STUDIES IN CATTLE OF *BRUCELLA* INFECTIONS, CONVENTIONAL AND MOLECULAR IDENTIFICATION OF THE ISOLATES FROM KACHIA GRAZING RESERVE AND JOS PLATEAU, NIGERIA

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AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

AUGUST, 2014

Declaration

I declare that the work in this Dissertation titled **"Studies in Cattle of *Brucella* infections, conventional and molecular identification of the isolates from Kachia Grazing Reserve and Jos Plateau, Nigeria"** has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Bertu, Wilson James

Student	**Signature**	**Date**

Certification

This dissertation entitled **"STUDIES IN CATTLE OF *BRUCELLA* INFECTIONS, CONVENTIONAL AND MOLECULAR IDENTIFICATION OF THE ISOLATES FROM KACHIA GRAZING RESERVE AND JOS PLATEAU, NIGERIA"** by Wilson James BERTU**,** meets the regulations governing the award of the Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to knowledge and literary presentation.

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Dedication

This dissertation is dedicated to God Almighty and to the entire Bertu family.

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Abstract

Brucellosis is an infectious disease of domestic animals and wildlife and one of the commonest human Zoonosis caused by slow-growing, small, Gram negative, cocobacilli and intracellular bacteria of the genus *Brucella*. The prevalence of brucellosis is high in sub-Saharan Africa. In Nigeria, brucellosis is endemic and wide- spread. Several serological studies carried out in various parts of the country indicated that brucellosis is prevalent, with prevalence rates ranging from low to high. A few studies on cultural isolation of *Brucella* organisms have also been conducted, all of which reported *Brucella abortus* biotype 1 as the species circulating in Nigeria. However, few or no studies have been done on brucellosis in the study areas. There has also been little or no work on the molecular identification of *Brucella* isolates from Nigeria. A cross-sectional study was carried out in Fulani cattle herds in Kachia Grazing Reserve (KGR) and the Jos Plateau to determine the prevalence of brucellosis, isolate *Brucella* strains and carry out conventional and molecular identification of the isolates from the two study areas along with some isolates from field submissions and from the archive. A total of 1,982 and 2,880 serum samples were collected from cattle from KGR and on the Jos Plateau respectively and tested for *Brucella* antibodies by standard RBPT and modified RBPT. A total of 229 samples made up of vaginal swabs, milk and hygroma fluids were collected from the two study areas and cultured for *Brucella* isolation according to standard methods. Classical biotyping was carried out on all the isolates in this study and those in the archive. Bruce-ladder multiplex polymerase chain reaction (PCR) was used to identify the isolates to species level. Geographic information system was used to map out the study areas. Based on standard RBPT, the individual animal prevalence and herd prevalence of brucellosis in KGR were 0.5% and 17. 5% respectively while the individual and herd prevalence of brucellosis on the Jos Plateau were 1.0% and 30.0% respectively. Fifty eight (2.9%)

individual animal prevalence and 21(52.5%) herd prevalence were recorded in KGR while 91(3.2%) individual animal prevalence and 23(63.9%) herd prevalence were recorded on the Jos Plateau respectively using the modified RBPT. Due to its higher sensitivity, the modified RBPT was used to determine the prevalence rates based on blocks, villages, sex, age and herd sizes. The prevalence of brucellosis was higher on the Jos Plateau compared to KGR but the difference was not statistically significant ($P>0.05$). Seropositivity was higher in larger herds compared to smaller herds, and this was statistically significant ($P<0.05$). The prevalence rates were significantly higher in females compared to males and in adults compared to the young ($P<0.05$). Three *Brucella abortus* isolates were obtained from KGR while four *Brucella abortus* isolates were obtained from the Jos Plateau. Classical biotyping of all the isolates including field submissions and those from the archive revealed that they were all *Brucella abortus* biotype 3. Molecular identification of all the isolates by Bruce-ladder multiplex PCR showed that they were all *Brucella abortus*. This is the first molecular identification of *Brucella* isolates from Nigeria to their species level and the first use of the Bruce-ladder multiplex PCR. This study may be the first to establish that *Brucella abortus* biotype 3 is the predominant *Brucella* biotype circulating in Nigeria. These findings are very significant as they established the endemicity of *Brucella* infection due to *Brucella abortus* biotype 3 in KGR and on the Jos Plateau. GIS enabled the spatial presentation of *Brucella* prevalence on the map of each study areas and provided a better understanding of the spread of the disease. There is urgent need for the institution and implementation of brucellosis control in these areas. Genotyping of the isolates should be carried out to determine their phylogenetic position in relationship with those in the data bases.

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List of Abbreviations

A.D	After Death
AMOS-PCR	Abortus, Melitensis, Ovis, Suis Polymerase Chain Reaction
A- antigen	Abortus-dominant antigen
bp	Base Pairs
BAB	Blood Agar Base
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
cELISA	Competitive ELISA
CFU	Colony Forming Unit
CFT	Complement Fixation Test
CFU	Colony forming unit
CITA	Centro Investigacione Techlologia Agroalimentaria
CMI	Cell Mediated Immunity
DNA	Deoxyribonucleic Acid
EC	European Commission
EDTA	Ethylene Diethyl Tetracetic Acid
ELISA	Enzyme-link Immunosorbent Assay
EU	European Union
FLD	Federal Livestock Division
FAO	Food and Agricultural Organization
FPA	Fluorescent Polarization Assay
GD	Gel Diffusion
GIS	Geographic Information System
GPS	Global Positioning System

H_2S	Hydrogen Sulphide
HCl	Hydrogen Chloride
iELISA	Indirect ELISA
IgA	immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IS	Insertion Sequence
I.U	International Unit
Iz	Izadnagar
KGR	Kachia Grazing Reserve
LFA	Lateral Flow Assay
LGAs	Local Government Areas
LPS	Lipopolysaccharide
LPU	Livestock Project Unit
MGB	Minor Groove Binding protein
MLVA	Multilocus Variable Number Tandem Repeat Analysis
M-antigen	Melitensis-dominant antigen
MRT	Milk Ring Test
MSAT	Microtitre Serum Agglutination Test
2-ME	2- Mercaptoethanol
NaCl	Sodium Chloride
NAD	Nicotinamide Adenine Dinucleotide
NH	Native Hapten
NVRI	National Veterinary Research Institute
OIE	Organization International des Epizooties

OMP	Outer Membrane Protein
PFGE	Pulsed-Field Gel Electrophoresis
PBS	Phosphate Buffered Saline
RBPT	Rose Bengal Plate Test
R/C	Rough Culture
RFLP	Restriction Fragment Length Polymorphism
RID	Radial Immuno-Diffusion
RB51	Rough *Brucella* 51
Rev.1	Revertant .1
RTD	Routine Test Dilutions
SAT	Serum Agglutination Test
SDA	Serum Dextrose Agar
SDS	Sodium Dodecyl Sulphate
S-LPS	Smooth Lipopolysaccharide
SNP	Single Nucleotide Polymorphism
S19	Strain 19
Tb	Tbilisi
TBE	Tris HCl, Boric acid and EDTA
TE	Tris HCl and EDTA
TPHI	Tropical and Public Health Institute
TSA	Trypticase Soy Agar
USA	United States of America
UV	Ultra Violet
VNTR	Variable Number of Tandem Repeat
Wb	Weybridge
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Members of the genus *Brucella* represent some of the world's major zoonotic pathogens responsible for enormous economic losses in livestock production and considerable human infection (Godfroid *et al.,* 2005; Pappas *et al.,* 2006a). It remains a major bacterial zoonotic disease of global importance (Cutler *et al*., 2005). While *Brucella* is sometimes cited as a re-emerging pathogen, often reflecting socioeconomic changes in particular locations, the organism has been recognised as a major scourge of mankind since its first isolation from humans (Bruce, 1887) as reported by Alton *et al*. (1988). There is considerable circumstantial evidence of the significance of brucellosis over human history (Evans, 1950; Hoover and Friedlander, 1997; Capasso, 2002). However, reflecting the insidious and non-specific nature of the disease and the difficulty associated with working with *Brucella* (Boschiroli, 2001), its importance is often overlooked in favour of more 'spectacular' diseases. It is also likely to be under-diagnosed and under-reported (Franco *et al*., 2007).

The World Health Organization (WHO) ranks brucellosis among the top seven 'neglected' zoonoses, a group of diseases that are both a threat to human health and a cause of poverty (Maudin and Weber, 2006; Seimenis, 2012). Ten species are currently recognized belonging to the genus *Brucella*. They are designated on the basis of differences in pathogenicity and host preferences. Eight of them that affect terrestrial animals are: *Brucella abortus* (cattle and buffalos*), Brucella melitensis* (sheep and goats), *Brucella ovis* (sheep), *Brucella suis* (pigs), *Brucella canis* (dogs), *Brucella neotomae* (desert wood rats), *Brucella microti* found in the common vole and *Brucella innopinata* isolated from breast implant of a woman (Verger *et al.,* 1987; Lopez-Goni *et al*., 2008;

Scholz *et al*., 2008a). Two species that affect marine mammals are *Brucella pinnipedialis,* found in pinepeds and *Brucella ceti* in cetaceans (Foster *et al*., 2007).

Five out of the nine *Brucella* species can infect humans and the most pathogenic and invasive for humans is *B. melitensis*, followed in descending order by *B. suis, B. abortus* and *B. canis* (Acha and Syzfres, 2003) and *B. ceti* (Sohn *et al*., 2003; McDonald *et al*., 2006).

The disease is highly contagious and is spread through contact with aborted foetuses, vaginal fluids, and milk, as well as congenitally and venerally (Ducrotoy, *at al*., 2014).

Brucellosis remains endemic in most parts of the world except in some countries where bovine brucellosis (*B.abortus*) has been eradicated following a lengthy and expensive control programmes involving vaccination and culling (Conde-Alvarez *et al*., 2013). These countries include; Australia, Canada, Cyprus, Denmark, Finland, The Netherlands, New Zealand, Norway, Sweden and the United Kingdom, most of the European Union countries (Seleem *et al*., 2008; Conde-Alvarez *et al.,* 2013).

The number of domestic animals susceptible to brucellosis in the world is estimated to be 4 billion and of these, less than 20% are in Europe (Conde-alvarez *et al.,* 2013). While diagnostic tests for brucellosis and methods for its control and elimination are well understood, this disease remains largely neglected, unrecognized, under-diagnosed and uncontrolled in a large number of pastoral areas of the world, especially in poor resource countries (Zinsstag *et al.,* 2011). The prevalence of brucellosis is highest in the Mediterranean region, the Middle East and South Asia (Pappas *et al.,* 2006). Brucellosis has been regarded as a major problem among ruminants in sub-Saharan Africa and its epidemiology and impact has been reported in several countries (McDermott and Arimi, 2002). It has also been reported in two of Nigeria's neighbouring countries namely; Chad and Cameroun (Schelling *et al.,* 2003; Bayemi *et al.,* 2009; Bronsvoort *et al*., 2009;

Scolamacchia *et al*., 2010). Other African countries that have reported brucellosis recently include Ethiopia, Zambia, Zimbabwe, Ivory Coast, Kenya and Ghana (Sanogo *et al.,* 2008; Chimana *et al*., 2010; Matope *et al*., 2011; Megersa *et al*., 2011; Muendo *et al*., 2012; Sanogo *et al.,* 2012;), indicating that the disease is prevalent on the continent. Although estimates of the cost associated with brucellosis in fection remain limited to specific countries, all data suggest that the worldwide economic losses due to brucellosis are extensive (McDermott *et al*., 2013). Direct losses are as a result of abortion, neonatal mortality, infertility, reduction in milk production, impediment to trade and exportation of animals and animal by-products (Lopez-Goni *et al*., 2008). Other losses include delayed conception, sterility, mortality and cost of veterinary care. Economic losses due to brucellosis in humans are cost of treatment and hospitalization and productivity loss (Seleem *et al.*, 2008).

In livestock, the main symptoms in female are abortion (especially at the second half of gestation), premature or full term birth of weak or dead offspring, retained placenta and metritis (Acha and Szyfres, 2003). In humans, the most common symptoms of brucellosis include undulant fever, in which the temperature can rise from 37^0C to in the morning, to 40^0C in the afternoon; night sweat with peculiar odour, chills and weakness. Common symptoms also include; malaise, insomnia, anorexia, headache, arthralagia, constipation, sexual impotence, nervousness and depression (Acha and Szyfres, 2003). Occupational groups like farm workers, veterinarians, ranchers and abattoir workers are considered to be at higher risk where the route of infection can be via cuts or abrasions, inhalation or conjuctival contamination by aerosols (Young, 2005; Tabak *et al.,* 2008).

Epidemiological studies of brucellosis have always relied on serology and bacteriological isolation of the bacteria followed by phenotypic characterization based on biological and physiological properties of the organism. This is usually laborious and time consuming,

sometimes inaccurate and very hazardous (Lopez-Goni *et al*., 2008; OIE, 2009) but remains the gold standard method for confirmatory diagnosis of brucellosis (Smirnova *et al*., 2013).

The advent of molecular methods, especially polymerase chain reaction (PCR) technique has enhanced disease diagnostic capacities to a point that identification of causative agents is less obscured (Colmenero *et al*., 2011). One of these methods is a multiplex conventional PCR called Bruce-Ladder (Garcia-Yoldi *et al.,* 2006; Lopez-Goni *et al*., 2008). The Bruce-ladder multiplex PCR is robust, performs excellently well and can differentiate all known *Brucella* species and the vaccine strains and is as sensitive as classical culture-based techniques. Furthermore, it is possible to detect dead bacteria and this reduces the necessity of sample preservation before analysis. The method is capable of accurately typing *Brucella* based on specific identification of *Brucella* nucleotide sequences associated with the genus, species, strains and biovars within the species.

1.2 Statement of the Research Problem

In Nigeria, brucellosis is recognized as an endemic disease (Ocholi *et al*., 1993). Serological studies of samples from parts of the country indicated that the disease is widespread among the cattle population particularly in ranches, livestock breeding centres, dairy farms and Fulani herds and trade cattle (Esuruoso, 1974; Falade and sellers, 1975, Falade, 1981; Bale *et al*., 1982**;** Chukwu, 1987; Ajogi, 1995; Halle and Ajogi, 1997; Ishola, *et al*., 2001; Ajogi *et al*., 2002, Ocholi *et al*., 2004b; Cadmus *et al.,*2006; Junaidu *et al*., 2008; Junaidu *et al*., 2011; Bertu *et al*., 2012). The reasons for the endemicity of brucellosis could be due to expansion of livestock flocks and herds with associated uncontrolled movement; lack of veterinary support services and vaccination and husbandry practices that favour the spread of the infection (FAO, 2003; Bertu *et al*., 2012; Mai *et al*.,

2012; Mai *et al*., 2013). Nigerian livestock population has been estimated at 15.2 million cattle, 23 million sheep, 28 million goats and 6.7 million pigs (FAO, 2006). This makes Nigeria one of the largest cattle rearing nations on the African continent. The vast majority of the cattle population (approximately 70%) are reared under traditional husbandry practices (Ducrotoy *et al*., 2014), and improvement of productivity has become a major challenge to meet the increasing demand for meat and dairy products due largely to diseases such as brucellosis (FAO, 2006). The country does not have a brucellosis control programme in place (Ocholi *et al*., 2004a), even though vaccine is produced at National Veterinary Research Institute (NVRI), Vom, for immunization of cattle against the disease. Epidemiological information about the disease is usually not properly documented (Zinsstag *et al.,* 2011). A combination of poor surveillance system, under reporting, inadequate diagnostic tools and lack of standardization of metrics of disease burden in animals and man leads to an inadequate knowledge base for informing policy (Zinsstag *et al.,* 2011). The approach to control, prevention and eradication of brucellosis in Nigeria is dependent on the understanding of factors such as the level of infection (incidence and prevalence) in the herds or flocks, type of husbandry, economic resources, public health impact and international trade implications (FAO, 2003).

Kachia Grazing Reserve (KGR) and Jos Plateau are among areas with high density of cattle, usually kept together with small ruminants under such husbandry systems and cultural life styles and practices that favour the spread of brucellosis among the different animal species (Ocholi *et al*., 2005; Godfroid *et al*., 2012).

The aim of this study was to determine the seroprevalence of brucellosis in the study areas, isolate the organisms and carry out phenotypic and molecular identification of *Brucella* isolates obtained and to recommend appropriate control measures.

1.3 Justification for the Study

Nigeria is one of the largest cattle-rearing nations on the African continent, and with vast majority of the cattle population reared under traditional husbandry practices. Improvement of productivity to meet the increasing demand for meat and dairy products due to brucellosis and other diseases has become a major challenge. Kachia Grazing Reserve (KGR) and Jos Plateau are among the areas in North Central geoecological zone of Nigeria with large number of cattle herds owned and managed under traditional husbandry system by nomadic Fulani cattle rearers respectively.

There is dearth of information on the prevalence of brucellosis in KGR, a very important and large Grazing Reserve with a very large population of cattle. Although there have been extensive studies on trypanosomosis, no such studies have been done on brucellosis in the area. There is also lack of information on *Brucella* species prevalent in the area.

Similarly, though there is a large concentration of nomadic cattle herds on the Jos Plateau, there is also a paucity of information on the current prevalence of brucellosis in this area. Migration and contact with other animals on common grazing grounds or at watering points form the major risk of transmission of brucellosis in these study areas. Other possible factors include poor farm and household hygiene, exchanges, purchases and borrowing of apparently healthy but brucellosis infected animals as these practices are common among herdsmen. The keepings of large herd sizes as well as keeping of mixed livestock species in the two study areas are contributing factors to the spread of brucellosis.

Given the close relationship between pastoralists and their cattle, their life styles and their habits of consuming raw milk, a study in order to determine the possible occurrence of the disease in catlle is important as the presence of the disease in animals may be indicative of its likely presence in the livestock owners in the areas. Variations in temperatures have

been reported to influence or affect the survival of *Brucella* organisms. The choice of KGR, a relatively hot area and the Jos Plateau which is relatively cold for this study is necessary in order to compare the prevalence of brucellosis based on differences in diurnal temperature in the two areas.

To date, and to our knowledge, no work seems to have been done on the use of bruce-ladder multiplex PCR for the identification of *Brucella* organisms isolated from Nigeria in general and in particular from these two communities. Thus the use of Bruce-ladder multiplex PCR which has been used for the molecular characterization of *Brucella* species due to its robust and capability of identifying all *Brucella* species as well as the vaccine strains will go a long way in identifying the *Brucella* species that could be isolated in these areas.

At present, there is no official national vaccination programme for brucellosis control in Nigeria. The lack of good understanding of the epidemiology of brucellosis in Nigeria can leads to improper determination of strategies for control and prevention of the disease, let alone its eradication. A better estimate of the true prevalence of the disease both at individual animal and herd levels and the identification of prevailing species of *Brucella* in these animals and humans is important for assessing the economic losses in animals and the public health implications. The findings of this study will provide useful insights for the establishment of appropriate intervention and control measures.

1.4 Aim of the Study

The aim of the study is to determine the prevalence of brucellosis in KGR and on the Jos Plateau, isolate and carry out phenotypic and molecular identification of the isolates by Bruce-ladder multiplex PCR.

1.5 Objectives of the Study

The objectives of the study are:

1) To determine the seroprevalence of brucellosis in cattle in Kachia grazing reserve and on the Jos Plateau.

2) To isolate and identify *Brucella* species from field samples (vaginal swabs, milk, hygroma, aborted foetuses, placentae) from the two study areas and carry out comprehensive characterization and biotyping of the isolates.

3) To Resuscitate, characterize and biotype archived *Brucella abortus* strains with a view to determining their phenotypic and molecular relationship with the extant isolates.

4) To carry out molecular identification of the various brucellae isolated using Bruce-ladder multiplex PCR.

5) To conduct Georeferencing and mapping of the study areas to show the spatial distribution of brucellosis in selected herds.

1.6 Research Questions

a) What is the prevalence of brucellosis in cattle in Kachia Grazing Reserve and on the Jos Plateau?

b) Which *Brucella* species and biotypes are prevalent in these areas?

c) Are the *Brucella* species and biotypes from this study different from those in the archive?

d) Can Bruce-ladder multiplex PCR be used to identify the species from this study and those from the archive?

