impact on the reduction of many zoonotic infections, including brucellosis. The results of the KAP study show that the majority of farm workers in the studied dairy farms were not aware of bovine brucellosis (96.1%). Farm workers with a primary and lower level of education were less likely to have heard of brucellosis when compared to those with secondary and higher level of education. Animal attendants with a primary and lower level of education are hence likely at a higher risk of exposure to the disease. Similar findings were reported by a study conducted in Tajikistan (246). Low awareness of the disease in the study area might be explained by the low prevalence of brucellosis in dairy cows. The majority (88%) of farm workers were male. This could be due to the fact that farm works in urban and peri-urban intensive diary is labour demanding, as a result of which most farm owners prefer to employ male farm workers.

Even though farm workers in over 50 % of surveyed farms refused to participate in the KAP study and this might limit the representation of the data to the entire population in the study area, the findings of this study indicated that practices posing a high risk of *Brucella* transmission were very common. Most participants reported assisting in animal parturition, disposing aborted foetuses/after birth in open environment without protective gloves or masks and consumption of raw meat and milk. The reason could be poor knowledge of the disease and risks of transmission but also lack of resources used for personal protection such as gloves, aprons and antiseptics. Similar results have been reported in a study from Tajikistan (246) and Egypt (145). Creation of awareness of the farm owners and attendants is important to control brucellosis in the area even though the prevalence in animals was low in this serological survey.

In the present study, bovine brucellosis at individual animal level was 0.06% (1/1550) and herd level prevalence was 0.8% (1/127) using c-ELISA whereas no seropositive animal was found on CFT in dairy farms of Addis Ababa. This observation is consistent

with previous reports made by Alem and Solomon (247) in Eastern and Western Showa zones of central Ethiopia using Rose Bengal Plate test (RBT), serum agglutination test (SAT) and complement fixation test (CFT) (n=564). This report is also consistent with (248) who reported no positive reactors in intensive dairy farms of the Addis Ababa area (n=747). Similarly, a study by (143) could not find positive reactors in selected dairy herds of Adama, central Ethiopia (n=52) and northern Ethiopia (Mekele and Gondar) (n=252). A study conducted in dairy herds in Debre Birhan and Ambo towns (249) reported that there was only one sero-reactor animal to *Brucella* infection using CFT (n=415).

In contrast, there are reports of a higher prevalence of antibodies to *Brucella* spp. in Addis Ababa dairy farms, 1.5% (164) and 2.2% (143). A similar study by (171) reported 1.4% in Asella and Bishoftu towns using the card test (CT), RBT, indirect Enzyme-Linked Immuno Sorbant Assay (i-ELISA) and Complement Fixation Test (CFT). In Ethiopia, brucellosis in animals has been reported from different localities of the country; with prevalence ranging from 0.4% to 8% particularly associated with cattle in both intensive and extensive management systems (143,154,169,170,196,250,159–162,165–168). A high seroprevalence of brucellosis (38%) in cattle in western Ethiopia has been reported (158), while most of the studies in Ethiopia suggested a low seroprevalence (below 5%) in cattle.

In the present study only one sero-reactor animal to *Brucella* spp. was found in cattle populations of Addis Ababa dairy farms. According to the individual animal record in the farm, this sero-reactor animal in the farm was purchased from outside the capital and had late abortion history at first calving. The very low seroprevalence of brucellosis in this study is remarkable, as bovine brucellosis is considered the world's most common bacterial zoonosis (43) and listed among top five zoonotic diseases in Ethiopia (241). The hypothesis that brucellosis is endemic in the investigated dairy farms of Addis Ababa could thereby not be confirmed in the present study. However, the presence of one or more positive reactors in the herd is a reliable predictor of the presence of infection (30). Seropositivity for *Brucella* spp. was found in only a very small percentage by c-ELISA test, although risk factors for obtaining *Brucella* infection

such as age variety, origin of animals, different level of parity, history of abortion, and herd size composition were present as revealed by farm characteristics analysis.

In epidemiological studies, the use of two or more tests applied serially is recommended to maximize the accuracy of test results. RBT is highly sensitive test and c-ELISA and CFT are highly specific and also sensitive and usually used as confirmatory tests (129). The combination of these tests in this study could therefore maximize the accuracy of the findings. False positive serological reactors in RBT are due to cross-reactions with Smooth Lipopolysaccharide (S-LPS) antigens of other bacteria. As there has never been history of vaccination, seropositivity in this case is assumed to be due to natural infection.

The difference in test results of C-ELISA and CFT is due to the variation in sensitivity of the tests. The C-ELISA test is more sensitive as compared to CFT for the diagnosis of brucellosis even though both tests have similar specificities of 99.9% (129). Moreover, CFT is prone to prozone effect (low dilutions of some titrated sera from infected animals do not fix complement) that could lead to a false negative result (39,251). The very low prevalence could be explained by the cross sectional study design if informal culling practices suggested by Tesfaye *et al* (164) had been instituted. These include culling of cows with abortion history of at least two times and above for any reason and removing seropositive reactors from the herd for economic reasons. The other possible explanation might be absence of infectious foci, such as *Brucella*-infected dairy farms or ranches in the surrounding areas, which could spread the disease among contact herds. Movement of infected animals to susceptible herds is a common route of transmission (30). The random selection used in our study design should have detected clustered infection if it were common (252).

2.5. Conclusions

Urban and peri-urban dairy farming offers an important opportunity to improve the livelihood of people in low-income countries. In the present study, there was only one seropositive animal found for bovine brucellosis by the c-ELISA test. This study also

showed poor knowledge of brucellosis and abundant high-risk behaviours among the farm workers that can have impact on disease transmission. Even though refusal of most farm workers to participate in the KAP study limit the representation of the data to the entire population, poor knowledge and high-risk practices identified in this study strengthen the argument for including health education as part of control programs. At present, there is no officially coordinated program for control of bovine brucellosis in Ethiopia. The disease is unlikely to be a significant limiter of dairy production in the Addis Ababa area due to the low prevalence, but it may be present in other animals and husbandry types and with the risk of future transmission. Since this favourable disease situation is not the result of informed policy, there is no guarantee that it will continue unchanged.

The current study warrants the need for a strategic surveillance program in case the prevalence rates do change. Feasible and cost-effective strategy for this would be having a surveillance programmes which are tailored to surveillances of other economically important zoonotic diseases of public health concern. This would help to better use of scarce resources available. Modernization of husbandry systems, improving the knowledge of farmers through trainings and testing of new animals before introducing them to dairy farms and quarantining and testing of aborting animals should be encouraged. A multi-sectorial framework should be promoted involving all stakeholders working in public and animal health in the context of a “One Health” approach. Since the current study was limited to cross sectional study design, future studies in the dairy farms should follow longitudinal study types to ascertain actual burden of the disease in the study area.

2. CHAPTER THREE

The cross-sectional study in this chapter was conducted in Borena zone, which is located over 500 km away from the capital in Southern Ethiopia. The study design in this chapter required that three livestock species (cattle, sheep and goats) and occupationally linked herders and animal attendants had to be sampled. Hence, two technical assistants from Yabello Veterinary Laboratory were hired to support in blood sample collection from animals and one medical laboratory technologist was hired from Yabello Medical hospital to collect blood sample from voluntary household members with frequent close contact with livestock for at least a year. The laboratory testing, data analysis and interpretation was conducted by the PI of this PhD project.

The work in this chapter has been published in PLOS Neglected Tropical Diseases Journal.

Bedaso Mammo Edao, Gobena Ameni, Zerihun Assefa, Stefan Berg, Adrian M. Whatmore, James L. N. Wood. (2020). Brucellosis in ruminants and pastoralists in Borena, Southern Ethiopia. *Plos Neglected Tropical Diseases* 14(7).

3.1. Introduction

Animal brucellosis constitutes a significant public health importance for a pastoral community where close intimacy with animals, raw milk consumption and low awareness on zoonoses facilitate zoonotic transmission of the disease. Milk is a major staple food and is an important source of protein and vitamins for households. Raw milk, which is the mode by which almost all the pastoral community consumes it, is also a source of infection with milk-borne zoonoses such as brucellosis (155). The overall infection risk is also influenced by the pattern of *Brucella* spp. present; as *B. melitensis* often represents a more serious public health hazard than *B. abortus* (51).

Serological evidence of brucellosis in Borena pastoral region, Southern Ethiopia was reported by a few studies (154,182). These studies, however, had limited geographic coverage and none of them included parallel study on human brucellosis in the study area. Large number of undiagnosed human cases with fever, neurological complications and other generalized complications in rural and pastoral communities are misdiagnosed and treated empirically as malaria or fever of unknown origin (142).

Cattle, camels, goats, and to some extent sheep are the principal livestock species that are reared by Borena pastoralists. Herding of these animals together, which is the normal practice of the traditional pastoral people, is one of the putative factors of transmission of *Brucella* infection. Comprehensive studies on brucellosis in different animal species sharing the same ecological zone, and zoonotic significance in occupationally linked humans are scarce. Documenting the risk profile of human–animal interface in Borena pastoral setting is, therefore, vital in developing feasible control strategies in Ethiopia. Hence, the objectives of this study were to estimate the sero-prevalence of *Brucella* infection in cattle, sheep and goats and their attendants and shepherds using the serological tests (RBT and C-ELISA), identify potential risk factors and assess the KAP of visited household members towards brucellosis.

3.2. Materials and methods

Borena pastoral area is located in Oromia Regional state, Southern Ethiopia. The capital of the zone, Yabello, is 575 km south of Addis Ababa. Borena Zone comprises thirteen districts and borders Kenya in the southern part at Moyale, Miyo, Dirre and Teltelle districts (Figure 3.1). According to the 2018 Borena Zone Pastoral Development Office (253), the zone has recorded livestock populations of 1,416,180 cattle, 1,262,782 goats, 776,870 sheep, 237,205 camels, 306,057 poultry, 102,767 donkeys, 1,841 horses and 4,433 mules; the human population was 1,283,925 in 2015 (254).

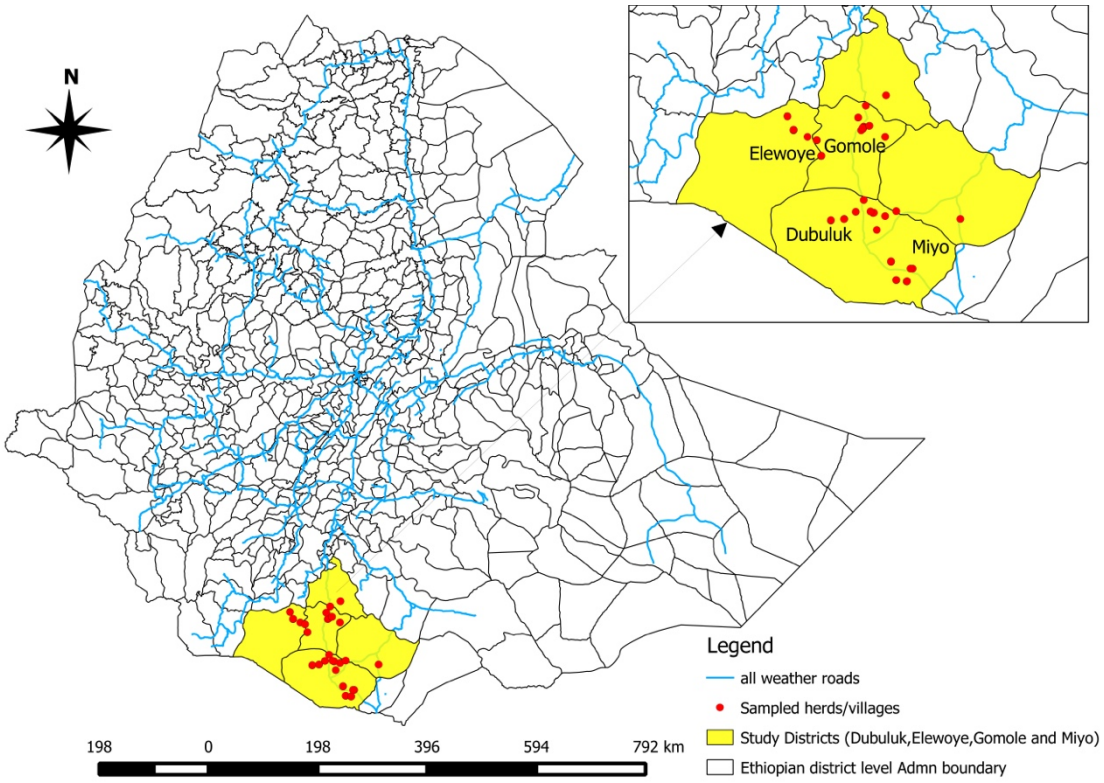

Figure 3.1: Map of Ethiopia and Borena pastoral zone.

Legend: This map is developed from Ethiopian shape files using QGIS Software,2013. The yellow shaded region represents study districts and red dots represent sampled villages.

Generally, the Borena plateau represents a lowland area where altitude gently slopes from the North (1650 m) to the South (1000 m) above sea level. The area has a bimodal rain pattern with annual average precipitation ranging from 300 mm to 700 mm. The main rainy season (65% of precipitation) extends from March to May, and a minor rainy season is between mid-September and mid-November. The main dry season extends from December to February (255). As surface water is very scarce in the area, deep wells, shallow ponds, and large machine-excavated ponds are important sources of water for both livestock and humans. Clans own traditional wells, while large ponds are communal and often responsible for aggregation of large numbers of animals at the water points.

The livestock production system is predominantly extensive, where animals are allowed to forage freely during daytime and kept in open enclosures during the night. (256). Livestock share common grazing areas and watering points, and probably

mingle at villages although separate enclosures are used for each species. Mobile herds are often maintained together with five or more village herds to reduce labour demand, a condition that facilitates transmission of the disease from infected to susceptible herds.

The pastoral village, *Olla* in Borena, is characterized by the clustering of households with close proximity of houses in a pastoral camp. A village chief, Abba Olla, who is an important contact person in facilitating cooperation between livestock owners, traditionally administers each village, which usually varies in size between 7 and 20 households. Keeping multiple livestock species and seasonal herd mobility are part of the dynamic nature of the pastoral production system. Livestock constitute the principal source of livelihood for Borena households. Nearly 70% of household cash revenues come from pastoral sources, mainly from livestock sales with sales from dairy products constituting only a small proportion (255).

3.2.1. Study Design and Population

Study design

A cross-sectional study was conducted to determine the prevalence of *Brucella* infection in cattle and sheep and goats and occupationally associated animal owners and attendants in four selected districts of Borena Pastoral region, to identify the potential risk factors associated with the seropositivity and to assess the KAP of visited household members towards brucellosis. The four districts (Gomole, Elewoye, Dubuluk, and Miyo) were randomly selected based on livestock species diversity, and close geographic location to regional veterinary laboratories. Study animals were grouped into different categories based on their sex, age, herd or flock size, physiological status and presence or absence of reproductive problems such as abortion history. Age determination and history for presence or absence of reproductive problems were obtained from animal owners and attendants. The target peasant associations (PAs) or villages from the four districts were selected based on

presence of at least three livestock species, accessibility of villages for vehicle and proximity of the villages to the main roads. Using simple random sampling, cattle and sheep and goats above six months of age were recruited for this study. Relevant individual animal biodata and herd level information were collected using a semi-structured questionnaire. Demographic information of voluntary participants and their KAP related to brucellosis were also recorded using a pretested structured questionnaire.

Study Populations

Cattle, Sheep and Goats: the target populations of cattle and sheep and goats were composed of local cattle breeds of Boran type, blackhead Somali sheep, and the long-eared Somali goats. Putative biological factors believed to be associated with epidemiology of brucellosis were recorded. These included, sex, age, species, herd size and physiological status.

Humans: as an inclusion criterion household members over five years of age who had frequent close contact with animals and animal products for at least one year were randomly sampled from the selected pastoral associations (PAs) or villages in the study area. A trained medical laboratory technologist from Yabello Hospital was used for this purpose. After the purpose of the study was explained and verbal consent to participate in the study was obtained from participants, blood samples were collected from volunteer livestock owners and animal attendants. The participants were interviewed using a structured questionnaire to collect demographic, epidemiological and KAP relating to brucellosis.

3.2.2. Sample size determination

A multistage sampling combined with the convenient sampling strategy was employed for sampling of individual animal species. A PA or a village is the smallest administrative unit in the study district. The PAs for the study were selected by randomization after obtaining the total number of PAs in the district. The total number of PAs within the four selected districts in Borena zone were listed and used as a

sampling frame. Households with two or more livestock species were identified and approached for permission to sample their animals. Factors such as Presence of three animal species per village, Species of animals per household, Willingness of herders to cooperate, and availability of herds during the visit were taken into consideration to estimate the number of each animal species to be sampled per village. Thus sample size (n) was determined based on the following formula previously published (245).

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

Where n = required sample size; P_{ex} = expected prevalence; d = desired absolute precision.

The average livestock holding per household was estimated to be 20 cattle, 15 goats, 6 sheep and 10 camels with possible variation between ethnic groups (255). As a result, with expected prevalence of 10.6 % in cattle (154), and 9.7 % in sheep and goats (177) with 80 % power and 5% desired absolute precision at 95% confidence level was assumed to calculate the desired samples size in cattle and sheep and goats. Accordingly, a minimum sample size of 150 cattle, 134 sheep and goats were required to be sampled from each of the four districts. Hence, this minimum target was reached by serum sampling a total of 750 cattle and 882 sheep and goat from the targeted villages. Similarly, with the expected prevalence of 3.7 % (257) in human with 5% desired absolute precision at 95% confidence level, a total of 341 blood samples were collected from occupationally linked humans.

3.2.3. Sample collection and laboratory tests

Blood samples (10ml) were collected from cattle, sheep and goats from the jugular vein and transported to Yabello Regional Veterinary Laboratory and stored at 4 ◦C. The following day the blood samples were centrifuged at 1500 × g for 10 min to obtain the serum. Sera were decanted into cryovials, identified and stored at −20 ◦C until being transported in cold chain using ice packs.

Rose Bengal test (RBT): All sera samples collected were initially screened by RBT using RBT antigen (Animal and Plant Health Agency (APHA), United Kingdom) according to described procedures (258). Briefly, sera and antigen were taken from refrigerator and left at room temperature for half an hour before the test to gain room temperature. RBT antigen (30 ml) was added onto a clean plate next to an equal volume of cattle and human serum sample. For sheep and goats, in order to improve the sensitivity of RBT as previously recommended (259), one volume of antigen and three volumes of serum (e.g. 25ul with 75ul) was used instead of an equal volume of each. The antigen and test serum were mixed thoroughly with a plastic applicator, shaken for 4 min, and the result (presence of agglutination or not) was read immediately.

Competitive ELISA: All RBT positive sera were further tested at Addis Ababa University, Aklilu Lemma Institute of Pathobiology (AAU-ALIPB) using the COMPELISA 160 and 400, a competitive ELISA kit for the detection of antibodies against *Brucella* in serum samples (Animal and Plant Health Agency, New Haw, Addlestone, Surrey, KT15 3NB, United Kingdom). The test was performed according to the manufacturer's instructions. The test was conducted in 96-well polystyrene plates that were pre-coated with *Brucella* species lipopolysaccharide (LPS) antigen. Twenty µl of each test serum was added to each well followed by 100 µl of prepared conjugate solution. The plates were then shaken vigorously for two minutes and incubated at room temperature for 30 minutes on rotary shaker, at 160 revs/min. The plates were washed five times and then dried. Hydrogen peroxidase substrate and chromogen solution was added and left to develop for 10 min. 100 µl of o-Phenylenediamine dihydrochloride (OPD) solution was added to all wells and the plates were incubated at room temperature for 10 to 20 minutes. The reaction was then being stopped using stopping solution. Optical densities (OD) were read at 450 nm using a micro plate reader. The lack of colour development indicated that the sample tested was positive. A positive/negative cut-off was calculated as 60% of the mean of the OD of the 4 conjugate control wells. Any test sample giving an OD equal to or below this value was regarded as being positive for *Brucella*.

3.2.4. Case definition

An animal or human case was considered positive if it tested seropositive on both RBT and c-ELISA in serial interpretation. Similarly, a herd or flock was considered seropositive when at least one animal in a herd or flock tested positive. Since there is no history of vaccination against brucellosis in Ethiopia, seropositivity observed in this study was considered to be due to natural infection of *Brucella*.

3.2.5. Ethics approval and consent to participate

Ethical clearance for this study was obtained from Department of Veterinary Medicine at University of Cambridge, and at Aklilu Lemma Institute of Pathobiology and at College of Veterinary Medicine and Agriculture (CVMA), both at Addis Ababa University Minutes of Animal Research Ethics and Review committee. Before conducting the research, verbal consent was obtained from the livestock owners to take samples from their animals and occupationally exposed people to be included in the study after they were informed with the objectives of the study.

3.2.6. Data analysis

Data generated from the questionnaire survey and laboratory investigations were recorded and coded using a Microsoft Excel spread sheet (Microsoft Corporation) and analyzed using STATA version 15.0 for Windows (Stata Corp. College Station, TX, USA). The association between explanatory and outcome variables was analyzed at individual animal level by using univariable and multivariable logistic regression. A multivariable logistic regression model was used to identify risk factors associated with *Brucella* infection, at individual and herd or flock level, with adjustment for clustering by village. Separate models were run for cattle, sheep and goats and humans. Choice of explanatory variables was based on previous evidence and association with outcome variable during data analysis. Variables with a p-value less than or equal to 0.10 (in univariable analysis) were included in the multivariable logistic model. For variables that showed co-linearity when tested using scatter plot and correlation coefficient, one of the two variables was excluded based on biological

plausibility to *Brucella* infection. Further selection of variables in the final model was based on stepwise backward elimination procedure with a likelihood-ratio test at p= 0.05 as a variable selection criterion. Prevalence in both livestock species, as well as human, was estimated with the chi-square and Fisher's exact test as appropriate. Odds ratio was used to assess the strength of association between exposure variables associated with seropositivity of the disease in both animals and human.

3.3. Result

3.3.1. Prevalence

Out of the total of 1632 ruminants sampled (750 cattle, 667 goats and 215 sheep), 2.8 % (CI= 2.1-3.7, n=46) ruminants were tested positive. An overall seroprevalence of antibodies to *Brucella* infection was 2.4 % (CI= 1.4-3.8) in cattle, and 3.2% (CI= 2.1-4.6) in sheep and goats (Table 1). In occupationally exposed household members, livestock attendants and herders a seroprevalence of 2.6 % (95% CI=1.2-4.9) was recorded.

The highest individual level seroprevalence of 3.7% (n=25) was recorded in goats, followed by cattle 2.4% (n=18) and sheep 1.4% (n=3). There was variation in the distribution of seroreactor animals and humans among the four districts. Eleweye district had the highest rate of seropositive animals and humans; cattle 6.3 % (n=11), sheep and goats 6.1% (n=13), and humans 5.1% (n=5). In Miyo district, no seroreactor cattle were detected whilst the proportion of seropositivity to *Brucella* infection in sheep and goats and humans was 4% (n=10) and 2.3% (n=2), respectively (Table 3.1).

Table 3.1: Distribution of seroreactor animals and humans among the four districts in Borena Zone, Southern Ethiopia (serial interpretation of RBT and C-ELISA).

District	Species tested	Nº Sampled	RBT Nº Positive (%)	C-ELISA Nº Positive (%)	95 % CI*
Dubuluk	Cattle	214	3(1.4)	3 (1.4)	0.3-4.0
	Sheep and goats	250	6(2.4)	3 (1.2)	0.2-3.5
	Human	93	1(1.1)	1 (1.1)	0.02-5.8
Eleweye	Cattle	176	11(6.3)	11 (6.3)	3.2-11.0
	Sheep and goats	215	15(7.0)	13 (6.1)	3.3-10.1
	Human	98	5(5.1)	5 (5.1)	1.7-11.5
Gomole	Cattle	140	7(5.0)	4 (3.0)	0.8-7.2
	Sheep and goats	166	3(1.8)	2 (1.2)	0.1-4.3
	Human	61	1(1.6)	1 (1.6)	0.04-8.8
Miyo	Cattle	220	0(0)	0 (0)	-
	Sheep and goats	251	14(5.6)	10 (4.0)	2.0-7.2
	Human	89	2(2.3)	2 (2.3)	0.3-8.0
Total	Cattle	750	21(2.8)	18 (2.4)	1.4-3.8
	Sheep and goats	882	38(4.3)	28 (3.2)	2.1-4.6
	Human	341	9(2.6)	9 (2.6)	1.2-4.9

95 % CI*= Confidence interval of prevalence in each species and district based on C-ELISA.

The distribution of *Brucella* seropositivity among the tested animals in the different pastoral villages in the four studied districts is shown in Table 3.2 and 3.3. Seropositive animals were found in 60% (12/20) and 15% (3/20) of the villages with at least one and two positive animal species, respectively. Village level seropositive reactors were more frequently detected in sheep and goats (23.3 %) than in cattle (11.4%). The average number of positive animals per positive herd was generally low, 1.4 in both cattle, sheep and goats, suggesting a slow within herd transmission of the disease.

Across the pastoral villages visited in the four study areas, the prevalence ranged from 0-23% in sheep and goats and 0-11.4% in cattle. In sheep and goats, the highest seroprevalence was recorded in Saba, 23.3% (n=10) followed by Rarewardelle, 12 % (n=6). The highest seroprevalence in cattle was also recorded in Saba, 11.4% (n=5) followed by 6.7% (n=2) in Harobake. (Table 3.2 and 3.3).

Table 3.2: Distribution of seroprevalence of *Brucella* antibodies among pastoral villages and cattle in the four studied districts of Borena zone (serial interpretation of RBT and C-ELISA).