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical Perspectives

Brucellosis is an infectious disease of many species of domestic animals and wildlife worldwide. It occurs most commonly in cattle, sheep, goats, dogs and pigs. Occasionally, it also affects other domestic animals such as camels and horses and wild animals such as bison, elk, and reindeer, caribou (Jensen *et al.,*1999) and marine animals like whales, dolphins, seals and porpoises (Foster *et al*., 2007).

The disease was first described by Hippocrates in 450 A.D. as a febrile disease (Alton *et al*., 1988). The causative agent was first isolated by Bruce in 1887 from the liver of a British soldier who died of undulent fever (Malta fever) on the Mediterranean Island of Malta. Then it was called *Micrococcus melitensis* (Bruce, 1887) as reported by Alton *et al*., 1988). Ten years later, Bang and Stribolt (1897) isolated a similar organism which they named *B. abortus* from an aborted bovine foetus and foetal membranes in Denmark. The relationship of this disease in animals and man was not well understood until Zammit (1905) demonstrated the organism in the blood of goats and the isolation of the organism from apparently healthy goats by Horrecks in the same year. This was followed by the isolation of a similar organism named *B. suis* from an aborted pig by Traun (1914) in the USA. Evans, (1918) showed that these organisms were related and they were therefore included in the same genus *Brucella* by Meyer and Shaw (1920), in honour of David Bruce. After these, three new species of the organism have been described as *B. ovis* was isolated from cases of epididymitis and abortions in sheep in New Zealand by Buddle and Boyles (1953). *B. neotomae* was isolated from a desert wood rat (*Neotoma lepida*) by Stoener and Lackman (1957) in Utah, USA and *B. canis* from cases of canine epizootic

abortion and epididymitis in Beagle dogs by Carmichael and Kenny in 1968 in the USA. There were then six recognized species in the genus *Brucella* namely, *B. abortus, B. melitensis, B. suis, B. canis, B. neotomae* and *B. ovis.*

Many years later, Corbel and Brinley-Morgan (1984) reported the isolation of *Brucella* species, which differ from the above mentioned ones from animals living in marine environment. Isolates of these *Brucella* species were obtained from free-living Seals and Cetaceans around the coast of Scotland and captive Dolphins in the USA (Ewalt *et al*., 1994). Their characterization showed that they could not be assigned to recognized species of *Brucella* but should be given a new species name called *Brucella maris* (Jahans *et al.,* 1997). However, based on molecular studies which indicate differences in the strains from cetaceans and seals, two new species, *B.ceti* and *B. pinipediae* were proposed and published, corresponding to cetaceans and seals respectively (Cloeckaert *et al*., 2001; Foster *et al*., 2007). Just as in terrestrial animals, host specificity in marine *Brucella* species does not appear to be absolute (Groussaud *et al*., 2007; Dowson *et al*., 2008). More recently also, additional *Brucella* species*,* named *Brucella microti,* initially isolated from Voles (Scholz *et al*., 2008a) and later in foxes and soil (Scholz *et al*., 2008b; Scholz *et al*., 2009) was fully described. The novel isolate from a human breast implant infection, strain BO1 was described recently, although the natural reservoir of this isolate remains unclear (De *et al*., 2008).

2.2 Etiology of Brucellosis

2.2.1 Description of the causative agents

Brucellosis in cattle, sheep, goats and camels is primarily caused by *B. abortus*, *B. melitensis* (Luchsinger and Anderson, 1979; Garin-Bastuji *et al., 1998),* or *B. ovis* which infects mainly sheep, *B. suis* (Paolicchi *et al*., 1993) infects pigs, *B. canis* (dogs), *B.*

neotomae (desert wood rat). Newly described species from marine mammals are *B. pinnipedialis* (seals) (Maquart *et al.,* 2008) and *B. ceti* (dolphins, porpoises) (Clavareau *et al*., 1998). Other species are *B. microti* (from the common voles), *B. inopinata* (from human breast implant but origin is unknown) (Whatmore, 2009). These make up the 10 well recognized and characterized *Brucella* species.

As a result of the use of modern molecular methods, potential novel *Brucella* species have been recognized. These were previously either unknown or were considered as *Brucella-like* organisms isolated from humans and various animal sources (Tiller *et al*., 2010a; Scholz and Vergnaud, 2013). These categories of *Brucella* species include:

(i) An unusual *Brucella* strain (BO2) was isolated from a lung biopsy specimen from a 52-year old human patient in Australia with chronic destructive pneumonia (Tiller *et al.,* 2010b).

(ii) Atypical *Brucella* strains were recently isolated for the first time from non-human primates associated with two cases of stillbirth (Schlabritz-Loutsevitch*, et al*., 2009).

(iii) Novel atypical *Brucella* strains isolated from red foxes in Austria (Hofer *et al.,* 2012).

(iv) Another potential novel *Brucella* species has been isolated from African bullfrogs (Eisenberg *et al*., 2012).

Extensive research is on-going to fully characterize these isolates as either new *Brucella* species or novel sub-lineages of some of the existing species (Eisenberg *et al*., 2012).

2.2.2 Morphological characteristics

Morphologically, brucellae are small coccobacilli or short rods 0.6 to 1.5µm long and 0.5 - 0.7µm in width. They are facultative, intracellular, non-motile organisms that lack capsules, flagellae, pili, endospores or plasmids **(**Alton *et al*.,1988; Corbel, 1997; Madkour, 2001; Whatmore, 2009; Araj, 2010). They are arranged singly and less frequently in pairs or small groups. The morphology of brucellae is frequently constant except in old cultures where pleomorphism is evident (Alton *et al*., 1988).

They are Gram negative and usually do not show bipolar staining. They are not truly acid-fast but resist decolorization by weak acids. They therefore stain red by modified Ziehl-Neelsen method that is sometimes used for the microscopic diagnosis of brucellosis from smears of solid or liquid specimens (Alton *et al.,* 1988).

2.3 Culture and Growth Requirements

2.3.1 *Brucella* selective media

In an attempt to achieve success in the isolation of *Brucella* from contaminated clinical veterinary specimens, development of selective media became paramount. Further modifications have also been made to *Brucella* selective medium by addition of antibiotics or dyes to the basal medium with the intention of eliminating other bacteria. For example, the Farrell medium used for *Brucella abortus* (Ryan, 1967; Farrell, 1974) is also recommended for *Brucella melitensis* (Alton *et al.,* 1988; Garin-Bastuji and Blasco, 2004). However, concentrations of Nalidixic acid and Bacitracin used in Farrell medium have inhibitory effects on *Brucella melitensis* strains (Marin *et al.,* 1996b) and Thayer-Martin medium is suitable for *Brucella melitensis* and *Brucella ovis* isolation (Garin-Bastuji, 2006) and isolation rate increases significantly by simultaneous use of both media (Marin *et al*., 1996a,b). A more suitable medium with more comparative advantages for the

growth of most *Brucella* species called modified Thayer-Martin medium or CITA medium was recently developed (de Miguel *et al*., 2011).

2.3.2 Carbon dioxide requirement

Members of the genus *Brucella* are aerobic but some strains require atmosphere containing 5-10% CO_2 added for growth especially on primary isolation (Alton *et al*., 1988). *Brucella melitensis,* however does not require CO_2 or serum and can be isolated on ordinary solid media under aerobic conditions at 37^0C (Garin-Bastuji, 2006). On repeated culture, isolates of *Brucella* can lose the requirement for added CO_2 for growth and may grow in air alone (Eze, 1981).

2.3.3 pH Requirement

The optimum pH for the growth of *Brucella* species is from 6.6-7.4 and culture media should be adequately buffered near pH 6.8 for optimum growth.

2.3.4 Growth temperature

The optimum growth temperature for *Brucella* organisms is 36-38^0C, but most strains can grow between 20^0C and 40°C (Anon, 2001; European Commission, 2001).

2.3.5 Colonial behaviour

Brucella requires biotin, thiamin and nicotinamide and the growth are improved by serum or blood. On suitable solid media (Thayer and Martins, 1964), *Brucella* colonies are visible after 2 days incubation. After four days, *Brucella* colonies become round, 1-2mm in diameter, with smooth (S) margins, transparent and pale honey colour when plates are viewed in transmitted light and have a bluish translucent appearance in reflected light

Alton *et al*., 1988). When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker. Rough *Brucella* isolates produce similar colony size and shape but are more opaque off-white in colour with a rather granular surface (Alton *et al.,* 1988). On blood agar, growth is slower than on serum dextrose agar (SDA) with the production of non-haemolytic, greyish- white glistening colonies after 48-72 hours incubation (Alton *et al.,* 1988). Growth in liquid media is usually poor unless the culture is vigorously shaken (Corbel and Brinley-morgan, 1984). On semi-solid media, CO_2-independent *Brucella* strains produce uniform turbidity from surface down to 3 millimetres depth while CO_2-dependent strains produce a disk of growth 2 millimetres below the surface of the medium (Corbel *et al*., 1984). Little or slow growth is produced by many *Brucella* strains on MacConkey agar, even after five days at 37^0C (Corbel *et al*., 1979).

The growth of most *Brucella* strains is inhibited by media containing bile salts, tellurite or selenite and does not require haeme (V-factor) and NAD (Alton *et al.,* 1988).

2.4 Biochemical Reactions and Sensitivity Tests

2.4.1 Biochemical tests

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

The production of H_2S from sulphur containing amino acid varies. *Brucella melitensis* does not produce H_2S (European Commission, 2001). Urease activity of *Brucella* species varies from fast to very slow. Indole and acetyl methyl carbinol are not produced from tryptophane and glucose respectively (Anon, 2001). Methyl red and Voges-Proskauer tests

are negative and *Brucella* neither liquefies gelatine nor lyses red blood cells (Alton *et al*., 1988).

2.4.2 Antigenic characteristics

Like other Gram-negative bacteria, *Brucella* has lipopolysaccharide (LPS) as a major component of their outer membrane and an important virulence factor (Cardoso *et al.,* 2006). Thus their colony morphology is termed as either 'smooth' or 'rough' depending on the LPS structure (Baldwin and Goenka, 2006). Structural variation in the LPS of smooth strains also defines the so called A and M antigens that have some a significant role in typing (Wilson and Miles, 1932; Godfroid *at al*., 2010). These antigens reflect differential O-side chain which is linked to α1,2 in A dominant strains but with every fifth residue linked α1,3 in M dominant strains (Bundle *et al.,* 1987). The O-PS is involved in bacterial virulence. It contributes in complement resistance and more importantly critically modulates bacterial entry into cells so that its removal causes attenuation (Conde-Alvarez *et al*., 2013). All smooth *Brucella* cross-react with one another in agglutination tests. They also cross-react with unabsorbed polyclonal antisera. This cross-reaction does not occur with non-smooth or the rough *Brucella* strains.

Lipopolysaccharide (LPS) comprises the major surface antigens of the corresponding colonial phase involved in agglutination. The (S-LPS) molecules carry the A and M antigens, which have different quantitative distribution among the smooth *Brucella* strains (Bundle *et al*., 1989). This is of value in differentiating biovars of the major species using absorbed monospecific A and M antisera (Anon, 2001). Serological cross-reaction has been reported between the smooth *Brucella* and various other Gram negative bacteria like, *E. coli* O: 116 and O: 157, *Salmonella* group N (O: 30) of Kaufmann-white and *Pseudomonas multophilia, Vibrio cholerae* and especially *Yersinia enterocolitica* (O: 9).

These organisms can induce significant antibodies, which cross-react with S-LPS of *Brucella* antigens in diagnostic tests (Corbel *et al.*, 1984; Anon, 2001).

2.4.3 Susceptibility to phages

There are about 40 phages which are lytic and specific to the genus *Brucella* and they are not known to be active against any other bacteria that have been tested. Therefore lysis by *Brucella* phages is a useful test to confirm the identity of *Brucella* species and for speciation within the genus (Anon, 2001).

Brucella phages currently in use for *Brucella* typing are Tbilisi (Tb), Weybridge (Wb), Izatnagar (Iz) and Rough Culture (R/C) (Alton *et al*., 1988). The first three phages are used for differentiation of smooth *Brucella* and R/C is used for rough B*rucella* (*B. ovis, B. canis*) (Corbel *et al*., 1987), (Table 2.1).

Table 2.1: Differential characteristics of *Brucella* species to *Brucella* phages involved in ruminant brucellosis

Species	Tb	IZ	R/C
B. melitensis	–	+	–
B. ovis	–	–	+
B. abortus	+	+	–

Source: Alton *et al*. (1988)

Key:
Tb = Tbilisi
Iz = Izatnagar
R/C = Rough culture

2.4.4 Susceptibility to dyes

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

2.4.5 Susceptibility to antibiotics

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

2.5 *Brucella* Evolution

By observing the evolutionary traits according to the ancestor-descendant rules of characters, it can be predicted that the ancestor of *Brucella* was an aerobic heterotroph free-living bacteria inhabiting the rhizosphere of plants (Moreno *et al.*, 2002). Complete chromosomal sequences of species belonging to these genera of alpha-proteobacteria allows genetic comparison with *Brucella* (DelVecchio *et al*., 2002; Moreno *et al*., 2002). It is accepted that the *Brucella* ancestor was a bacterium that evolved into an animal intracellular parasite by losing structures, modifying existing ones and generating or acquiring new traits (DelVecchio *et al*., 2002). These characteristics are reflected in the genome as well as in the phenotype and life cycle of the extant *Brucella* (Moreno *et al*., 2002). Commensurate with these features are the genome size and the presence of two chromosomes with the same G+C content of *Brucella*, which is almost identical with its free-living plant and obligate intracellular alpha-proteobacteria relatives such as the free-living and opportunistic pathogen, *Ochrobacterium* (Michaux-Charachon *et al*., 1997).

Some of the orthologous characteristics identified as or predicted to be relevant to virulence did not probably undergo substantial modifications, as they are shared by the phylogenetic neighbours (Michaux-Charachon *et al*., 1997). Other modifications that are likely the result of the adaptive evolution to pathogenicity include several changes in the outer membrane features. These include changes in the lipopolysaccharide (LPS) lipid, a structure in *Brucella* which was not substantially modified with respect to that of the phylogenetic neighbours (Velasco *et al*., 2000). Similarly, the high conservation of the two-component BvrR/BvrS sensory regulatory system seen in *Brucella* and the striking difference in its periplasmic sensing region from those of the phylogenetic neighbours support the idea that this adaptation has preceded speciation. Also the type IV secretion system in plant endosymbionts functions in the establishment of the bacteroid condition,

whereas in *Brucella* they may be devoted to transference of molecules inside phagosomes for controlling intracellular trafficking (Moreno *et al*., 2002). By comparing dendrogram and phylogenies, it seems feasible to hypothesize that *B. abortus* and *B. melitensis* shared the same ancestor while *B. canis* and *B. suis* shared a common ancestor (Moreno and Moriyon, 2001). Based on nutritional requirements, *B. suis* is hypothesized the closest species to the *Brucella* ancestor (Plommet, 1991) and it is the most diverse of the classical brucellae in genomic structure and host preferences (Moreno and Moriyon, 2001). The strains of *B. suis* are the most closely related to *Ochrobacterium* (Malek *et al*., 2002) and the most diverse among all the *Brucella* species that parasitize terrestrial mammals and the most closely related to the heterotroph ancestor (Moreno, 2002).

2.6 Phylogenetic Position and Taxonomy of *Brucella*

The genus *Brucella* belongs to the order *Rhizobiales* within the class alpha-proteobacteria (Gupta, 2005; Williams *et al*., 2007). The family *Brucellaceae* consists of the genera that include *Brucella*, *Mycoplana* and *Ochrobacterium* according to the latest edition of Bergey's Manual of Systemic Bacteriology (Whatmore, 2010). However, in recent years, at least three additional genara, *Pseudochrobactrum* (Kampfer *et al*., 2006; 2007; Romanenko *et al*., 2008), *Daeguia* (Yoon *et al*., 2008) and *Crabtreella* (Xie and Yokota, 2006) with close phylogenetic relationships to *Brucella*, and which likely expand the family, have been identified. *Brucella* has been proposed to be a monospecific genus in which all types should be regarded as biovars of *Brucella melitensis* (Verger *et al.*, 1985; 1987) in view of their high degree of DNA homology (>90% for all species). This proposal has not yet been fully accepted and, therefore the old classification of the genus into six species, *Brucella melitensis, Brucella abortus, Brucella ovis, Brucella suis, Brucella neotomae, Brucella canis* (Corbel and Brinley-Morgan, 1984) is still in use worldwide. Studies with a limited number of strains of classical species and biovars

showed that they can be differentiated by restriction fragment length polymorphism (RFLP), analysis of whole DNA (Allardet-Servent *et al*., 1988; Jensen *et al*., 1999), the Bru-RS 2 elements (Halling and Bricker, 1995), the insertion sites of IS711 (Bricker and Halling 1994; Ouahrani-Betteche *et al*., 1996; Clavareau *et al*., 1998; Bricker, 1999), the REP and ERIC sequences (Mercier *et al*., 1996; Tcherneva *et al*., 2000) and DNA sites (Cloeckaert *et al*., 1996). *Brucella melitensis, Brucell abortus, Brucella suis and Brucella neotomae* occur in the smooth form while *Brucella ovis* and *Brucella canis* are being encountered in the rough form.

For the species infecting cattle and small ruminants, *Brucella melitensis* has 3 biovars; *Brucella abortus* has seven biovars (1-6 and 9) while *Brucella ovis* has one (rough) (Table 2.2). Species differentiation is routinely based on lysis by phages and simple biochemical tests like oxidase and urease tests. Differentiation at biovar level between *Brucella melitensis, Brucella abortus, and Brucella suis* is currently performed by four main tests which include CO_2 requirement, production of H_2S, sensitivity to thionin and basic fuchsin dyes and agglutination with monospecific A and M antisera (Alton *et al*., 1988). A recently developed agglutination test using latex beads coated with a pair of monoclonal antibodies directed against the rough lipopolysaccharide (R-LPS) and the 25 kDa outer membrane protein (OMP25) respectively (Bowden *et al.,* 1997) makes it possible to accurately differentiate *Brucella ovis* from *Brucella canis,* and the occasional rough isolates of the smooth *Brucella* species.

Table 2.2: Biovar differentiation of *Brucella* species involved in cattle brucellosis

Species	Biovar/ morphology	CO_2 Required.	H_2S Production	Dye sensitivity		Aggl. in Polyclonal antiserum		R-LPS	Latex mAb
				Thionin	B/fuchsin	A	M		OmP 25
B. melitensis	1	-	-	+	+	-	+	-	-
	2	-	-	+	+	+	-	-	-
	3	-	-	+	+	+	+	-	-
	R	+	-	+	-	-	-	+	-
B. ovis	R	+	-	+	-	-	-	+	-
B. abortus	1	+	+	-	+	+	-	-	-
	2	+(2)	+	-	-	+	-	-	-
	3	+(2)	+	+	+	+	-	-	-
	4	+(2)	+	-	+	-	+	-	-
	5	-	-	+	+	-	+	-	-
	6	-	-	+	+	+	-	-	-
	9	±	+	+	+	-	+	-	-
	R	+or- (3)	± (3)	± (3)	± (3)	-	-	+	+

Source: Alton *et al. (*1988)

2.7 Epidemiology of Brucellosis

2.7.1 Geographical distribution

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

2.7.2 Survival of *Brucella* in the environment

Under appropriate conditions, *Brucella* can persist in the environment for very long periods (Corbel, 1988). The survival of *Brucella* organisms in the environment plays an important role in the epidemiology of the disease. Temperature, humidity and pH influence the organism's ability to survivein the environment (Bercovich, 1998). The organisms resist drying, especially in organic substrates and can remain viable in the dust or soil for up to 10 weeks (Corbel, 1988). In damp soil, *Brucella* can survive for 60 days at 20^0C and 144 days at 40% relative humidity (Bercovich, 1998). In tap water, *Brucella* can survive for several months at 4-8^0C, 2½ years at 0^0C and several years in frozen tissues or medium (Seifert, 1996). In butter, *Brucella* survives for up to 4 months, 6 weeks in milk and 2 weeks in cooled meat and up to 30 days in ice cream (Seifert, 1996). The organism can survive for 30 days in urine, at least 75 days in aborted foetuses and more than 60 days in uterine discharges (Corbel, 1988; Bercovich, 1998). They can also survive in cattle faeces for at least 1 year and in liquid manure and frozen soil for 2½ years (Seifert, 1996). Contaminated straw with *Brucella* organisms remains infectious for more than 1 month (Weidmann, 1991). Seasonal climatic variations may play an important role in the persistence of brucellosis in communities.

Thus in situations of low temperatures, *Brucella* may persist for longer periods in the environment, leading to the risk of direct transmission (Zinsstag *et al.,* 2013). *Brucella* organisms are sensitive to direct sunlight, and under dry conditions, they survive only when embedded in proteins (Davies and Casey, 1973). They are sensitive to wide variety of disinfectants including strong acids and alkali, hypochlorites, iodophors, 1% Lysol and formaldehyde (Corbel, 1988; Seifert, 1996). However, the presence of organic matter and exposure to low temperatures drastically reduces the efficacy of these chemical disinfectants (Corbel, 1988). Pasteurisation of milk at 68^0C for 10-15 minutes can destroy the *Brucella* organisms (Merchant and Packer, 1975).

According to Corbel (1988), brucellae show high sensitivity to a wide range of antibiotics and chemotherapeutic agents including some penicillins, aminoglycosides, chloramphenicol, tetracyclines and sulphonamides. They are, however, resistant to vancomycin, bacitracin, polymyxins, and metronidazole, nalidixic acid and antifungal agents.

2.8 Transmission

In animals, brucellosis is usually a herd or flock problem. It is spread within the herd or flock primarily by ingestion of contaminated material (European Commision, 2001; Mcdermott and Arimi, 2002). Congenital (in-utero) or pre-natal infection may occur with the ensuing development of latent infections (Bale and Nuru, 2001). Spread within herd a usually occurs by the introduction of asymptomatic chronically infected animals (FAO, 2003).

In cattle, the excretion of *Brucella* may start 39 days after exposure (Phillipon *et al.*, 1970). Cow shade or pasture becomes contaminated after the infected animal aborts or has full-term parturition and this may continue for 15 days (FAO, 2003). . Although the

infectious materials from the genital tract usually clear after 2-3 months, the cattle becomes a carrier of *Brucella* and sheds it intermittently for many years (Herr *et al.*, 1990). In goats, excretion of the organism from the vagina is usually prolonged and copious (2 to 3 months) while in sheep, excretion is generally less prolonged, usually ceasing within 3 weeks after abortion or full-term parturition (Okoh, 1980). Shedding of *Brucella* is also common in udder secretions and semen, and may be isolated from various tissues such as lymph nodes, especially those associated with reproductive organs and from arthritic lesions (Alton *et al*., 1988). The persistent infection of the mammary glands and supra-mammary lymph nodes leads to constant or intermittent shedding of the organisms in milk in succeeding lactations. It provides an important source of infection for humans and young animals (Anon, 2001).

Susceptible animals become infected through consumption of infected materials or by aerosols as animals tend to lick aborted foetuses or the genital discharges of an aborting animal (Bercovich, 1998). Exposure to *Brucella* infection also occurs *in-utero* or when animals born to healthy dams are fed with colostrum or milk from infected dams (Catlin and Sheehan, 1986; Grillo *et al*., 1997; Bale and Nuru, 2001). Grazing animals on pastures where infected animals have grazed or coming in contact with infected premises or manure can lead to infection (Anon, 2001). Infected males shed the organisms in semen especially during the acute phase of the infection. Such males could transmit the infection to females through coitu (Bercovich, 1998). This is the reason why infected semen used for artificial insemination, could introduce brucellosis to a free herd (Bale and Nuru, 2001; Blood and Radostits, 1989). Dogs have been found to be mechanical and biological vectors of brucellosis (WHO, 1986), as they are involved in transportation of infected materials from one place to another thereby introducing the infection to new areas and also infecting themselves.

Transmission in man is principally by contact with infected materials such as carcasses, aborted foetuses, placentas, vaginal discharges, manure and urine (Anon, 2001). Man to man infection is very rare (Currier, 1989). Humans get infected through contact with infected animals or consumption of unpasteurized milk or milk products (Solare, 2010). Occupational groups like livestock farmers, farm workers, veterinarians, ranchers and abattoir workers are considered to be at higher risk where the route of infection can be via cuts or abrasions, inhalation of air droplets containing the oorganism or conjunctiva contamination by aerosols (Currier, 1989; Young *et al*., 2005; Tabak *et al*., 2008). Accidental self-inoculation with Strain 19 *Brucella abortus* vaccine or organisms in culture has been demonstrated (Weidmann, 1991).

2.9 Animal Reservoirs of *Brucella*

Epidemiological evidence shows that *Brucella* species usually have definitive host preferences (Bercovich, 1998), even though interspecies transmission does occur. For example, *Brucella abortus* is recognized as the main cause of contagious abortion in cattle but can also cause abortion in sheep, goats, dogs, horses, camels, buffaloes as well as wild animals that have contact with them (FAO, 2004). Serological and bacteriological studies show that cattle may acquire infection due to other *Brucella* species from other animals (Nicoletti, 1980; Corbel, 1988; Forbes, 1990; Weidmann, 1991). *Brucella melitensis* is highly contagious to goats and sheep, but can as well infect cattle, dogs, camels, and pigs (Zowghi and Ebadi, 1988). *Brucella suis* commonly infects pigs but may also infect other animals like cattle, horses, dogs, and wild animals. *Brucella ovis* infects sheep while *B. canis* infects dogs primarily. Wild animals like buffaloes, wild pigs, deers, foxes, rodents, bison, elk, and antelopes are susceptible to *Brucella* infection and may serve as reservoir for domestic animals (Boer *et al*., 1980;

Tessaro, 1986; Cook *et al.,* 1988; Davis, 1990; Ocholi, 1990) especially in nomadic Fulani herds than in intensive farming system.

2.10 Resistance to the Infection

Resistance to brucellosis is influenced by age, sex, breed and stage of pregnancy of affected animal. Young animals tend to be less susceptible to infection than mature animals (European commission, 2001). Data also indicate that pregnant animals are more susceptible than non-pregnant ones and males because gravid uterus sustains the organism due to the presence of erythritol, thus influencing the progression of the disease (Crawford *et al.,* 1990). It also appears that the younger the foetus is at the time of infection, the longer the incubation period (Radodtits *et al*., 2007). Data from naturally occurring and experimentally induced brucellosis suggest that natural resistance to brucellosis also influence the course and severity of the disease (Harmon *et al.,* 1989; Ho and Cheers, 1982).

2.11 The Disease in Cattle

Brucella infection due to *B. abortus* in cattle is similar to that of *B. melitensis* in sheep and goats. Nevertheless, differences are significant and each species of *Brucella* causes a different disease (OIE Manual, 1996). The organisms are facultative intracellular parasites of the reticutoenlothelial system. The virulence of *Brucella* varies considerably according to the species, strains and the size of infecting inoculum. Host susceptibility also varies and is associated with the reproductive status of the animal. Therefore in the field, all intermediate stages between typical acute infection and complete resistance may be observed and in addition, vaccinal immunity may modify the parasite-host relationship OIE Manual, 1996; Olsen, 2010).

2.11.1 Pathogenesis of the infection in cattle

The major route of infection is through mucous membranes of the oropharynx and upper respiratory tract or conjunctiva (Tabak *et al*., 2008). Another route is through the mucous membrane of the male and female genital tract. On entering into the body of the host, the organism encounters the cellular defences of the host but generally succeed in arriving via the lymph vessels at the nearest lymph node after escaping the cellular defenses (Ko and Splitter, 2003). The fate of the invading bacteria is mainly determined by cellular defences of the host chiefly macrophage and T-lymphocytes though specific antibody also plays a part (Radostits *et al*., 1997). The outcome depends on the ruminant species infected, age, immune status of the host, pregnancy status, and the virulence and the number of invading *Brucella* (Seifert, 1996).

When the bacteria prevail over the host's defences, a bacteraemia is generally established. The bacteraemia is always detected after 10 to 20 days and persists from 30 days to more than two months. If the animal is pregnant, bacteraemia often leads to the invasion of the uterus (Olsen, 2010). At the same time, infection becomes established in various lymph nodes and organs, often in the udder and sometimes in the spleen (WHO, 1986).

The major clinical sign in the first stage of the disease is abortion, but other signs due to localization of the organism may be observed ((Radostits *et al*., 1997). These signs include orchitis, epididymitis, hygroma, arthritis, metritis and subclinical mastitis among others (Radostits *et al*., 1997). However, numerous animals develop self-limiting infection or they may become asymptomatic latent carriers and potential excretors (WHO, 1986). Infection is not established if the female is exposed to the organism at the end of the pregnancy (Fensterbank, 1987). The second stage is characterized by

either elimination of *Brucella* or more frequently, by persistent inflammation of mammary gland and supramammary and genital lymph nodes, with constant or intermittent shedding of the organisms in milk and genital secretions (Fensterbank, 1987). Animals generally abort once during the mid- third of gestation but re-invasion of the uterus occurs in subsequent pregnancies with shedding in fluids and foetal membranes. The pregnancy can also get to full- term (Oyedipe *et al*., 1981). Females that are born into an infected area and get infected generally abort less than others. This explains the high level of abortions in newly infected herds and their relatively low frequency in herds where infection is enzootic (Radostits, *et al*., 2007). The udder is a very important predilection site for *Brucella* organisms. Infection in lactating, non-pregnant goats is likely to lead to colonization of the udder with excretion of *Brucella* organisms in the milk (Renoux *et al.,* 1953).

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

2.11.2 *Brucella melitensis* infection in sheep and goats

In endemic areas such as Mediterranean region, South West Asia, and parts of Latin America, brucellosis constitutes a serious zoonosis. Increased prevalence has been noted in areas with increased sheep farming (European Commission, 2001). Pathologically and microbiologically, *B. melitensis* infection in sheep and goats is very similar to *B. abortus* infection in cattle. Most breeds of goats are fully susceptible to the infection but

the susceptibility in sheep varies from one breed to the other (Radostits *et al*., 1997). For example the Maltese sheep are very resistant while the fat tailed sheep of South West Asia are very susceptible (Corbel, 2006). These form a reservoir of infection that give rise to widespread brucellosis outbreaks in man.

In most countries around the Mediterranean and South West Asia, the brucellosis problem largely affects sheep, while in Latin America; goats are chiefly involved (WHO, 1996). In goats, about two thirds of acute infections acquired naturally during pregnancy leads to infection of the udder and excretion of the bacteria in milk during subsequent lactation (Alton, 1985). Although a variety of symptoms have been described in artificially infected goats, abortion in females and orchitis in males appear to be the main symptoms seen in natural cases of brucellosis in small ruminants. High mortality rate in kids/lambs, mastitis and reduced milk production are other consequences of brucellosis in samall ruminants. The lesions on the placenta are more prominent with *B. melitensis* infection than when *B. abortus* is the causal organism. Following infection of *B. ovis* in small ruminants, epididymitis and inflammation of the vesicular glands are the most prominent symptoms in the male animals and these have a significant influence on the herd fertility (WHO, 1986).

2.11.3 Transmission and pathogenesis in sheep and goats

The pathogenesis and transmission in sheep and goats are generally the same as in cattle. Materials excreted from the female genital tract form the supply source of organisms for transmission to other animals. In goats, excretion of the organisms from the vagina is prolonged and copious. In both sheep and goats, the persistent excretion of *B. melitensis* in milk forms or provides an important source of human infection (FAO / OIE/ WHO, 2006).

2.11.4 *Brucella ovis* infection in sheep

Brucella ovis has greater affinity for the reproductive tract of the male than the female. Thus, epididymitis and sterility are the most frequent and serious consequences of infection with *B. ovis* (European Comission, 2012).

In rams, natural infection occurs over a long incubation period (6-17 weeks) during which time the animals are asymptomatic (European Commission, 2001). The organism remains confined to the exposure sites and regional lymph nodes for 10-14 days before the infection advances to the bacteraemic stage with a more generalised infection that involves the spleen, kidneys and lymph nodes distal from the exposure sites. During the later part of the bacteraemic stage, the infection begins to localise in the genital organs. The localised lesions appear in the seminal vesicles, ampullae, testes, tail of epididymis and then less frequently the head of the epididymis. Some organisms may remain in the kidney and such animals become shedders of *B. ovis* in their urine. These organisms can cause chronic interstitial nephritis in sheep (FAO / OIE / WHO, 2006).

In ewes under conditions stimulating natural infection, it was found that organisms disappear from the local site shortly after exposure, bacteraemia is prolonged and the organisms reappear in the genital tract at the third month of gestation (European Comission, 2001). Abortion occurs when there is sufficient accumulation of necrosis of the placenta and separation from the caruncles. The slow progress of the infection probably accounts for the frequent finding of positive complement fixation test (CFT) titres but infrequent occurrence of abortion either by experimental or natural infection (FAO / OIE / WHO, 2006).

Foetal lambs can survive long periods *in-utero* in the presence of the infection and the primary consequences of the infection is not abortion but a placentitis that interferes

with foetal nutrition and results in lambs with lowered birth weights. Rams are more susceptible than ewes and are more actively responsible for the transmission of the organisms as well as serving as reservoir for the maintenance of the disease. Contaminated semen from infected rams is the source of the organism for the ewes (FAO / OIE / WHO, 2006).

In breeding flocks, the organisms are spread from infected rams to non-infected rams through passive venereal transmission. This occurs when the infected ram mates with an ewe followed by a non-infected ram within the same oestrous cycle. Ram to ram transmission of infection can occur by direct contact or by housing a non-infected ram in a barn or shed previously occupied by an infected ram through the contamination of the barn by the urine from the infected ram (Radostits, 1989). Sodomy is the major source of spread in young rams. Young rams are also susceptible to infection with *B. ovis* and in some flocks, up to 80 per cent of unmated young rams have been found to have clinical and serological evidence of the infection (Radostits, 1989). Experimental transmission has been demonstrated (Anon, 2001). Other means of infection is through the nasopharynx since rams often muzzle the genital organs of other rams (Smith *et al*., 1972). Ewes are more resistant compared to rams. In rare cases, direct contact during abortion between ewes can lead to infection. Although following abortion or parturition, an infected ewe may excrete *B. ovis* for almost 10 days; it does not maintain the infection from one breeding season to the other. The ewe may however transmit the infection if she comes into oestrus. *B. ovis* infection in rams has a deleterious effect on their semen quality, breeding efficiency and breeding capacity (FAO / OIE / WHO, 2006).

In flocks where abortion has occurred, lamb yields have been known to drop from 100% to 25%. About 20% of the ewes may remain barren and up to 16% of lambs born alive die before six weeks of age. There is also a prolongation of the lambing period as a result of interference with normal breeding operation.

2.11.5 *Brucella abortus* infection in sheep and goats

Brucella abortus has predilection for uterus and placenta (Smith *et al.,* 1972; Blood and Radostits, 1989). This special predilection has been attributed to the presence of erythritol (in the placenta, uterus, cotyledons and chorions) which has been shown to stimulate the growth of *Brucella* both *in-vivo* and *in-vitro* (Smith *et al.* 1972; Corbel, 1988). Erythritol is utilized by *Brucella abortus* as an energy source and trace amounts can stimulate its intracellular growth (Corbel, 1988). Erythritol is present in the normal and infected placenta of cattle, sheep, goats, and swine as well as the seminal vesicles and testes of the males of these species, but has not been found in the human placenta or in that of rabbits, guinea pigs, and rats (Corbel, 1988). Thus, cases of abortion are very much reduced in humans and these laboratory animals. Erythritol favours the multiplication of *Brucella abortus* where it causes degeneration and necrosis of the cotyledons leading to abortion (Williams *et al.,* 1962; Smith *et al.,* 1972; Blood and Radostits, 1989). Following abortion, there is a further heavy invasion of the blood stream by the organism, which is followed by a rising titre. The stage in gestation when abortion occurs depends on the degree of infection, virulence of the *Brucella* and the stage of pregnancy when the infection occurs (Morgan, 1970). During pregnancy the disease is much more acute and tremendous multiplication occurs not only in the placenta but also in the lymph nodes leading to bacteraemia and vigorous antibody response (Corbel and Brinley-Morgan, 1984).

The pathogenesis of the disease in the males is similar to that in the females except that the seminal vesicles and testes are involved. The predilection site in the females is mainly the uterus and the presence of erythritol, (2R, 3S) - butane- 1, 2, 3, 4, tetraol , a low calorie sugar alcohol produced naturally by the developing foetus (Smith *et al.*, 1972; Radistits *et al*.,1997).

2.12 Clinical Manifestation of Brucellosis

The incubation period of brucellosis varies from a few days to several weeks. According to Seifert (1996), the incubation period varies from 14 to 120 days. The disease could run an acute or chronic course. When brucellosis is introduced into a clean herd of susceptible animals, it may run an acute course causing "abortion storm" in 50% or more of the pregnant animals. The incubation period is often tied up with the stage of pregnancy at the time of exposure (Seifert, 1996). In female animals, the major clinical signs are: abortion at third trimester of pregnancy, stillbirth, birth of weak offspring or dead full-term calves, retained placenta, infertility and sterility and decreased milk production (Corbel, 1988; Seifert, 1996; Anon, 2001). The aborted foetuses are covered by yellowish, slimy exudates. The placenta is oedematous and the cotyledons are covered with sticky odourless brownish exudates. Hygroma of the knee joint has been reported as a significant sign of brucellosis in the tropics, especially among nomadic animals (Corbel, 1988; Blood and Radostits, 1989). This occurs in both sexes as a result of chronic brucellosis (Fensterbank, 1987). In the male animals, the major signs include; orchitis, epididymitis, inflammation of the seminal vesicles and swelling of the scrotum (Seifert, 1996).

In sheep and goats, the disease is characterized by high mortality rate in lambs and kids, mastitis, reduced milk production, abortion, epididymitis, orchitis and arthritis.

Persistent infection of the supramammary lymph nodes is common in goats (Anon, 2001).

In horses, the disease is characterized by arthritis, fistulus withers and poll evil, bursitis and resultant lameness (Radostits *et al.,* 1997). In the mare, abortion and infertility may occur and the organisms have been isolated from mares which have aborted (Ray *et al.,* 1988; Ocholi *et al.,* 2004a). Some horses may present with general stiffness, fluctuating temperature and lethargy (Macmillan *et al*., 1985). Brucellosis in dogs is characterized by abortion and infertility in bitches, epididymitis and scrotal dermatitis in male dogs (Eze, 1978b; Okoh, 1978; Corbel, 1988).

In man, the disease is characterized by intermittent or irregular fever, weakness, depression, night sweating, anorexia, constipation, myalgia and stiffness of the vertebral column (Falade, 1974; Thornes and Morton, 1977; Currier, 1989 and Weidmann, 1991). The first report of orchitis in man caused by *Brucella abortus* biovar 1 was reported in Ecuador (Ron-Roman *et al*., 2012).

2.13 Pathology

In the pregnant animal, the disease is principally characterized grossly by necrosis of the placenta and ulcerative endometritis. The placental cotyledons are covered by sticky exudates, swollen and necrosed tissues (Radostits *et al*., 2007). The intercotyledonary areas are frequently thickened and opaque with a leathery appearance (Corbel, 1988). Histologically, the disease is characterized by marked leucocytic infiltration especially with leucocytes and plasma cells, congestion and cellular proliferation leading to necrosis (Radostits *et al.,* 2007).

Infection in foetal lungs produces bronchopneumonia with congestion, fibrinous exudation and cellular infiltration. In males the lesions are confined to the genital organs. The testes, seminal vesicles and the epididymis are enlarged and there may be multiple granulomas with infiltration of lymphoid and plasma cells in the epididymis (Smith *et al.,* 1972).

2.14 Immune Response

The chronicity of infection with *Brucella* reflects the fact that it is a facultative intracellular parasite capable of surviving within host cells following phagocytosis by adapting to intracellular conditions and overcoming the host's normal immune defences (Kohler *et al.*, 2002; Kohler *et al*., 2003; Baldwin and Goenka, 2006).The organism can enter and replicate in a variety of cells including epithelial cells, placenta trophoblasts, dendritic cells and macrophages (Gorvel, 2008). Pathogenesis of *Brucella* infection has been the focus of past reviews (Baldwin and Goenka, 2006; Guerra, 2007; Gorvel, 2008, Seleem *et al*., 2008).