District	Village	Species	Nº Sampled	Nº Positive (%)
Gomole	Dhadacha Quufaa	Cattle	26	1 (3.8)
	Harboro	Cattle	30	2 (6.7)
	Dasie Gora	Cattle	21	0 (0)
	Bildim	Cattle	32	0 (0)
	Harobake	Cattle	31	1 (3.2)
Elewoye	Elewoye Golba	Cattle	32	2 (6.3)
	Elewoye magala	Cattle	40	2 (5.0)
	Saba	Cattle	44	5 (11.4)
	Sarite	Cattle	30	1 (3.3)
	Ada Galchati	Cattle	30	1 (3.3)
Dubuluk	Lafto	Cattle	40	0 (0)
	Higo	Cattle	44	1 (2.3)
	Arbale	Cattle	24	1 (4.2)
	Dhoqolle	Cattle	50	0 (0)
	Jigessa	Cattle	55	1 (1.8)
Miyo	Baha	Cattle	50	0 (0)
	Arda Jila	Cattle	41	0 (0)
	Rarewardale	Cattle	50	0 (0)
	Boku	Cattle	25	0 (0)
	Chari Turura	Cattle	54	0 (0)
	Total	**Cattle**	**750**	**18 (2.4)**

Table 3.3: Distribution of seroprevalence of *Brucella* antibodies among pastoral villages and sheep and goats in the four studied districts of Borena zone (serial interpretation of RBT and C-ELISA).

District	Village	Species	Nº Sampled	Nº Positive (%)
Gomole	Dhadacha Quufaa	Sheep and goats	31	0 (0)
	Harboro	Sheep and goats	32	0 (0)
	Dasie Gora	Sheep and goats	34	0 (0)
	Bildim	Sheep and goats	32	0 (0)
	Harobake	Sheep and goats	37	2 (5.4)
Elewoya	Elewoye Golba	Sheep and goats	34	0 (0)
	Elewoye magala	Sheep and goats	48	3 (6.3)
	Saba	Sheep and goats	43	10 (23.3)
	Sarite	Sheep and goats	50	0 (0)
	Ada Galchati	Sheep and goats	40	0 (0)
Dubuluk	Lafto	Sheep and goats	45	0 (0)
	Higo	Sheep and goats	35	0 (0)
	Arbale	Sheep and goats	58	2 (3.4)
	Dhoqolle	Sheep and goats	65	1 (1.5)
	Jigessa	Sheep and goats	47	0 (0)
Miyo	Baha	Sheep and goats	50	2 (4.0)
	Arda Jila	Sheep and goats	50	1 (2.0)
	Rarewardale	Sheep and goats	50	6 (12.0)
	Boku	Sheep and goats	50	0 (0)
	Chari Turura	Sheep and goats	50	1 (2.0)
	Total	**Sheep and goats**	**882**	**28 (3.2)**

3.3.2. Risk factors for Brucella spp. seropositivity in cattle

Table 3.4 shows the prevalence and univariate logistic regression analysis of associations of risk factors for *Brucella* seropositivity in cattle. The major exposure variables that were considered to predict the response of the outcome variable includes, District, Herd size, Age, Parity, Physiological status, and History of Abortion. The result showed that most of the recorded variables showed a high degree of association with seropositivity to *Brucella* infection.

Table 3.4: Univariate logistic regression analysis of the risk factors for *Brucella* seropositivity in cattle.

Risk factor	**Level**	**Nº Sampled**	**Nº Positive (%)**	**OR**	**95% CI**	**p-value**
District	Dubuluk	214	3 (1.4)	-	-	
	Eleweye	176	11(6.3)	4.6	1.3-17.0	0.02
	Gomole	140	4(2.9)	2.0	0.4-8.6	0.4
Herd Size*	Small	234	4(1.7)	-	-	
	Medium	455	8(1.8)	1.0	0.3-3.5	0.96
	Adult	61	6(9.8)	6.4	1.7-23.4	0.005
Age**	Age in years	678	18(2.6)	1.2	0.9-1.4	0.067
Physiological status	Heifer	106	2(1.9)	-	-	
	Lactating	343	8(2.3)	1.2	0.3-6.0	0.78
	Not pregnant	155	3(1.9)	1.0	0.1-6.2	0.98
	Pregnant	74	5(6.8)	3.8	0.7-20.0	0.12
Parity	≤ 2	393	6(1.5)	-	-	
	> 2	285	12(4.2)	3.6	1.2-10.5	0.01
History of Abortion	No	582	11(1.9)			
	Yes	96	7(7.3)	4.1	1.5-10.8	0.000

*Herd size: <20=Smallholder, 20-50= Medium, and >50 = Large

**Age: age in years used as a continuous variable.

The variables with a p-value < 0.10 from univariable logistic regression analyses were included in the final multivariable logistic model. Age of animals showed co-linearity with parity and were not included in the multivariable logistic regression model. As no animal from Miyo district tested positive, district was not considered in subsequent multivariable analysis.

The final multivariable logistic regression model (Table 3.5) showed that animals kept in a large herd were more likely to be exposed to *Brucella* infection than those maintained in a medium and small herd (OR=8.1, 95% CI= 2.1-32, P=0.003). The result also showed that animals with parity greater than two were more likely to acquire infection than those with parity less than two (OR=4.7, 95% CI=1.5-14.1, P=0.005). Similarly, cows with history of abortion were more likely to be seropositive for *Brucella* infection than cows without such history (OR= 4.3, 95% CI= 1.6-12.0, P=0.000).

Table 3.5: Multivariable logistic regression model of risk factors for *Brucella* seropositivity in cattle at individual and herd level.

Risk factor	**Level**	**Nº sampled**	**Nº positive (%)**	**OR**	**95% CI**	**P-value**
Herd Size*	Small	234	4(1.7)	Ref		
	Medium	455	8(1.8)	1.1	0.3-3.6	0.942
	Large	61	6(9.8)	8.0	2.0-31.3	0.003
Age**	Age in years	678	18(2.6)	1.1	0.8-1.3	0.805
Parity	≤ 2	393	6(1.5)	Ref		
	> 2	285	12(4.2)	4.7	1.5-14.1	0.005
History of	No	582	11(1.9)	Ref		
Abortion	Yes	96	7(7.3)	4.3	1.6-12.0	0.000

Herd size*: <20=Smallholder, 20-50= Medium, and >50 = Large, Age**: age in years used as a continuous variable. Variance of seropositivity between villages visited was 1.39

3.3.3. Risk factors for Brucella spp. seropositivity in sheep and goats.

The prevalence and univariate logistic regression analysis of associations of explanatory variables for *Brucella* seropositivity in sheep and goats was shown in Table 3.6. Seropositivity was found to be significantly associated with district, Age > 3 years, increased flock size, and with history of abortion (P< 0.05). The odds of *Brucella* seropositivity was 2.8 times higher in goats compared to sheep.

Table 3.6: Univariate logistic regression analysis of the risk factors for *Brucella* infectivity in sheep and goats.

Risk factor	Level	Nº Sampled	Nº Positive (%)	OR	95% CI	P-value
District	Dubuluk	250	3 (1.2)	-	-	
	Eleweye	215	13 (6.1)	5.2	1.5-18.8	0.01
	Gomole	166	2 (1.2)	1.0	0.2-6.0	1.0
	Miyo	251	10 (4.0)	3.4	0.9-12.5	0.06
Flock Size*	< 39	432	7 (1.6)	-	-	
	≥ 39	450	21 (4.7)	3.0	1.3-7.0	0.01
Species	Ovine	215	3 (1.4)	-		
	Caprine	667	25 (3.7)	2.8	0.8-9.2	0.10
Age**	Young	292	2 (0.7)	-	-	
	Adult	590	26 (4.4)	6.7	1.6-28.3	0.01
Physiological status	Weaner	51	1 (2.0)	-	-	
	Lactating	449	9 (2.0)	1.0	0.1-8.2	0.98
	Not Pregnant	89	3 (3.4)	1.7	0.2-17.2	0.63
	Pregnant	228	15 (6.6)	3.5	0.5-27.3	0.23
Parity	≤ 2	221	4 (1.8)	-	-	
	> 2	596	24 (4.0)	2.3	0.8-6.6	0.13
History of Abortion	No	538	10 (2.0)	-	-	
	Yes	279	18 (6.5)	3.6	1.6-8.0	0.001

*Median flock size was 39, ** Age: young is ≤ 3 years whereas adult is > 3 years.

Explanatory variables with P< 0.10 in univariate logistic regression analyses were subjected to a multivariate logistic regression model. Variables such as district, flock size, age, and history of abortion were included in the multivariable logistic regression model (Table 3.7). Thus, further selection of variables in the final model was based on stepwise backward elimination procedure. The multivariable logistic regression model indicated that sheep and goats from Eleweye district were 6 times more likely to be seropositive for *Brucella* infection than other districts of the study area (OR=6.0, 95% CI=1.7-22, P=0.006). Increase in flock size ≥ 39 was significantly associated with *Brucella* seropositivity (OR: 3.3, 95% CI= 1.3-8.4, P=0.01). Mature animals (> 3 years) were 4.8 times more likely to be seropositive for *Brucella* infection than young sheep and goats (OR=4.8, 95% CI= 1.1-20.7, P=0.04). Having a History of abortion was significantly associated with *Brucella* seropositivity (OR=3.1, 95% CI= 1.4-6.9, P=0.006).

Table 3.7: Multivariable logistic regression analysis of the risk factors for *Brucella* infectivity in sheep and goats.

Risk factor	**Level**	**№ Sampled**	**№ Positive (%)**	**OR**	**95% CI**	**P-value**
District	Dubuluk	250	3 (1.2)	Ref	-	
	Eleweye	215	13 (6.1)	6.0	1.7-22.0	0.006
	Gomole	166	2 (1.2)	1.6	0.3-10.2	0.6
	Miyo	251	10 (4.0)	3.0	0.8-11.3	0.11
Flock Size*	< 39	432	7 (1.6)	Ref	-	
	≥ 39	450	21 (4.7)	3.3	1.3-8.4	0.01
Age**	Young	292	2 (0.7)	Ref	-	
	Adult	590	26 (4.4)	4.8	1.1-20.7	0.04
History of Abortion	No	538	10 (2.0)	Ref		
	Yes	279	18 (6.5)	3.1	1.4-6.9	0.006

*Median flock size was 39, ** Age: young is ≤ 3 years whereas adult is > 3 years. Variance of seropositivity in sheep and goats between villages visited was 6.5

3.3.4. Serological survey for human Brucellosis

Seroprevalence of brucellosis in occupationally linked household members and its association with demographic factors in the four districts using Fishers exact test is shown in Table 3.8. An individual seroprevalence of 1.5% (n=5) in Eleweye, 0.6 % (n=2) in Miyo, and 0.3 % (n=1) in both Dubuluk and Gomole districts were recorded. Numbers of positives were generally too small to allow useful statistical comparison. Seroprevalence was similar across genders, areas, age groups and other demographic factors.

Table 3.8: Prevalence of brucellosis in occupational risk groups and its association with demographic risk factors.

Risk factor	**Level**	**Nº Sampled**	**Nº Positive (%)**	**p-value**
District	Dubuluk	93	1 (0.3)	
	Eleweye	98	5 (1.5)	0.36
	Gomole	61	1 (0.3)	
	Miyo	89	2 (0.6)	
Sex	Male	165	5 (1.5)	0.7
	Female	176	4 (1.2)	
Age	≤ 19	65	2 (0.6)	0.7
	20-60 years	222	5 (1.5)	
	> 60 years	54	2 (0.6)	
Marital status	Single	68	1 (0.3)	0.7
	Married	273	8 (2.4)	
Educational Level	Illiterate	265	8 (2.4)	0.9
	Primary	57	1 (0.3)	
	Secondary	14	0 (0)	
	College diploma	3	0 (0)	
	University degree	2	0 (0)	
Number of people in household	1-5	103	5 (1.5)	
	6-10	203	2 (0.6)	0.07
	> 10	35	2 (0.6)	
Species of animals in household	1	10	0 (0)	0.54
	2	30	0 (0)	
	>3	292	9 (2.6)	

As 98.8 % (337/341) of participants that includes all seropositive individuals had no knowledge of brucellosis and only 4 (1.2 %) had knowledge of brucellosis, this variable was not considered in the subsequent logistic regression analysis. On univariate logistic regression analysis, assisting during calving or birthing (P=0.02) and Presence of seropositive animal at household (P=0.000) were significantly associated with increased risk of brucellosis in humans (Table 3.9). Participants from Eleweye districts were 3.6 times more likely to be seropositive for *Brucella* infection than other districts in the study area. Individual who consumed raw milk mixed with blood had 4 times higher odds of *Brucella* seropositivity than those who had not (OR= 4.0, 95% CI=0.7-23, although this was not significant). Similarly, household members who disposed of dead foetus and retained foetal membranes (RFM) were 3.6 times more likely to be seropositive for *Brucella* infection (OR= 3.6, 95% CI=0.7-13), but again this was not significant.

Table 3.9: Univariable logistic regression analysis of K-A-P related risk factors for *Brucella* seropositivity in humans.

Risk factor	Level	Nº Sampled	Nº Positive	OR	95% CI	P-Value
District	Dubuluk	93	1(0.3)	-	-	
	Eleweye	98	5 (1.5)	3.6	0.6-22.6	0.17
	Gomole	61	1 (0.3)	1.5	0.2-15.0	0.72
	Miyo	89	2 (0.6)	1.7	0.2-13.6	0.59
Raw milk consumption	No	54	1 (1.8)	-	-	
	Yes	287	8 (2.8)	1.1	0.2-6.3	0.93
Consume raw meat	No	181	4 (2.2)	-	-	
	Yes	160	5 (3.1)	1.4	0.4-5.0	0.61
Consume raw milk mixed with blood	No	139	1 (0.7)	-	-	
	Yes	202	8 (4.0)	4.0	0.7-23.2	0.12
Assist during birthing/calving	No	198	1 (0.5)	-	-	
	Yes	143	8 (5.6)	8.3	1.4-47.5	0.02
Dispose dead foetus or RFM	No	171	2 (1.2)			
	Yes	170	7 (4.1)	3.6	0.7-13.2	0.12
Presence of seropositive animals at household	No	317	3 (1.0)			
	Yes	24	6 (25.0)	31.5	7.9-126	0.00

Explanatory variables with P< 0.15 in univariate logistic regression analyses were subjected to a multivariate logistic regression model. On multivariate logistic regression analysis, assisting during calving or birthing and Presence of seropositive animal at household were significantly associated with increased risk of *Brucella* seropositivity in humans ($p<0.05$) (Table 3.10).

Table 3.10: Multivariate logistic regression analysis of factors associated with brucellosis in humans.

Risk factor	Level	Nº Sampled	Nº Positive (%)	OR	95% CI	p-value
Consume raw milk with blood	No	139	1 (0.7)	-	-	
	Yes	202	8 (4.0)	6.0	0.7-50.4	0.098
Assist during birthing/calving	No	198	1 (0.5)	-	-	
	Yes	143	8 (5.6)	9.9	1.4-72.0	0.024
Dispose dead foetus or RFM	No	171	2 (1.2)			
	Yes	170	7 (4.1)	3.4	0.7-19.1	0.169
Presence of seropositive animal at household	No	317	3 (1.0)			
	Yes	24	6 (25.0)	45.1	8.7-233.5	0.000

* Variance of seropositive humans between villages visited was 0.4.

3.4. Discussion

The present study documented serological evidence of brucellosis in cattle, sheep and goats and occupationally exposed household members in four selected districts of Borena pastoral region in Southern Ethiopia. An overall individual animal and herd level prevalence of 2.4 % (95% CI=1.4-3.8) and 16 % (95% CI=8.6-26.2) was recorded in cattle. In sheep and goats, individual animal and flock level prevalence of 3.2% (95% CI= 2.1-4.6) and 22.7% (95% CI=13.8-33.8) was recorded, respectively. The prevalence of *Brucella* infection in occupationally exposed household members was 2.6 % (95% CI=1.2-4.9).

As no single serological test is appropriate in all epidemiological situations, the use of two tests applied serially is usually recommended for maximal specificity and ruling out of false positive cross-reactions (259,260). A combination of RBT and C-ELISA test is the most widely used serial testing scheme. In cattle and humans, we used a combination of RBT and C-ELISA, and for sheep and goats, a modified RBT and C-ELISA was used serially. RBT is selected as a screening test based on low cost, easy

performance and high sensitivity, especially in endemic areas (261). However, C-ELISA is selected due to its high specificities to discriminate between false positive cross–reactions and *Brucella* infections (262,263). False positive serological reactions in RBT could be due to cross-reactions with smooth lipopolysaccharide (S-LPS) antigens of other Gram-negative bacteria. As there has never been history of vaccination, seropositivity in all cases assumed to be natural infection.

The animal level prevalence detected in cattle in the present study was comparable with the report of 2.9% (95 % CI:2.0-4.0) by Jergefa *et al* (159) in central Ethiopia, 3.1% (95 % CI: 2.2-4.0) by Ibrahim *et al* (161) in Jimma zone, Southwest Ethiopia, 1.4 % (95% CI: 0.7-2.4) by Gumi *et al* (264), and 1.3 % (95 % CI:0.5-3.0) by Degefu *et al* (162) from Agro–pastoral region in Somali regional state. A consistent prevalence with the present study was reported in Ethiopia by Asgedom *et al* (170) who reported a prevalence of 2.4% (95 % CI:1.4-3.6) in cattle in Alage district.

Compared to the present study, higher prevalence of 9.7% (95 % CI: 7.0-14.7) by Chaka *et al* (173) in Nechisar National Park, Southern Ethiopia and 8% (95 % CI: 5.9-10.5) by Megersa *et al* (154) in Borena region was reported. Similarly, higher prevalences were recorded in other African countries; 6.6% (95 % CI: 3.4-11.2) in Ghana, by Kubuafor *et al* (265), 6.6% (95 % CI: 4.6-23.5) in Chad by Schelling *et al* (155) and 6.8% (95 % CI: 5.4-8.5) in Tanzania by Assenga *et al* (266). The difference in the prevalences recorded in the different study area may be associated with the differences in agro ecology, management system, tests used to detect *Brucella* seropositivity and sample sizes used in each study.

Our finding of 16% herd level seroprevalence in cattle was similar to 15% reported by Ibrahim *et al* (161) and 13.6 % by Jergefa *et al* (159) whilst other studies in Ethiopia showed a low seroprevalence (163,166,267,268). Conversely, other authors have reported higher herd level seroprevalences; 45.9 % from Ethiopia by Kebede *et al* (231), 55.5 % from Uganda by Bernard *et al* (269) and 62% from Zambia by Muma *et al* (261). Such contrasting findings could be either related to the overall individual animal level prevalence status of the disease or the size of studied herds.

The highest seropositivity observed in the large herds by multivariable logistic regression analysis is in accordance with previous findings (150,161,166,172), and 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 (270).

Association of *Brucella* seropositivity with increase in parity number greater than two was consistent with the findings of earlier studies (163,265,271). This has been attributed to increased chance of infection with increasing age (272). Seroprevalence of brucellosis may increase with age as a result of prolonged duration of antibody responses in infected animals and continued exposure to pathogen, particularly in pastoral cattle production systems where cattle are maintained in herds over long period of time. On a different note, delayed age at first calving (5 to 7 years) as evident from animal biodata could indicate that age is not a true explanatory variable in this case. In our data analysis, the fact that older animals showed higher seropositivity to *Brucella* infection than young ones, and this variable (Age) showed collinearity with parity substantiates this fact.

In this study, the odds of *Brucella* seropositivity in pregnant cows were not significantly different to those in in non-pregnant and lactating cows, in agreement with the findings of Omer *et al*., (270) in Eritrea and Tolosa *et al* (267) and Adugna *et al* (166) in Ethiopia. History of abortion of cows was significantly associated with *Brucella* seropositivity, as reported elsewhere (161,268) and as expected for *Brucella* spp. infection (155).

The overall individual animal level seroprevalence of *Brucella* infection in small ruminant recorded in this study was in agreement with Teklue *et al* (183) and Tsehay *et al* (185) who reported prevalence of 3.5% (95 % CI: 2.4-4.7) and 3.6% (95 % CI: 2.0-5.8) in small ruminant in southern Tigray and Somali pastoral region, respectively. Conversely, in Afar pastoral region, a higher individual animal level prevalence of 12.4 % (95 % CI: 10.5-14.4) and 13.7 % (95 % CI: 11.0-15.2) were reported by Tegegn *et al* (188), and Tadeg *et al* (176), respectively.

The present study revealed that individual species level seroprevalence was 3.7 % (95 % CI: 2.4-5.5) in goats and 1.4 % (95 % CI: 0.3-4.0) in sheep. The seroprevalence recorded in goats was similar to that in Ashagrie *et al* (179) and Deddefo *et al* (273), in goats in South Omo zone and Arsi Zone, respectively; as well as in Morocco (274) and Eritrea (270). Higher prevalence has however been reported in Afar region of Ethiopia (176,188) and Sudan (274). A seroprevalence of 1.4 % recorded in sheep in the present study was consistent with that of Teklu *et al* (183) in Southern Tigray and Yesuf *et al* (275) in South Wollo. Likewise, similar seroprevalences were recorded in Morocco (274) and Eritrea (270). In contrast, higher seroprevalence than the current study was reported in Afar region (176,188) and Dire Dawa (181), Eastern Ethiopia.

In sheep and goats, a flock level seroprevalence of 22.7 % was recorded in the present study. Other authors reported comparable results in Arsi and East shoa zones, central Ethiopia (273) and in pastoral regions of Guji and Borena, Southern Ethiopia (184). Similar seroprevalences were reported in flock of sheep in Egypt (276).The differences in seroprevalences observed could be due to variations in sensitivity and specificity imparted by the various test used, agro-ecological location, and sample size and production systems.

Significant difference in seropositivity to *Brucella* antibodies in sheep and goats among the four districts studied was observed in the current study (Table 3.7). Even though all districts in Borena have similar agro ecological conditions and livestock production systems, higher seroprevalence observed in Eleweye district could be associated with the livestock auction market at Eleweye town. This auction market receives animals from various districts from Borena zone including a few peasant associations from Konso district of Southern Nations Nationalities and People's region (SNNPR). This may be coupled with traditional use of communal grazing and watering points mixing susceptible and infected populations, a situation that facilitates *Brucella* transmission between animals. Due to the fact that Eleweye district borders agro-pastoral districts of the SNNPR, Borena pastoralists trek their animals towards Eleweye district during

dry seasons for better pasture and watering points, a condition that causes animals from various pastoral associations and villages to intermingle at Eleweye. Moreover, domestic animals in Borena share pasture and watering points with various wild animals, which could facilitate emergence of infectious foci and spill over of various infectious agents from wildlife to domestic animals including brucellosis.

Larger flock sizes were found to be significantly associated with *Brucella* seropositivity in sheep and goats, as previously reported (183,184). The recovery of higher seroreactors in large flock size could be due to intermingling of flocks in communal grazing area and at watering points, which have been suggested to be major factors responsible for high transmission risk of brucellosis in pastoral production systems (141,277).

Borena pastoralists rear their sheep and goats together, where both species graze and are corralled together at night in the same pen. Goats are browsers and can utilize trees and shrubs more efficiently during dry season than sheep, which are grazers and are dependent mainly on grasses for their energy and nutritional needs(278). This could be one of the reasons why Borana pastoralists keep relatively lower number of sheep than goats within a flock of sheep and goats. The difference in seroprevalence between sheep (1.3%; CI: 0.3-4.0) and goats (3.7%, CI: 2.4-5.5) could therefore be associated with the difference in proportion of sheep and goats in the flock, which were included in the present study.

As previously reported, we found that older animals (>3 years) were more likely to be seropositive than younger animals (142,178–180,184), consistent with cumulative risk of exposure increasing over time. Reproductive loss due to abortion, birth of weak offspring, and infertility are recorded as the common clinical signs of brucellosis in natural hosts (279,280). The major complaints of abortion in farm animals is ascribed to *Brucella* infection (35,155,261). In this study, seropositivity to *Brucella* infection was significantly associated with history of abortion as previously reported in Ethiopia (196) and Uganda (281).

In general, the distribution of *Brucella* antibodies among different animal species and pastoral villages was found to be variable. This could be associated with variability of the herd sizes and samples tested per visited households. Short drought cycles caused by climate changes drive Borena pastoralists to trek their livestock, with the exception of lactating and few pregnant animals, to different villages, districts, or even crossing national borders by traveling several kilometres. This results in massive concentration of animals in areas with relatively better pasture and watering points. This in turn, may contribute to the increased transmission of *Brucella* organisms among different herds resulting in emergence of new infectious foci creating variation in distribution of *Brucella* infections among different villages and districts. Mobility also increases the opportunity of interactions with wild animals. Sharing the same ecology with wildlife was shown to be an important risk factor for brucellosis in domestic animals kept under traditional livestock production systems (261,282).

In two villages, Bildim and Boku no positive case was detected in all animal species tested (Table 2). This could be explained by absence of seropositive cases due to the small sample sizes. Besides, the average number of positive animals per positive herd was generally low, 1.4 both in cattle and sheep and goats, suggesting a slow within herd transmission of the disease. The practice of culling breeding animals with weak reproductive performance and of old age animals could reduce the risk of within – herd transmission of brucellosis and subsequently its zoonotic hazard to humans.

Even though attempts to isolate *Brucella* species circulating in the region was not successful, these results reveal more than one seroreactor animal species in villages and household visited, raising the possibility of cross-species transmission of *Brucella* infections. In areas where control measures for brucellosis are not in place, the disease caused by the classical species (*Brucella abortus* in cattle, *B. melitensis* in sheep and goats, *B. suis* in pigs etc.) can be endemic in herds or flocks, characterized by high seroprevalence (69). However, in cases of spillover to the accidental host from the preferential host, onward transmission is not likely to be so efficient and a lower

seroprevalence record would be expected (154). *B. melitensis* biovar 1 infection has been reported in cattle in Kenya (148), *B. abortus* biovar 6 infection in sheep in Eastern Sudan (283), and *B. melitensis* biovar 2 and 3 from cattle in South Africa (284) which would be consistent with cross-species transmission in Borena pastoral region.