Infection with *Brucella* usually results in the induction of both humoral and cell-mediated immune responses, in which magnitude of response is affected by various factors including virulence of the strain, size of inoculum, pregnancy, sexual and immune status of the host (Joint FAO/WHO Expert Committee on Brucellosis, 1986). Though the pattern of response has not been extensively studied in sheep and goats, information suggests that it is similar to that of cattle in which IgM is fast produced followed within 1-2 weeks by IgG. Both immunoglobulins fall to a low level in more chronic state of infection but IgG level is higher (European Commision, 2001).

Brucella melitensis Rev. 1 vaccine strain when used under standard conditions may induce a long lasting serological response to the agglutination test that seriously

interferes with serological screening for infected animals (Alton, 1990; Macmillan, 1990; Elberg, 1996). As no differences have been found between the diagnostic antigens from field strains of *Brucella melitensis* and those from Rev. 1 vaccine, serological test capable of distinguishing antibodies arising from infection and vaccination respectively has not been developed (Kolar, 1995).

Cellular defences of the host normally determine the fate of the invading bacteria especially macrophages and T-lymphocytes (Moreno-Lefont, 2003). The most widely used correlates of cell mediated immunity (CMI) are lymphocytes stimulation, macrophage inhibition, delayed-type hypersensitivity and Gamma interferon induction. The response can be expected as rapidly as few weeks just as in humoral immunity but could be variable and may not be detected. Studies in experimental models have provided important insight into host defences that eventually control infection with *Brucella* organisms. Serum complement effectively lyses some rough strains like those that lack the O-polysaccharide side chain on their LPS but little effect on smooth strains (those that have a long O-polysaccharide side chain).

Administration of antibody to mice prior to challenge with rough or smooth strain of *Brucella* was found to reduces the number of organisms that appeared in the liver and spleen. The effect is due mainly to antibodies directed against LPS with little or no contribution of antibodies directed against other cellular components (Montaraz and Winter, 1986). Reduction in intensity of infection in mice can be transferred from immune to non-immune animals by both cluster of differentiation CD4+ and CD8+ T-cells (Araya *et al.*, 1989) or by immunoglobulin fractions in the serum. Administration of antibodies to interferon gamma worsens the outcome in experimental infection (Zhan and Cheers, 1993). Moreover, macrophages treated with interferon *in-vitro* were seen to

inhibit intracellular bacterial replication (Moreno-Lefont, 2003). In ruminants, vaccination with killed vaccines has been shown to provide some protection against challenge but live vaccines are much more effective (Anon, 2001).

2.14.1 The role of T-cells in brucellosis

The major role of T-cells in *Brucella* immunity is secretion of interferon Gamma for the activation of bactericidal function of macrophages and cytotoxic T-lymphocytes activity as well as IgG_{2a} and IgG_3 isotope switching. The significance of CD4+ and CD8+ T-cells in *Brucella* immunity has been controversial (Araya *et al*., 1989).

In brucellosis, the different effectors of the immune system namely antigen presenting cells, NK cells, CD4, CD8 cells and B-cells act together to provide a coordinate response (Golding *et al.,* 2001). However, the primary mechanism of control involves cell mediated immunity, rather than antibodies although, some immunity against re-infection is provided by serum immunoglobulins (Cheers, 1984; Araya *et al.,*1989; Golding *et al.,* 2001; Young, 2005;). Large numbers of *Brucella* are present in the endothelial cells and chorions. Histologically, the disease is characterized by a marked leukocytic infiltration, with lymphocytes and plasma cells, congestion and cellular proliferation leading to necrosis (Laing, 1979).

2.14.2 Evaluating cellular immune response

T-cell counting is the standard method for evaluating the cellular immune response against intracellular pathogens such as *Brucella* (Celik and Akbutut, 2005). Previous studies on murine models have shown that cytokine secreting CD4+ T cells are the fundamental components of immunity against *Brucella*. Nevertheless, it is known that CD8+ cells are very important in protective immunity (Cheers, 1984). More recently, optimal protection against intracellular *Brucella* has been regarded as a coordinated

interaction between different T- cell subsets (Araya *et al.,* 1989). Earlier on, murine brucellosis studies indicated the importance of T- cell subsets in the resolution of infection but produced conflicting data suggesting that either CD4 and or CD8 T-cells were responsible for protection (Pavlov *et al.,* 1982; Araya *et al.,*1989; Meilke, 1991). In murine experiment, depletion of CD8 T-cells prevented the elimination of *Brucella* as did treatment with anti Thy 1.2 monoclonal antibodies, suggesting that CD8 T-cells are critical for eliminating intracellular infection (Mielke, 1991).

The paradigm of disease resolution was founded on adoptive T-cells transfer or *in-vivo* subpopulation depletion using monoclonal antibodies both with potential pitfalls caused by contamination by unwanted cell population or the unknown elimination of certain cells. To clarify the controversy over T-cell subsets responsible for *Brucella abortus* disease resolution, gene knock out mice have been used. As expected in an immune response to a complex bacterium, CD4 as well as CD8 T-lymphocytes recognize and proliferate to numerous antigens of *Brucella* (Cheers, 1984).

2.15 Diagnosis of Brucellosis

Diagnosis depends on a combination of clinical examination, bacteriological detection and serology:

2.15.1 Clinical signs

Brucellosis could be suspected in any herd with history of abortion during the third trimester of pregnancy, infertility, orchitis, epididymitis, stillbirths, neonatal mortality and hygroma (Poester *et al*., 2010). These signs are however not pathognomonic to brucellosis (Bercovich, 1998).

2.15.2 Culture, isolation and biotyping

The only 'gold standard' method for the diagnosis of brucellosis is the cultural isolation or detection of *Brucella* organisms from the infected host (Alton *et al.,* 1988; Smirnova, *et al*., 2013). This can be made by means of microscopic examination of smears stained with the modified Ziehl-Neelsen method from vaginal swabs, placenta, or aborted foetuses (Stamp, 1950). However, morphologically related microorganisms such as *Chlamydia psittaci* and *Coxiella burnetii* can mislead one in the diagnosis (Radostits *et al*., 1997; Garin-Bastuji, 2006).

Isolation of *B. abortus*, *B. melitensis* and *B. ovis* requires 50 ml of serum per litre of medium with addition of appropriate quantities of new born calf serum and antibiotics and incubation at 37^0C in 5-10% CO_2 or in the absence of CO_2 (for *B. melitensis*) in the atmosphere for growth of the organism (Garin-Bastuji *et al*., 2006). The suspect materials that are suitable for cultures are; semen from rams, and vaginal discharge, milk and aborted foetus and foetal membranes from female animals that have aborted or delivered lambs prematurely (Poester *et al*., 2010).

Vaginal swabs and milk from aborted animals are the best materials/samples for the isolation of *Brucella* species, while spleen and lymph nodes (iliac, mammary and pre-femoral) are the most reliable samples for isolation purposes in necropsied animals (Marin *et al.,* 1996a).

The Farrell's selective medium developed for the isolation of *B. abortus* from milk (Farrell, 1974) is also recommended for the isolation of *B. melitensis* and other species (Alton *et al.,* 1988). However, Nalidixic acid and Bacitracin concentrations in this medium may have inhibitory effects on some *B. melitensis* strains (Marin *et al.,* 1996a). Thus its sensitivity for the isolation of *B. melitensis* from naturally infected sheep is

sometimes lower than that obtained with the less selective Thayer – Martin's modified medium (Marin *et al*., 1996a). The sensitivity of bacteriological diagnosis is significantly increased by the simultaneous use of these two media (Marin *et al.,* 1996b). A novel isolation method which included homogenization and addition of homogenized tissues to an isolator (1.5ml microbial tube) (Oxoid, Basingstoke, England), and host cells lysed with saponin to release intracellular bacteria has been developed. The homogenized and lysed suspension was inoculated onto *Brucella* selective agar plates and incubated at 37^0C in 5-10% CO_2 for 5 days. This method yielded higher number of *Brucella* isolates than the conventional methods (Abril *et al*., 2011).

The identification of *Brucella* involves Gram's reaction, colonial and cellular morphology and routine biochemical tests (Corbel, *et al*., 1987). Species are distinguished on the basis of lysis by bacteriophages and oxidative reactions on amino acids and carbohydrate substrates. Typing of isolates into biovars is based on CO_2 requirement, sensitivity to thionin and basic fuchsin dyes, H_2S and urease production (Alton *et al.,* 1988).

2.15.3 Animal inoculation

Laboratory animals can be used for the diagnosis of brucellosis. Guinea pigs are reported to be the most susceptible to brucellosis than any other laboratory animal (Golding *et al*., 2001). However, mice and rabbits are also susceptible and may be used. Two guinea pigs per sample are recommended for inoculation (Morgan *et al.,* 1978).

A suspension of suspected material (foetal lungs, abomasal content, placenta, blood, uterine discharges, lymph nodes and milk) is injected intraperitonially or intramuscularly into guinea pigs. Such guinea pigs are then sacrificed and necropsied 3-

6 weeks after inoculation (Morgan *et al.,* 1978). If positive, *Brucella* organisms produce septicaemia in the guinea pigs (Smith *et al.,* 1972). The recovery of *Brucella* from the spleen or lymph nodes cultured on appropriate media and a positive SAT at 1/10 or above is evidence of infection (Morgan *et al.,* 1978; Alton *et al.,* 1988).

2.15.4 Serological diagnosis

Despite the development of numerous serological tests, no single test identifies all infected animals and a wide variation exists in estimates of their diagnostic accuracy (Abernethy *et al.,* 2012; Adone and Pasquali, 2013). Serological tests cannot differentiate between *Brucella* species and cannot therefore identify which species has induced host antibodies. Thus, only isolation of the species or specific DNA detection by polymerase chain reaction (PCR), allows identification of the infecting strain (Godfroid *et al.*, 2013; Plumb *et al*., 2013). Within the European Union, (EU), legislation prescribes the permitted tests which prior to 2006, included the serum agglutination test (SAT), complement fixation test (CFT), Rose Bengal plate test (RBPT), indirect enzyme-linked immunosorbent assay (iELISA), competitive enzyme-linked immunosorbent assay (cELISA) and ethylene diamine tetracetic acid (EDTA) test (Abernethy *et al*., 2012).

The current serological tests used for the diagnosis of *Brucella melitensis* and *Brucella ovis* in sheep and goats were initially developed for the diagnosis of *Brucella abortus* in cattle (OIE, 2012). Although not formally validated for use in sheep and goats, these tests, especially Rose Bengal Plate test, Complement fixation test and more recently ELISA, have been widely used for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; Macmillan, 1990). They are also the official tests for international trade (European Commission, 2002; OIE Collective Manual, 2004). The outer

membrane of the bacteria contains the main antigens involved in *Brucella* antibody detection. The antigenic suspensions (whole cell) used in both tests are made with A-dominant *Brucella abortus* biovar 1. Theoretically, infections due to M-dominant strains (i.e. *Brucella melitensis* biovar 1) are thought to be misdiagnosed (Alton *et al.,* 1988; Macmillan, 1990). However, existence of a common (C) epitope in the immunodominant S-LPS of *Brucella abortus* and *Brucella melitensis* can account for the high sensitivity of the *Brucella abortus* biovars 1 antigens to detect *Brucella melitensis* biovars 1 infections and vice versa (Macmillan, 1990; Diaz-Aparicio *et al.,* 1993). In fact, no significant differences have been found in the sensitivity of the classical *Brucella abortus* 1 RBPT antigen (AC) and ovine population infected either with *Brucella melitensis* biovars 1 (MC) or 3 (AMC) (Blasco *et al.,* 1994a; b). Moreover, the indirect ELISA (iELISA) sensitivity in sheep, goats and cattle is not affected by epitopic composition (AC or AM) of the antigens used (Alonso-Urmeneta *et al*., 1998). The immunoelectrophoretical pattern of cytoplasmic proteins, considered specific for the genus *Brucella* shows little differences between the several *Brucella* species when assayed with polyclonal sera (Diaz *et al*., 1968).

The ELISA test has been found to be highly sensitive and specific in brucellosis diagnosis (Ocholi, 1990) while plate and capillary agglutination tests have been used for the diagnosis of ovine and caprine brucellosis. Several factors influence the outcome of serological diagnosis of brucellosis such as long and variable incubation period (Smith *et al.,* 1972; Blood and Radostits, 1989) vaccination status (Morgan *et al.*, 1969) and type of exposure. The RBPT, CFT, iELISA and fluorescent polarization test (FPA) are suitable standard tests for diagnosis of brucellosis (OIE, 2012; Adone and Pasquali, 2013).

2.15.4.1 Rose Bengal plate test (RBPT)

This test was developed by Rose and Roekpe (1957) for the diagnosis of bovine brucellosis to differentiate specific *Brucella* agglutinins from non-specific factors. When the antigen was buffered at pH 4.0 they observed that agglutination of *Brucella abortus* cells by non-specific agglutinins of bovine serum was inhibited whereas the activity of specific *Brucella* antibodies was not affected. Despite the scanty and sometimes conflicting information available (Alton, 1990; MacMillan, 1990; Blasco *et al., 1994a; b*), this test is internationally acknowledged as the test of choice for the screening of brucellosis in cattle as well as in small ruminants (WHO, 1986; Garin-Bastuji and Blasco, 2004). However, the standardization conditions suitable for diagnosing cattle infection (MacMillan, 1990; European Commission, 2002; Garin-Bastuji and Blasco, 2004) are not adequate in sheep and goats (Blasco *et al* 1994a, b) and account for the low sensitivity of RBPT in small runinants (Blasco *et al*., 1994a). If the antigen is standardized differently, to give a higher analytical sensitivity, the diagnostic sensitivity to *B. melitensis* infection will be improved. The Rose Bengal test is based on the detection of specific antibodies of the IgM and IgG types but more effective in detecting antibodies of the IgG_1 type than the IgG_2 and IgM types (Levieux, 1974; Levieux, 1978). Although the low pH (3.65) of the antigen enhances the specificity of the test by inhibiting non-specific agglutinins (Corbel, 1997; Rose and Roekpe; 1957)**,** the temperature of the antigen and the ambient temperature at which the reaction takes place may influence sensitivity and specificity (Macmillan, 1990).

The RBPT could be modified for testing of sera in endemic, low prevalence areas to increase the sensitivity of the test. This simple modification is achieved by increasing slightly the amount of sera for the test dose from 25µl to 75µl, at the same time maintaining the antigen volume at 25µl. This results in significantly increase in the

sensitivity of the test without affecting the specificity (Blasco *et al*., 1994a; Ferreira *et al*., 2003).

2.15.4.2 Serum agglutination test (SAT)

The original form of the SAT was established by Huddleson and Abell in 1928 and then Welch and Mickle in 1933 who prepared the antigens. The SAT has been the most widely used serological test for the diagnosis of brucellosis in animals and man. The microtitre SAT (MSAT) has been used as a second serological test for the diagnosis of bovine brucellosis (Herr, *et al*., 1982). Serum agglutination test has been used for the control and eradication of brucellosis in most countries of the world such as Britain, United States of America, Canada, Israel and Germany among others (Nicoletti, 1969; Morgan, 1977; Stemshorn *et al.*, 1985; Dahoo *et al*., 1986). The SAT contributed to the international harmonization of brucellosis control and eradication programme, although it is not the most sensitive. The test measures agglutinating antibodies of the IgM, IgG_1, IgG_2, IgA types (Levieux, 1974) but the IgM types are more reactive (Bercovich, 1998). Thus it is superior to the CFT in the diagnosis of early phases of infections as it has the capacity to detect IgM which predominates at the beginning of the infection (Chappel, 1989). Since SAT may yield both false positive and false negative results, it effectively detects brucellosis only on a herd basis (Corbel *et al.*, 1984a; Nielsen *et al*., 1988). The SAT has been widely used for the serological tests in Nigeria (Ajogi *et al.,* 1996; Olayinka and Ogundipe, 2001; Ojo *et al*., 2007; Bertu *et al.,* 2010b; Gusi *et al*., 2010).

2.15.4.3 Immunochromatographic lateral flow assay

The *Brucella* lateral flow assay is a one-step immunochromatographic lateral flow assay, (Smits *et al*., 2003; Smits, *et al*., 2009; Franco *et al*., 2007). It is made up of a porous nitrocellulose detection strip flanked at one end by a reagent pad and by an

absorption pad at the other end. A sample application pad flanks the reagent pad in turn. The composite detection strip is contained in a plastic assay device with a round sample well positioned above the sample application pad and a test window positioned above the detection zone. The detection zone contains two distinct lines, a test line and a control line. The test line was obtained by spraying *Brucella*-specific antigen onto the nitrocellulose strip. A crude *Brucella* lipopolysaccharide (LPS) preparation prepared from a culture of *Brucella abortus* strain 1119-3 was used as antigen immobilized in a discrete line on the porous nitrocellulose membrane located in the test zone to capture specific antibodies. It utilizes a dried detection reagent deposited within the device. The mobile detection reagent consists of anti-IgG total antibodies labelled with red colloidal gold particles. The assays for the different animal species differ in the species specificity of the conjugate (Smits *et al*., 2009).

To perform the assay, a serum or whole blood is placed in the sample port. Running fluid (supplied with kit) is added to solubilise the detection reagent and to carry the detection reagent together with antibodies present in the sample through the porous nitrocellulose membrane of the test strip. Brucella- specific antibodies attach to the LPS antigen line in the test zone in the kit and these antibodies will be stained by binding of the detection reagent.

2.15.4.4 Milk ring test

Milk Ring Test (MRT) was first developed by Fleischner in 1937 for detecting the presence of *Brucella* antibodies in milk using stained standard *Brucella* antigen (Morgan, 1967). It is a valuable screening test both in the periodic testing of brucellosis–free dairy herds and in locating potentially infected herds (Corbel, 1988). The diagnostic significance of MRT is based on the presence of lacteal anti-*Brucella* IgM and IgA bound to milk fat globules, which rises to the cream layer of the milk. The

Brucella antigen is stained blue with haematoxylin. When the stained antigen is added to the milk, a stained antigen-antibody complex is formed (if antibody is present) which adheres to the fat globules and rise to form a blue coloured cream layer seen as a ring at the top of the tube. If there are no antibodies in the milk, the stained *Brucella* antigen remains in suspension in the milk column (Morgan *et al.*, 1978).

2.15.4.5 Complement fixation test (CFT)

Complement fixation test is the most widely used confirmatory test and it a recommended by OIE (Garin-Bastuji *et al*., 2006)**.** As in cattle brucellosis, there is agreement that this test is effective for the serological diagnosis of brucellosis in sheep and goats despite the complexity and the heterogeneity of the techniques used in different countries, (Farina, 1985; Alton, 1990; Macmillan, 1990). The CFT is based on the detection of specific antibodies of the IgM and IgG_1 that fix complement (Levieux, 1974). It is highly specific but laborious and requires highly trained personnel as well as suitable laboratory facilities. Its specificity is very important for the control and eradication of brucellosis but may test negative when antibodies of the IgG_2 type hinder complement fixation (Macmillan, 1990).

2.15.4.6 Antiglobulin (Coombs) test

This test is an extension of SAT, used for the detection of incomplete, blocking or non-agglutinating IgG (Alton *et al.,* 1988). Those SAT tubes containing serum dilutions and whole *B. abortus or B. melitensis* cells (as antigens) that showed negative reaction after incubation for 24 hours are centrifuged, the supernatant decanted and the cell pellets resuspended and washed with phosphate buffered saline. This is repeated three times. Species- specific antiglobulin reagent (anti-IgG) is added to the last pelleting in each test tube. The pellet is resuspended and incubated in a water bath at 37^0C for 24 hours

(Araj, 2010). Agglutination can be determined visually as for SAT by using an agglutinoscope or a drop on a slide examined under the microscope. The test is good for complicated and chronic cases and misses around 7% of cases compared with ELISA (Araj and Awar, 1997).