In the present study, an overall human *Brucella* seropositivity of 2.6% (95% CI= 1.2-4.9) was recorded (Table 1). This finding is comparable with previous reports in Western Tigray zone, Northern Ethiopia (201) and in Adamu Tullu Jido Kombolcha district, central Ethiopia (61). Similar findings were also reported in Eritrea (285) and Chad (155). Conversely, a higher seroprevalence than the current study was recorded in Kenya (286) and in Kyrgyzstan (287). The variations observed in different studies could be associated with prevalence of brucellosis in the livestock population, duration of exposure, sample size epidemiological settings of the study population and variability related to diagnostic test and method applied.

The present study determined risk factors for human brucellosis among occupationally linked household members in Borena pastoral region. Studies in Kenya by Namanda *et al* (288) and in Tanzania by John *et al* (289) have reported occupation as a risk factor for acquiring brucellosis, whereby animal handlers and associated professionals were the most susceptible groups. In our study, it was revealed that 98.8 % of participants had no knowledge of brucellosis. Therefore, there is a clear need to promote health education about transmission, prevention and risk factors for brucellosis to occupational risk groups to reduce the risk of acquiring the disease.

Consumption of unpasteurized milk was reported to be a risk factor for acquiring brucellosis in human (288,290,291). Practices of consuming raw milk among Borena pastoral communities is due to a belief that boiling a milk would reduce its nutritional content. Our study indicated that 84% (n=287) of participants had consumed raw milk, 59 % (n=202), raw milk mixed with blood collected from domestic livestock, and 47 % (n=160) consumed raw meat. However, none of these practices were significantly associated with seropositivity, although numbers were low. Variations in number of

human seroreactors among the four districts followed the same pattern as seropositivity in cattle and goats, although again, results were not significant (Table 3.1).

The multivariable logistic regression analysis of potential risk factors indicated that assisting during birthing or calving was significantly associated with *Brucella* seropositivity (OR=9.9, 95% CI=1.4-72). Assisting in calving or birthing was associated with increased risk of brucellosis in similar settings in Northern Tanzania (292) and in Kenya (293). *Brucella* species are known to have a predilection for reproductive organs particularly placenta and aborted foetuses, it is reasonable that assisting animals in delivery would increase risk of infection (286).

Our study revealed that human seropositivity was associated with presence of seropositive animal at household. The odds of human seropositivity were 45 times higher in households with a seropositive animal as compared to those without. Similar finding were reported in Kenya by Osoro *et al* (286) and Kyrgyzstan by Bonfoh *et al* (287). This study thus contributes to the evidence base that human brucellosis is often transmitted from livestock in close contact (49,265). In many developing countries including Ethiopia, brucellosis continues to be a major public and animal health problem as there is no control strategies put in place, although a One-Health strategy is now being developed in Ethiopia.

3.5. Conclusions

The current study revealed that antibodies to *Brucella* spp. are detected in cattle, sheep and goats sharing the same ecological zone and occupationally linked pastoralists in Borena, Ethiopia. The study also showed associations between human and animal seropositivity at household level. Convenience sampling of villages and not including children less than 5 years of age may limit the representation of the data to the entire population. Adult age group, larger herd/flock sizes, greater parity in cattle and history of abortion were found to be risk factors for brucellosis in cattle and sheep

and goats. Assisting during calving without using protective equipment was also an explanatory variable associated with *Brucella* seropositivity in humans. The traditional mixed livestock farming system in Borena supplemented with recurrent livestock mobility triggered by climatic changes and other factors will likely continue to enhance the prevalence of the disease in the area.

The occupational risks for pastoralists such as contact with infected animals, particularly assisting during calving without protective equipment and the tradition of raw dairy product consumption facilitates zoonotic transmission. Further extensive epidemiological studies involving one health approach needs to be undertaken to isolate and characterize circulating *Brucella* species among humans and livestock so as to identify the transmission dynamics of *Brucella* species. Raising public awareness regarding traditional practices that could potentially cause exposure to *Brucella* infection and prevention methods is a clear need. A socioeconomic study to provide a societal perspective of the burden of the disease is highly warranted as this would help in determining feasible control measures to be undertaken in different settings.

4.CHAPTER FOUR

In this chapter brucellosis outbreak investigation was conducted in one Government owned Agricultural Research Centre Dairy Farm (Adamu Tullu Agricultural Research Centre) in central Oromia, Ethiopia. Farm visit, serological testing, post-mortem examination and tissue sample collection for bacterial culture was conducted by principal investigator of this PhD project along with a technical assistant from College of Veterinary Medicine, Addis Ababa University. Due to lack of high containment level three laboratory for *Brucella* culture in Ethiopia, the tissue samples were shipped to Animal and Plant Health Agency (APHA), OIE *Brucella* reference laboratory. Brucella culture and molecular characterisation at APHA and Whole Genome Sequencing and subsequent bioinformatic analysis at Department of Veterinary Medicine, University of Cambridge were conducted in collaboration with experts at respective institutes.

The work in this Chapter has been drafted for publication and is being reviewed by supervisors and co-workers.

4.1. Introduction

The dairy sector in Ethiopia is highly heterogeneous comprising of the traditional pastoral/agro-pastoral and mixed crop–livestock production systems and the market-oriented intensive specialized producers. There are at least 13 million cattle keeping households (243) in the country. Ethiopian dairy production systems can be classified into four sectors, including the commercial, the urban/peri-urban, the mixed crop-livestock, and the pastoral/agro-pastoral systems (294). Commercial dairy farms are specialised dairy farms which are run as a full-time business; located mainly in and around the major cities and produce milk exclusively for sale. Urban and peri urban dairy are a smallholder dairy farms produce milk for both home use and sale either as full time or part time business in urban and peri-urban areas (143). Mixed crop-livestock production is a subsistence-oriented farming system in the mid-and high-altitude agro-ecological zones of Ethiopia where cereals and cash crops are dominant farm activities and milk production is an integral part of the production system.

Pastoral and agropastoral production is the major system of milk production practiced in the lowland regions of Ethiopia (294).

The Ethiopian Government's livestock road map is ambitious and aspires to increase domestic cow milk production by about 93% by 2020 through improvements in genetics, health and feeding, so as to meet consumption demand, and start export of cow milk and milk products (295). In the road map, cross breeding the local zebu breeds with high milk yield producing exotic breeds such as Holstein Friesian and Jersey is considered a priority. It is hoped that implementation of the road map will greatly improve the potential of small holder dairy farmers so that they can fulfil the demand for milk. In order to accomplish this task, the Government of Ethiopia is using its agricultural research centres as breeding centres for the production and dissemination of genetically improved dairy cattle breed and genetically improved semen for artificial insemination.

The Adami Tullu Agricultural Research Centre (ATARC) is one of the government agricultural research centres which is producing crossbred dairy cattle by cross breeding high yielding Holstein Friesian and Jersey dairy breeds with the zebu breed, and thereafter distributing the crossbred dairy cattle to farmers. However, the Centre has been constrained by the occurrence and prevalence of infectious diseases including bovine brucellosis, which has frequently affected the dairy cattle in the Centre. Bovine brucellosis is endemic in Ethiopia and has been reported from various regions and different livestock production systems in the country(143,296).

To better understand the epidemiology of the disease, isolation and characterisation of circulating *Brucella* species or genotypes among livestock systems will play a crucial role in future planning and designing of feasible control strategies. Knowledge of genetic diversity of *Brucella* would also help to better understand transmission dynamics across different species and geographic regions. In April 2018, an "abortion storm" occurred in the ATARC during which a significant number of cows aborted. Bovine brucellosis was suspected to be the cause of the outbreak of abortion, and further investigation of the outbreak of the abortion was necessary. Hence, this study was conducted with the aim answering the following questions.

1. What was the causative agent of the outbreak?
2. What particular genotypes, sequence types and SNP types of the causative agent were circulating in the farm?

In addition to the above questions, the study was also aimed to investigate phylogenetic placement of the causative agent relative to the global diversity of the same species.

4.2. Materials and methods

4.2.1. Study centre and animals

Adami Tullu Agricultural Research Centre (ATARC) is one of the seventeen branch centres of Oromia Agricultural Research Institute (OARI). The objectives of the centre are to generate, adapt and transfer feasible technologies that could improve agricultural productivity and contribute to economic and socio development of East Shewa and West Arsi zone, central Oromia. It is located in the mid Rift Valley, central Ethiopia, 167 km South of Addis Ababa in Oromia National Regional State. It lies at latitude 7°9'N and longitude 38°7'E at an elevation of 1650m above sea level (Fig 4.1). The area has a relative humidity of 60% and receives average annual rain fall of 760.9 mm with minimum and maximum average annual temperature of 12.7°C and 27.2°C, respectively(297).

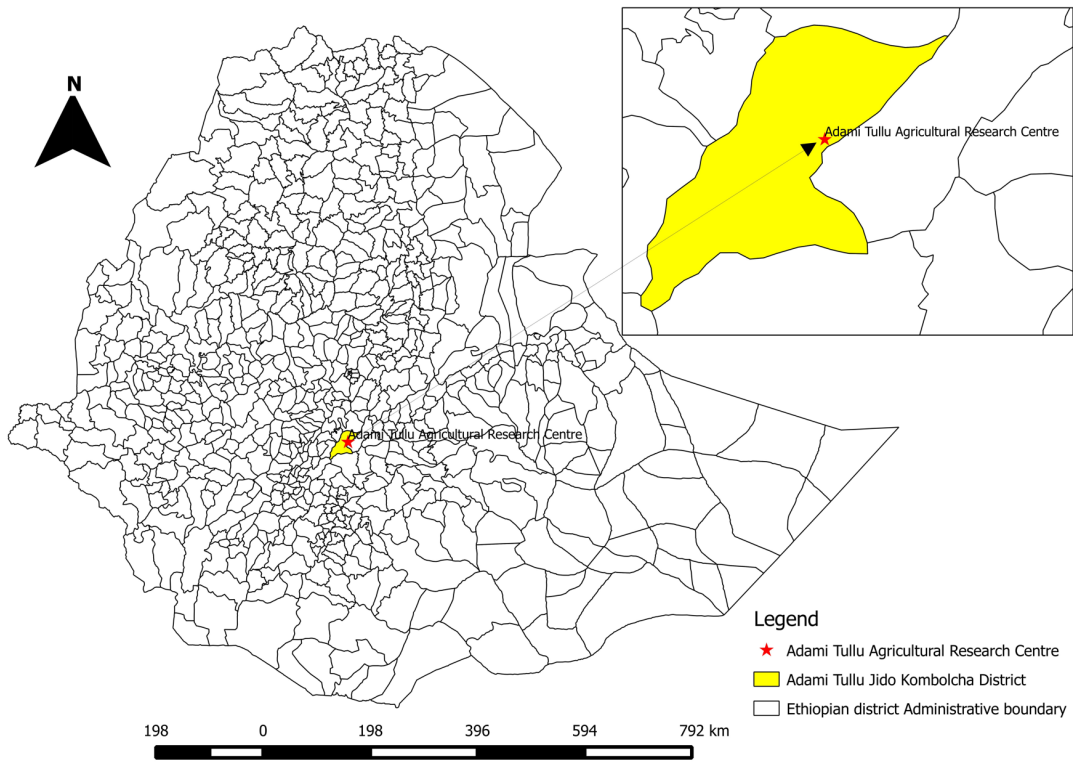

Figure 4.1: Map of Ethiopia, Admi Tullu Jido Kombolcha district and Adami Tullu Agricultural Research Centre.

Legend: This map is developed from Ethiopian shape files using QGIS software, 2013.The yellow shaded region is representing Adamu Tullu Jido Kombolcha district and the red start represents ATARC.

Dairy cattle in the Centre were kept semi-intensively where cows were allowed to graze in a protected grazing area in the Centre during the daytime and managed in separate pens established for calves, lactating, pregnant and dry cows during the nights. A river crossing the Centre is used as a watering point. The study population included a total of 547 dairy cows of cross and local breeds that were maintained semi intensively at the Centre.

A network of veterinarians working in district veterinary offices, regional veterinary laboratories and government agricultural research centres was established. Its purpose was to collect information and gain access to dairy farms or herds with a history of recent abortions to make further investigation using serological, bacteriological and molecular tools. A report of an abortion storm in dairy cattle of cross and local breeds at ATARC was obtained from Assela Veterinary Regional Laboratory. This was the only opportunistic report received during the study period.

On April 2018, a research team lead by the principal investigator of this study travelled to the ATARC and conducted a cross-sectional investigation on the outbreak. Investigation was conducted on crossbred and zebu dairy cattle kept in the Centre.

4.2.2. Data collection

During the first visit in April 2018 permission was obtained from the manager of ATARC to further investigate the cause of the outbreak in the affected farm after explaining the objective of the study. The second visit was made in May 2018. Blood samples (10 ml) from the jugular vein of each animal were collected, using sterile needles and plain vacutainer tubes labelled with individual animal identification numbers. The clotted blood samples were transported to microbiology laboratory at ATARC and centrifuged at 3000 × g for 10 min to obtain the serum within 12 hours of collection. Sera were decanted into cryo-vials, identified and stored at -20°C until screened for antibodies against natural *Brucella* exposure using serological analysis. The biodata of seropositive animals was obtained from each individual animal record book. Permission was not obtained to access the biodata of seronegative animals.

Rose Bengal plate test (RBT): All sera samples collected were initially screened by RBT using RBT antigen (Animal and Plant Health Agency (APHA), Surrey, UK) according to OIE (2016) procedures at the Aklilu Lemma Institute of Pathobiology, Addis Ababa University (ALIPB-AAU). Sera and antigen were taken from the refrigerator and left at room temperature for half an hour before the test to reach room temperature. Briefly, 30 µl of RBT antigen was added onto a glass slide next to an equal amount of cattle sera. The antigen and test serum were mixed thoroughly with a plastic applicator, shaken for 4 min, and agglutination was read immediately. Any agglutination observed with the naked eye was considered to be a positive reaction.

Competitive ELISA: All RBT positive sera were further tested using the COMPELISA 160 and 400, a competitive ELISA kit for the detection of antibodies against *Brucella* in serum samples (APHA, Surrey, United Kingdom) at ALIPB-AAU. The test was

performed according to the manufacturer's instructions and was conducted in 96-well polystyrene plates that are pre-coated with *Brucella* species lipopolysaccharide (LPS) antigen. An animal was considered positive if it tested seropositive on both RBT and c-ELISA in serial interpretation.

4.2.3. Postmortem examination and bacteriological sample collection

The postmortem examination was conducted in June 2018. Not all seropositive animals were postmortem examined. As all seropositive animals had history of abortion, animals for postmortem examination were selected randomly. Assuming that the outbreak occurred in a single farm and depending on resources and logistics we have, and maximum number of animals allowed to be postmortem examined by the research Centre, only 30 animals were postmortem examined and samples collected for bacteriology. Protective clothing, closed-toed disinfectable footwear and disposable gloves were used. For each sample individual sterile scalpel blade was used to minimize the risk of cross contamination between samples. Tissue samples such as cross-section of uterine tissues and mammary gland lymph nodes were collected into 20 ml sterile polystyrene centrifuge tubes with normal saline. Vaginal swabs were collected using Amies sterile media swabs (Deltalab, Spain). Thereafter, all samples were transported with ice packs to the ALIPB-AAU and stored at −20°C until shipped to the *Brucella* Reference Laboratory at the Animal and Plant Health Agency (APHA) in the United Kingdom (UK). All samples including 3 milk samples collected from goats with history of abortion in Borena during the field survey were shipped to the APHA, the UK following the International Air Transport Association (IATA) rules for shipment of samples suspected of category B agents.

4.2.4. Bacteriology culture

All *Brucella* culture work was conducted following standard operating procedures recommended by the OIE/FAO Reference Laboratory for Brucellosis at APHA. Readymade and APHA quality tested Farrell's (*Brucella* selective), and serum–dextrose

agar (SDA) solid media plates and Brodie and Sinton broth (298) were used. A total of 88 samples (3 milk, 38 swab, 43 tissue and 4 culture positive isolates in glycerol stocks) were cultured. Briefly, tissue samples were macerated using a sterile scalpel blade and chopped using an electric blender with Brodie and Sinton broth inside a Class-III microbiological safety cabinet. The tissue suspensions were then inoculated into Farrell's and SDA agar plates. Swab samples were streaked onto a duplicate of Farrell's and SDA plates, after which the swab tip was cut off and incubated in Brodie and Sinton broth. All the cultured plates and broths were then incubated with 10 % CO_2 at 37 °C for 3 to 5 days. The plates were checked for any growth of *Brucella* every 24 hours until suspect colonies were observed. Figure 4.2 indicates a flow chart that indicates the steps from serological testing of animals to isolation of *Brucella*.

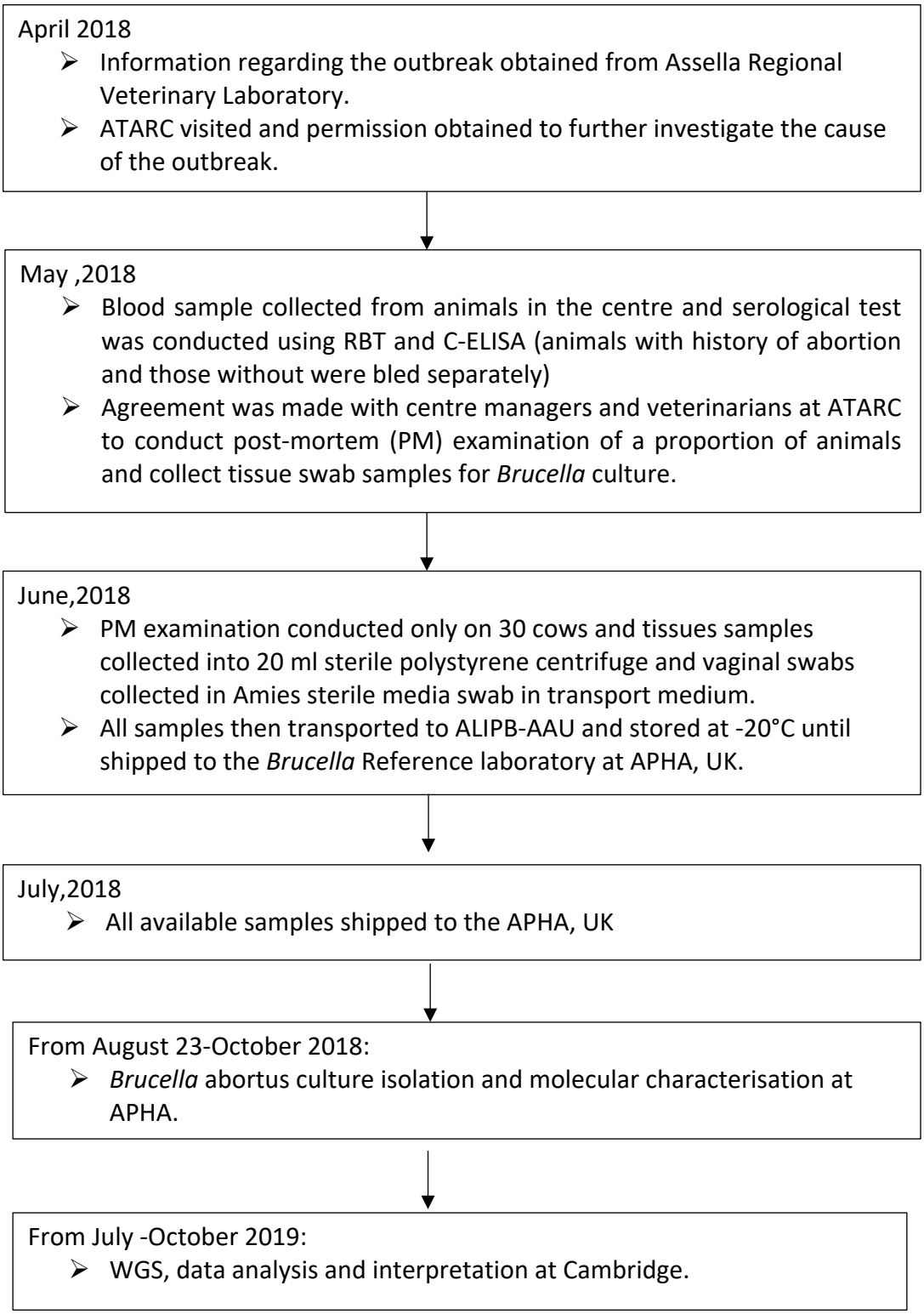

Figure 4.2: A flow chart that indicates the steps from serological testing of animals to isolation and molecular charactersiastion of *Brucella.*

4 .2.5. Isolation and Identification

Colonies with round shape and pale honey colour were suspected to be *Brucella* if observed on Farrell's media plates after 72 hrs of incubation. These suspect colonies were sub-cultured into new Farrell's media plates and incubated with 10 % CO_2 at 37 °C for 3 to 4 days. Suspect colonies were further tested using a slide agglutination test with a polyclonal anti-*Brucella* serum and anti-A and anti-M monospecific sera. To further characterise the isolates using polymerase chain reaction (PCR), PCR-ready DNA was harvested by boiling a single colony from the Farrell's agar plate in 200 µl nuclease-free water at 100°C for 10 minutes, then centrifuging at 13,000 rpm for 30 seconds. This boiled supernatant was used for all PCR assays.

4.2.6. Real-Time PCR

This assay was based on two markers used for specific identification of *Brucella* species. These are the multiple copy insertion sequence *IS711* and the gene *bcsp31* encoding a 31-kDa antigen conserved among *Brucella* spp. The assays were conducted according to the protocols previously described by Matero et al (76) for *IS711* and by Probert et al (299) for *bcsp31*. A commercial exogenous Internal Positive Control (IPC) assay was multiplexed with the *Brucella* spp. specific *IS711* assay. An IPC (Provided with Taqman® Exogenous Internal Positive Control Kit from Applied Biosystems) assay with a different fluorescent dye (VIC) than the FAM-labelled *IS711* assay was used to allow multiplexing and ensure a high sensitivity. Oligonucleotide primers and probes used in the real-time multiplex PCR are shown in Table 4.1.

Table 4.1**:** *IS711***/**BCSP31 primer/ Probe sequences.

Name	Sequence (5’ to 3’)
IS711 Forward	GGCCTACCGCTGCGAAT
IS711 MGB Probe	FAM – AAGCCAACACCCGGC – MGBNFQ
IS711 Reverse	TTGCGGACAGTCACCATAATG
BCSP31 Forward	GCTCGGTTGCCAATATCAATGC
BCSP31 Probe	FAM - AAATCTTCCACCTTGCCCTTGCCATCA - BHQ1
BCSP31 Reverse	GGGTAAAGCGTCGCCAGAAG

Amplification was performed in a total volume of 25 μl reaction mixture. The IS*711* real time TaqMan PCR assay contained 12.5 μl of TaqMan Universal PCR Master Mix, 2.5 μl of primer/probe mix (0.3 μM forward primer, 0.9 μM reverse primer and 0.25 μM probe), 2.5 μl of Exogenous Internal Positive Control, 5 μl of nuclease free water and 2.5 μl of template DNA. The reaction mixture for *bcsp31* Real time TaqMan PCR assay included 12.5 μl of TaqMan Universal PCR Master Mix, 2.5 μl of primer/probe mix (0.2 μM forward and reverse primers, and 0.1 μM probe), 7.5 μl of nuclease free water and 2.5 μl of template DNA. All samples and controls were tested in duplicate wells. Nuclease free water and the DNA of *Brucella* reference strain (*B. abortus* 544) were used as a negative and positive control respectively. The amplification reactions were performed in duplicate in an optical 96 well PCR microplate (Thermo Scientific) using a real time thermocycler (Agilent Technologies).

Cycling conditions for the IS*711* assay were as follows: one cycle for uracil-N-glycosylase (UNG) digestion at 50 °C for 2 minutes, initial denaturation at 95 °C for 10 minutes, followed by 45 cycles at 95 °C denaturation for 15 seconds, and 1 minute for annealing and extension at 60 °C. The conditions for the *bcsp31* assay were the same as the IS711 assay except that annealing and extension was done at 57 °C.

4.2.7. Real time PCR based Single Nucleotide Polymorphism (SNP) typing

This method was conducted to identify *Brucella* isolates to the species level. As described by Gopaul *et al* (77), SNP typing is based on species defining single nucleotide polymorphisms (SNPs) in specific loci in different *Brucella* species, which enable these species to be distinguished. Individual assays for each SNP are based on the use of two alternative DNA Minor Groove Binding probes, one that preferentially binds to the species-specific polymorphism, while the other binds to the allele state present in all other members of the genus. In each case the species-specific probe is labeled with VIC while the alternative state probe is labeled with FAM. The SNP species-specific assay was conducted according to the protocols described by Gopaul *et al* (77) and (300) for the following known *Brucella* species; *B. abortus, B. melitensis, B. ovis, B. suis (bv1-4), B. ceti, B. pinnipedialis, B. canis, B. neotomae, B. microti,* and *Brucella* reference strain (*B.abortus* 544) was used as a positive control. A reaction mixture without template DNA was used as a negative control. Sequences of the primers and MGB probes used for the individual species defining assays are listed in Table 4.2.

Table 4.2: Sequences of primers and MGB probes used for the individual species defining assays.

			Primers			Probe	
Species	Gene	Location		Sequence (5'-3')	5' Dye	Sequence (5'-3')	3' Dye
B. abortus	*fbaA*	AEO17224	F	TGACATCATGCTCCGTCACATG	VIC	ATGCCGTGGCGGAA	NFQ
		360225-	R	CAGACCGGAATATGCGGATAGAT	FAM	ATGCCGTGACGGAA	NFQ
B. melitensis	*gap*	AEO17223	F	GGCTCAGGTTCTCAACGATACTATC	VIC	CGTGGTCATAAAGC	NFQ
		1684721-	R	TCGCCCGTATAGGAGTGGAT	FAM	CGTGGTCATGAAGC	NFQ
B. ovis	*aroA*	AEO17223	F	CGACCACCGCATCGC	VIC	CCATGACAAGGAAAC	NFQ
		29246-30598	R	CCGGCTTTTCCGATGCAA	FAM	CATGACGAGGAAAC	NFQ
B. suis bv1-4	*prpE*	AEO17223	F	GCGACCGCATCCTCATCTATATG	VIC	CAAGCGTGGCAACC	NFQ
		1687718-	R	CGCCGAATACGACGGAATGAAT	FAM	CAAGCATGGCAACC	NFQ
Marine spp	trpE	AEO17223	F	CGAGGATTCCTTCGTCCATACG	VIC	CCAATTATTTCCACCAGACG	NFQ
		1537355-	R	ACGCACGGTGGAAACCTT	FAM	CCAATTATTTCCGCCAGACG	NFQ
B. canis	*omp25*	AEO17223	F	GCTGGCGCCTTTGCT	VIC	AACTTCCAGAAGGACC	NFQ
		710024-	R	GGCCGTCCTTGGACTTCTTG	FAM	AACTTCCAGCAGGACC	NFQ
B. neotomae	Putativ	AEO17223	F	GGTTTTCCATGCGGTTTATTTGC	VIC	CATTGAGTGGCCCGAT	NFQ
		1989869-	R	GGCATCATGCACAGTGATATCGA	FAM	ATTGAGCGGCCCGAT	NFQ
B. microti	*aroA*		F	CGTCACCATCCGCAATGT	VIC	ATGAACCCAACCCGC	NFQ
			R	CCCCCATTTCCTGCAACG	FAM	ATGAACCCGACCCGC	NFQ

The assay for each sample was performed in duplicate in an optical 96 well PCR microplate. A total of 12.5 μl reaction mixture was made from 6.25 μl of TaqMan Genotyping Master Mix, 1.25 μl of primer/probe mix, 4 μl of nuclease free water and 1 μl of template DNA. The reaction conditions used were initial denaturation for 10 minutes at 95 °C followed by 40 cycles at 92 °C denaturation for 15 seconds, and 1 minute for annealing and extension at 60 °C.

4.2.8. Multiplex PCR

This assay is based on polymorphisms arising from species-specific localisation of the insertion sequence *IS711* region in the *Brucella* chromosome and was conducted according to Mayer-Scholl *et al.* (106) and Lopez-Goni *et al.* (107). In multiplex PCR using DNA from *B. abortus*, four fragments should be amplified: 794, 587, 450, and 152 bp in size; with *B. melitensis* DNA, an additional 1,071 bp fragment should be amplified. The primer sequences utilized for the amplification of the fragments are indicated in Table 3. A total of 25 μl reaction mixture was made that consisted of 12.5 μl of Qiagen multiplex PCR master mix, 2.5 μl of Bruce-Ladder primers working dilution (0.2 μM of each primer), 9 μl of nuclease free water and 1 μl of template DNA. The amplification was run in a thermocycler (Eppendorf Master Cycler) with the following conditions: initial denaturation at 95 °C for 15 minutes, followed by 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute and 30 seconds and extension at 72 °C for 3 minutes with final extension at 72 °C for 10 minutes. PCR products were analysed by standard 2% agarose electrophoresis. Sequences of primers used in this assay are listed in Table 4.3.

Table 4.3. Sequences of primers used during Multiplex PCR assay.

Primers Pair	Sequences (5'-3')	Amplicon size (bp)	Reference
BMEI0998f	ATCCTATTGCCCCGATAAGG	1682	(301)
BMEI0997r	GCTTCGCATTTTCACTGTAGC		
BMEI0535f	GCGCATTCTTCGGTTATGAA	450	(301)
BMEI0536r	CGCAGGCGAAAACAGCTATAA		
BMEII0843f	TTTACACAGGCAATCCAGCA	1071	(301)
BMEII0844r	GCGTCCAGTTGTTGTTGATG		
BMEI1426	TCGTCGGTGGACTGGATGAC	774	(302)
BMEI1427	ATGGTCCGCAAGGTGCTTTT		
BMEII0428f	GCCGCTATTATGTGGACTGG	587	(301)
BMEII0428r	AATGACTTCACGGTCGTTCG		
BR0953f	GGAACACTACGCCACCTTGT	272	(301)
BR0953r	GATGGAGCAAACGCTGAAG		
BMEI0752f	CAGGCAAACCCTCAGAAGC	218	(301)
BMEI0752r	GATGTGGTAACGCACACCAA		
BMEII0987f	CGCAGACAGTGACCATCAAA	152	(301)
BMEII0987r	GTATTCAGCCCCCGTTACCT		
BMISPECF	AGATACTGGAACATAGCCCG	510	(303)
BMISPECR	ATACTCAGGCAGGATACCGC		

4.2.9. Whole genome sequencing (WGS) of Brucella isolates

DNA extraction

DNA was extracted using a Qiagen genomic DNA purification kit (Qiagen DNeasy Blood and Tissue Kit) following the manufacturer's instruction. One to two colonies from each agar plates were harvested in 500 μl nuclease-free water and boiled at 100 °C for 10 minutes. The contents were then pelleted by centrifugation (10,000 g for 10 minutes) and resuspended in 180 μl ATL buffer. Twenty microlitres of proteinase K was added into the tube and mixed thoroughly by vortexing then incubated at 56 °C in a thermomixer until the cells were completely lysed. The samples were then vortexed for 15 seconds and 200 μl of AL buffer was added to each sample and mixed thoroughly by vortexing. Two hundred microlitres of ethanol (96-100%) was added immediately afterwards and mixed again thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini Spin column placed in a 2 ml collection tube and centrifuged at 6000 g. After discarding the flow-through and collection tube, the DNeasy Mini Spin column was placed in a new 2 ml collection tube and 500 μl AW1 buffer was added and centrifuged at 6000 g for 1 minute. The flow-through and collection tube was discarded and DNeasy Mini Spin column was again placed in a new 2 ml collection tube and 500 μl AW2 buffer was added. This was then centrifuged at 20,000 g for 3 minute and the flow-through and the collection tube were discarded. The DNeasy Mini Spin column was then placed in a 1.5 ml microcentrifuge tube and 200 μl AE buffer was pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 minute. To elute the DNA, it was centrifuged at 8,000 rpm for 1 minute. DNA concentrations were quantified using the Qubit 2.0 fluorometer and a double-stranded DNA high-sensitivity assay kit (Thermo Fisher Scientific, MA).

DNA library preparation

Genomic libraries were constructed using a NEB Next Ultra II library preparation kit (NEB) according to the manufacturer's instructions. Briefly, 250 ng DNA was fragmented in Tris-EDTA (TE) buffer by sonication using a Misonix XL 2020 Ultrasonic Liquid processor with 600 bp fragments being most prevalent. A reaction mixture of 60 μl containing 3 μl of NEBNext Ultra II End Prep Enzyme Mix, 7 μl of NEBNext Ultra II End Prep Reaction Buffer was added to

50 μl of fragmented DNA and thoroughly mixed. The reaction mixture was then placed in a thermocycler with the heated lid set to ≥ 75 °C and run at 20 °C for 30 minutes and at 65 °C for 30 minutes.

Adaptor Ligation

For adapter ligation, a total of 93.5 μl reaction mixture of 60 μl End Prep Reaction Mixture, 30 μl of NEBNext Ultra II Ligation Master Mix, 1 μl of NEBNext Ligation Enhancer and NEBNext Adaptor for Illumina was prepared and mixed thoroughly. After incubating the ligation mixture at 20 °C for 15 minutes in a thermocycler with the heated lid off, 3 μl of USER Enzyme was added, mixed and incubated at 37 °C for 15 minutes with the heated lid set to ≥ 47 °C.

Clean up or size selection

For size selection or cleanup of Adaptor-Ligated DNA, 15 μl of vortexed and resuspended NEBNext Sample Purification Beads (SPRIselect) was added to the 96.5 μl ligation reaction. The reaction was mixed well by pipetting up and down at least for 10 times and care was taken to expel all of the liquid out of the tip during the last mix. After incubating the samples at room temperature for 5 minutes, the tubes containing the samples were placed on a magnetic stand to separate the beads from the supernatant. When the solution was clear (after 5 minutes), the supernatant containing the DNA was transferred to a new tube and the beads containing the unwanted large fragments were discarded. Ten microlitre of resuspended NEBNext Sample Purification Beads was added to the supernatant and mixed at least 10 times before the samples were incubated at room temperature for 5 minutes. The tube was then placed on a magnetic stand to separate the beads from the supernatant. After 5 minutes, when the solution was clear, the supernatant containing unwanted DNA was carefully removed and discarded. While the tube was on the magnetic stand, 200 μl of freshly prepared 80 % ethanol was added and incubated at room temperature for 30 seconds. The supernatant was carefully removed without disturbing the beads that contain the DNA targets and discarded. The above washing step with ethanol was repeated a second time and all the visible liquid after the wash was removed. The beads in the tube were air dried with the lid open for 5 minutes while on the magnetic stand. The tube was removed from the magnetic stand and the DNA target was eluted from the beads into 17 μl 0.1xTE. The tube was mixed

well on a vortex mixer and incubated at room temperature for 2 minutes and placed on a magnetic stand. When the solution was clear after 5 minutes, 15 μl was transferred to a new PCR tube for amplification.

PCR Enrichment of adaptor-ligated DNA

For this a total of 50 μl reaction mixture containing 15 μl of Adaptor ligated DNA fragments, 25 μl of NEBNext Ultra II Q5 Master Mix, 5 μl of Index Primer/i7 Primer and 5 μl of Universal PCR Primer/i5 Primer was prepared and mixed thoroughly. The tube was then placed on a thermocycler and PCR amplification was conducted with following cycling conditions: 1 cycle of initial denaturation at 98 °C for 30 seconds, 3 cycles of denaturation at 98 °C for 10 seconds, 3 cycles of annealing/extension at 65 °C for 75 seconds and 1 cycle of final extension at 65 °C for 5 minutes.

Clean-up of PCR reaction

NEBNext Sample Purification Beads were vortexed to resuspend and 45 μl of resuspended beads was added to the PCR reaction. The reaction was then mixed thoroughly by pipetting up and down for at least 10 times and incubated at room temperature for 5 minutes. The tube was then placed on a magnetic stand to separate the beads from the supernatant. After 5 minutes, when the solution was clear, the supernatant was carefully removed and discarded. While the tube was on a magnetic stand, 200 μl of 80% freshly prepared ethanol was added and incubated at room temperature for 30 seconds. The supernatant was then carefully removed and discarded. The washing step with 80% ethanol was repeated once with utmost precaution to remove all visible liquid after the second wash. The beads were then air dried for 5 minutes while the tube with the lid open was on the magnetic stand. The tube was removed from the magnetic stand and the DNA target was eluted from the beads by adding 33 μl of 0.1X TE. After mixing the contents by vortex mixer, the tube was incubated at room temperature for 2 minutes. The tube was then placed on the magnetic stand for 5 minutes until the solution became very clear. Finally, 30 μl of the clear solution was transferred into a new PCR tube and the size distribution of the library was checked on an Agilent Bioanalyzer High Sensitivity DNA chip (Agilent Technologies) according to the manufacturer's instruction.

Library size selection and sequencing

The library size selection was 550 bp, and a paired-end (PE) sequencing strategy (2 X 250 bp) was performed using the Miseq platform and MiSeq Reagent Kit v2 following the manufacturer's recommended protocol. Briefly, the Miseq reagent cartridge was thawed overnight at 2°C to 8 °C. It was then inverted ten times to mix thawed reagents. When the cartridge was fully thawed, the prepared library was loaded onto the cartridge. The run and BaseSpace was set up. After rinsing the flow cell with laboratory grade water and ensuring it was thoroughly dried, it was placed on the flow cell stage and the flow cell compartment door was closed. The wash bottle was then removed and the PR2 bottle was loaded. The reagent chiller door was opened, and the reagent cartridge was pushed into the reagent chiller until the cartridge stopped. The run parameters were reviewed, and the pre-run check was performed before starting the run. A total of 301 cycles were run.

4.2.10. WGS quality control

Basic quality control metrics for the raw sequence data were generated using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/); these included read counts, sequence quality and GC content. The sequence reads were classified into multiple levels in the taxonomic tree using Kraken, a tool for the taxonomic classification of high-throughput sequence data (304), and a database of prokaryote reference sequences (minikraken_20171019_8GB). The abundance of those classifications at a single level was estimated by Bracken. Bracken (Bayesian re-estimation of abundance after classification with KrakEN) uses the taxonomic assignments made by Kraken to estimate abundance at the species level, rather than the genus level or higher estimated by Kraken (305). At least 90% of the reads in each sequence file mapped to a *Brucella* species reference. Fastq reads were then trimmed using trimmomatic, an efficient pre-processing tool used to remove low quality reads and adapter sequences from Illumina sequence data, using the following parameters: -phred33 (use phred33 quality scores), ILLUMINACLIP (cut Illumina adapter and other sequences), LEADING:10 (remove leading low quality or N bases below quality 10), TRAILING:10 (Remove trailing low quality or N bases below quality 10), SLIDINGWINDOW:5:20 (scan each read with a 5-base wide sliding window, cutting when the

average quality per base drops below 20), MINLEN:20 (remove reads shorter than 20 bases) (306). Example commands for running FastQC, Kraken, Bracken and trimmomatic are provided in Appendix 5.

4.2.11. Data collection, assembly and annotation

All *B. abortus* and *B. melitensis* genome assemblies available in National Center for Biotechnology Information (NCBI) genome database were downloaded. In addition, published Illumina paired end sequenced fastq files of *B. abortus* and *B. melitensis* were downloaded from the European Nucleotide Archive (ENA). All *B. melitensis* fastq files downloaded from the ENA were also available in NCBI database and were excluded from the final dataset. The Ethiopian *B. melitensis* and *B. abortus* genomes, and *B. abortus* fastq files downloaded from the ENA were *de novo* assembled using SPAdes v3.13.1, an assembly toolkit containing various assembly pipelines (307) using the following parameters: -careful (reduce the number of mismatches and short indels) and -k 21, 25, 29, 33, 37, 41, 45, 49, 53, 57, 61, 65, 69, 73, 77, 81, 85, 89 (list of k-mer sizes to be used to find the best assembly). The quality of the final assemblies assessed using Quast, a quality assessment tool for genome assemblies (308). These assemblies, along with the *B. melitensis* and *B. abortus* assemblies downloaded from the NCBI, were annotated using Prokka using the *Brucella* genus database. Prokka is a rapid prokaryotic genome annotation tools that works on preassembled genomic DNA sequences in FASTA format (309). Example commands for running SPAdes v3.13.1, Quast and Prokka are provided in the Appendix 5.

4.2.12. Core genome phylogenetic analysis

Pan genome analysis for *B. melitensis* and *B. abortus* using the Ethiopian assemblies and assemblies retrieved from the ENA and NCBI was conducted using Roary (Appendix 5). Roary is a high speed stand-alone pan genome pipeline, which takes annotated assemblies like those produced by Prokka and calculates the pan genome (310). A blast minimum percentage identity threshold of 90% (-i 90) was chosen as this would account for nucleotide differences amongst core genes from different populations and genes identified as being part of the core genome (-cd 99; present in >99% of genomes) were extracted from each genome and aligned.

Variable sites were extracted from the resulting core gene alignments using SNP-sites removing sites with 'N's (-c) (311) (Appendix 5). IQ-TREE (312) was run on the variable site alignments using the model finder (MFP) and ascertainment bias flags (ASC; -m MFP+ASC) and 1000 bootstraps (-bb 1000) to build maximum likelihood phylogenetic trees that were then visualised and annotated using iTOL (313).

4.2.13. Core SNP Analysis

The phylogenetic tree of *B. abortus* revealed that the nearest genome to the Ethiopian *B. abortus* genomes in the tree was a strain downloaded from the NCBI: *B. abortus*_88_226_V1 (Accession number: GCF_000366445.1) collected in Mozambique in 1988. This sequence was selected as a reference to map the Ethiopian sequence data to using Snippy with default settings (https://github.com/tseemann/snippy). Snippy is a pipeline which finds SNPs between a haploid reference genome and sequence reads and outputs a core SNP alignment. The resulting core SNP alignment was then used to construct a phylogenetic tree with IQ-TREE using the model finder (MFP) and ascertainment bias flags (ASC) and 1000 bootstraps to build a maximum likelihood phylogenetic tree that was then visualised and annotated using iTOL. A similar process was followed for the Ethiopian *B. melitensis* sequence data using *B. melitensis* 16M (Accession number: GCF_000007125.1) as the reference.

4.2.14. In silico MLST and MLVA

All sequences of *Brucella* isolates underwent *in silico* multi-locus sequence typing (MLST) with 9 loci (314) and multiple locus variable number tandem repeat analysis (MLVA) with 16 loci (MLVA-16) (315) to further understand the epidemiological and geographic clustering of the strains in the context of globally recognized *B.abortus* and *B.melitensis* lineages and clades. *In silico* MLST (Appendix 5) was also performed on *B. abortus* (414) and *B. melitensis* (297) genomes retrieved from NCBI and ENA using mlst (https://github.com/tseemann/mlst) and the *Brucella* 9 loci scheme database from pubMLST (316). The resulting sequence types (STs) were compared to 218 *B. melitensis* and 210 *B. abortus* profiles recovered from the *Brucella* MLST database (https://pubmlst.org/brucella/) and a minimal spanning tree showing the relationships between the STs was generated using GrapeTree (317). In silico MLVA typing

was undertaken using a purpose-written script (MLVA finder; https://github.com/dpchris/MLVA) (Appendix 5) applied to the *de novo* genome assemblies, as described by Vergnaud et al (116). The genotypes obtained by MLVA-16 analysis were compared with 2493 entries of *B. melitensis* and 1667 entries of *B. abortus* accessed online at http://microbesgenotyping.i2bc.paris-saclay.fr and a minimal spanning tree showing the relationships between the MLVA types was generated using GrapeTree (317). The Hunter and Gaston diversity index (HGDI) is a widely used estimator of discriminatory power of genotyping methods and diversity of molecular markers in bacterial pathogens (318). It is available online at (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl) and was used to describe the discriminatory capacity of each MLVA locus. HGDI varies from 0 (all strains identical) to 1 (all strains different) (319).

4.3. Results

4.3.1. Outbreak investigation and culture results

From a total of 547 dairy cows tested for anti-*Brucella* antibodies on the ATARC farm, 180 were positive in serial testing using RBT and C-ELISA. The bio data of 125 seropositive animals as noted from a dairy cattle record of ATARC is given in Table 4.4. Permission to access the biodata of all seronegative animals from dairy cattle record keeping section was not obtained and hence not included in the table 4.4. Out of 84 samples consisting of 3 milk, 38 vaginal swabs, and 43 tissue samples collected from uterus, placenta, spleen, and mammary gland lymph nodes collected during *postmortem* examination of 30 seropositive dairy cows, 15 samples (9 mammary gland lymph node, 3 uterine tissues and 3 vaginal swabs) were culture positive (Table 4.5). In addition, from unrelated year-old glycerol stocks of *Brucella* isolates from aborted goats in Afar, Ethiopia, two isolates were recovered.

Table 4.4: Summary of the biodata of seropositive cows during outbreak investigation.

District	Species/ Category	No Sampled	C-ELISA Positive
Breed	Jersey	1	1
	Jersey x Arsi	1	1
	HF X Arsi	15	15
	Arsi	108	108
Age	≤ 3 years	25	25
	> 3 years	100	100
Origin	ATJK*	7	7
	Habura	8	8
	Meki	10	10
	Batu	10	10
	Shashemene	13	13
	Bulbula	16	16
	ATARC	17	17
	Assela	44	44
Parity	≤ 2	108	108
	> 2	17	17
Physiological Status	Pregnant	50	50
	Not Pregnant	75	75
Breeding	Natural	6	6
	AI	119	119
Frequency of Abortion	Once	63	63
	Two times	62	62

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Table 4.5: Summary of positive samples based on culture, real time PCR and real time PCR based SNP.

Sample Code	**Host**	**Sample Type**	**Recovered Species**	**IS*711* RT-PCR Ct Values**	***bcsp31* RT-PCR Ct Values**	**RT-PCR based SNP typing**
16_S16_L001	Caprine	Culture broth	*Brucella melitensis*	11.43	14.62	*B. melitensis*
17_S17_L001	Caprine	Culture broth	*B. melitensis*	11.48	14.73	*B. melitensis*
7_S7_L001	Bovine	Mammary gland lymphnode	*Brucella abortus*	14.08	16.15	*B. abortus*
6_S6_L001	Bovine	Mammary gland lymphnode	*B. abortus*	13.05	15.44	*B. abortus*
3_S3_L001	Bovine	Mammary gland lymphnode	*B. abortus*	13.3	15.78	*B. abortus*
2_S2_L001	Bovine	Mammary gland lymphnode	*B. abortus*	13.37	15.93	*B. abortus*
5_S5_L001	Bovine	Mammary gland lymphnode	*B. abortus*	13.06	15.71	*B. abortus*
1_S1_L001	Bovine	Mammary gland lymphnode	*B. abortus*	14.36	17.04	*B. abortus*
4_S4_L001	Bovine	Mammary gland lymphnode	*B. abortus*	13.2	15.68	*B. abortus*
15_S15_L001*	Bovine	Mammary gland lymphnode	*B. abortus*	13.97	16.54	*B. abortus*
11_S11_L001	Bovine	Mammary gland lymphnode	*B. abortus*	13.68	16.62	*B. abortus*
13_S13_L001	Bovine	Uterine tissue	*B. abortus*	13.45	16.15	*B. abortus*
10_S10_L001	Bovine	Uterine tissue	*B. abortus*	14.18	16.88	*B. abortus*
12_S12_L001	Bovine	Uterine tissue	*B. abortus*	13.5	15.81	*B. abortus*
9_S9_L001	Bovine	Vaginal Swab	*B. abortus*	13.92	16.36	*B. abortus*
8_S8_L001	Bovine	Vaginal Swab	*B. abortus*	13.42	16.05	*B. abortus*
14_S14_L001*	Bovine	Vaginal Swab	*B. abortus*	13.59	16.41	*B. abortus*

***** Isolates with code 14_S14_L001 and 15_S15_L001 are isolated from the same animal from two different samples.

4.3.2. Molecular Characterization

Real Time PCR

Real time PCR assay targeting IS*711* and *bcsp31* for the identification of the genus *Brucella* revealed amplification of the target sequences in 17 boilate DNA samples from suspect colonies. The individual assay was considered positive when amplification was seen at Ct of 35 cycles or less for IS*711* and at 40 cycles or less for the *bcsp31*. Heat-inactivated culture samples generated Ct values of between 14.62 and 17.04 for *bcsp31* and 11.43 and 14.36 for IS*711*, confirming that all isolates belong to the genus *Brucella*. Amplification plots for real time PCR assay targeting IS*711* and *bcsp31* are given in Fig 4.3 and 4.4.

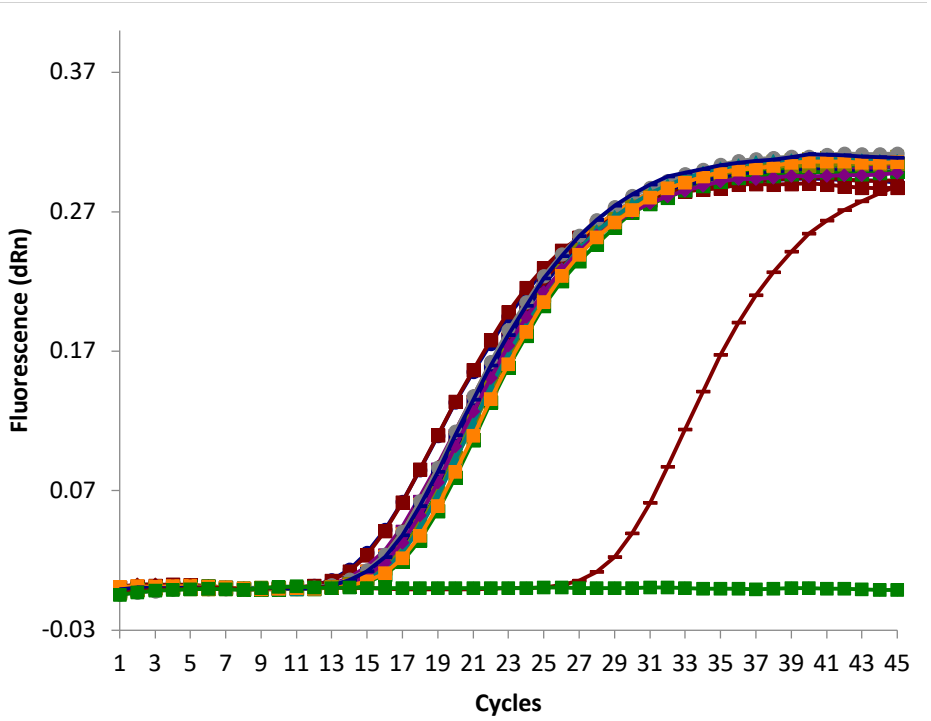

Figure 4.3: Real Time PCR targeting *bcsp31*, showing amplification plots for 17 Ethiopian *Brucella* spp. isolates.