Coombs test detects incomplete *Brucella* antibodies of the IgG_2 type and it is used to confirm SAT results. Although the test is laborious, it is particularly important when the SAT and CFT results are negative or inconclusive (Kiss, 1971). The main limitation of this test is that the test results are indicative of infection only when its titres are at least two times the titre of the SAT.

2.15.4.7 Skin hypersensitivity test

Data from countries that have eradicated brucellosis indicate that only testing with several serological tests identifies infected herds (Stemshorn, 1985; Dahoo *et al.,* 1986; WHO, 1986; Weidmann, 1991). However since the reliability of serological tests depend on the presence of antibodies; infected animals with low antibody will not be detected. To improve on brucellosis diagnosis therefore, researchers have used various brucellins to elicit an allergic response in infected animals (Fensterbank, 1978; Cunningham *et al.*, 1980; Chukwu, 1985).

The test involves injecting the allergin into the flank or intra-palpebrally and measuring the thickness of the skin (Weidmann, 1991). The test is of great importance in animals with latent infection of *Brucella* in areas with low prevalence and areas known to be free from brucellosis (Bercovich, 1998).

2.15.4.8 2-Mercaptoethanol test (2-MET)

This test is aimed at eliminating IgM but IgG is not affected (Morgan *et al.*, 1967). It is done by adding equal volumes of 0.2M Mercaptoethanol to serum diluted 1:5 and conducting a serial dilution tube test (Morgan *et al.,* 1978). Seifert (1996) reported an observation that the activity of IgM is destroyed after the serum has been treated with Mercaptoethanol. The test is a simple modification of the tube agglutination tests (TAT) in which the non-specific IgM antibodies are eliminated. It detects chronic infections with fewer false positives. Diagnosis of brucellosis is established with 2-ME titre $\geq$ 1:80 while titres < 1: 80 are considered negative. The 2-mercaptoethanol and the Rivanol tests detect specific IgG (Rossi and Cantini, 1969) and are usually used to differentiate between infected and vaccinated cattle.

2.15.4.9 Brucellacapt test

This test is based on an immuno-capture agglutination technique and in a single step detects non-agglutinating IgG and IgA antibodies as well as agglutinating antibodies. The test is performed according to manufacturer's instructions: a specified volume of each serum dilution is added to a microplate with U shaped wells pre-coated with specified anti-immunoglobulin. Then a whole cell antigen suspension is added. The plate is incubated for 18-24 hours at 37^0C before reading visually. Positive reactions show agglutination over the bottom of the well. Negative reaction presents a pellet in the centre of the bottom of the well (Orduna *at al*., 2000). Brucellacapt offers a valuable alternative to Coomb's test, since it is performed in just a single step. It shows similar sensitivity, specificity and similar performance with Coomb's test but it is more rapid and less cumbersome to carry out compared to Coomb's test and CFT. Another

advantage of this test is its ability to diagnose complicated and chronic cases cases of brucellosis (Orduna *at al*., 2000).

2.15.4.10 Enzyme-link immunosorbent assay (ELISA)

The most widely used enzyme immunoassay in detecting brucellosis is indirect ELISA (iELISA). It is the test of choice for complicated, focal and chronic cases, especially when other tests are negative in cases that are under high clinical suspicion (OIE, 2004). It can reveal total and individual specific immunoglobulins (IgG, IgM and IgA) rapidly in 4 to 6 hours with high sensitivity and specificity (Araj and Azzam, 1996). The method involves binding of the antigen on a solid phase usually a polystyrene microtitre plate so that antibody if present in a sample, binds to the immobilized antigen-enzyme conjugate which in combination with a chromogenic substrate gives a coloured reaction indicative of the presence of antibody in the sample. It is this method that is now familiar to most researchers. The test is carried out in a 96-well microtitre plate that is pre-coated with pre-determined *Brucella* antigen (whole-cell or purified LPS). A standardized volume and dilution of serum are added to react with the antigen in the wells and the plates are incubated and washed. An enzyme- conjugate antispecies IgG, IgM, IgA is then added to designated wells. After addition of enzyme substrate and incubation, the optical density of the wells can be read at the appropriate enzyme wavelength. Cut-off values for seropositive samples can be extrapolated from incorporated negative and positive controls and or standardized assay.

The indirect ELISA, using more or less purified S-LPS of *Brucella* as antigen and polyclonal conjugate (anti-IgG H+L) has been reported to be sensitive enough for the diagnosis of infection in cattle, sheep and goats (Jimenez de Bagues *et al.,* 1995; Diaz-Aparicio *et al.*, 1994; Blasco *et al.,* 1994b; Delgado *et al.*, 1995). While the use of

ELISA for diagnosis of bovine brucellosis is well developed, more extensive field trials are required to validate the test for use in sheep and goats.

As mentioned before, the use of a purified periplasmic protein (26kDa) in iELISA or c-ELISA protocols could be useful for differentiating infected from vaccinated animals (Debbarh *et al.*, 1996).

2.15.4.11 Native hapten gel precipitation test (NHGPT)

In case of recent vaccination with S19, only the gel diffusion (GD) or radial immunodiffusion (RID) tests with native hapten antigen (Diaz *et al*., 1979) and the cELISA (Nielsen *et al*., 1996) appear to be useful for diagnosis, allowing differentiation of infection by *Brucella abortus* from S19 vaccination. However, only the gel difusion with NH (Diaz *et al.,* 1979) was able to discriminate, at a given time after vaccination, the immune responses of *Brucella melitensis* infected from vaccinated sheep (Marin *et al.,* 1999).

2.15.4.12 Fluorescence polarization assay (FPA)

This test, already regarded as an official test for the diagnosis of bovine brucellosis by the (McGiven *et al*., 2003; OIE, 2004), is currently under evaluation in sheep and goats (Garin- bastuji *et al*., 2006).

2.16 Molecular Typing

2.16.1 Pulsed-field gel electrophoresis

Early characterization of the genomes of *Brucella* took advantage of pulsed-field gel electrophoresis (PFGE) where infrequently cutting restriction enzymes are used to generate high molecular weight fragments. These are then separated in a size-dependent

manner with the relative orientation of the gel and electric field being periodically altered to allow efficient fractionation of large fragments. Initial studies showed that all five species of *Brucella* tested gave distinct profiles when digested with XbaI although biovars could not be distinguished (Allardet-Servent *et al.,* 1988).

Later studies used PacI and SpeI to confirm that PFGE profiles and physical maps of all *Brucella* species while highly conserved, were distinguishable with the exception of the *B. suis* and *B. canis* reference strains. This confirmed earlier observations (Michauxet *et al*., 1993) showing that all six classical species reference strains harbour two chromosomes (Michaux-Charachon *et al*., 1997).

PFGE-based approaches were thus invaluable in understanding genome structure. However, unlike many other bacterial groups where PFGE is a widely used diagnostic and epidemiological tool (Swaminathan *et al*., 2006), PFGE has not found widespread use as a routine typing tool for *Brucella*. This likely reflects the very limited diversity identified at the sub-species level. The application of this technique has been limited to small studies examining areas such as minor variants of *Brucella ovis* (Ridler *et al*., 2005), differentiating a vaccine strain from field isolates (Jensen *et al.*, 1995) and differentiating marine mammal isolates from porpoises, seals and dolphins (Jensen *et al*., 1999; Bourg *et al*., 2007).

2.16.2 Insertion sequence- based typing

Insertion sequences (IS) are short DNA sequences that can transpose between prokaryotic genomes causing mutations and genomic rearrangements. Reflecting their tendency to insert randomly and occupy multiple genomic locations IS elements can be used as probes for discrimination between isolates. In many bacteria, IS localization is sufficiently stable to facilitate the use of this approach for typing and epidemiological purposes. In *Brucella,* an insertion sequence IS711 (Halling *et al*., 1993), also known as

IS6501 (Ouahrani *et al*., 1993), appears to represent a major source of diversity in the genus. The IS711 copy number varies greatly from around 6 to 12 in most *Brucella* species through to >25 in *Brucella ovis* and marine mammal *Brucella* (Ouahrani *et al.,* 1993; Bricker *et al*., 2000). Characteristic IS711 profiles can be generated by probing genomic digests with a labeled IS711 probe and, in the presence of a suitable database; these can be useful for identifying members of particular species. However, there are some difficulties as reference strains of different species, such as *Brucella abortus* biovar 3, *Brucella suis* biovars 3 and 4, and *Brucella canis*, share identical profiles (Ouahrani *et al*., 1993) and, while there is evidence of limited subspecies level diversity, no examination of the usefulness of the approach using a large number of isolates has ever been published. Two additional repeated DNA elements of 103 and 105 bp have been reported in *Brucella* and designated Bru-RS1 and Bru-RS2 (Halling and Bricker, 1994). Hybridization patterns for the elements among single isolates of the six 'classical' species were unique but no further investigation of these elements as typing tools has been reported.

2.16.3 Polymerase chain reaction (PCR)

Although culturing is a specific method and is confirmatory, its sensitivity depends on the viability and numbers of *Brucella* within the sample, the nature of the sample (foetal organs, foetal membranes, lymph nodes, etc.) and the number of specimens tested from the same animals (Hornitzky and Saerson, 1986). The time required for culturing field specimens can be long and tissues or fluids that are only contaminated with low number of *Brucella* may not be detected. Therefore in the case of fluids or tissues contaminated with nonviable or a low number of *Brucella*, PCR could be a potentially useful method for the diagnosis of brucellosis. Several authors reported good sensitivity of PCR for detection of *Brucella* DNA from pure cultures (Baily *et al.,* 1992; Romero *et al.,* 1995;

Da Costa *et al.,* 1996). Others showed that PCR could be a potentially useful tool when used alone or in combination with labelled probes to differentiate some *Brucella* species and biovars (Mercier *et al.,* 1996; Ouahrani-Betteche *et al*., 1996).

The possibility of PCR techniques to detect the DNA of dead bacteria, or in pauci-bacillary samples and even in samples highly contaminated with other microorganisms, could increase the rate of detecting animals infected with *Brucella*.

In recent years, a number of assays have been developed employing strain-specific targets, especially the IS711 element described above, to differentiate *Brucella* mostly to the species level (Tcherneva *et al.,* 1996). Unlike the previous applications many of these have found widespread use in diagnostic laboratories worldwide. Historically, the most widely used assay is known as the AMOS-PCR, named after the *Brucella* species it can identify (*Brucella abortus* biovars 1, 2, and 4, *Brucella melitensis, Brucella ovis* and *Brucella suis* biovar 1) (Bricker and Halling, 1994). The assay makes use of one common primer anchored in the IS711 element as well as a species-specific primer that binds to unique sequence flanking that insertion site. This results in species discrimination due to different amplicon sizes. The assay was subsequently modified to include the vaccine strains S19 and RB51 (Bricker and Halling, 1995). This is a significant improvement, given the critical need to distinguish field and vaccine isolates in eradication programmes (Bricker, 2002) and some later modifications to primers were reported to improve performance (Ewalt and Bricker, 2003; Bricker, 2002). However, despite some later additions (Ocampo-Sosa *et al.,* 2005) this assay still has the disadvantage that it fails to detect all species or all biovars of some species.

The recent availability of genome data has enabled the development of a new generation of multiplex PCR assays with wider scope. 'Bruce-ladder', a single tube PCR assay that

can differentiate all the six classical species, *Brucella* isolates from marine mammals and the vaccine strains *Brucella abortus* RB51 and S19 and *Brucella melitensis* Rev 1, was recently described (Garcı´a-Yoldi *et al*., 2006). The test uses eight primer pairs designed based on species-specific differences identified historically or by genomic approaches (Sangari and Aguero, 1994; Vizcaino *et al.,* 1997; Vemulapalli *et al*., 1999; Cloeckaert *et al.,* 2000; Cloeckaert *et al*., 2002; Rajashekara *et al*., 2004; Garcı´a-Yoldi *et al*., 2005; Halling *et al*., 2005; Rajashekara *et al*., 2008) to generate amplicons of different sizes resulting in a unique profile for each species following agarose gel electrophoresis. A large validation exercise has recently been published examining over 600 strains and showed that the assay is very effective with the exception of *Brucella canis* where almost half of the isolates examined were erroneously identified as *Brucella suis* (Lo´pez-Goni *et al*., 2008).

A similar multiplex approach based on species-specific differences was recently described as being able to distinguish among the six classical species (Hinic´ *et al*., 2008). However, this is not as comprehensive as 'Bruce-ladder'. Also, it has some problems with *Brucella canis* and *Brucella suis* differentiation as it reported identical profiles for *Brucella suis* biovar 4 and *Brucella canis* reference strains. It has also not been as rigorously validated as 'Bruce-ladder. Ratushna *et al*. (2006) also recently reported the identification of molecular targets for the identification of *Brucella* species based on genomic comparisons. The report has identified a series of PCR primers that can be used in a stepwise manner to differentiate all the classical *Brucella* species. This includes a primer pair that differentiates the *Brucella suis* biovar 4 reference strain from *Brucella canis*. Once again, however, this lacks the scope of 'Bruce-ladder' failing to include marine mammal *Brucella* or differentiation of vaccine strains and no extensive validation using field isolates has yet been reported.

In addition to the multiplex PCRs discussed above, some single target PCRs have proven particularly useful. Of note here is the so called bp26 PCR which utilizes a copy of IS711 located downstream of the bp26 gene in marine mammal isolates to distinguish marine mammal *Brucella* from those associated with terrestrial mammals (Cloeckaert *et al.,* 2000). This is in fact the marker for marine mammal isolates utilized in 'Bruce-ladder' but has proven useful as a stand-alone assay to distinguish marine mammal *Brucella* in a number of studies and notably as an unexpected cause of human infection (Sohn *et al*., 2003; McDonald *et al*., 2006). Additional useful PCRs applied particularly to *Brucella* from marine mammals are a series of four specific PCR reactions based on fragments identified by infrequent-restriction site PCR (Cloeckaert *et al*., 2003). All the four PCRs are specific for *Brucella* from marine mammals but it divided them into one profile specific for *Brucella pinnipedialis* and two profiles specific for *Brucella ceti*.

2.16.4 Restriction fragment length polymorphism-based approaches

One common typing approach is the use of restriction fragment length polymorphism (RFLP) applied to genes known to be polymorphic in *Brucella* (Al Dahouk *et al*., 2005a). This approach has particularly been applied to various outer membrane protein (omp) encoding genes and has become a popular method for differentiation of *Brucella* species and for descriptions of new strains (Viscaino *et al*., 2000; Bricker, 2002). Much work has focused on the omp2 locus originally characterized by Ficht *et al*. (1989) and shown to consist of two genes designated omp2a and omp2b, displaying around 85% sequence identity, and arranged in opposite orientations. Further studies indicated the presence of species-specific polymorphisms that have been exploited to develop PCR-RFLP approaches (Ficht *et al.,* 1990; 1996). Cloeckaert *et al*. (1995) used these changes to distinguish *Brucella* species, with the exception of *B. abortus* biovars 3, 5, 6 and 9 from some *Brucella melitensis* strains and *Brucella canis* from *Brucella suis* biovars 3

and 4. Furthermore, species-specific markers were identified in omp25 including a marker for *Brucella melitensis* in the form of absence of an EcoRV site though *Brucella suis* biovars 3 and 4 and *Brucella canis* could still not be distinguished (Cloeckaert *et al*., 1995). Other omp genes examined include omp31, known to be deleted in *Brucella abortus* (Vizcaino *et al*., 1997), but which has markers for *Brucella canis*, *Brucella suis* biovar 2 and *Brucella ovis* (Garcı´a-Yoldi *et al.,* 2005). DNA polymorphism at the omp2 locus was also instrumental in the suggestion of the division of the new marine mammal *Brucella* isolates into two species (Cloeckaert *et al.,* 2001).

2.16.5 Tandem repeat- based typing

Tandemly repeated sequences display allelic hypervariability related to variation in the number of repeats caused by slipped strand mispairing or replication slippage (Levinson and Gutman, 1987) or DNA recombination between homologous repeat sequences (van Belkum *et al*., 1998). These markers are known as variable number of tandem repeats (VNTRs) and are considered High - speed molecular clocks (van Belkum, 1999). Schemes based on such markers are sometimes referred to as MLVA (multilocus VNTR analysis) and they have proven particularly useful for discrimination of bacterial species that, like *Brucella*, display very little genomic diversity as is seen in *Bacillus anthracis* (Keim *et al.,* 2000), *Yersinia pestis* (Klevytska *et al*., 2001) and *Mycobacterium tuberculosis* (Mazars *et al.,* 2001).

The first application of VNTR based typing to *Brucella* was the so-called 'HOOF-Prints' scheme (Hypervariable Octomeric Oligonucleotide Finger-Prints) as reported by Bricker *et al*. (2003). The approach was based on a comparison of the newly completed genome sequences of *Brucella suis* and *Brucella melitensis* (DelVecchio *et al*., 2002; Paulsen *et al*., 2002) along with a draft *Brucella abortus* sequence which identified an eight base pair tandem repeat sequence at nine distinct genomic loci. Eight of the nine

loci were variable among the three genome sequences. Thus, allowing the development of a PCR based method to identify the number of repeat units at each locus. The technique successfully differentiated all type strains for all *Brucella* species and biovars. It also differentiated among unrelated *Brucella abortus* biovar 1 field isolates while isolates from the same herd or from short term *in vitro* passage showed little or no variation. In the following year, two additional VNTR typing schemes for *Brucella* were published. One scheme included eight of the original 'HOOF-Prints' loci as well as an additional 13 newly described VNTR loci to give a 21 locus scheme, VNTR-21 (Whatmore *et al.,* 2006). In contrast to the original 'HOOF-Prints' scheme, this approach includes loci with a much broader range of evolutionary speeds. At around the same time a scheme labelled MLVA-15 was published (Le Fle`che *et al.,* 2006), taking a very similar approach. The unprecedented level of discrimination offered by VNTR based approaches has meant they have rapidly become a method of choice in the field.

The highly discriminatory multilocus variable number tandem repeats (VNTR) analysis (MLVA) allows both species delineation and differentiation of individual isolates and thus represents a perfect first-line tool for molecular epidemiological studies within outbreaks investigations (Scholz and Vergnaud, 2013). Both 'HOOF-Prints' (Valdezate *et al.,* 2007) and MLVA-16, a slight modification of MLVA-15, (Al Dahouk *et al.,* 2007b; Kattar *et al.,* 2008) have been used to recognize human outbreaks that relate to a common source or to confirm relapse (Al Dahouk *et al.*, 2005b). MLVA16 has also been used to confirm the source of a laboratory infection (Marianelli *et al*., 2008). It has also been used to demonstrate heterogeneity in profiles even in a restricted area of endemicity (Marianelli *et al*., 2007) and identify that human *Brucella melitensis* isolates from Peru form a distinct cluster from previously described European isolates (Smits *et al.,* 2009). MLVA-16 typing of 295 marine mammal Brucella isolates from different

geographical regions identified 7 major groups within Brucella ceti and Brucella pinnipedialis (Marquart *et al*., 2009). MLVA-15 has also been used to assess the [

-stability of a live vaccine (Garcı´a-Yoldi *et al.*, 2007a) and to show that wild boar and domestic pigs sharing localities can have identical *B. suis* genotypes (Garcı´a-Yoldi *et al.,* 2007b). The 'minisatellite' elements of both VNTR-21 and MLVA- 15 mean they have also found use as tools at the taxonomic level. Thus VNTR-21 has been used to help identify at least three distinct groups within the marine mammal *Brucella* that are inconsistent with the currently accepted species and to identify a particular genotype of marine mammal *Brucella* associated with human disease (Groussaud *et al.,* 2007; Whatmore *et al*., 2008). Likewise MLVA-15 has added to the body of evidence for identification of the recently described *Brucella* species such as *B. microti and B. inopinata* (Scholz *et al.,* 2008a).