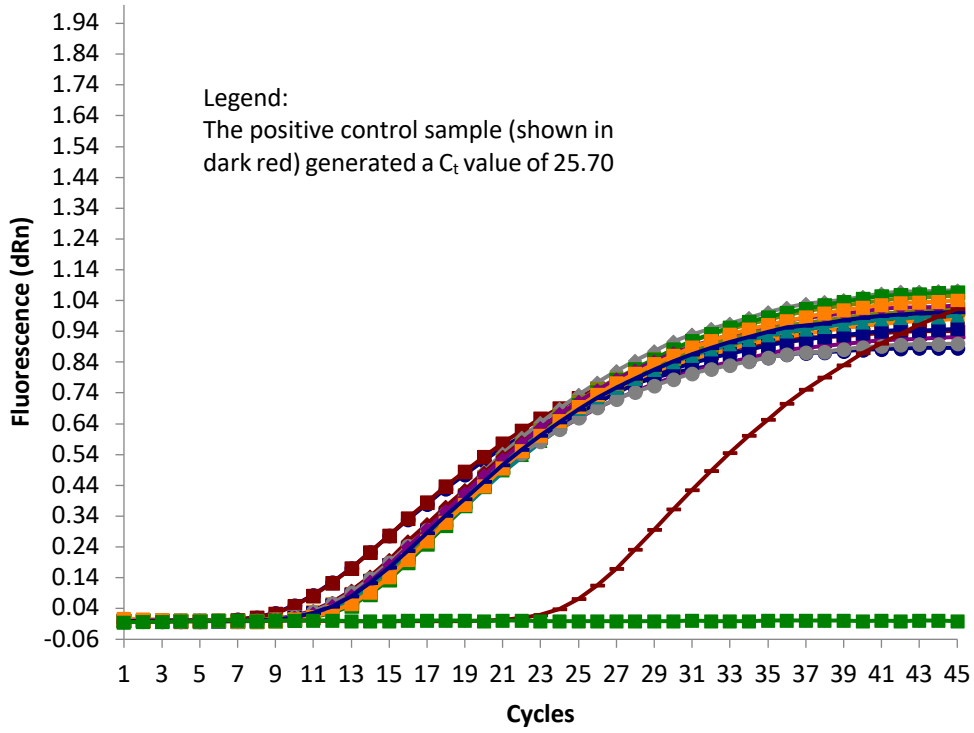

Figure 4.4:Real Time PCR targeting *IS711*, showing amplification plots for 17 Ethiopian *Brucella* spp. isolates.

Real Time PCR-based single nucleotide polymorphism

Fifteen *Brucella* isolates from dairy cattle were confirmed to be *B. abortus* and two *Brucella* isolates from goats were confirmed to be *B. melitensis* using the real time PCR-based single nucleotide polymorphism typing assay (Fig 4.5-Fig 4.8).

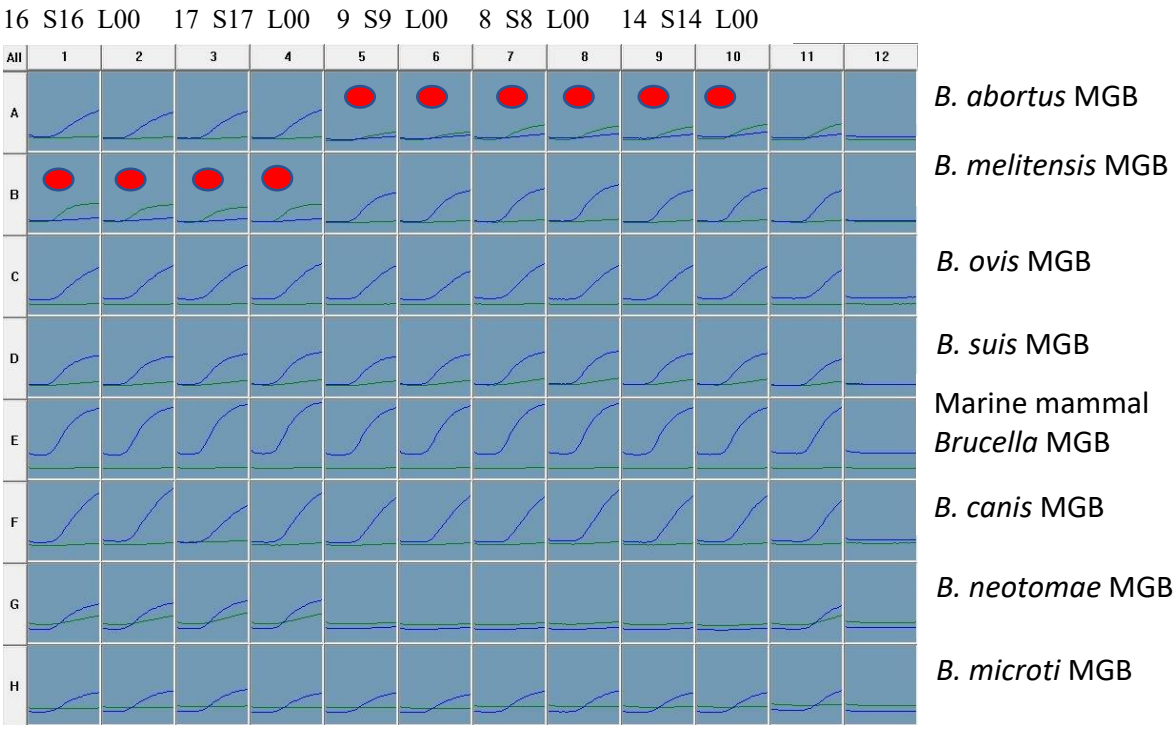

Figure 4.5: SNP assay (Plate 1).

Legend: On the top of the columns are the sample names. The 11th well in each row represents a positive control (*B. abortus* 544) and 12th well is negative control, which is a reaction mixture without template DNA. Each species-defining assay is run in rows (A-H) with samples run in columns in duplicates. The green PCR profiles represent reactions with the VIC labelled probe, representing the species-specific probe in each probe pair. The blue PCR profiles represent reactions with the FAM labelled probes, representing the reaction with the alternate state (non species-specific) allele probe in each probe pair. The identity of each of the isolates 1–12 is indicated by a red dot where an isolate generates a positive PCR reaction with a VIC-labelled probe.

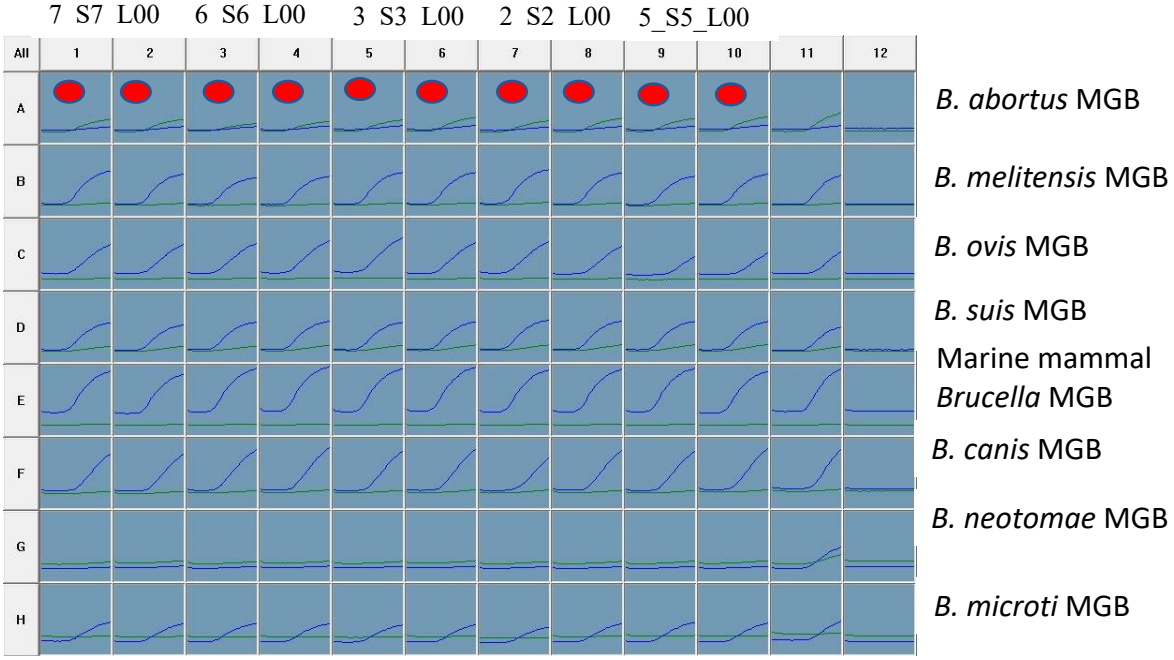

Figure 4.6: SNP assay (Plate 2).

Legend: On the top of the columns are the sample names. The 11th well in each row represents a positive control (*B. abortus* 544) and 12th well is negative control, which is a reaction mixture without template DNA. Each species-defining assay is run in rows (A-H) with samples run in columns in duplicates.

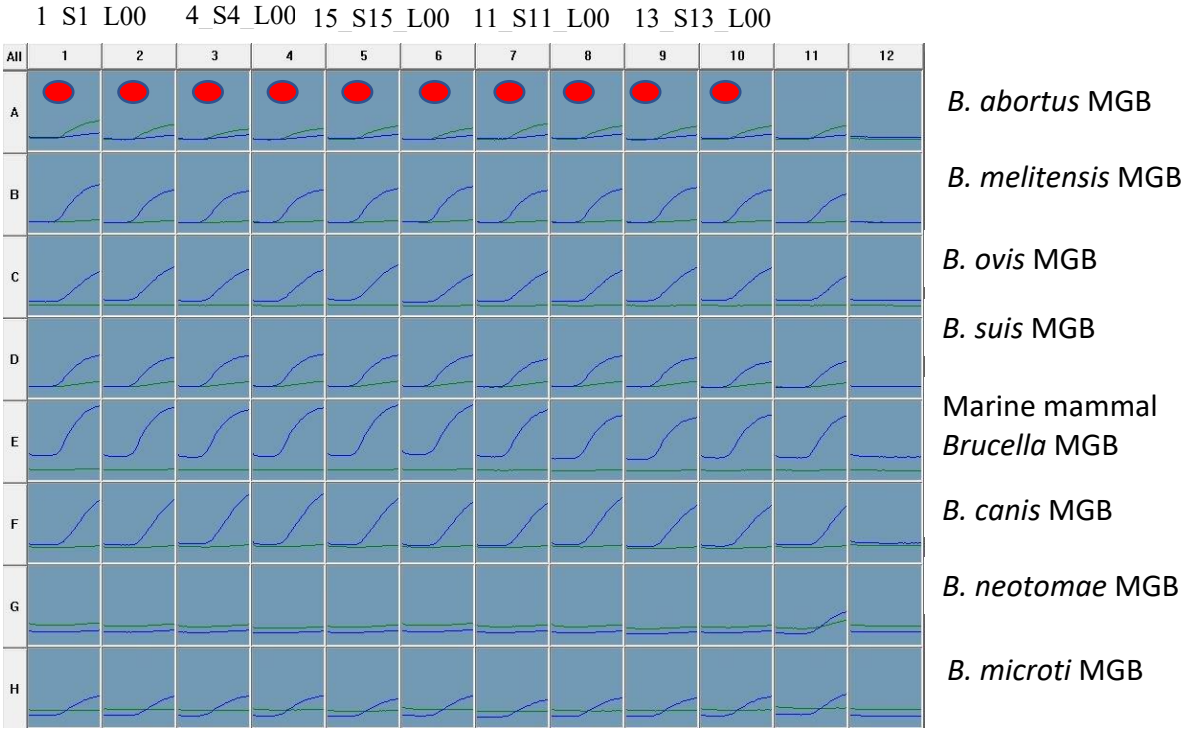

Figure 4.7: SNP assay (Plate 3).

Legend: On the top of the columns are the sample names. The 11th well in each row represents a positive control (*B. abortus* 544) and 12th well is negative control, which is a reaction mixture without template DNA. Each species-defining assay is run in rows (A-H) with samples run in columns in duplicates.

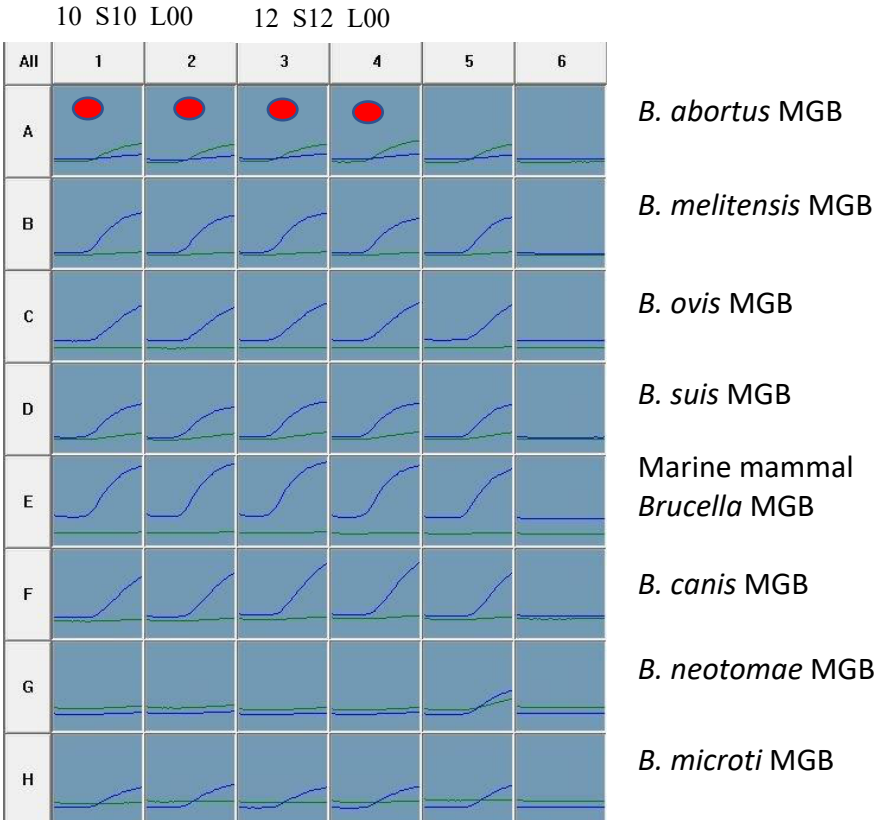

Figure 4.8: SNP assay (Plate 4).

Legend: On the top of the columns are the sample names. The 5th well in each row represents a positive control (*B. abortus* 544) and 6th well is negative control, which is a reaction mixture without template DNA. Each species-defining assay is run in rows (A-H) with samples run in columns in duplicates.

Multiplex PCR Results

Agarose gel electrophoresis of the Multiplex PCR products indicated the amplification of fragments sizes of 794 bp, 587 bp, 450 bp, and 152 bp in *B.abortus*, and 794 bp, 587 bp, 450 bp, 152 bp and 1071 bp which are respectively descriptive of *B.abortus* and *B.melitensis* (Fig 4.9)

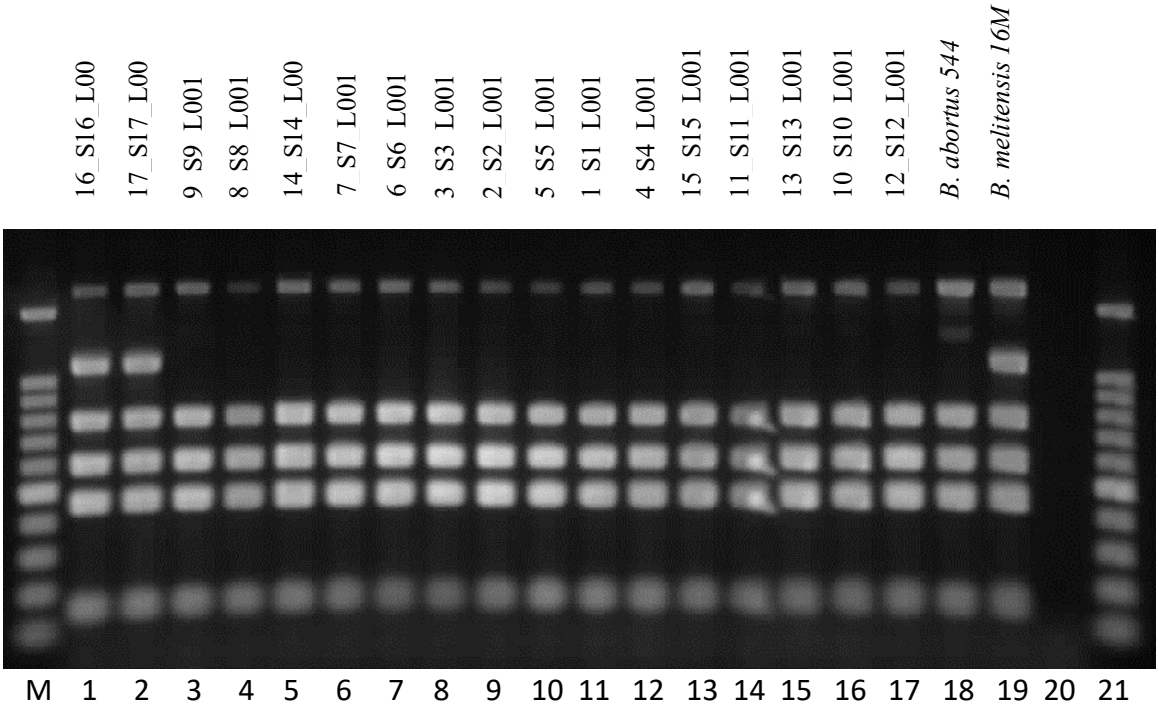

Figure 4.9: Gel electrophoresis of multiplex PCR amplification products showing various amplicon sizes specific to *B. abortus* and *B. melitensis*.

Legend:

Lane M & 21 are 100 bp markers, Lane 1 &2 are *B. melitensis,* Lane 3-17 are *B. abortus* and lane 20 is a negative control. Sample ID 14_S14_L001 (lane 5) and 15_S15_L001 (lane 13) were isolated from vaginal swab and mammary gland lymph node, respectively of the same animal.

4.3.3. WGS and analysis results of B. abortus

Whole genome sequence assemblies

A total MiSeq library yield of 70,841,238 reads with an average coverage of 131-fold (73-243) per sample was generated. An average total of 65,615,420 reads passed the quality filter and showed an average quality score above Q30 in more than 79.64%. *De novo* assembly of *B. abortus* Fastq files using SPAdes resulted in an average of 31 contigs (range:24-54), average total length of 3,272,887 bp with an average GC content of 57.23 %, and average N_{50} value of 388,250.6, L50 value of 4 and L75 value 7.

Core genome alignment and phylogenetic analysis

The Roary results for *B. abortus* genomes revealed a pan-genome of 2885 core genes. Phylogenetic analysis based on core genome SNPs of *B. abortus* isolates including 433 *B. abortus* genomes downloaded from ENA and NCBI databases indicated that *B. abortus* from

Ethiopia were clustered closely with African *B. abortus* strains from Mozambique (Gene Bank accessions GCF_000366445.1) and Kenya (Gene Bank accessions GCF_000370065.1) (Fig 4.10). These two strains, along with the fifteen Ethiopian isolates, formed a distinct clade, basal to all other *B. abortus* genomes included in the analysis. Within the fifteen Ethiopian *B. abortus* isolates there was very little diversity evident in WGS SNP based analysis. Outside of this basally branching clade, a second cluster contained a larger number of isolates also of primarily African origin, including strains from Senegal (78/32 and 78/33), Nigeria (80/101), Togo (BCCN 80-211), Zimbabwe (F1 06-B21 and 60/28) and Sudan (F6/05-2, 87/28 and F6/05-4). Outside of these two exclusively or predominantly African clades, two much larger clusters of strains were evident. The largest of these contained the *B. abortus* type strain (*Brucella abortus* 544) and was characterised by very low levels of diversity amongst the majority of isolates.

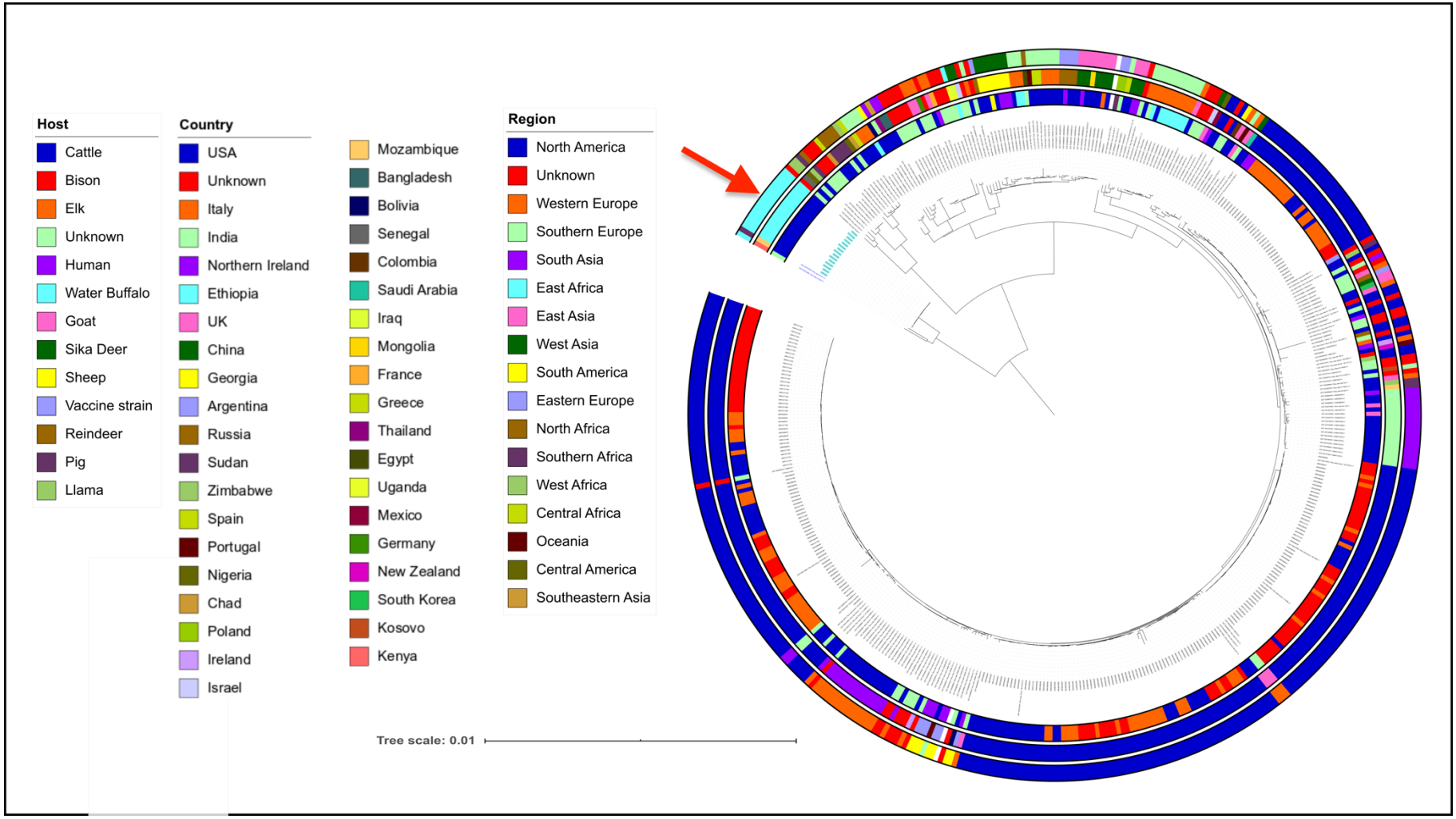

Figure 4.10: Maximum likelihood phylogeny of 458 *B. abortus* genomes using core genome SNPs.

Legend: The outer colored ring shows the geographic region of the strains, the middle colored ring shows the country of isolation, and the inner colored ring shows the host species from which sequenced isolates were derived. Ethiopian isolates (n=15) are light blue highlighted as indicated by the arrow. The scale bar show nucleotide substitutions per site.

Whole genome SNP Analysis

Mapping of Ethiopian *B. abortus* sequence data against *B. abortus*_88_226_V1 (Accession number: GCF_000366445.1) showed that all *B. abortus* isolates are at least 841 core SNPs away from the reference strain (Fig 4.11). Within the fifteen Ethiopian *B. abortus* isolates there was very little diversity evident in WGS SNP based analysis, with no more than five SNPs identified between any two strains within the panel. Pairwise SNP distance identified between any two isolates of the fifteen Ethiopian *B. abortus* strains is indicated in Table 4.6.