2.16.6 Multilocus sequencing

Multilocus sequencing has become the major approach applicable to studying the global epidemiology of bacteria and is also frequently used for phylogenetic studies. In its classical form multilocus sequence typing involves the sequencing of short fragments (c. 500 bp) of a number of housekeeping genes which are subject to purifying selection and slow evolution and within which variation is nearly neutral (Enright and Spratt, 1999). While there are few polymorphic sites in individual housekeeping genes, the use of combined sequences of multiple housekeeping genes can give high discriminatory power while retaining signatures of longer term evolutionary relationships. It also buffers against potentially skewed evolutionary pictures obtained by single-locus analyses (Margos *et al.,* 2008). The only application of multilocus sequence analysis published to date in this field examined nine discrete genomic loci, equating to 4.396 bp from 160 *Brucella* isolates of all species and biovars known at the time (Whatmore *et*

al., 2007). The study confirmed the genetic uniformity of *Brucella* with only 1.5% of sites found to be polymorphic across all 160 isolates. In all, 27 distinct sequence types (STs) were identified and concatenated sequence data were used to construct an unrooted neighbour-joining tree representing the relationships between STs. This showed that the four previously characterized classical *Brucella* species*, B. abortus, B. melitensis, B. ovis and B. neotomae* corresponded to well-separated clusters. With the exception of biovar 5, *B. suis* isolates cluster together, although they form a more diverse group than other classical species with a number of distinct STs corresponding to the remaining four biovars. *Brucella canis* isolates are located on the same branch very closely related to, but distinguishable from, *B. suis* biovar 3 and 4 isolates. Marine mammal isolates represented a distinct, though rather weakly supported cluster (Whatmore, 2009).

2.16.7 Single nucleotide polymorphism (SNP) typing

An additional approach to typing has been developed on the basis of multilocus sequence data as, for bacteria with a clonal population structure such as *Brucella*. SNPs can be used to accurately describe the phylogenetic framework of a species. An attractive typing approach is thus to use a rapid SNP based assay to identify a broad grouping (species) (Whatmore, 2009) while tandem repeat based typing can provide finer resolution in a hierarchical typing scheme such as proposed for *Bacillus anthracis* (Keim *et al*., 2004), an organism similarly genetically conserved. Two alternative approaches have been used both of which will identify any *Brucella* isolate as a member of one of the six classical species or as a marine mammal *Brucella* isolate. While an initial publication (Scott *et al*., 2007) described a primer extension-based approach to identify SNPs, two later publications (Gopaul *et al*., 2008; Foster *et al*., 2008) described

an approach based on Minor Groove Binding protein (MGB) probes applied on a real-time PCR platform. The real-time platform has certain benefits of speed and technical simplicity with the advantage that amplification and detection occur concurrently rather than in individual steps.

2.16.8 Methods for differentiating vaccine strains

In addition to the enhanced *abortus, melitensis, ovis and suis* (AMOS) and the recently developed 'Bruce-ladder' multiplex PCR (Lopez-Goni *et al*., 2008) and SNP typing approaches described above (Whatmore, 2009), there are a number of stand-alone molecular assays that have traditionally been used to differentiate vaccine isolates. The resistance of *Brucella melitensis* Rev.1 to streptomycin is attributed to a point mutation in rpsL and this is the basis of a PCR-RFLP method to differentiate this vaccine from field strains (Cloeckaert *et al*., 2002; Whatmore *et al*., 2005). Differentiation of *Brucella abortus* RB51 from field isolates uses a differential PCR based on the insertion of an IS711 copy into the wboA gene (Vemulapalli *et al*., 1999; Conde-Alvarez *et al*., 2013). Differentiation of *Brucella abortus* S19 from field strains relies on a differential PCR based on a 702 bp deletion within the eryCD locus (Sangari and Agu¨ ero, 1994), even though this deletion is apparently not conserved in all S19 variants (Mukherjee *et al.,* 2005).

To date, at least 400 reports have been published dealing with various PCR-based methods for *Brucella* detection and differentiation (Scholz and Vergnaud, 2013) and further details are contained in the report by Yu and Nielsen (2010).

2.17 Prevention of Brucellosis

Currently, three vaccine strains (*Brucella abortus* S19, RB51 strains and *Brucella melitensis* Rev.1 strain) are recommended by World Organization for Animal Health

(Office International des Epizooties, OIE) for use in control of brucellosis in livestock (Garin-Bastuji and Blasco, 2008; Nielson and Ewalt, 2008).

2.17.1 *Brucella melitensis* Rev.1 vaccine

This vaccine is used for the immunization of sheep against infection by *Brucella melitensis*. In most countries where *B. melitensis* infection is endemic, immunization is the most appropriate first step, either as a preliminary approach to eradication or as sole eradication measure. The live attenuated *B. melitensis* Rev.1 strain is presently recognized as the best available vaccine for the prophylaxis against brucellosis in sheep and goats. The value of the vaccine for this purpose has been confirmed through various independent field and controlled experiments (Alton, 1990; Blasco, 1997). The ability of Rev.1 to produce a high level of immunity against both artificial and natural challenge has been convincingly demonstrated both for sheep and goats (Alton, 1990).

The duration of immunity conferred by vaccinating with Rev.1 was investigated by vaccination of Maltese goats at 4 to 12 months of age and challenged them at about $2^{1}/_{2}$ years later and others at $4^{1}/_{2}$ years after vaccination. The resistance of the animals challenged at $2^{1}/_{2}$ years and those at $4^{1}/_{2}$ were found to be the same, thus the immunity was considered to be life long.

More recent work has demonstrated the efficacy of Rev.1 in sheep either vaccinated as lambs conjunctivally or subcutaneously and challenged 9-10 months (Fensterbank *et al.*, 1985) or $7^{1}/_{2}$ - $15^{1}/_{2}$ months later, respectively (Verger *et al.*, 1995) or vaccinated as adult conjunctivally and challenged $2^{1}/_{2}$ years after. Likewise, good results of protection were obtained when young goats were vaccinated at 4 months of age conjunctivally and subcutaneously and challenged $8^{1}/_{2}$ - $12^{1}/_{2}$ months later (Fensterban, 1987). To avoid

stimulating serological reactions that would interfere with subsequent diagnosis, immunization is usually restricted to lambs and kids aged 3-8 months. Similarly a reduced dose (10^3-10^6 CFU) has been used subcutaneously in field trials and reported as an effective method to control brucellosis in small ruminants and being relatively safe in pregnant sheep and goats (Kolar, 1984; Gasca *et al.*, 1985; Al Khalaf *et al.*, 1992; Sales-Henriques *et al.,* 1992; Delgado *et al.,* 1995; Al Shamkh, 1995; Uysal, 1995).

2.17.2 *Brucella abortus* strain 19 vaccine

The S19 vaccine for *Brucella abortus* provides life long immunity against field strain of *Brucella* following natural infection in cattle. This is the most commonly used vaccine for the prevention of bovine brucellosis (Nicoletti, 1990). This strain was isolated from the milk of a Jersey cow in 1923. It was left at room temperature in the laboratory for one year and was found to have become attenuated. The strain was found to be capable of inducing protective immunity in cattle though effectiveness may vary depending on age at vaccination, dose and route of administration and prevalence of brucellosis in vaccinated herd (Nicoletti, 1990). *Brucella abortus* Strain 19 is an attenuated organism of smooth morphology which normally does not grow in the presence of erythritol. The strain is of low virulence such that vaccination with 40-120 x 10^9 CFU (classical dose) of living *Brucella abortus* organisms gives a good protection. However, it has disadvantages such as induction of abortion in pregnant cows, induction of persistent antibody response due to the presence of LPS with an O-chain on Strain 19 that cannot be serologically differentiated from that due to natural infection and its excretion in milk which can serve as a source of infection to man (Plommet, 1991). This vaccine possesses LPS with O-chain that makes the production and persistence of antibodies possible in serum following the administration of the vaccine. The presence

of these antibodies makes the continued use of the vaccine incompatible with simultaneous application of test and slaughter procedures in the control of brucellosis. This undesirable effect can be eliminated by adopting the following procedures.

1. Calves are vaccinated with a reduced dose of 3-10 x 10^9 CFU between 4 and 8 months of age and then the same dose is repeated when they are adults.

2. Calves are vaccinated conjucntivally with 2 drops of the vaccine containing 4-10 × 10^9 CFU at the age of 4-10 months and a second conjucntival vaccination with the same dose six months later (Nicoletti, 1990).

2.17.3 *Brucella abortus* RB51

Another classically obtained, live attenuated but rough (SLPS) *Brucella abortus* strain vaccine is the RB51 (Schurig *et al*., 1991). The RB51 vaccine is another effective vaccine for vaccinating cattle and gives a lifelong immunity (OIE, 2004).

It was selected by growth of *B.abortus* strain 2308 in the presence of rifampicin (Schurig *et al*., 1991). The "R" means rough; "B" refers to *Brucella* while "51" refers to an internal laboratory nomenclature used at the time the vaccine was derived. Strain RB51 is devoid of the O-chain, its roughness being very stable after multiple passages in vitro and in vivo through various species of animals (Colby, 1997). This vaccine strain has been reported to be equally effective as S19 vaccine in protecting against *B. abortus* cattle, without inducing anti-O chain antibodies as detected by serological tests (Palmer *et al.,* 1997). Preliminary experiments suggest that this vaccine can be effective for the prophylaxis *B. melitensis* infection in goats (Suarez *et al.,* 1998). In contrast, it has been demonstrated that this vaccine does not confer protection against *B. ovis* in controlled experiments in rates (Jemenez de Bagues *et al*., 1995). The optimum age of

vaccination is between 4 and 8 months. It is also permitted and efficacious for adult cattle to be vaccinated using a reduced dose of the vaccine, such as one-tenth and one-twentieth of the recommended dose (Nicoletti, 1990).

2.18 Control of Brucellosis

2.18.1 Immunisation to reduce the rate of infection in specific herds

The control of brucellosis can be achieved by using vaccination to increase the population's resistance to the disease. Vaccination practically eliminates the clinical signs of brucellosis and is accompanied by a reduced contamination of the environment as well as exposure of the population at risk to the infectious agent (Nicoletti, 1993). Mass vaccination of a herd or flock helps to rapidly establish a relatively immune stock, reduces the level of abortions and and excretors of *Brucella,* thus reducing contamination of the environment and disease transmission (Kolar, 1995).

Vaccination of all animals (young and old) in a herd or region is an important approach for the control of brucellosis in livestock (European Commission, 2001). This has been applied with success in many countries under different socio-economic conditions (Blasco, 1997). In general, mass immunization is indicated where the prevalence of infected animals is high. Thus it should be based on knowledge of the prevalence in the herd/flock or region (Blasco, 1997). However, this strategy has the limitation that pregnant animals should not be vaccinated because the vaccine is not innocuous enough for pregnant animals and the efficacy of the strategy depends on the continous availability of the vaccine (WHO, 1986).

2.18.2 Elimination of infected animals by test and slaughter

This approach is usually carried out to obtain brucellosis free herds/flocks or region. It is usually accepted that a programme of eliminating brucellosis by tets and slaughter policy is justified on economic grounds only when the prevalence of infected animals is about 2% or less (Nicolletti, 1993). For the implementation of this programme, it is essential that the herds / flocks or region are under strict surveillance and movement control (OIE, 2004). Animals must be individually identified and an efficient and well organized veterinary service for surveillance and laboratory testing must be in place (OIE, 2004). Before embarking on the implementation of test and slaughter policy, it is necessary to ensure that the epidemiological situation is favourable, the necessary facilities and financial resources are available. It also necessary that a pool of healthy replacement animals is available and that the resources exist for continuing surveillance for a considerable period of time (Nicoletti, 1993). It also essential that full co-operation of farmers is obtained as slaughter of seropositive animals can be resisted by the owners because of lack of clinical signs or inadequate compensation or lack of replacement animals (Robinson *et al.,* 2003).

A brucellosis control and eradication plan based on test and slaughter strategy can either be voluntary or compulsory (Mcdermott *et al*., 2013; Smits, 2013). Voluntary schemes which apply to individual herds/flocks may be useful in the early stages of the campaign but may need to be supported by adequate incentives such as bonus on sale of milk from brucellosis-free herds/flocks or per capita payments (WHO, 1986). Compulsory eradication is required in the final stages, although it is often advisable from the start (WHO, 1986).

2.18.3 Prevention of spread between animals and monitoring of brucellosis-free herds or zones.

In herds/flocks or regions where brucellosis-free status had been established, or where such a status is suspected on epidemiological grounds, the risk of importing the disease by means of animal movementmust be curtailed (OIE, 2004). Movement of potentially infected animals into such areas, regions or herds should be prohibited. Only animals from brucellosis-free certified areas or regions should be allowed entry (OIE, 2004). This applies to transport of animals and certain animal products within countries, regions as well as between countries, following the general principles and procedures as specified in the international zoo-sanitary code of the OIE which also describes the essential testing of animals and quarantine measures (WHO, 1986; Robinson *et al.,* 2003).

In addition to the strategy selected, simple non-specific measures of prevention of the spread of the infection should be applied in a systematic way. These include general hygiene measures and provision of clean accommodation that can be disinfected at parturition. All parturitions should be regarded as potential sources of infection and all the nonliving products and contaminated materials must be incinerated or buried (Alton, 1990; McDermott*, et al*., 2013). The provision of information and education concerning the disease to farmers and local communities is essential. The farmer must be informed of all the advantages of the control programme, such as the economic benefits and the elimination of risks to the health of his family and himself (WHO, 1986).

2.19 Public Health Importance

Brucellosis in humans is a zoonosis caused by the facultative intracellular Gram-negative bacteria of the genus *Brucella*. Six classical species exist of which three cause disease in humans, namely, *Brucella abortus* (from cattle) *Brucella melitensis* (sheep

and goats), *Brucella suis*, (pigs) *B. canis* (dog). Infection due to *B. melitensis* and *B. suis* often give rise to a severe and prolonged form of the disease (Solare, 2010).

Recently, marine mammals have been recognized as additional animal reservoirs for *Brucella* species with zoonotic potentials (Foster *et al.,* 2007). These new species are called *Brucella ceti* and *Brucella pinnipedialis* (Lopez-Goni *et al*., 2008; Solare, 2010).

The disease also known as undulent fever or Malta fever is transmitted through direct contact or by aerosol. Rarely fatal, infection of human can be severely debilitating and disabling. It is a multi-systemic, acute to chronic and wasting disease characterized by diverse nonspecific clinical signs including undulant fever, fatigue, depression, headache, night sweating, join pain, lumber pain, muscular pain, weight loss, hepatomegaly, splenomegaly and arthritis (Alausa, 1979; Adekolu-John, 1989; Currier, 1989; Falade, 2002; Corbel, 2006; Smits *et al.,* 2009). About 500, 000 cases of human brucellosis are reported annually worldwide (Pappas *et al*., 2006; Franco *et al*., 2007). Despite its incidence, brucellosis is one of the neglected endemic zoonoses in the world (WHO, 2012).

Humans are accidentally infected by *Brucella* organisms and are normally dead-end hosts. The disease primarily is an occupational risk in exposed professionals such as veterinarians, farmers, laboratory workers, abattoir workers and others who work with animals and their products (El Sherbini *et al.,* 2007). The maximum danger being the lambing, calving or kidding period. Others get infected through dairy products consumption without necessarily having contact with the animals. Symptoms of acute brucellosis are intermittent fever and flu-like conditions (McDermott and Arimi, 2002). The incubation period is 1-4 weeks. The clinical diagnosis of brucellosis in humans

remains a considerable challenge, and to the unaware physician, the diagnosis becomes a protracted problem for months and sometimes years (Araj, 2010).

The economic losses due to human brucellosis although difficult to estimate, result from physical and psychological suffering due to infection, hospitalization, cost of drugs and the loss of work or income due to illness, physical incapacity and reduction in productivity (WHO, 1986; OIE, 2004). In West Africa, knowledge of the actual impact of the disease in humans is limited and human cases remained under- reported. However, serological evidence has been reported in some of the countries including Nigeria, Benin Republic, Guinea, Mali, Guinea Bissau, Ivory Coast, Togo and Burkina Faso (Pappas *et al*., 2006; Akakpo *et al.*, 2010), with prevalence ranging from 10%-26% in Nigeria and Burkina Faso (McDermott and Arimi, 2002). Due to little interest in brucellosis or lack of knowledge of the disease in hospitals in this part of the continent, acute brucellosis might be misdiagnosed in favour of other febrile illnesses similarly encountered as other endemic human diseases like malaria and typhoid fever (McDermott and Arimi, 2002; Pappas *et al*., 2006; Akakpo *et al*., 2010).

2.20 Economic Importance of Brucellosis

Brucellosis is one of the most frequent bacterial zoonotic diseases in low income countries where control programmes have not been established or have not succeeded in eradicating it. It is a 'multiple burden' disease with economic impacts attributable to human, livestock and wildlife disease. The epidemiology and economic impact of brucellosis vary by geography and livestock system (McDermott *et al*., 2013). It is widely distributed in low-income regions of the world with high endemicity in Mediterranean, Middle East, Latin America, sub-Saharan Africa and South Asia. It is a major cause of direct economic loss and impediment to trade and exportation (Lopez-

Goni *et al*., 2008). Brucellosis causes economic losses through abortion, temporary infertility due to metritis, decreased milk production in dairy cows, deaths of young animals due to stillbirths, weak offspring and sterility, increased cost of animal replacement and lowered sale value of infected animals (Rikin, 1988; Ajogi *et al*., 1996; Radostits, *et al.,* 2007; FAO, 2004).

There are also indirect economic losses due to enforcement of control and eradication measures. These include cost of vaccines and vaccination, personnel cost and logistics. There are also losses due to compensation paid for slaughtered infected animal (FAO, 2004). The economic loss due to brucellosis in Nigerian livestock was assessed to be 224 million U.S Dollars by Esuruoso (1979). Ajogi and Akinwumi (2001) estimated losses in Wase and Wawa-Zange cattle reserves at N9.38 Million and N3.38 Million per annum respectively. Others were by Chukwu, (1985b) and Rikin, (1988). Indirect losses especially those that require brucellosis-free status to access regional or international livestock markets, have not been estimated in sub-Saharan Africa but could be a constraint to future trade (McDermott and Arimi, 2002).

2.21 Occurrence of Brucellosis in Nigeria

2.21.1 Bovine brucellosis

Brucellosis is an endemic disease in Nigeria having been reported sporadically all over the country as far back as 1927. More of the reports come from established farms than from the nomadic cattle rearers who control about 90% of the animal population. Due to poor disease reporting system in Nigeria, the distribution of the disease in accurate proportion is not well documented. Similarly, surveillance has not been very efficient leading to poor determination of the incidence and prevalence of the disease in Nigeria. The earliest reference to the occurrence of the disease in Nigeria is contained in the

annual report of the Veterinary Department for the year 1927 in which 10 cases of contagious abortions were recorded (Anon, 1927). This was followed by Earshaw and O'Brien (1928) who reported cases of knee-joint hygroma in indigenous cattle. Hall (1934) diagnosed contagious bovine abortion in a government ranch where sero-agglutination revealed a positive reactor rate of about 15 per cent. The annual reports of the Veterinary Department in Nigeria for the year 1946 indicated an increase in reactor rates among government owned cattle herds (Mettam, 1948).

Various serological studies over the years have proved that brucellosis is widespread in Nigerian livestock (Macgregor, 1964; Esuruoso, 1965; Adams and McKay, 1966; Banerjee and Bhatty, 1970; Esuruoso and Hill, 1971; Esuruoso and Van Blake, 1972; Esuruoso, 1974; Nuru, 1974; Nuru and Dennis, 1975; Marrison *et al*., 1975; Eze, 1978a; Bale and Kumi- Diaka, 1981; Eze, 1978a; Agunloye *et al.,* 1988; Ocholi, 1990; Bertu *et al*., 2010a, 2010b, Bertu *et al*., 2010c). There are also reports from slaughter houses (Nuru and Dennis, 1975; Chukwu, 1987; Cadmus *et al*., 2006; Gusi *et al*., 2010) corroborating with the field studies. Prevalence rates vary between 20-60%. Gusi *et al*. (2010) in a recent study in slaughtered cattle at the Jos abattoir, reported a prevalence of 5.8% while another study in cattle herds in three LGAs of Plateau north geo-ecological zone revealed a prevalence of 3.8% (Wungak *et al*., 2011). More recently, Farouk *et al*. (2013) demonstrated *Brucella* antibodies in cattle milk marketed in Jigawa State where they reported 7.4% prevalence in marketed milk and 3.4% in fresh milk.

There were also bacteriological studies over the years that reported the isolation of *Brucella abortus* in cattle in Nigeria (Eze, 1978b; Bale and Kumi-Diaka, 1981; Falade, 1981; Ocholi *et al.,* 2004a; Ocholi *et al.,* 2004b, Ocholi *et al*., 2005).

2.21.2 Caprine brucellosis

The first evidence of brucellosis in goats was presented by Adams and McKay (1966) and later by Kramer *et al.* (1967). Since then little information was available until the first comprehensive reports of brucellosis among goats in Nigeria was presented by Falade (1974).). Other reports on caprine brucellosis presented from various areas of Nigeria were, (Falade *et al.,* 1975; Falade, 1978; Bale, 1980; Falade, 1981; Bale *et al.,* 1982, Ogundipe *et al*., 1994 and Brisbe *et al*., 1996) all revealing prevalence rates between 2.1-14.5%. A study carried out by Cadmus *et al.* (2006) in Ibadan, Oyo State revealed a prevalence of 0.86% while a similar study by Tijjani *et al.* (2009) in slaughtered goats in one abattoir in Yobe State recorded 14.0% prevalence. In a survey of brucellosis in goats in a prison farm in Sokoto State, Junaidu *et al*. (2008) reported a prevalence of of 30.76%. In a recent survey of small ruminant brucellosis in Plateau State, 14.5% prevalence was recorded in goats (Bertu *et al*., 2010b) while Adamu *et al*. (2012) reported a revalence of 3.2% by RBPT and 1.6% by CFT in goats in the semi arid zone of Northern Nigeria. In an abattoir survey of brucellosis carried out by Gusi *et al.* (2010) in goats slaughtered at Jos abattoir, 7.5% prevalence was recorded.

2.21.3 Ovine brucellosis

Early efforts at ascertaining causes of abortion in sheep failed until Okoh (1980a) confirmed the isolation of *B.abortus* from sheep in an investigation farm in Kano State of Nigeria. Subsequent serological (Okoh, 1980; Falade and Shonekan, 1982; Okewole *et al.,* 1988; Ogundipe *et al.,* 1993; Brisbe *et al*., 1996) and culture and isolation studies (Bale *et al.,* 1982; Ocholi *et al*., 2005a) have confirmed ovine brucellosis in Nigeria. In a study conducted in aborting sheep on a farm mixed with cattle, Ocholi *et al.* (2005) reported a prevalence of 14.5%. Similarly, Junaidu *et al.* (2008) reported a prevalence

of 22.35% in sheep in a prison farm in Sokoto State while Tijjani *et al*. (2009) reported 6.0% in an abattoir survey of slaughtered sheep in Yobe State. In a recent survey of small ruminant brucellosis in Plateau state, 16.0% prevalence was recorded in sheep (Bertu *et al*., 2010b). Gusi *et al*. (2010) reported 5.5% prevalence in sheep slaughtered at the Jos abattoir.

2.21.4 Equine brucellosis

Horses have recently gained popularity in Nigeria due to increasing interest in polo games and horse racing. There are very few reports available on the presence of the disease in horses. Bale and Kwanashie (1984) demonstrated *Brucella* antibodies in horses in Northern Nigeria where 14 (8%) out of the 166 horses were reactors. The isolation of *B. abortus* from an aborted equine foetus confirmed the presence of the disease in horses in the country (Oladosu *et al*., 1984). Recently, *B. abortus* was also isolated from horses on a government-owned farm (Ocholi *et al*., 2004a). This is a further confirmation of the existence of equine brucellosis in Nigeria. In a recent serological study carried out on sera obtained from 75 horses from three horse stables belonging to the Nigerian Police and Nigerian Army in Kaduna and Plateau States, 14.7% prevalence was recorded (Ehizibolo, 2011).

There appears to be a dearth of information on the prevalence of brucellosis in donkeys in Nigeria. Athough a lot of studies have been done for most domestic animals in Nigeria including horses, studies on brucellosis in donkeys seems to have been neglected. The only study on the prevalence of brucellosis in donkeys in Nigeria available to us is that reported by Sadiq *et al.* (2013), where they tested 300 donkeys in three LGAs of Yobe State for *Brucella* antibodies by RBPT, MSAT and cELISA. They

reported an overall brucellosis prevalence rate of 15%. They also reported 6%, 3%, 3% for Machina, Gaidam and Bursari LGAs respectively.

2.21.5 Porcine brucellosis

The first report of swine brucellosis in Nigeria is contained in reports by Mettam (1947) and Adams and McKay (1966). Since then, there has been no sufficient information to indicate the distribution and prevalence of the disease in Nigeria. It is, however, certain that the disease is present in the country as a serological study from 6 piggeries in Northern States of Nigeria showed that 12 (2.7%) out of 443 pigs sampled had *Brucella* antibodies, (Bale and Nuru, 1985).

2.21.6 Canine brucellosis

In the past there were doubts as to the presence of canine brucellosis in Nigeria when serological survey by Falade (1977) found no antibodies to *Brucella* in dogs. However *B. canis* had been detected through serology where a prevalence rate of 28.6% was obtained (Adesiyun *et al.,* 1986) and through cultural recovery from a case of abortion in an imported Boxer (Okoh, 1978). With the current upsurge in dog breeding using imported dog species and the attendant random movement of dogs through trade, the spread of brucellosis among dogs in the country is most likely to be increased. This is because puppies or adult dogs from infected kennels may be sold to non-infected kennels.

2.21.7 Camel brucellosis

Brucellosis in camels in Nigeria was first diagnosed based on serological survey by Okoh *et al*. (1979) where 1.0% prevalence of *Brucella* antibodies was obtained. Subsequent serological tests by Adamu *et al.* (1997) and Adamu and Ajogi (1999) showed increasing rates of 19.1% in three surveys and 12.5% in one abattoir survey

respectively. A recent investigation of brucellosis in the one humped camels in the Lake Chad area of Borno State by Sadiq *et al*. (2008) revealed prevalence of 12.13%.

2.21.8 Human brucellosis

The distribution of human brucellosis is directly related to the distribution of animal brucellosis. Brucellosis is a widespread disease with millions of individuals at risk worldwide, especially in developing countries where a significant animal reservoir exists (Conde-Alvarez *et al.,* 2013). Therefore the risk in humans is a function of the risk in livestock and the livestock- human infection rate. In these countries, milk pasteurization is not routine and the consumption of raw milk and milk products is common (Pappas *et al*., 2006). Transmission of brucellosis from mother to child is possible through breast milk (Palanduz *et al*., 1999).

The first case of brucellosis in a human being reported in Nigeria was in 1962 (Collard, 1962) when *Brucella* antibodies were demonstrated in the sera obtained from healthy persons in various parts of Nigeria. Between 1962 and 1967 records obtained from the University College Hospital, Ibadan indicated 9 cases of human brucellosis and *Brucella* species were isolated from 4 blood samples and one bone marrow culture (Falade, 1981). Most cases of human brucellosis were essentially an occupational hazard, occurring amongst workers in the livestock industry. Epidemiological studies revealed significantly higher prevalence of infections among occupationally exposed persons including herdsmen, abattoir workers, laboratory workers and veterinarians than occupationally non-exposed population such as blood donors and pregnant women (Falade, 1974; Alausa and Osoba, 1975; Alausa and Awoseyi, 1976; Alausa, 1979; (Al-majali and Shorman, 2009)). Brucellosis in humans presents as a febrile flu-like illness with intermittent fever (Berman, 1981; Chukwu, 1987; Seifert, 1996). A recent

serosurvey of brucellosis in butchers at the Jos abattoir revealed a prevalence of 5% (Gusi *et al*., 2010).

2.22 Clinical Manifestation of Human Brucellosis

Human brucellosis is a debilitating disease characterized by a long-term progression and the disorders of the nervous and cardiovascular systems and osteoarticular apparatus (Smirnova *et al*., 2013). The disease often leads to a partial or complete loss of physical efficiency (Poester *et al*., 2010). The common clinical signs of human brucellosis include intermittent or irregular fever, weakness, depression, night sweating, anorexia, constipation, myalgia, endocardiitis and stiffness of the vertebral column (Falade, 1974; Thornes, 1977; Currier, 1989 and Weidmann, 1991).

2.23 Treatment of Human Brucellosis

Human brucellosis can be treated by the use of antibiotics that are effective against *Brucella* organisms. Effective treatment depends upon long periods of antibiotic therapy, often requiring combinations of antibiotics (Anon, 1986; Ersoy *et al.,* 2005). Despite extensive studies for many years, the optimum antibiotic therapy for brucellosis is still in dispute. The WHO recommends rifampicin and doxycycline daily for a minimum period of six weeks for adults and children above 8 years in acute cases. Some still claim that the long established combination of intramuscular streptomycin with an oral tetracycline gives fewer relapses. Infections with complications such as meningoencephalitis and endocarditis require a combination of therapy with rifampicin, tetracycline, and aminoglycosides. A combination of rifampicin and cotrimoxazole or trimethoprim-sulfamethoxazole orally twice daily for 45 days plus gentamicin, daily for 7 days or rifampicin orally daily for 45 days plus gentamicin, intravenously or intramuscularly daily for 7 days is the best treatment for uncomplicated cases in

children below 8 years old ((Solera, 2010). In pregnany, the recommended dosage regiment is rifampicin, orally for 45 days or rifampicin orally, daily, for 45 days plus trimethoprim-sulfamethoxazole one double strengh tablet twice daily for 45 days. In focal infections (complicated cases such as spondylitis, endocarditis, meningitis, paraspinous abscesses), Doxycycline orally twice daily and rifampicin orally daily for 6-52 weeks plus either streptomycin, intramuscularly daily or gentamicin intravenously, daily for 14-21 days. Alternatively, trimethoprim-sulfamethoxazole, ciprofloxacin orallyS twice daily, ofloxacin orally twice daily as a substitute for doxycycline or rifampicin (Solera, 2010) (Appendix V). However relapses may not be completely ruled out. With no human vaccine in sight and the possibility of relapses after antibiotic therapy, control of human brucellosis remains dependent on control of animal brucellosis and appropriate food safety regulations being in place (Whatmore, 2009).

2.24 Brucellosis in some African Countries

Brucellosis has been reported in many of African countries including those bordering Nigeria such as Benin Republic, Niger, Chad, Cameroun, Burkina Faso, The Gambia, Ghana, Ivory Coast, Guinea, Mali, Sierra Leone,Togo, Ethiopia, Zambia, Zimbabwe, Kenya, Tanzania, Uganda and Kenya (Kang'ethe, *et al*, 2000; Kabagambe *et al*., 2001; Mangen *et al.,* 2002; Shelling *et al*., 2003; Ungeret *et al*., 2003; Kunda *et al*., 2007; Muma, *et al.,* 2007; Bayemi *et al*., 2009; Bronsvoort *et al*., 2009; Bankole., 2010; Chimana *et al*., 2010; Scolamacchia *et al*., 2010; Matope *et al.,* 2011; Megersa *et al*., 2011; Tesfaye *et al.,* 2011; Sanogo *et al*., 2012; Sanogo *et al*., 2013; Yohannes *et al.,* 2013). This indicated that brucellosis is widespread on the African continent. The fact that *Brucella abortus* biotype 3 is the circulting strain in most of these countries is not

surprising, considering the porous nature of borders between neighbouring countries where infected trade cattle could easily be transported from one country to another.

Besides sedentary production system, cattle are also reared under a nomadic production system known as transhumance system where herds are moved across international boundaries to find better pasture and secured places (Blassett and Turner, 2007; and OECD, 2008). The implication of this is that brucellosis can easily spread from one country to the other. Nigerian cattle are at risk of brucellosis infection considering the large number of cattle coming into the country from Chad and other neighbouring countries and transported to the southern part of the Nigeria for slaughter. Cadmus *et al.* (2010) reported brucellosis seroprevalence ranging from 5.3% to 6.2% in trade cattle slaughtered between 2004 and 2006 at Bodija Municipal abattoir, Ibadan, southwestern Nigeria. A high brucellosis seropositivity was also observed among trade cattle meant for slaughter in Benue state, central Nigeria (Bertu *et al*., 2012). Majority of these trade cattle were brought from the North where some of them could have originated from Niger, Chad or Cameroun.

Apart from the serological evidence of brucellosis in various parts of Africa, *Brucella abortus* has also been isolated from cattle in many of the countries. Some of these countries include; Ivory Coast, Guinea Bisau, Mali, Niger, the Gambia, Senegal and Togo, further confirming the endemicity of brucellosis in sub-Saharan region (Sanogo *et al*., 2013). This situation is not surprising as it is consistent with the absence of sustained and efficient control programme for brucellosis in the region.

CHAPTER THREE

MATERIALS AND METHODS

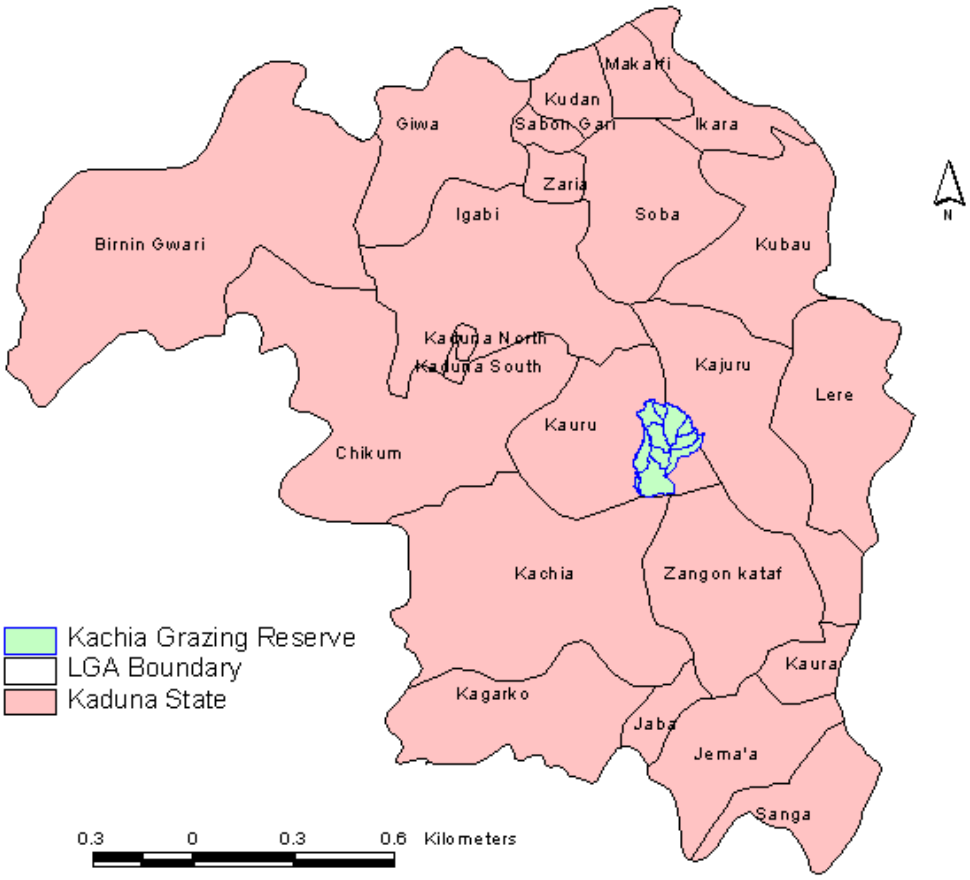
3.1 Study Areas

3.1.1 Kachia grazing reserve

This study was conducted in Kachia Grazing reserve (KGR) in Kaduna state It is a wide span of flat lowland area covering about 33, 411 hectares within three LGAs in the present Kaduna State, namely; Kachia, Kajuru and Zangon Kataf. It is located in the tropical sub-humid climate of the Guinea Savannah Zone of Nigeria. It is located between Latitude 10.10112-10.29477^{0}N and Longitude 8.00708-8.15154^{0}E. The vegetation is made up of forested areas and shrubs, with undergrowth grassland. The annual rainfall in the area is between 1000 and 1200mm. The total population of Fulani herdsmen in the area is about 18, 000 occupying various settlement areas known as blocks. Based on the result of a census conducted in June 2011, there are six blocks in all with a total of 777 households. The animal population in the Grazing Reserve is made up of 41,234 cattle, 10,161 sheep and 4,828 goats. Record of previous brucellosis prevalence in the area was lacking but a prevalence of 8.0% has been reported for Kaduna State (Ocholi *et al*., 1993). The area experiences two distinct seasons. Dry season is experienced between November and March while rainy season starts from month of April to October. Temperature ranges between a minimum of 19^{0}C in January and a maximum of 39^{0}C in April with an average of 28^{0}C.

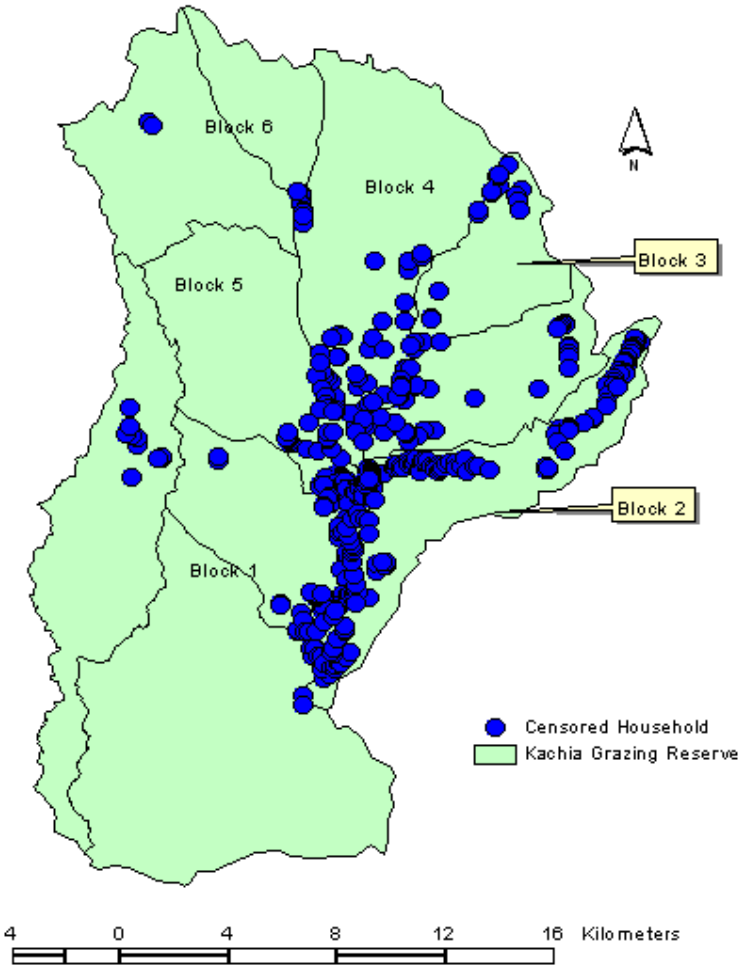
The KGR was established in 1968, but developmental work did not commence until 1970. During this period, 13 earth dams were constructed; six of the dams were constructed by the Kaduna State government, six by Livestock Project Unit (LPU) and one by Federal Livestock Department (FLD). The dams were to serve as water

reservoirs for both human and animal use. After further developmental work was carried out by the World Bank, settlement of nomads started in 1988.



Source: ICONZ Project (FP7/2007-2013)

Figure 3.1: Map of Kaduna State showing the location of Kachia Grazing Reserve



Source: ICONZ Project (FP7/2007-2013)

Figure 3.2: Spatial map of KGR showing the location of 777 households enumerated during the census.

3. 1. 2 Jos Plateau

The Jos Plateau study area is in Plateau State of Nigeria. It is located within the Guinea Savannah Zone of Nigeria. It is the highest landmass in Nigeria and it is approximately 250km by 150km in size. It comprises high plains of about 1,300m above sea level and a number of granite hill ranges over 1,900m. Temperatures on the Jos Plateau are lower than the surrounding plains with a minimum of 15.5^0C and a maximum of 30^0C. The maximum rainfall is 2000mm and the minimum is 1500mm, with an average of 1411mm per year.