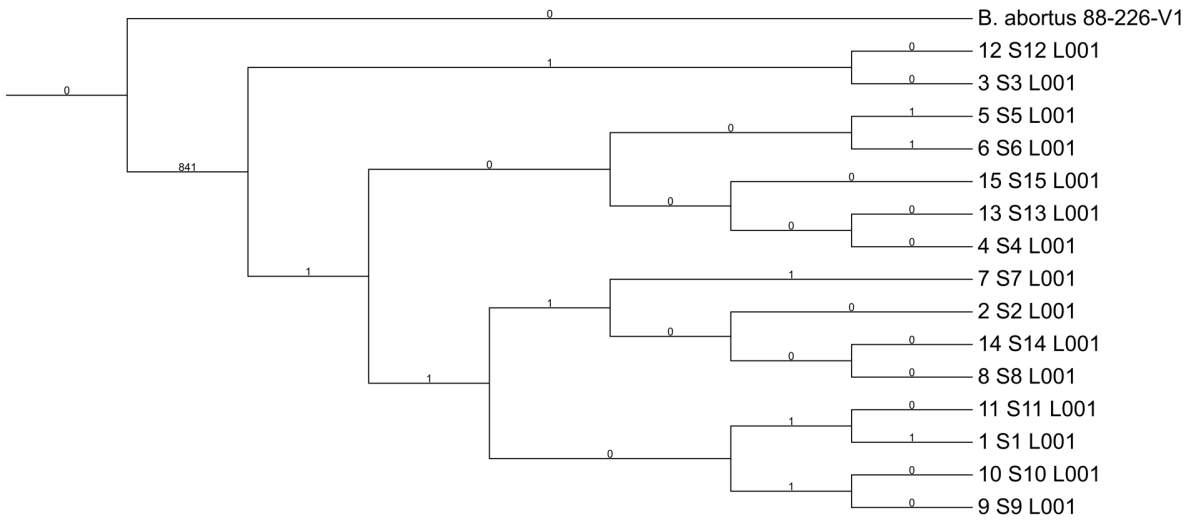

Figure 4.11 : Maximum likelihood phylogeny of Ethiopian *B. abortus* genomes (n=15) when mapped against *B. abortus* 88-226-V1 reference strain from Mozambique.

Legend: Isolate numbering is as given in Table 4.5. Branch labels show the number of SNPs identified.

Table 4.6: Pairwise SNP distance identified between any two isolates of the fifteen Ethiopian *B. abortus* strains.

key	Access_number	10_S10_L001	11_S11_L001	12_S12_L001	13_S13_L001	14_S14_L001	15_S15_L001	1_S1_L001	2_S2_L001	3_S3_L001	4_S4_L001	5_S5_L001	6_S6_L001	7_S7_L001	8_S8_L001	9_S9_L001	Reference*
1	10_S10_L001	0	2	4	2	2	2	3	2	4	2	3	3	3	2	0	844
2	11_S11_L001	2	0	4	2	2	2	1	2	4	2	3	3	3	2	2	844
3	12_S12_L001	4	4	0	2	4	2	5	4	0	2	3	3	5	4	4	842
4	13_S13_L001	2	2	2	0	2	0	3	2	2	0	1	1	3	2	2	842
5	14_S14_L001	2	2	4	2	0	2	3	0	4	2	3	3	1	0	2	844
6	15_S15_L001	2	2	2	0	2	0	3	2	2	0	1	1	3	2	2	842
7	1_S1_L001	3	1	5	3	3	3	0	3	5	3	4	4	4	3	3	845
8	2_S2_L001	2	2	4	2	0	2	3	0	4	2	3	3	1	0	2	844
9	3_S3_L001	4	4	0	2	4	2	5	4	0	2	3	3	5	4	4	842
10	4_S4_L001	2	2	2	0	2	0	3	2	2	0	1	1	3	2	2	842
11	5_S5_L001	3	3	3	1	3	1	4	3	3	1	0	2	4	3	3	843
12	6_S6_L001	3	3	3	1	3	1	4	3	3	1	2	0	4	3	3	843
13	7_S7_L001	3	3	5	3	1	3	4	1	5	3	4	4	0	1	3	845
14	8_S8_L001	2	2	4	2	0	2	3	0	4	2	3	3	1	0	2	844
15	9_S9_L001	0	2	4	2	2	2	3	2	4	2	3	3	3	2	0	844
16	Reference*	844	844	842	842	844	842	845	844	842	842	843	843	845	844	844	0

- Reference strain from Mozambique (*B. abortus*_88_226_V1, Accession number: GCF_000366445.1)

MLST

In silico BruMLST09 analysis identified all *B. abortus* isolates as ST 72. Minimum spanning trees showing clustering of all *B. abortus* MLST sequence types identified in this study, including those retrieved from the public database, is illustrated in Fig 4.12. The Ethiopian *B. abortus* isolates identified as ST72 were placed with the single previously described isolate of the same ST (isolate 88/218) from Mozambique. This ST and two others (ST37 and ST38) formed a distinct cluster, divergent from other *B. abortus* sequence types. Sequence type ST37 is represented by three isolates only, all of which originate from Mozambique, whilst ST38 is represented by a single isolate from Kenya (63/294).

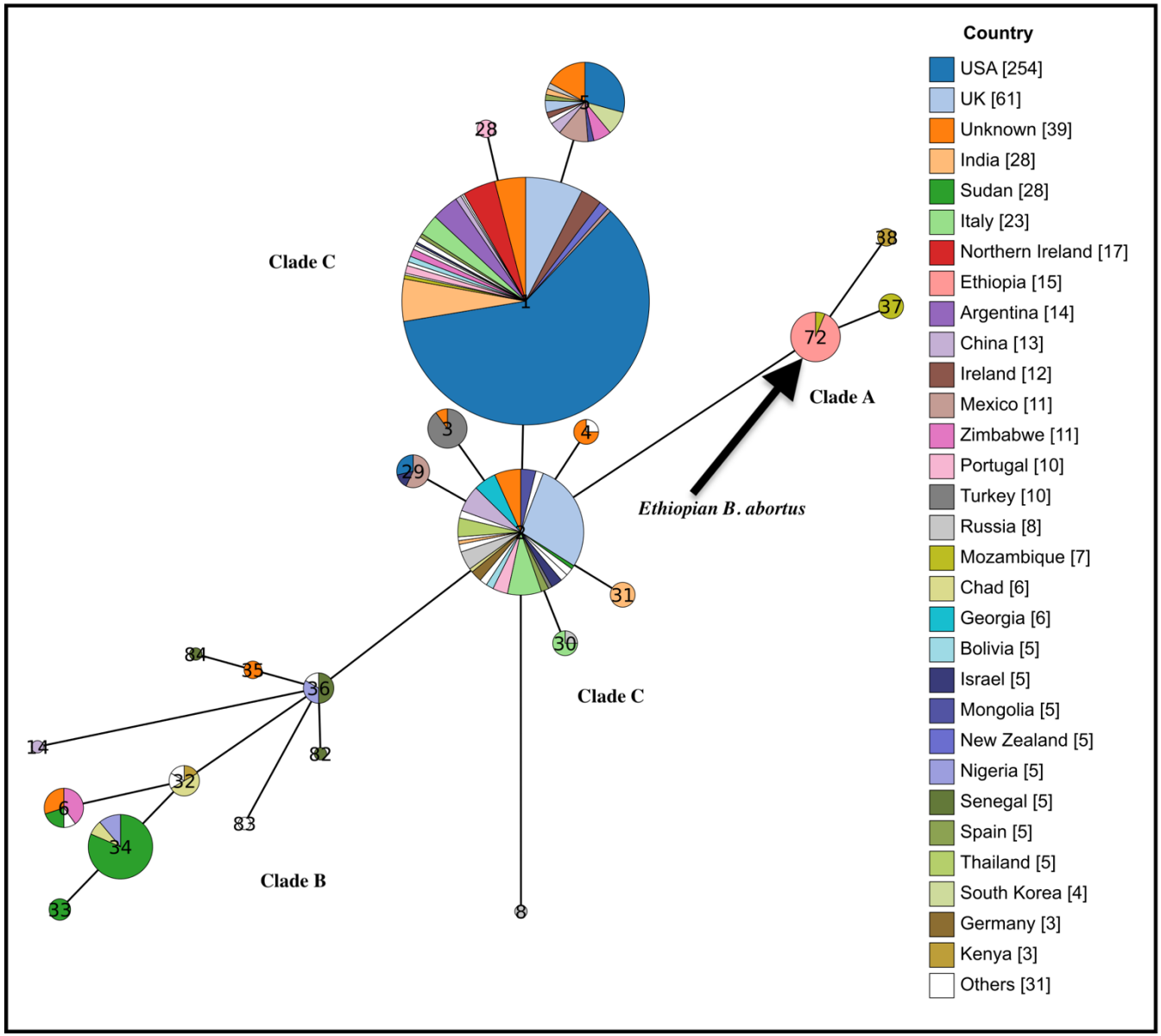

Figure 4.12: Minimum spanning tree showing global genetic diversity of *B. abortus* described by a nine-locus MLST scheme.

Legend: the figure shows 15 Ethiopian *B. abortus* isolates (indicated by the arrow), relative to 639 (414 from NCBI and 210 from pubMLST databases) *B. abortus* isolates. Colouring indicates the country of isolation, with numbers in square brackets giving the number of isolates from each location. Each circle denotes a particular ST type with the size of the circle illustrating the number of isolates of that particular type. The number in each circle represents STs.

MLVA

MLVA-16 typing showed that the 15 *B. abortus* isolates were clustered into 8 genotypes. The *B. abortus* genotypes were comprised of single, double and triple locus variants with the variable markers being bruce04, bruce16 and bruce30 (Table 4.7). The HGDI was calculated and used to describe the discriminatory capacity of each locus (Table 4.8).

Minimum spanning tree showing the clustering of all *B. abortus* MLVA-16 genotypes found in this study, including those retrieved from the public database is illustrated in Fig 4.13. The fifteen Ethiopian *B. abortus* isolates from the current study formed a distinct cluster with isolates from Mozambique (*B. abortus* 82 217 V1) and Kenya (*B. abortus* 63 294 V1). Outside of this grouping the majority of other isolates originating from Africa incorporated within this analysis formed a distinct cluster, containing primarily isolates of West African origin, including Guinea-Bissau, Senegal and Togo. Minimum spanning tree showing regional genetic diversity of African *B. abortus* strains including Ethiopian *B. abortus* described by MLVA16 is illustrated in Fig 4.14. The other cluster contained two sister clades containing the majority of isolates from around the world.

Table 4.7: Summary of MLVA-16 pattern of 15 *B. abortus* isolates.

		Panel 1								**Panel 2A**			**Panel 2B**									
key	Access_number	Bruce06-1322	Bruce08-1134	Bruce11-211	Bruce12-73	Bruce42-424	Bruce43-379	Bruce45-233	Bruce55-2066	Bruce18-339	Bruce19-324	Bruce21-329	Bruce04-1543	Bruce07-1250	Bruce09-588	Bruce16-548	Bruce30-1505	Genotype	Species	Host	Origin	Farm	Year of Isolation
1	10_S10_L001	2	4	2	12	3	2	3	1	5	45	8	9	2	3	7	9	02	*B. abortus*	Cattle	Assella	ATARC	2018
2	11_S11_L001	2	4	2	12	3	2	3	1	5	45	8	8	2	3	8	9	01	*B. abortus*	Cattle	Assella	ATARC	2018
3	12_S12_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	9	9	06	*B. abortus*	Cattle	Habura	ATARC	2018
4	13_S13_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	9	8	07	*B. abortus*	Cattle	Assella	ATARC	2018
5	14_S14_L001*	2	4	2	12	3	2	3	1	5	45	8	7	2	3	7	10	04	*B. abortus*	Cattle	Bulbula	ATARC	2018
6	15_S15_L001*	2	4	2	12	3	2	3	1	5	45	8	7	2	3	10	9	08	*B. abortus*	Cattle	Bulbula	ATARC	2018
7	1_S1_L001	2	4	2	12	3	2	3	1	5	45	8	9	2	3	7	9	02	*B. abortus*	Cattle	Assella	ATARC	2018
8	2_S2_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	7	9	05	*B. abortus*	Cattle	Batu	ATARC	2018
9	3_S3_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	9	9	06	*B. abortus*	Cattle	Assella	ATARC	2018
10	4_S4_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	9	9	06	*B. abortus*	Cattle	Batu	ATARC	2018
11	5_S5_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	10	9	08	*B. abortus*	Cattle	Bulbula	ATARC	2018
12	6_S6_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	6	10	03	*B. abortus*	Cattle	Batu	ATARC	2018
13	7_S7_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	7	9	05	*B. abortus*	Cattle	Assella	ATARC	2018
14	8_S8_L001	2	4	2	12	3	2	3	1	5	45	8	7	2	3	7	10	04	*B. abortus*	Cattle	Bulbula	ATARC	2018
15	9_S9_L001	2	4	2	12	3	2	3	1	5	45	8	9	2	3	7	9	02	*B. abortus*	Cattle	Bulbula	ATARC	2018

*Isolates with code 14_S14_L001 and 15_S15_L001 are isolated from the same animal from two different samples.

Table 4.7: Hunter and Gaston diversity index (HGDI) for each loci of MLVA-16 typing for 15 *B. abortus* strains.

Locus	HGDI (*B. abortus*)	Number of isolates compared
Panel 1		
Bruce06	0.00	15
Bruce08	0.00	15
Bruce11	0.00	15
Bruce12	0.00	15
Bruce42	0.00	15
Bruce43	0.00	15
Bruce45	0.00	15
Bruce55	0.00	15
Panel 2A		
Bruce18	0.00	15
Bruce19	0.00	15
Bruce21	0.00	15
Panel 2B		
Bruce04	0.50	15
Bruce07	0.00	15
Bruce09	0.00	15
Bruce16	0.73	15
Bruce30	0.50	15

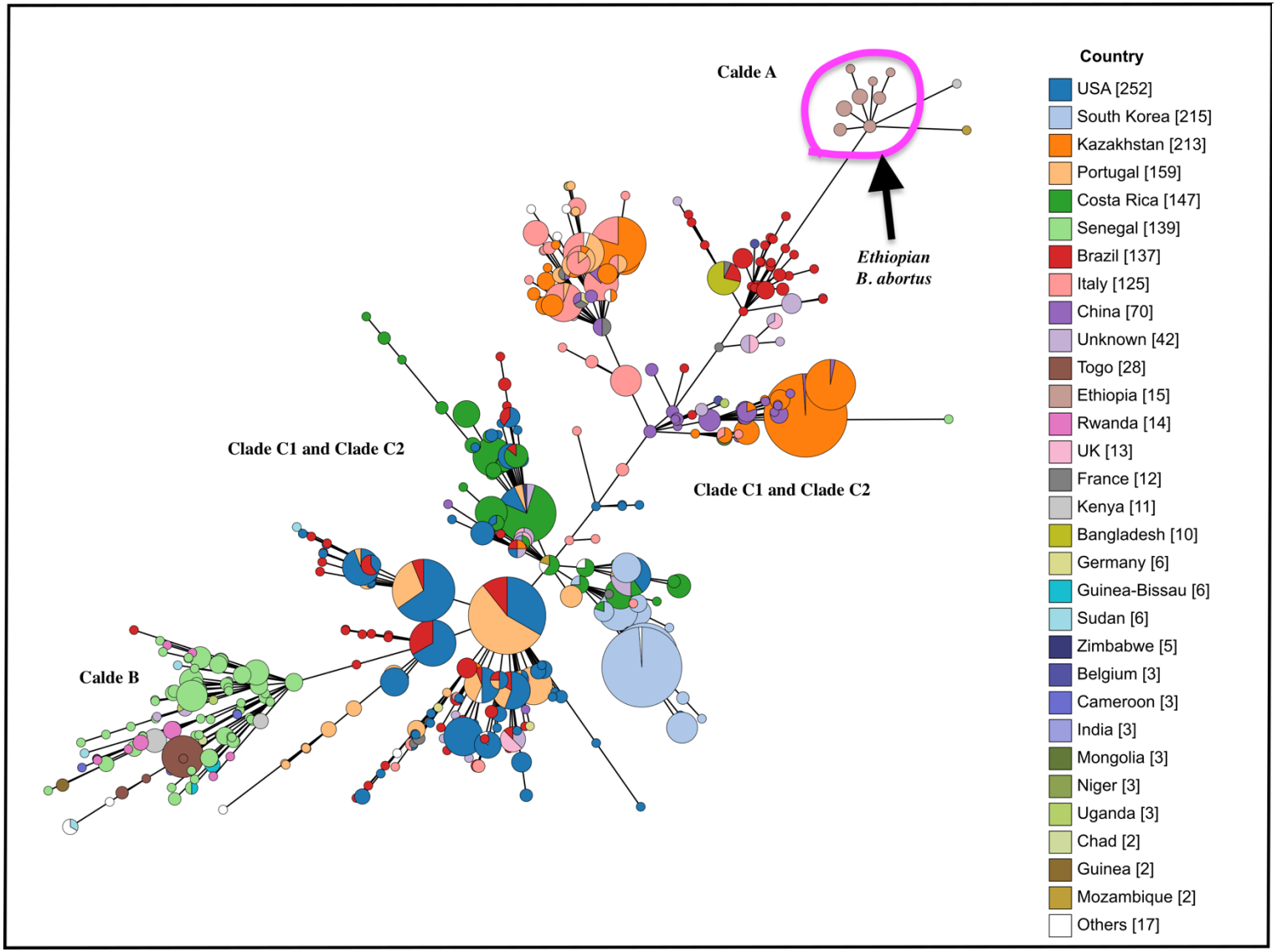

Figure 4.13: Minimum spanning tree showing global genetic diversity of Ethiopian *B. abortus* described by MLVA16.

Legend: Ethiopian *B. abortus* isolates (n=15), as indicated by the arrow, are shown relative to 1667 *B. abortus* isolates available in the global *Brucella* MLVA database. Colouring indicates the country of isolation, with numbers in square brackets giving the number of isolates from each location.

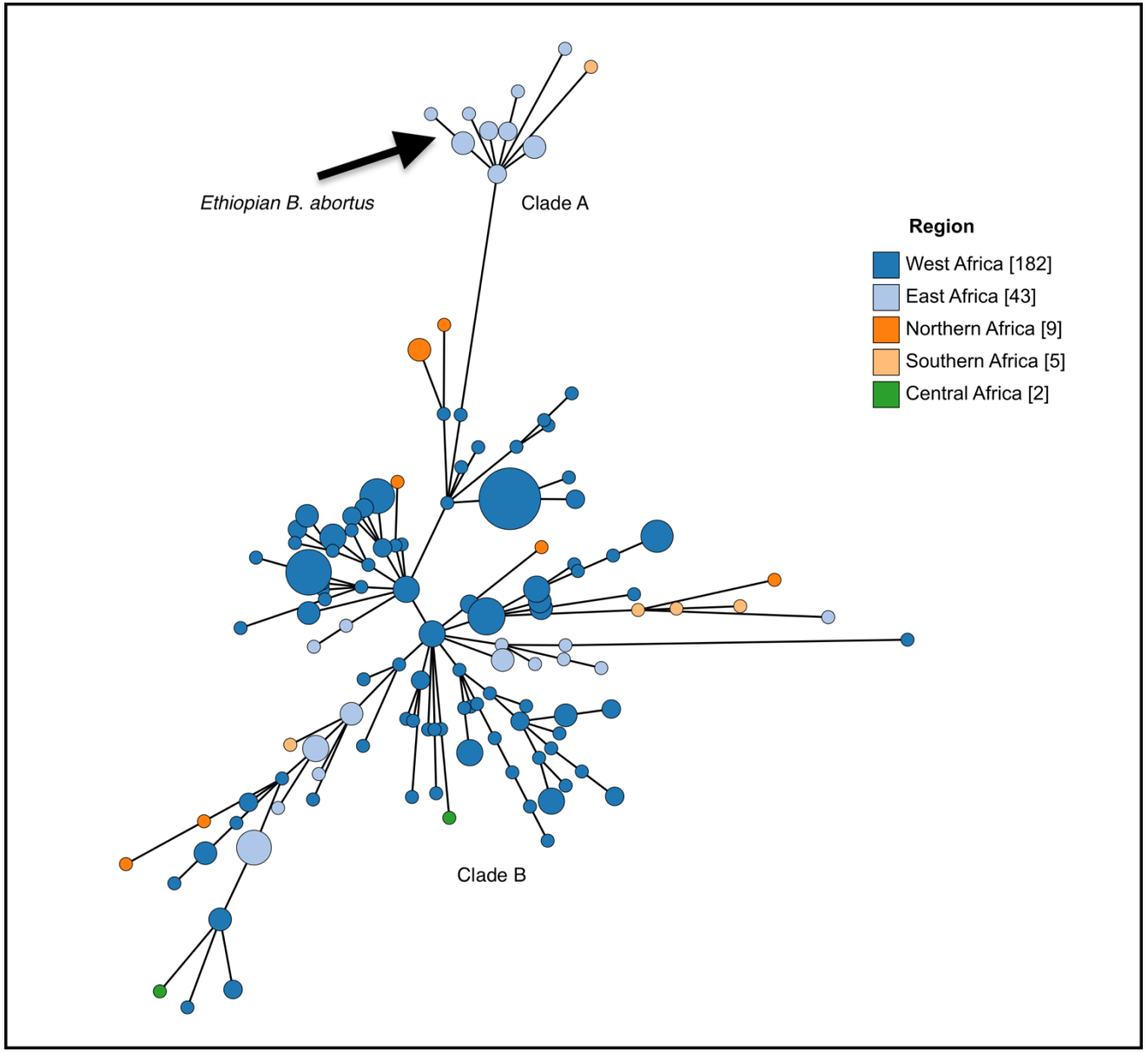

Figure 4.14: Minimum spanning tree showing regional genetic diversity of African *B. abortus* strains including Ethiopian *B. abortus* described by MLVA16.

Legend: Ethiopian *B. abortus* isolates (n=15), as indicated by the arrow, are shown relative to 226 African *B. abortus* isolates available in the global *Brucella* MLVA database. Colouring indicates the geographic region of the strains, with numbers in square brackets giving the number of isolates from each location.

4.3.4. WGS and analysis results of B. melitensis

Whole genome sequence assemblies

De novo assembly of *B. melitensis* Fastq files using SPAdes resulted in an average of 26 contigs, total length of 3,264,021 bp, a GC content of 57.24 %, an N_{50} value of 292,262.5, L50 value of 4 and L75 value 7.

Core genome alignment and phylogenetic analysis

The Roary results for *B. melitensis* genomes revealed a pan-genome of 2952 core genes. The core genome alignment and SNP analysis of 303 *B. melitensis* genomes from the NCBI database and the two *B. melitensis* isolates from Ethiopia showed that *B. melitensis* clustered into three distinct clades. The Ethiopian isolates from the current study were clustered in an African sub-clade comprising strains from Somalia, Zimbabwe and Nigeria. An Ethiopian origin *B. melitensis* strain isolated from a human in Norway were also clustered in this sub-clade. (Fig 4.15). Outside of this sub-clade the Ethiopian isolates were placed within a diverse clade of relatively few isolates, containing isolates from Argentina, USA and the *B. melitensis* type strain (*B. melitensis* 16M). The second cluster contained a large group of strains, primarily from Italy and France. The other cluster contained diverse collection of strains predominantly from Asian continent and Eastern and South-eastern European countries such as Albania, Kosovo, Bulgaria and Russia.

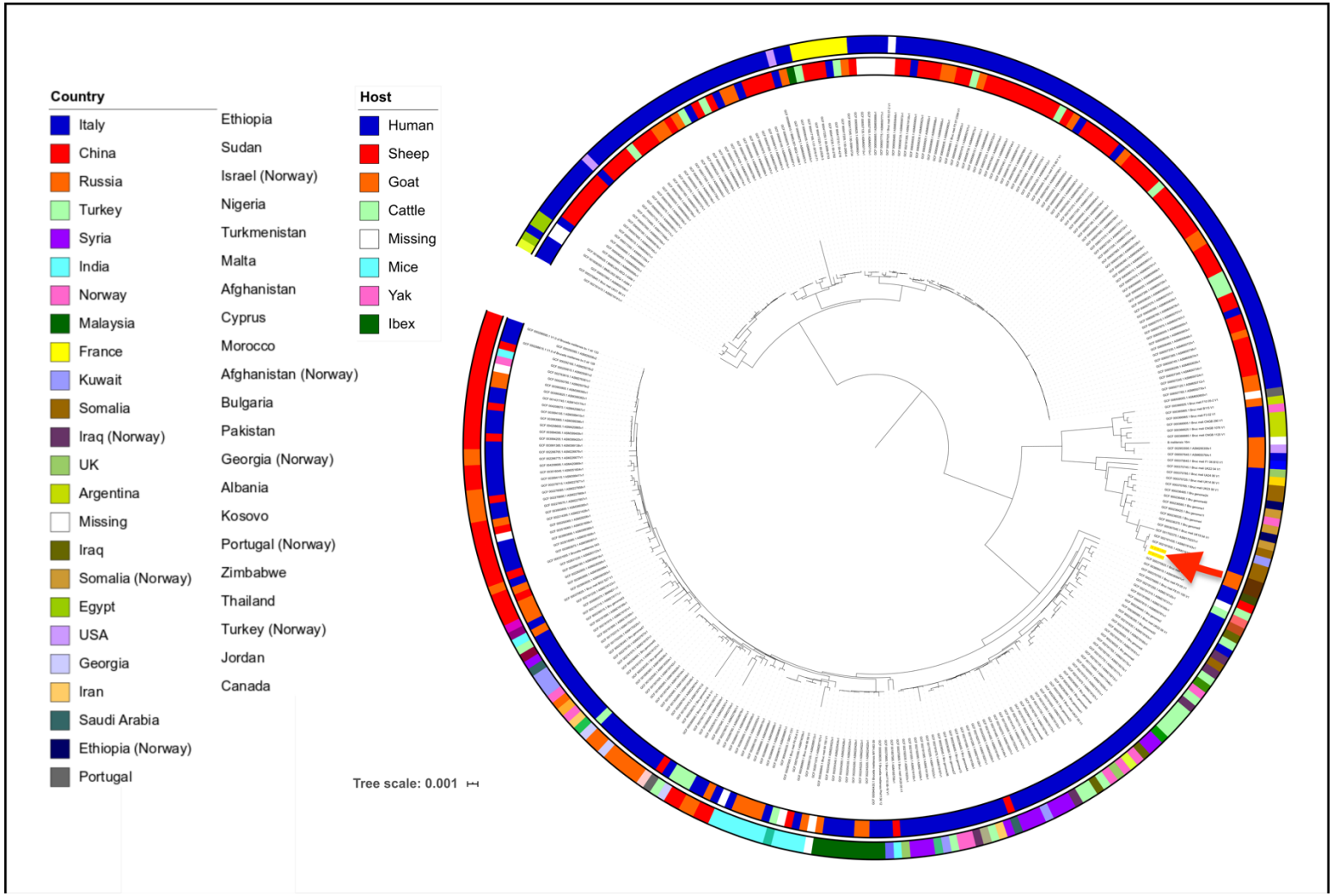

Figure 4.15 : Maximum likelihood phylogeny of 305 *B. melitensis* genomes using core genome SNPs.

Legend: The outer coloured ring shows the country of isolation, and the inner coloured ring shows the host species from which sequenced isolates were derived. Ethiopian isolates (n=2) are highlighted by a yellow colour as indicated by the arrow. The scale bar shows nucleotide substitutions per site.

Whole genome SNP Analysis

Mapping of Ethiopian *B. melitensis* sequence data against *B. melitensis* 16M revealed that Ethiopian *B. melitensis* isolates had a difference of 1927 SNPs to the reference strain (Fig 4.16). The two Ethiopian *B. melitensis* isolates exhibited slightly higher diversity than observed amongst the *B. abortus* isolates in this study and were separated by 10 SNPs.

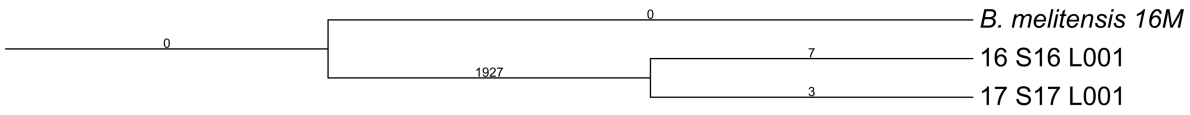

Figure 4.16 : Maximum likelihood phylogeny of Ethiopian B. melitensis genomes when mapped against B. *melitensis* 16M reference strain.

Legend: Isolate numbering is as given in Table 4.5. Branch labels show the number of SNPs identified.

MLST

In silico BruMLST09 analysis identified Ethiopian *B. melitensis* isolates as ST12, alongside a large number of other isolates, primarily of African origin. These include a significant number of isolates from eastern Africa (Kenya, Tanzania and Uganda) and in particular the Horn of Africa (Ethiopia and Somalia). The majority of ST12 isolates within the *Brucella* pubMLST database are recorded as being isolated from humans, with only three isolates originating from livestock. Minimum spanning trees showing clustering of *B. melitensis* MLST sequence types identified in this study, including those retrieved from the public database, is illustrated in 4.17.

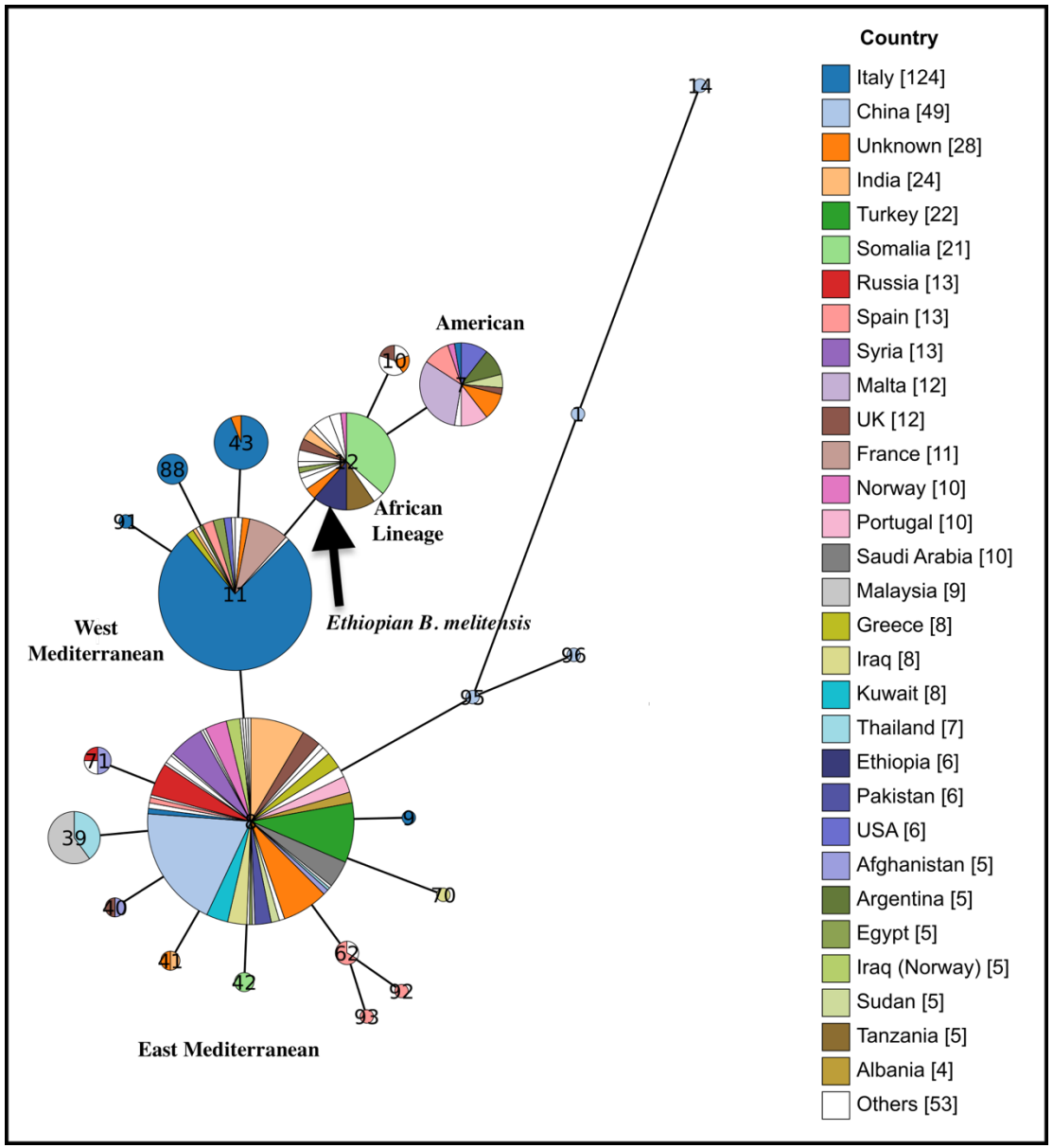

Figure 4.17: Minimum spanning tree showing global genetic diversity of *B. melitensis* described by a nine-locus MLST scheme.

Legend: It shows 2 Ethiopian *B. melitensis* isolates (indicated by the arrow), relative to 513 (297 from NCBI and 218 from pubMLST database) *B. melitensis* isolates. Colouring indicates the country of isolation, with numbers in square brackets giving the number of isolates from each location. Each circle denotes a particular ST type with the size of the circle illustrating the number of isolates of that particular type. The number in each circle represents STs.

MLVA

MLVA-16 typing showed that the two *B. melitensis* strains were grouped into two genotypes. *B. melitensis* genotypes were double locus variants with bruce09 and bruce04 being variable markers (Table 4.9). The HGDI was calculated and used to describe the discriminatory capacity of each locus (Table 4.10). Minimum spanning trees showing the clustering of *B. melitensis* MLVA-16 genotypes found in this study, including those retrieved from the public database is illustrated in Fig 4.18. The two Ethiopian *B. melitensis* isolates from the current study form a sub-African cluster in Americas clade. Other African isolates in this clade are isolates from Somalia, Sudan, Kenya and South Africa. Isolates from the northern African countries such as Algeria and Tunisia were clustered under Western Mediterranean clade.

Table 4.8: Summary of MLVA-16 pattern of 2 *B. melitensis* isolates.

		Panel 1								Panel 2A			Panel 2B										
key	Access_number	Bruce06-1322	Bruce08-1134	Bruce11-211	Bruce12-73	Bruce42-424	Bruce43-379	Bruce45-233	Bruce55-2066	Bruce18-339	Bruce19-324	Bruce21-329	Bruce04-1543	Bruce07-1250	Bruce09-588	Bruce16-548	Bruce30-1505	Genotype	Species	Host	Origin	Farm	Year of Isolation
1	16_S16_L001	2	5	3	14		2	3	4	4	36	8	5	4	7	5	5	09	*B. melitensis*	Goat	Amibara	Pastoral	2016
2	17_S17_L001	2	5	3	14		2	3	4	4	36	8	6	4	6	5	5	10	*B. melitensis*	Goat	Amibara	Pastoral	2016

Table 4.9: Hunter and Gaston diversity index (HGDI) for each loci of MLVA-16 typing for 2 *B. melitensis*.

	Panel 1							Panel 2A			Panel 2B					
	Bruce06-1322	Bruce08-1134	Bruce11-211	Bruce12-73	Bruce42-424	Bruce43-379	Bruce45-233	Bruce55-2066	Bruce18-339	Bruce19-324	Bruce21-329	Bruce04-1543	Bruce07-1250	Bruce09-588	Bruce16-548	Bruce30-1505
HGDI	0.0	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0
No of isolates	2	2	2	2		2	2	2	2	2	2	2	2	2	2	2

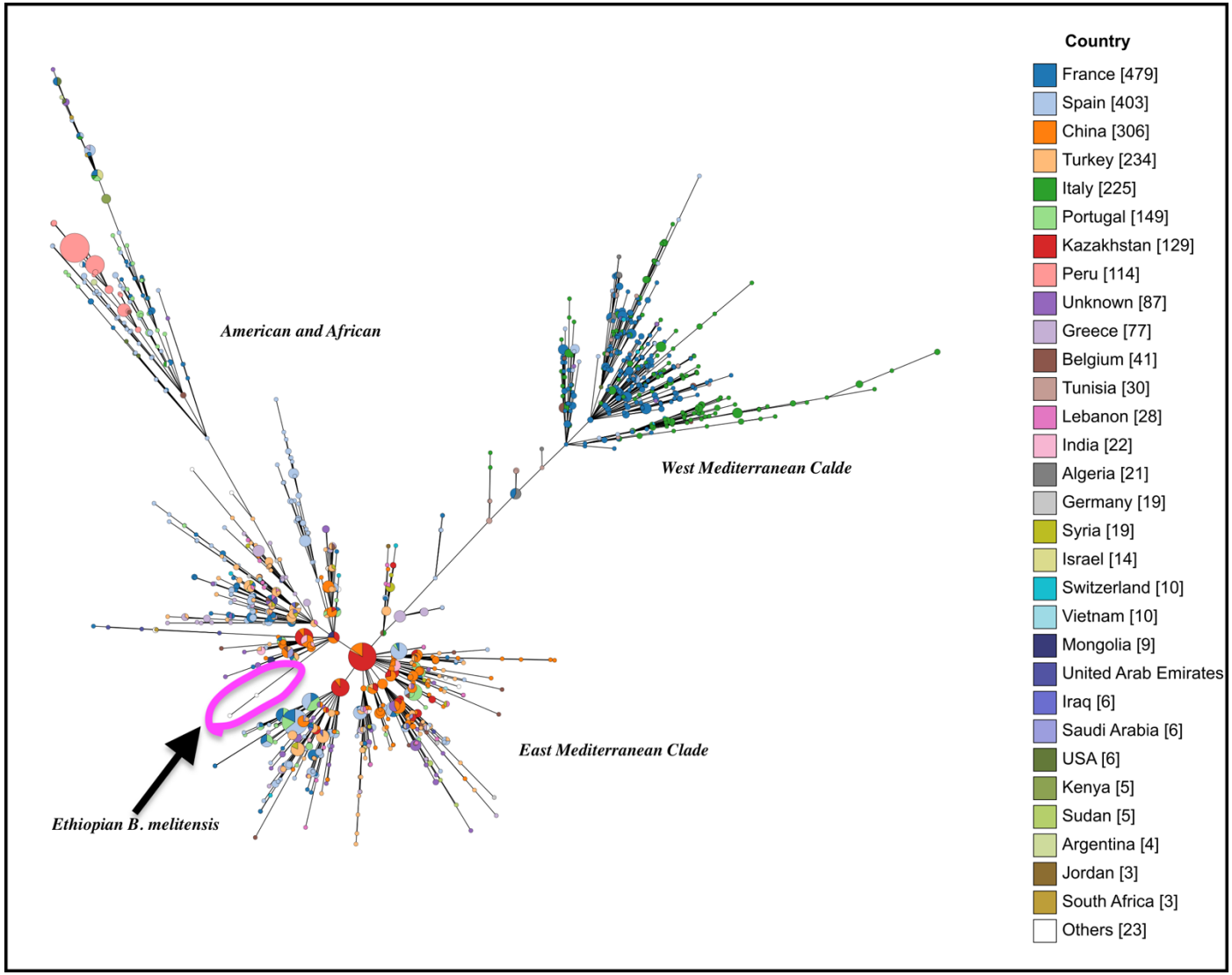

Figure 4.18: Minimum spanning tree showing global view of genetic diversity of Ethiopian *B. melitensis* provided by MLVA16.

Legend: Ethiopian *B. melitensis* isolates (n=2), as indicated by the arrow, are shown relative to 2493 *B. melitensis* isolates available in the global *Brucella* MLVA database. Colouring indicates the country of isolation, with numbers in square brackets giving the number of isolates from each location.

4.4. Discussion

In this study, an abortion storm in a dairy farm at Adami Tullu Agricultural Research Centre (ATARC), Central Oromia, Ethiopia was investigated. The cattle in the centre comprised mainly of local Zebu cattle of Arsi breed. For the purpose of producing a crossbreed, a parent stock of Holstein Friesian and Jersey breeds, and their crosses were also maintained. The record maintained in the farm indicated that all seropositive animals had abortion history. The abortion storm was noticed after an introduction of new animals purchased from a local market without any prior testing. The government dairy research centre at Assela, brucellosis endemic region (168,171), which comprised of local zebu of Arsi and Borena breeds was closed and the animals were moved to ATARC that could have brought infectious animals into the centre. As there were no *Brucella* vaccination introduced in Ethiopia, the outbreak was due to natural infection.

Even though serological evidence of brucellosis in Ethiopia was known since 1970's, brucellosis continued to be endemic in Ethiopia, and information on the type of circulating *Brucella* species is scarce. This study has revealed the first report of isolation of *B. abortus* from an outbreak of brucellosis in local Zebu cattle in Ethiopia. The knowledge of the epidemiology and clinical features of commonly circulating *Brucella* species is vital for better diagnosis, prevention and control of the disease (39,320). The knowledge of epidemiology and type of circulating *Brucella* species identified in this study could therefore play a crucial role in the plan and design of feasible control and intervention strategies in Ethiopia. The type *B. abortus* lineages identified in this study were previously reported only from Kenya and Mozambique (321); hence this would increase the representation of this lineage of *B. abortus* in the global *Brucella* database.

Nine out of 15 *B. abortus* isolates were recovered from *post mortem* collected mammary gland lymph nodes signifying the importance of *Brucella* transmission through colostrum feeding to new-born calves (51). It also indicates a public health hazard as much of the

milk produced in the centre were utilized by the farm workers beside selling it to Adami Tullu town residents.

Molecular typing has been used as a replacement to biochemical typing for the rapid identification and characterisation of *Brucella species* (76,105,322) so as to reduce handling of live cultures ,which poses risk of laboratory exposure and infection. In this study 15 strains from cattle were identified as *B. abortus* and 2 strains from goats were identified as *B. melitensis* using *Brucella* genus specific real-time PCR, and a combination of SNP-based discriminatory assays and multiplex PCR to distinguish species. Subsequent analyses applied a number of approaches for sub-species typing, using whole genome sequencing data.

Phylogenetic analysis based on SNPs in the core genome identified that Ethiopian *B. abortus* isolates from the current study form a distinct clade with two previously described isolates (identified as 88/217 and 63/294), branching basally to all other isolates included within the analysis. These two isolates were isolated in Mozambique and Kenya respectively. The existence of a basally branching *B. abortus* clade has been previously described, using an expanded 21-locus MLST scheme applied to a comprehensive panel of *Brucella* isolates (321). This clade, referred to by Whatmore *et al.*,(321) as *B. abortus* Clade A, was shown to branch basally to the other main *B. abortus* groupings (referred to as Clade B, Clade C1 and Clade C2). Clade A was previously represented by only a small number of isolates, and thus the placement of Ethiopian isolates from the current study within this group substantially increases the representation of these basal *B. abortus* strains in genomic databases.

Whole genome core-SNP based analysis in the current work additionally identified a second clade containing isolates of predominantly African origin (Figure 4.10). Again, this is consistent with previous findings based on expanded 21-locus MLST (321), where Clade B formed a sister group to Clade C, and was described as comprising isolates originating

from across a broad geographic range within Africa (including Senegal, Nigeria, Zimbabwe, Sudan, Mozambique, Kenya, Chad, and Uganda). Two larger clades, containing the majority of isolates incorporated into the current core genome SNP analysis consisted of isolates widely distributed across many continents. This is also in agreement with Whatmore *et al*.,(321) who explained Clade C1 and C2 based on expanded 21-locus MLST as clades with global distribution.

Based on the phylogenetic tree drawn in this study using core genome SNPs, Ethiopian *B. melitensis* isolates in the current study were clustered into a lineage with a subclade containing isolates primarily from Somalia, Nigeria and Zimbabwe. This subclade was previously reported as Genotype III based on full genome SNP-based phylogenetic analysis (323–325) and phylogeographically described to spread towards the south of Mediterranean region to form African lineage (323). The other subclade within this lineage contained isolates from USA and Argentina. This subclade, referred to as genotype V, also known as American clade (321) was believed to be introduced into American continent from Europe by infected animals (323,324). In agreement with previous designation, the isolates primarily from Italy and France formed West Mediterranean clades (321,326). The diverse collection of isolates from Asian continent included in the analysis formed East Mediterranean clade. This clade has previously been described as genotype II (323,324).

Whole-genome sequencing (WGS) has been used as a robust tool for accurate typing of *Brucella* spp., due to the fact that the entire genome of the bacteria can be studied, thus providing better resolution (325,327). In this study, the results of WGS and SNP based phylogenetic analysis revealed that all *B. abortus* clustered into a distinct African clade with an average of 844 SNPs distance from the selected *B. abortus* reference strain from Mozambique (GeneBank accessions GCF_000366445.1). In addition, they have a different ancestral lineage compared to West African *B. abortus* isolates from Nigeria, Chad and

Senegal. Similarly, the two *B. melitensis* strains had common ancestral lineages with *B. melitensis* isolates from Somalia and Norwegian isolates from patients infected in Ethiopia (325) using phylogenetic analysis based on core-genome SNPs.

Multiple locus variable number tandem repeat (VNTR) analysis (MLVA) and multi-locus sequence typing (MLST) have been reported to be highly discriminatory in identifying subtypes within *Brucella* species, associated in some cases with geographic origin (314,315). In this study, *in silico* analysis of BruMLST-09 from WGS data identified two genotypes: ST72 (*B. abortus)* and ST12 (*B. melitensis*) . BruMLST09 profiles obtained from 15 *B. abortus* and 2 *B. melitensis* were compared using the web-based *Brucella* pubMLST database (https://pubmlst.org/brucella/). According to the database, ST12 has been well described and most of the strains were mainly originated from Eastern African countries including Ethiopia such as Somalia, Kenya, Eritrea, and Tanzania (Figure 4.17). Using expanded 21-locus MLST scheme, this ST has previously been described to cluster under Americas clade and more than 60 % of the isolates of this clade are reported to be originated from the African continent (321). *B. abortus* ST72 has not been reported from the African continent except a single strain in the database reported to be originated from Mozambique. The clustering together of Ethiopian *B. abortus* ST72 with *B. abortus* ST37 (Mozambique strain) and *B. abortus* ST38 (Kenyan strain) to form a distinct cluster (Figure 4.12) and isolates from the rest of African continent (Togo, Senegal, Nigeria, Chad, Cameroon, Sudan, Rwanda and Zimbabwe) forming a separate cluster in the BruMLST09 phylogeny is congruent with results of core genome SNP analysis.

MLVA results for *B. abortus* isolates also revealed that the 15 Ethiopian isolates, Mozambique and Kenyan strains fall into a distinct cluster. This cluster was described earlier as Clade A using core genome SNP analysis. Most other African isolates fall into a different cluster containing isolates from e.g. Togo, Senegal, Chad and Nigeria. This clade has been described previously by Vergnaud *et al.,* (116) as Clade B using similar typing methods. Hence, the results of MLVA-16 typing were congruent with the results of core

genome SNP analysis and MLST analysis. The clustering of Ethiopian *B. melitensis* from the current study into the Americas clade based on MLVA16 typing and when compared to a diverse collection of *B. melitensis* isolates from *Brucella* MLVA database is congruent with MLST data and in agreement with the findings of Whatmore *et al.,* (321).

The MLVA-16 typing revealed two *B. melitensis* and eight *B. abortus* genotypes (Table 4.6). Panel 1 and Panel 2A markers in MLVA-16 loci displayed no diversity among the genotypes. The loci which varied most among the isolates were those from the rapidly evolving panel 2B and displayed a moderate diversity. This rapidly evolving loci within Panel 2B has previously been described to be highly polymorphic and sufficient for a rapid identification of genotypes in a local outbreak investigation (120,315,326). On the other hand some epidemiologically unlinked isolates were reported to have identical MLVA-16 profiles (119).

None of the genotypes observed in this study were described before in the public database. Even though Panel 1 and Panel 2A markers were homogeneous, the Hunter and Gaston diversity index (HGDI) of Panel 2B markers such as bruce04 (HGDI = 0.5), bruce16 (HGDI = 0.73) and bruce30 (HGDI = 0.5) for *B. abortus* genotypes suggest moderate diversity and these could be early branching genotypes of the same strain circulating in the region. Cows from two or more districts were found to be infected by the same genotype. Genotype 02 was isolated from of cows from Assella and Bulbula, genotype 05 from Batu and Assella, and genotype 06 from Habura, Batu and Assella. On the other hand, five genotypes (genotype 01, 02,05,06, and 07) were recovered from cows of Assella origin, 3 genotypes (02, 04, and 08) from Bulbula and 3 genotypes (03, 05 and 06) from Batu. It is very likely that infection was introduced into the farm from other regions and within herd transmission of *Brucella* could have resulted in abortion storm. Moreover, it seems more likely that some animals acquired infection post introduction to the farm. As this was the first report from Ethiopia, it was not possible to traceback the source of infection for the outbreak.

It was interesting to note that two genotypes with moderate diversity, genotype 04 and 08 that corresponds to two WGS SNP types, 14_S14_L001 and 15_S15_L001 were isolated from vaginal swab and mammary gland lymph node, respectively of the same animal. MLVA-16 typing revealed that these two genotypes are two allele variants (Bruce16 and Bruce30, which are the most unstable and evolving loci) with the rest of fourteen markers being homogenous. This suggests that these strains could be an early branching genotypes of the same strain circulating in the farm.

Minimum spanning tree analysis of BruMLST09 and MLVA-16 genotypes identified in this study and those retrieved from public database demonstrated a global clustering of the strains in accordance with currently known *B. abortus* and *B. melitensis*. It also revealed a similar spatial clustering when compared to the core-genome-SNP analysis with classifying *B. melitensis* into an African clade as previously described (323,324), which in some cases classified into Americas clade (321). *B. abortus* on the other hand formed a distinct African clade that has been described by Whatmore *et al.,* (321) to be confined to African continent and limited global spread. The SNP types identified during the whole genome-SNP analysis of *B. melitensis* were congruent with the two MLVA genotypes identified whereas SNP types identified during whole genome-SNP analysis of *B. abortus* isolates did not correspond to the eight MLVA genotypes identified. This could be due to different diversity and mutation rates of different types of genetic markers (SNPs and VNTR loci) (328).

In this study we have elucidated for the first time in Ethiopia the type of *Brucella* species and the genotypes circulating in ATARC, central Oromia and Amibara district, Afar. Given the outbreak nature of the samples, WGS SNP analysis revealed that very little diversity was evident with the fifteen Ethiopian *B. abortus* isolates, with no more than five SNPs identified between any two strains within the panel. The diversity observed during MLVA-16 typing involves highly polymorphic and rapidly evolving loci (Panel 2B) suggesting moderate diversity of early branching genotypes of a single strain circulating in the region.

It should also be noted that none of the MLVA-16 genotypes found in this study were identical to any of the genotypes in the *Brucella*2019 database. The diversity observed by core genome phylogeny and MLVA-16 typing suggested that the epidemiological status of brucellosis in ATARC is the result of the introduction of a single lineage, which have subsequently diversified at the most unstable rapidly evolving loci. Although the number of isolates in this study is limited and the study did not cover a broad geographic area, it has critical implication that brucellosis will continue to be endemic hampering livestock productivity and posing a public health hazard if feasible control strategies involving One Health is not put in place. Future studies should aim to investigate the epidemiology of the disease in districts and towns believed to be the sources of these animals. This would help to better understand the molecular epidemiology of *Brucella*.

5. CHAPTER 5

5.1. General discussion and future perspectives

Ethiopia is believed to have the largest livestock population in Africa. According to the 2018 Agricultural Sample Survey report by the Central Statistical Agency of Federal Democratic Republic of Ethiopia the livestock population is comprised of about 60.4 million cattle, 31.3 million sheep, 32.7 million goats, 2 million horses, 8.8 million donkeys, 461,665 mules, 1.4 million camels and 56 million poultry. Livestock contributes to more than 30% of the agricultural gross domestic product and to 19% in export earnings. Despite these huge livestock resources and the critical role, the livestock sector plays in Ethiopia's economy, the economic return gained from this subsector is very low, partly because of prevalent infectious diseases, including brucellosis. Moreover, the livestock sector has not received the policy-level priority it deserves. This is largely explained by lack of in-depth analytical research and policy tools that would inform decision-making and priority setting at sectoral, regional, or national levels (329).

With the second largest human population in Africa, the Ethiopian economy is largely dependent on agriculture (236) and about 80% of its households live in close contact with domestic animals, increasing the risk of spill over of zoonotic pathogens that can cause infections and spread of diseases (238,239). Having a large population of poor livestock keepers ranks Ethiopia very high in the health risk of zoonotic diseases (240). The lack of coordination between veterinary and public health sectors coupled with limited resources in the country have also been major factors that have contributed to the high health burden of zoonotic diseases(241).

Ever since the first serological evidence of brucellosis in Ethiopia in the 1970's (193,230), the disease has been reported from various regions of the country. Most studies since then have involved passive surveillance published by academic institutions, the results of

which have rarely been used to inform animal and public health sectors. Variability in the sensitivity and specificity of serological tests used for diagnosis of brucellosis, coupled with lack of standard case definition, make the interpretation of the results of brucellosis serological surveillance difficult.

Serological tests commonly used in Ethiopia include, RBT, SAT, CFT, i-ELISA, c-ELISA, and FPA. As no single serological test is appropriate in all epidemiological situations (83), most studies in Ethiopia have involved serial testing of animals using RBT as screening test and further confirmation of RBT positive cases using CFT even though there are studies where a single test was used such as RBT or i-ELISA to estimate the prevalence of the disease. In the latter cases cross reacting antibodies raised against LPS of other Gram-negative bacteria could have resulted in false positive reaction of RBT and there by affecting the true estimate of the prevalence of the disease in a population. The reproducibility of the diagnostic protocols and reagents developed for testing in the developed world is also challenged by the level of expertise and storage and maintenance conditions available in Ethiopia. In this study a combination of RBT and c-ELISA (both procured from Animal and Plant Health Agency, UK) were used. In the outbreak investigation (Chapter 4), a combination of serological, bacteriological, and molecular methods were used for definitive diagnosis of brucellosis as described by OIE (83).

This thesis set out to investigate the epidemiology of brucellosis in two different livestock production systems and occupational risk groups. The study began by determining the prevalence of the disease in urban and peri-urban dairy systems in Addis Ababa, and also examined cattle, small ruminants and occupationally associated livestock herders and animal attendants in Borena. The knowledge attitude and practices of farmers and animal attendants relating to brucellosis has also been determined. *Brucella* species associated with abortion in cattle and goats were isolated and characterised to add to knowledge of the nature of strains circulating in Ethiopia.

Studies involving estimation of seroprevalence of brucellosis in cattle, small ruminants, and camels since 2007 were reviewed (Chapter 1). These studies were fragmented in time and place and a few hospitals based studies involving human were also reported. The seroprevalences in cattle varied from 0.06% in commercial intensive dairy production (Chapter 2) to 9.7% in extensive production system at the livestock wild-life interface (173). In small ruminants on the other hand, seroprevalences varied from 0.4 % in small holder extensive production system in and around Bahir Dar, Northern Ethiopia (175) to 13.7 % in pastoral production system of Afar Region, Eastern Ethiopia (176). Compared to cattle and small ruminants, brucellosis in camels is understudied. However, the published literature indicates that seroprevalences of brucellosis in camels ranges from 1.8- 4.4% (193,195).

The effect of a type of livestock production system in the epidemiology of brucellosis has not been studied in Ethiopia. Summary of studies conducted in the last decade (Table 1.2 and 1.3, in Chapter 1) showed that the seroprevalence of brucellosis was higher in cattle and small ruminants managed in extensive and pastoral livestock production systems compared to those kept under intensive management systems. This study also revealed that there was a higher prevalence of bovine brucellosis in pastoral systems compared to cattle kept under intensive dairy production systems in and around Addis Ababa. A review of published studies in Kenya on animal brucellosis in the last century have also indicated that seroprevalences were higher in animals kept under pastoral grazing systems compared to smallholder mixed crop or dairy farming systems (330). As mentioned in Chapter three, this could be due to mixing of large number of animals at watering and grazing points, movement of livestock in search of better pasture during the drought seasons, and presence of wildlife sharing grazing areas, which are conditions that increase the risk of transmission between infected and susceptible animal populations(331).

In urban and peri-urban dairy farming systems in Addis Ababa and its environs animals are completely housed and supplied with commercial feeds. The absence of communal grazing with neighbouring herds coupled with informal culling practices of animals with history of abortion (often due to multiple unknown reasons) (164) and repeat breeding for economic reasons are believed to play role in decreasing the prevalence of brucellosis in these settings. Better husbandry practices in urban and peri-urban dairy farming as compared to traditional production systems as a result of veterinary extension education is also likely to reduce the disease incidence(332,333).

Semen from infected bulls can transmit *Brucella* (8), hence semen for AI should only be collected from bulls free from *Brucella* infection. AI is the common breeding strategy in most urban and peri-urban dairy farming in Addis Ababa. In this region semen for AI is produced and distributed by the National Artificial Insemination Centre, Addis Ababa, which routinely test their bulls for *Brucella* infection. There were also farmers who utilized both AI and bulls in their farm (Chapter 2, Table 2.3). When artificial inseminators were not available or the cows failed to conceive after repeated AIs, farmers preferred to either use home grown bulls or bulls from neighbouring herd that could potentially transmit the disease if infected (39). In traditional livestock production systems, such as in Borena pastoral region, no AI is used.

Just above 80 % of smallholder and large-scale dairy farmers in Addis Ababa and almost all herders in Borena reported dumping of dead foetus and aborted foetal membranes into the environment. This could represent a risk for disease transmission to other animals, especially in traditional systems such as in Borena, as *Brucella* could survive in wet soil and manure during rainy season for up to two months (39). The role of dogs as symptomatic carriers and reservoirs of *Brucella* has been shown in Egypt by Wareth *et al*

(334) and in Korea by Baek *et al.* (335) where they isolated *B. abortus* from dogs housed in a dairy farm. Dogs consuming aborted foetus and foetal membranes could play a role in transmission and spill over of brucellosis to other livestock and to humans.

It has been indicated that a good knowledge of brucellosis among farm owners and herders has a crucial effect in preventing and controlling of the disease both in animal and human populations (336,337). Findings from the KAP survey (Chapter 2 and Chapter 3) illustrate that the majority of the participants (> 90 %) in Addis Ababa and Borena had no knowledge about brucellosis. As a result of this, they regularly participated in one or more high risk practices such as assisting in animal parturition and disposing aborted foetus and foetal membranes without using protective gloves, thereby posing a risk for brucellosis transmission. The study in Borena (Chapter 3) revealed that assisting in calving was a significant risk factor for transmission of brucellosis to humans. This finding is consistent with a studies in Kenya (293) and Tanzania (292).

Consumption of unpasteurized milk has been described as a risk factor for brucellosis transmission from animals to humans (44,336). This study found that 67% of participants in Addis Ababa (Chapter 2) and 84% in Borena (Chapter 3) consumed unpasteurised milk and milk products. If milk from infected herd is consumed, this suggests a higher risk of infection via raw milk consumption, which is consistent with studies in Pakistan (338). The study in Borena has also revealed that participants who consumed raw milk mixed with raw blood had increased risk of seropositivity for *Brucella* infection (OR=4.0, CI: 0.7-23.2). The misconception by most pastoralists that boiling or pasteurizing milk would reduce the nutritional quality of milk could also increase the risk of transmission of brucellosis in Borena as previously described(339).

The ultimate sources of infection for human are infected animals (39). The current study in Borena and others have also shown that direct contact with infected animals, such as assisting during calving, milking, feeding etc and indirect contact such as consumption of raw milk and milk products from infected animals, are risk factors for zoonotic transmission of brucellosis from infected animals to humans (286,340,341). The strong association between presence of seropositive animal in the households and seropositivity in humans revealed in this study (Chapter 3) could be due to the direct and indirect contact with the infected animal in the household(342).

As a strategy to cope with a drought, Borena pastoralists rear a group of livestock species such as cattle, sheep, goats and camels together. This study in Borena revealed that there were more than one seroreactive animal species in some of the households visited suggesting the possibility of cross-species transmission of *Brucella*. Both in cattle and small ruminant herds in Borena, herd size was found to be a risk factor indicating that an increase in a stocking density may increase the risk of infected animals contacting susceptible populations(154). Similarly, the association of *Brucella* seropositivity with adult age group could be linked with frequent exposure of the animal to the pathogen over a long period of time in pastoral settings where different livestock species are corralled together during communal grazing (211).

One of the economic impacts of brucellosis is the loss of calves or lambs due to abortion during the last trimester of gestation (204). The current study in Borena revealed that history of abortion was found to be significantly associated with *Brucella* seropositivity. On the other hand, the presence of seronegative animals with a history of late abortion both in Addis Ababa and Borena could be due to other infectious and non-infectious causes of abortion(143). In addition to abortion, repeat breeding was reported by most farmers as one of the common reproductive disorders in small, medium and large-scale dairy herds in Addis Ababa (Chapter 2). Even though repeat breeding occurs as a sequel

of brucellosis, most farmers in Addis Ababa reported that artificially inseminated cows had more repeat breeding problems than those inseminated by bulls. This could be due to lack of early heat detection and proper timing of AI (343,344).

Apart from the cross-sectional surveys in different livestock management systems (Chapter 2 and 3) , the outbreak investigation (Chapter 4) revealed the first isolation and molecular characterisation of *Brucella* from local zebu breeds in Ethiopia maintained at ATARC for cross breeding and composite breed production so as to distribute the crossbreed cows to the local farmers. ATARC purchased local zebu breeds from the nearby districts and villages without having the animals serologically screened for brucellosis. It is therefore likely that some of the purchased animals were infected and introduced into the herd and possibly causing the investigated outbreak.

Core genome based phylogenetic analysis of the *B. abortus* strains isolated from local zebu cattle in the research centre and other *B. abortus* genomes available in the global genomic databases revealed that Ethiopian *B. abortus* isolates formed a distinct clade basal to all other *B. abortus* genomes included in the study (Chapter 4). This clade has only previously been reported from Mozambique and Kenya (345), and represents a cluster which is under sampled and underrepresented in the global *B. abortus* database. The findings of this study could, therefore, substantially increase the representation of these basal *B. abortus* strains in the genomic databases.

The core genome phylogenetic analysis of *B. melitensis* isolated from goats with history of abortion (Chapter 4) including other *B. melitensis* genomes in the public databases revealed that the Ethiopian isolates clustered into an African clade containing isolates primarily from Somalia, Nigeria and Zimbabwe. The *B. melitensis* strains isolated previously from a human patient in Norway with history of travel to Ethiopia (325) were

also clustered in this clade. This clade has been described by various authors as Genotype III, an African lineage, based on whole genome SNP-based phylogenetic analysis (323,324).

The results of subsequent global phylogeny of *B. abortus* and *B. melitensis* including Ethiopian *B. abortus* and *B. melitensis* isolates from the current study based on MLST and MLVA, were congruent with the core genome based phylogenetic analysis as described in Chapter 4. In agreement with previous findings, the map of global genetic diversity of *B. abortus* (Figure 4.9, Chapter 4) showed that the isolates were represented by four clades; Clade A and B, that are African lineages and Clade C1 and C2 comprising lineages with global distribution (116,345). Similarly, the global genetic diversity and phylogeography of *B. melitensis* isolates in the current study (Figure 4.10, Chapter 4) on the basis of core genome SNPs were represented by four lineages as described earlier (323,324) Strains from Italy, France and Egypt formed a West Mediterranean clade. However, strains from Egypt and Italy have previously been classified as Mediterranean strains and identified as genotype I. Strains primarily from the Asian continent formed an East Mediterranean clade. The *B. melitensis* isolates from Ethiopia and other African countries formed an African lineage whereas strains from USA and Argentina including the commonly used *B. melitensis* 16M reference strain formed an American clade. In another study, strains from Africa, Europe and America were identified as genotype III, IV and V, respectively (323,324).

Even though brucellosis is considered as a priority animal disease of socioeconomic and trade significance by the Ethiopian Ministry of Agriculture, there are no brucellosis control policies and strategies put in place; this might partly be due to lack of better understanding of the magnitude of the disease both in livestock and public health sector. This study has revealed the magnitude of brucellosis and risk factors in two different livestock production systems and in three livestock species in Ethiopia. The burden of

the disease in occupationally linked farmers and animal attendants and level of their knowledge, attitudes and practices towards the disease has also been assessed for the first time. In addition, this study has revealed the first report of isolation of *B. abortus* from an outbreak of brucellosis in local Zebu cattle in Ethiopia, which represents *B. abortus* lineages previously underreported in public databases. The findings of this study can, therefore, contribute with information to the public and animal health sector so as to design and implement feasible control strategies in the country. Moreover, the isolation of circulating *Brucella* species in Ethiopia adds enormous understanding on the epidemiology of *Brucella* and is a crucial step if vaccination is to be introduced to prevent transmission of the disease from infectious foci towards disease free area.

Introducing a health policy that increases public awareness regarding brucellosis and its transmission routes will help reduce disease risks in occupationally linked farmers or herders, farm workers and animal attendants. The individual animal level prevalence of the disease was found to be low (< 5 %) in the study sites. The variation of prevalence of brucellosis among districts, villages and herds in Borena and the outbreak at ATARC are an indication that brucellosis is a herd or a regional epidemiological problem rather than being an individual animal problem as previously described (140). Given this epidemiological situation of the disease, careful identification of infectious foci and maintaining biosecurity measures around an infected herd would play an important role in reducing spillover of the disease into non-infected areas. Conducting active national surveillance for a single livestock disease is not realistic in Ethiopia due to limitation of resources. However, combined surveillances involving multiple zoonoses in similar emerging livestock production systems and settings will be helpful to better use of resources as witnessed by this PhD project linked to ongoing bovine TB project in Ethiopia. This will also help in designing feasible control strategies that would be useful to control multiple zoonoses. In line with this, surveillance systems should be strategic so as to identify clustered infectious foci to contain the spread of the infections to disease free areas as a result of market-oriented livestock movement. As there is no functional One health policy in Ethiopia, establishment and reinforcement of One-Health policy towards

the control of brucellosis in particular and other zoonoses in general is highly recommended.

5.2. Limitations of this study

This study has some limitations. Seasonal migration of livestock in Borena in search of good pasture and watering points could be associated with temporal variation of prevalence of the disease that was not assessed due to the cross-sectional design of the current study. Convenience sampling of villages and not including Children less than 5 years of age in Borena may limit the representation of the data to the entire population. Security problems related to political instability during the course of this study limited the number of districts surveyed. As the survey was conducted in drought season, some of the pastoralists refused to allow their herds to be sampled contending that collecting blood sample from their animals could impede productivity. The hypothesis developed in this study that finding of more than one sero-reactive animal species in Borena pastoral households could indicate cross-species transmission of *Brucella* infection should be strengthened by further research. Isolation and molecular characterisation of circulating *Brucella* species for the first time is a significant step in the knowledge of the epidemiology of brucellosis in Ethiopia, however, further studies should be made to isolate and characterise additional *Brucella* strains for better understanding of the epidemiology and population structure of circulating strains both in livestock and occupational risk groups.

6. APPENDICES

Appendix 1: Individual animals data recording sheet.

No	Animal ID	Species	Age	Sex	Origin (O, P)	Parity	Physiological Status (PR, NPR, L, Dry)	Breeding Strategy (AI, B)	Hx of Abortion	Hx of Retained Fetal membranes (RFM)	History of Still birth	Remark
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
17												
16												
17												
18												
19												
20												

Appendix 2: Individual human data collection sheet

No	Sample ID	Sex	Age	Marital status (S, M)	Educational level	No of people at home	Animals at home (Y, N)	Species of animals at home (Cattle, Shoats, Camel)	Know brucellosis (Y, N)	Had illness such as Malaria and Typhoid	Consumption behaviour (Yes, No)			Assist during calving /Parturition (Y, N)	Dispose dead and aborted fetus and FM (Y, N)	Animal attendant or shepherd (Y, N)	Milking cows and nanny goat (Y, N)	Remark
											Raw milk	Raw meat	Raw Milk with blood or alone					
1																		
2																		
3																		
4																		
5																		
6																		
7																		
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Appendix 3: Farm/herd epidemiological data collecting questionnaire.

Dear participant,

This survey is an investigation of risk factors precipitating the transmission of brucellosis within herd, between herds and to humans. The results of this study will help veterinarians and public institutions in designing control strategies.

There is no need to tell your name to the enumerator. Your responses will be kept confidential. You will in no way be personally linked to any of the final outcomes of the survey. There is no risk to you from participating in this questionnaire, and there is no anticipated direct benefit. Thank you in advance for your participation!

Questions related to risk factors for bovine brucellosis in dairy farms.

1. Have you ever seen reproductive problems in your farm in your farm/herd in the last 12 months ? A) Yes B) No
2. If the answer is "yes" to the above question, list the local name or symptom of the disease (rank the most common first) a) …… b) ……..…… c) ……
3. What type of breeding services do you use when the cow is on heat? A) AI B) Bull C) Both
4. Where do you get the replacement stock? a. Buy in b. Raise own replacement c. Both
5. If you buy a new animal, do you take any actions to ensure the animal is healthy? Do not read out. Yes ☐No ☐ If yes, how? Trust in own experience ☐ use veterinary inspection ☐ require laboratory tests ☐ demand immunization certificate for brucellosis ☐ buy from persons you trust have healthy animals ☐ other, specify…
6. Is there separate calving pen? a. Yes b. No
7. Do you separate cows during parturition? a. Yes b. No

8. What do you do to the calving pen after parturition? a. Flushing with water b. disinfecting with detergents c. Both d. other……..
9. Do you know brucellosis "*Wurja Beshita*" in Amharic? A) Yes B) No
10. How do you judge the bovine brucellosis status of your farm currently? A) endemic B) unknown C) Free
11. Is your farm tested for bovine brucellosis during the past three years?

A) Yes, B) No, if yes, a) When was the test done? _____ b) what was the percentage positivity? _____ c) Which measure have you taken on positive animals? A) Slaughtered, B) sold, C) segregated, D) No action - remained in the herd,

12. Have you observed abortion/still birth in your farm? a. Yes b. No
13. Do you have separate pen for aborted animal? a) Yes b) No
14. Do you sterilize instruments or appliances used during abortion? a) Yes b) No
15. If yes to the above questions, what kind of sterilization/disinfection do you use?
16. How many abortions/still births or retained after birth have you encountered during the last five years? a. Number of abortions_____ b. Number of still births _____c. Number of retained fetal membrane _____d. repeat breeder cow
17. How do you dispose aborted fetus and fetal membranes? A. Burning B) Burying C) Damped to the environment (open dump) D) Feeding to dogs
18. How do you dispose of contaminated straw/bedding.?

    A) Burning B) Burying C) Damped to the environment (open dump)
19. At what stage of pregnancy do you face abortion? a. First trimester b. Second trimester c. Third trimester
20. In which stage of parity abortion is observed?
21. What kind of grazing system do you employ? a) Communal b) own grazing c) both
22. Water supply for the dairy cattle? A) communal b) own c) both
23. What are your culling criteria?

    A) Reproductive problems B) Non-reproductive problems C. Logistics D) Others
24. Do you share vets, AI technician or attendants with neighboring farms? A) Yes, B) No

25. Contact of bovines with other animal species? A) Sheep B) Goats C) Others please specify _____

Appendix 4: Knowledge-Attitude and Practices (KAP) Questionnaire

Dear participant,

This survey is an investigation of risk factors and assessment of knowledge practices and attitudes of farm workers toward bovine brucellosis. The results of this study will help veterinarians and public institutions in designing control strategies.

There is no need to tell your name to the enumerator. You will in no way be personally linked to any of the results of the survey. There is no risk to you from participating in this questionnaire, and there is no anticipated direct benefit. Thank you in advance for your participation!

Part I: Demographic issues

1. Sex: Male ☐ Female ☐
2. Age: Below 13 ☐, 13-19 ☐ 20-59 ☐ above 60 ☐
3. Residence: Urban ☐ Periurban ☐ Rural ☐
4. Marital status: Married ☐ Single ☐ Widowed ☐ Divorced ☐
5. Animals at home: Yes ☐ No ☐
6. What the last grade of formal education you completed?

No formal school ☐ Some primary ☐ Completed primary ☐ Some secondary school ☐ Completed secondary school ☐ Technical /vocational ☐ Some/completed pre-university ☐ Completed diploma degree ☐University ☐ Don't know ☐ Refused ☐

7. How many people live in your household (including children, relatives)?

Part II: Awareness of Brucellosis

1. Have you heard of the disease brucellosis? Yes ☐ No ☐ If yes, from where did you get the information? Veterinarian ☐ public health workers ☐ newspapers ☐ TV ☐
2. Which animal can get infected with brucellosis? ……
3. Can humans be infected with brucellosis? Yes ☐ No ☐ If yes, what symptoms …….
4. Do you know how spread occurs between animals? Yes ☐ No ☐
5. Do you know brucellosis as a zoonotic disease? Yes ☐ No ☐
6. Does brucellosis present like any other illnesses? Yes ☐ No ☐
7. If "yes" to the above question, which other illnesses look like brucellosis? A) Malaria b) typhoid c) tuberculosis d) others e) I don't know
8. Do you know how humans can be infected with brucellosis from an animal? Do not read the options

Insect bites ☐ by close contact with infected animals☐ by consumption of raw milk/milk products ☐ by consumption of raw meat ☐ handling aborted fetuses and placentas, offal ☐ Assisting during animal during calving/abortion ☐ Contact with infected people ☐ Others ☐, please specify …… Don't know ☐

9. Do you know if there is any treatment for brucellosis in cows/sheep/goats? Yes ☐ No ☐ If yes, what kind and for how long?
10. Is brucellosis treatable in human? Yes ☐ No ☐
11. Who do you talk to most regularly about animal health issues?

Family member/friend ☐ neighbour ☐ veterinarian ☐ village chief /community leader ☐ other, please specify ……

Part III: Attitudes

Skip question 1 and 2 if the answer was NO on the question "have you heard of the disease Brucellosis" (Part I: 1)

1. Do you believe any family members are at risk of acquiring brucellosis? Yes ☐ NO ☐
2. If yes, to the above questions, which family member(s) do you think is /are most susceptible to infection? .....

3. If any animal in your household gets brucellosis, how serious do you consider this to be?

    Cattle: Not serious ☐ quite serious ☐ Very serious ☐

    Sheep: Not serious ☐ quite serious ☐ Very serious`

    Goats: Not serious ☐ quite serious ☐ Very serious

Do you need/would you like more information on brucellosis? Yes ☐ No ☐ if yes, how would you like to receive that information? .....

Part IV: Practices

Skip question 6 if the answer was NO on the question "Do you involve in delivery of pregnant cow?"

1. How often do you milk cows per day?
2. How often do you wash your hands after milking the cows? Every time ☐ Frequently ☐ sometimes ☐ Rarely ☐ Never ☐
3. If answering to above question sometimes, rarely, never –why?

    Not important ☐ No soap/not enough soap ☐ No clean water ☐ other reason ☐ specify
4. What do you do with dead fetuses (Calf, lamb? kid) ……
5. Do you take any specific actions to protect yourself when dealing with cows having an abortion or with retained placenta/dead fetuses? Don't read out.

    Use gloves ☐ use mask ☐ wash hands ☐ others ☐ please specify……
6. Do you involve in delivery of pregnant cow? Yes: ☐ NO: ☐
7. If yes to the question number 5 above, how do you involve?

    Birth aid ☐ Assistance to veterinarian ☐ Giving IU medication ☐
8. Do you consume fresh milk/ raw meat? Yes ☐ No ☐

Appendix 5: Scripts used during bioinformatic analysis.

Trimmomatic

java -Xmx8000m -jar trimmomatic-0.33.jar PE -threads 8 -phred33 <forward fastq> <reverse fastq> <trim forward_fastq> <unpaired forward fastq> <trim reverse fastq> <unpaired reverse fastq> ILLUMINACLIP:illumina-adaptors.2.fasta:2:30:10 LEADING:10 TRAILING:10 SLIDINGWINDOW:5:20 MINLEN:20

fastqc

ls *.fastq.gz | parallel -j 4 "fastqc -q {}"

kraken/bracken

run_kraken_farm5.py -f fastq_ids.txt -c 5 -o Brucella_QC

Spades

spades.py -o <output directory> -1 <trim forward_fastq> -2 <trim reverse fastq> --careful -t 8 -m 24 -k 21,25,29,33,37,41,45,49,53,57,61,65,69,73,77,81,85,89

Quast

ls *.fasta | parallel -j 4 "python quast.py {}"

Prokka

prokka --genus Brucella --outdir <output directory> --locustag <locus tag> <fasta file>

Roary

roary -p 16 -i 90 -e -n -cd 99 *.gff

snp-sites

snp-sites -c -o <roary core gene snp alignment> <roary core gene alignment>

iqtree

iqtree -s <roary core gene snp alignment> -nt AUTO -ntmax 8 -mem 8G -bb 1000 -m MFP+ASC

pyjar

python pyjar.py -a <roary core gene snp alignment> -t <iqtree treefile> -o <output prefix>

snippy

snippy-multi <input file> --ref <reference file> --cpus 16 > runme.sh

./runme.sh

```
# MLST-

mlst --scheme brucella *.fasta | cut -f1,3 > B_abortus_ST.tsv

# MLVA

python MLVA_finder.py -i <MLVA directory> -o . -p Brucella_primers.txt

# MLVA

# run_kraken_farm5.py - this is the script to run kraken and bracken on the Sanger cluster

# MLVA_finder.py
```

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