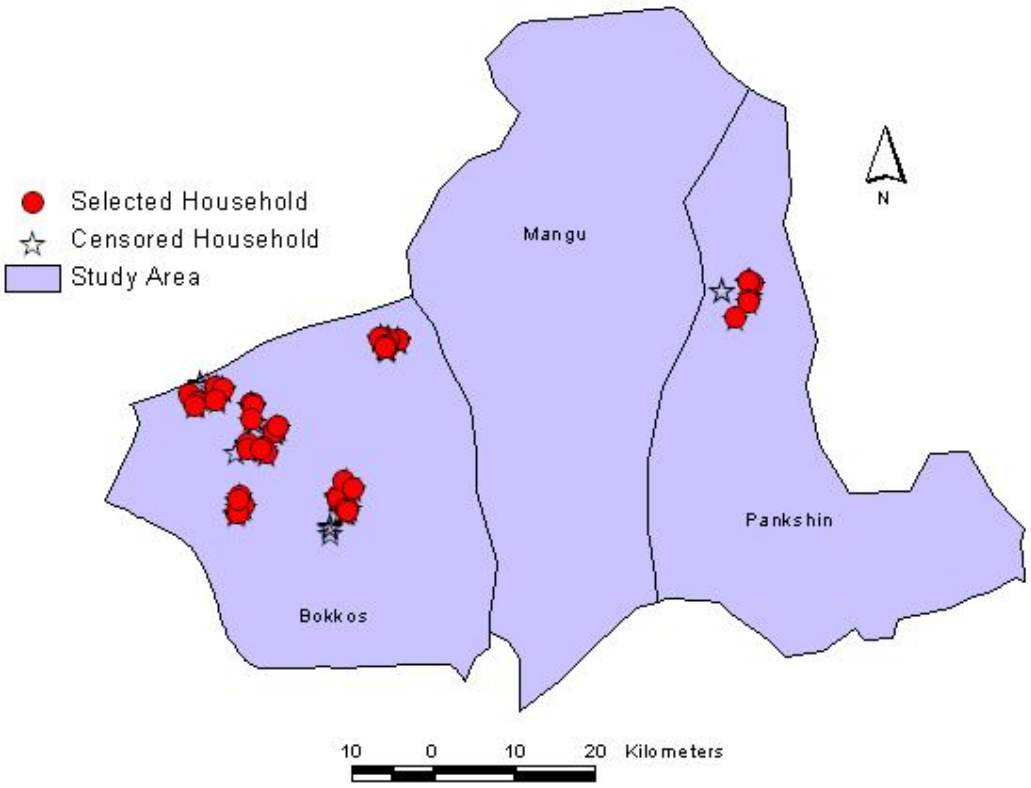
The Jos Plateau study area comprises 10 selected villages, with a total of 210 farmers (households) and 2,539 people. The median household size of the 206 farmers in the sampling frame is 10 people, with a range of 1-50 people. All households own cattle, of which there are 10,264 animals. The median number of cattle per household is 37.5, with a range of 5-200.

One hundred and forty nine of the 206 households own sheep, totalling 3,607 animals. The median number of sheep per sheep-owning household is 20, with a range of 2-100. Ninety seven of the 206 households own a total of 1,302 goats. The median number of goats per goat-owning household is 9, with a range of 1-70.



Source: ICONZ Project (FP7/2007-2013)

Figure 3.3: Map of Plateau State showing study area.



Source: ICONZ Project (FP7/2007-2013)

Figure 3.4: Map of the Jos Plateau study area showing the selected households in the six villages where sampling was carried out.

3.2 Census and Georeferencing of Study Areas

A 5-day general census of each of the study areas was conducted prior to the time of sampling. During this period, every household was enumerated. The population of all the livestock owners was obtained. The total population of cattle, sheep and goats was also taken. Geo-referencing of all the households in the two study areas were conducted by recording the coordinates of every household in both KGR and on the Jos Plateau using the global positioning system (GPS).

A list of the forty selected households and their GPS coordinates for KGR are presented (Appendix I) while the list of the thirty six selected households and their GPS coordinates for the Jos Plateau is also presented, (Apendix II).

3.3 Study Design and Criteria for Choosing Study Areas

The study was a cross-sectional study in KGR in 2011 and the Jos Plateau in 2012. The list of all blocks and households and the population of cattle were obtained during the census. The criteria for choosing the study areas were cattle population of at least 5000 and accessibility to the areas. All herds were eligible for sampling in KGR irrespective of herd sizes. On the Jos Plateau only herds with eighty anomals and above were eligible for sampling. This is because we used the design that was done in a previous study which required herds. However, herds whose owners did not agree to participate in the study were excluded. The target population were cattle of all ages, sexes and breeds.

3.4 Sampling Design

A 2-level multistage clustered sampling method was used in this study as previously described by Bennett *et al.* (1991) and Thrusfield (2005).

A household unit was considered as a cluster. A random sample of herds / households from both study areas made up the first sampling level. The second sampling or the ultimate sampling unit was the individual cattle. All the animals in selected herds in KGR were sampled while eighty animals were sampled from each herd on the Jos Plateau. The design in this study area was done previously by a project group that required a minimum of eighty animals for three groupings. Therefore, two or more herds with few animals were combined as one to make up 80 animals, thereby becoming qualified for sampling.

3.5 Sample Size

Sample size was calculated using C-Survey software (Version 2.0) UCLA (2008). Based on a calculated minimum total cluster/herd of 40 and based on the median herd size of 49 for cattle to be sampled. The sample size of 1,960 was obtained in KGR. The sample size for the Jos Plateau was 2,880 based on a calculated minimum total cluster / herd of 36 and a median herd size of 70. This calculation uses the following parameters in cluster survey software:

Confidence level: 95%

Assumed prevalence: 10%

Absolute precision: 5%

Design effect: 2.18 (for *B.abortus*, (Otte and Gumm, 1997).

3.6 Sample Collection and Handling

About 5 ml of blood was collected from the jugular vein of all animals in selected herds into sterile vacutainers and kept in a slanting position to clot. Every sample container

was well labelled with animal number, household number and block number. Serum was separated by gently decanting it into another clean sample tube leaving the clotted red blood cells in the first tube. Sera were tested by standard RBPT on the field.

A total of 229 samples (vaginal swabs, hygroma, milk and placentae) were collected from animals in selected herds with history of abortion or any animal that had abortion at the time of sampling (Appendix III).

The samples were collected into sterile sample bottles containing tryptone broth as transport medium and properly labelled. The serum samples and samples meant for culture were transported on ice packs to the Brucellosis Research Laboratory at the National Veterinary Research Institute, Vom. Sera were stored at -20^0C for further testing while samples for isolation were cultured immediately on serum dextrose agar.

3.7 Serological Testing of Samples by RBPT

All sera were first tested for *Brucella* antibodies in the field by standard Rose Bengal plate test (RBPT) as described by Alton *et al.* (1988). They were later tested in the laboratory by the modified RBPT according to the method described by Blasco *et al*. (1994a) and Ferreira *et al*. (2003). Briefly, 25µl of serum was placed on a white ceramic tile and 25µl of *Brucella abortus* antigen (*Brucella abortus* strain 99, Spain) was placed beside it. For the modified method, 75µl of serum and 25µl of antigen were used. They were mixed and the plate rocked for 4 minutes. The presence of agglutination was considered positive reaction while the absence of agglutination was considered negative**.** *Brucella* Positive and negative control sera were also tested along with the test sera to guide in the reading of the results. The results were recorded and stored in Microsoft Excel spreadsheet.

3.8 *Brucella* Isolation from Field Samples

3.8.1 Culturing of samples

All samples for *Brucella* isolation were incubated for 24 hours in peptone broth after which they were inoculated on serum dextrose agar or Trypticase soya agar for isolation of *Brucella* organisms as described previously (Alton *et al*., 1988; de Miguel *et al*., 2011). The media plates were prepared by weighing 4g of Trypticase soy agar or dextrose agar in 100ml of sterile distilled water and autoclaved. Two ml of *Brucella* antibiotic supplement (Oxoid) containing a cocktail of antibiotics (Polymyxin B, 2,500 IU; Bacitracin, 12,500 IU; Cycloheximide, 50.0 mg; Nalidixic acid, 2.5mg; Nystatin, 50, 000 IU; Vancomycin, 10.0 mg) was added per 100 ml of media. Five ml of newborn calf serum was also added before the media was poured into Petri dishes (Appendix VII). Samples were inoculated onto two sets of the agar lates. One set of inoculated plates were incubated at 37^0C for 3-7 days in 5-10% CO_2 atmosphere while the other set without CO_2.

3.8.2 Observation of colonial morphology

On serum dextrose agar or Trypticase soya agar media, *Brucella* colonies are visible after 3-5 days incubation. After four days, *Brucella* colonies become round, 1-2 mm in diameter, with smooth (S) margins, transparent and pale honey colour when plates are viewed in transmitted light and have a bluish translucent appearance in reflected light. When viewed from above, colonies appear convex and pearly white. Later, colonies become larger and slightly darker.

3.8.3 Microscopic examination of *Brucella* isolates

Suspected *Brucella* colonies were picked using sterile wire loop and mixed with a drop of sterile distilled water on a clean glass slide to make a smear. The smear was heat fixed on the slide and air-dried. The slide was stained by Gram staining method for subsequent microscopic identification of the organisms. Isolates were Gram negative, very tiny, coccobacili that were arranged mostly in singles but some in pairs and also in clusters. These typical *Brucella* organisms observed under the microscope were from seven samples, three from KGR and four from Jos Plateau and were further subjected to various biochemical tests and characterization.

3.8.4 Characterization of the isolates in NVRI laboratory

On further confirmatory tests, organisms produced hydrogen sulphide (H_2S), they were urease positive, oxidase positive, positive with positive *Brucella* sera but negative with negative *Brucella* sera. They produced agglutination with *Brucella* monospecific antisera A but negative with *Brucella* monospecific antisera M. All the isolates were lysed by Weybridge (Wb), Tbilisi (Tb), Izatnagar (Iz) which are smooth *Brucella* phages at routine test dilution (RTD). The isolates did not grow on media containing 20µg/ml and 40µg/ml concentrations of thionin. They grew on media containing 20µg/ml and 40µg/ml basic Fuchsin dye. They were considered as *Brucella abortus* biotype 1. The agar plates with the isolates were wrapped in tin foil and stored in the refrigerator at 4^0C ready for freeze-drying.

3.9 Preservation of Isolates

All positive isolates were lyophilized (freeze-dried) and stored at -20^{0}C and aliquoted into PCR tubes and stored in 15% glycerol at -80^{0}C until required for PCR in Spain.

3.10 Packaging of Isolates for Classical Biotyping and Bruce-ladder PCR Work in Spain

A list of all the *Brucella abortus* Isolates including full information on the identity of each isolate was made and sent along in a well packaged box to University of Navarra, Pamplona, Spain (Appendix IV).

Eighteen of the isolates were taken in duplicates but for three isolates that were not in sufficient quantity in the archive, one ampoule each was taken. The 16 pairs of isolates (7 pairs from this study and 9 pairs from field submissions) have been freeze-dried in vials while the archive isolates (2 paired and three singles) have been freeze-dried in ampoules. After completing all the required Import and export docements, the isolates were packaged according to standard procedure and shipped to Spain.

3.11 Reconstitution of the Isolates in Spain.

Each of the 32 lyophilized vials (both extant and archived isolates) were reconstituted with 1ml of Phosphate buffered saline (PBS) using a 1000µl microtitre pipette and allowed to stand without shaking for 10 minutes. Phosphate buffered saline was used because the isolates were freeze-dried with lactoalbumin as a constituent. Due to differences in the sizes of the 7 ampoules, different quantities of PBS were used for their reconstitution. One ml of PBS was used for the reconstitution of the 5 ml ampoules while 200µl was used for the reconstitution of the 1ml ampoules.

3.12 Inoculation of *Brucella* Suspensions on Media Plates

Thirty nine Trypticase soy agar (TSA) plates equivalent to the 39 vials of reconstituted *Brucella* strains were labelled appropriately. A loopful of each reconstituted bacterial suspension was aseptically taken and smeared on one half of the plate and then streaked to the second half until the entire surface was covered. This procedure was done for all the *Brucella abortus* isolates. The inoculated plates were incubated at 37^0C in 5% CO_2 and the vials and ampoules securely covered with parafilm tape in a rack and stored in the refrigerator at 4^0C.

The plates were checked on daily basis to monitor the growth of the organism as well as the presence of any contaminants and were re-incubated. When contaminants were observed in a plate, the sample was purified by subculturing the *Brucella* colonies onto a new agar plate.

3.13 Co-agglutination Test with *Staphylococcus aureus* Cowan 1 Strain

This test is used to differentiate *Brucella* colonies and contaminants growing on a plate. The *Staphylococcus aureus* Cowan 1 strain has been sensitized with *Brucella* antiserum and therefore readily produces agglutination when it is emulsified with a colony of suspected *Brucella* organisms. The test was carried out by dropping 10µl of the sensitized *Staphylococcus aureus* Cowan 1 suspension on a clean glass slide with a microtitre pipette. Using a wire loop, one or two colonies of the test organism on the plate was taken and emulsified with the suspension. The reaction was observed over a bright light source. Agglutination was observed after 2-5sec to confirm the organism as *Brucella*. The absence of agglutination showed that the organism was not *Brucella* and the reaction was recorded as negative. All the isolates were tested for co-agglutination

in the *Staphylococcus aureus* Cowan 1 test. Freshly grown colonies of *Brucella melitensis* 16M strain was tested along with the isolates as control.

Five of the 21 *Brucella* isolates were negative to this test due to contamination and were kept aside for further purification.

Therefore, sixteen of the twenty one *Brucella abortus* isolates (five from the study, eight from field submissions and three from archived isolates) were confirmed as *Brucella* (Appendix V) by their co-agglutination with the *Staphylococcus* Cowan. 1. They were packaged for classical biotyping and Bruce-ladder multiplex PCR (Appendix V).

3.14 Characterization and Biotyping *Brucella* Isolates in Spain

Phenotypic characterization of the *Brucella* isolates were carried out as described by Alton *et al*. (1988) based on the following tests:

3.14.1 Growth in presence of CO_2

Some *Brucella* species grow both in air and in atmosphere containing 5-10% CO_2, while others grow only under CO_2 supplemented atmosphere. A loopful of *Brucella* suspension was sub-cultured onto serum dextrose agar plates in duplicates. One set was incubated in anaerobic jar containing carbon dioxide generating pack while the other set was incubated in air in the incubator. Growth on plates containing CO_2 and absence of growth in plates in air indicates positive CO_2 requirement.

3.14.2 Hydrogen sulphide production

The *Brucella* colonies to be tested were inoculated onto a serum-dextrose agar slope and a strip of lead acetate paper was placed in the tube such that it did not touch the inoculated media in the tube, and incubated at 37^0C and observed daily for 24-48 hours.

The lead acetate paper was blackened from its tip down to its length when hydrogen sulphide was produced. Its colour remained unchanged if hydrogen sulphide was not produced.

3.14.3 Urease test

A slope of Christensen's medium with addition of 20% urea solution (Appendix VII) was inoculated with a loopful of *Brucella* cultures. The bottle was incubated at 37^0C and observed for colour change. When the medium was observed to change to purple-pink, it showed urease activity and it indicated positive reaction.

3.14.4 Agglutination with positive and negative *Brucella* sera.

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

3.14.5 Agglutination with monospecific antisera A and M

A drop of diluted anti-A and anti-M sera were placed on a clean glass slide. A loopful of *Brucella* culture was emulsified with each of the sera, rocked gently for one minute and observed for agglutination. *B. abortus* will agglutinate with monospecific antisera A while *B. melitensis* will agglutinate with antisera M. This test included reference strains of *Brucella abortus* (S19) and *Brucella melitensis* (16M) as controls.

3.14.6 Sensitivity to thionin and basic fuchsin dyes

Three sets of 300ml blood agar base (BAB) media were prepared in three 500ml bottles by dissolving 40g of the media per litre of distilled water and dispensing 297ml, 294ml and 188ml of media in three bottles. The media were autoclaved and allowed to cool to 56^0C. Three ml of freshly prepared thionin dye solution was added to the first bottle, 6 ml to the second bottle and 12ml to the third bottle respectively to obtain 10, 20 and 40 percent concentrations of thionin in the media. The bottles were rocked gently to allow for proper mixing of media and the dye. The media was then poured into Petri dishes and allowed to stand on the bench to set. The plates were labelled with the respective concentrations.

Two other sets of 300ml media were prepared in two 500ml bottles as above and dispensing 294 and 188ml media in the bottles. After autoclaving and allowing the media to cool to 56^0C, 6ml and 12ml basic fuchsin dye was added to the first and second bottle to obtain media with 20% and 40% basic fuchsin dye respectively. The bottles were rocked gently to mix well. The dye media was then poured into Petri dishes and allowed to stand on the bench to set. The plates were labelled with the different concentrations.

All the dye plates (three sets for thionin and two for Basic fuchsin) were marked for inoculating with 4 different *Brucella* isolates. They were arranged from the most concentrated to the least concentrated plate for each dye so that inoculation will start from the lowest to the highest concentration.

A suspension of each of the *Brucella* strains to be inoculated was made by suspending a loopful of a 24- hour culture in 0.5ml of saline in a sterile tube and covered with its sterile cover. Following the numbers on the Plates and using a sterile cotton swab soaked in the suspension, a horizontal line was streaked across the media using the same

side of the swab for the same type of dye but changing to another side for the other dye. This was done for each of the isolates and the five control strains which were *Brucella abortus* S19, *Brucella melitensis* 16M, *Brucella suis* 1330, *Brucella ovis* (Bow) and *Brucella melitensis* Rev.1. The plates were incubated at 37^0C in atmosphere of 5-10% CO_2 and another set of plates of the same dyes, concentrations, same number of *Brucella* samples and controls were inoculated and incubated at the same conditions but in the absence of CO_2.

The absence of *Brucella abortus* growth on any of the dye media at any concentration was used to indicate its sensitivity to the action of the dye at the particular concentration while the growth of *Brucella abortus* was used to indicate non-sensitivity of the organism to the action of the dye at the given concentration.

3.14.7 Sensitivity to *Brucella* phages (Wb, Tb, Iz, R/C)

The *Brucella* isolates to be tested were grown on media along with the control strains for 24 hours. Care was taken to ensure all suspensions had uniform turbidity. As for the dye test above, four horizontal lines were made on each of the agar plates so that four isolates were streaked on each plate along the horizontal lines. Five horizontal lines were made on the agar plates to be used for inoculation with the reference strains as controls. The Trypticase soy agar (TSA) plates containing 1% yeast extract and 5% sterile newborn calf serum were well dried. The plates were first of all inoculated with the *Brucella* isolates and then the controls strains in similar pattern as in the dyes tests. However, in this case the swabs sticks used for streaking the agar plates with the isolates were changed after streaking each isolate. There were five sets of four plates each. The first four sets of four plates were labelled for inoculation with four isolates. Each of the four isolates was streaked in straight line on the agar plate. Therefore all the

16 test *Brucella* isolates were represented in each set of the agar plates. The last set of four agar plates was used for inoculation with five different *Brucella* reference strains as controls. Each plate was labelled for streaking with each of the five control strains. The control plates were shared among the first four sets to obtain four sets of five agar plates. Each set of five agar plates was assigned to one phage as there were four phages to be used, namely, Tb, Wb, Iz and R/C.

Using a Pasteur pipette, 1 drop of Routine test Dilution (RTD) of the phage was carefully applied on each streaked line in alternating pattern to avoid coalescing of the drops. This was done for all the five agar plates per phage until the four phages were applied. These first sets of phage agar plates were for incubation at 37^0C in 10% CO_2. A second four sets of five agar plates were also inoculated similarly with the four phages for incubation at 37^0C without CO_2. The plates were left to stand for the phages to fully absorb into the media before they were packed and incubated at 37^0C both in 10% CO_2 and in absence of CO_2 and observed after four days.

The plates were then removed from the incubator and observed for lysis in the streaked areas where phages were applied. A zone of lysis on the streaked areas where phages were applied indicated that the organism was sensitive to the particular phage while absence indicated that the organism was not sensitive to the phage. *Brucella melitensis, Brucella canis and Brucella ovis* are always not lysed by all phages at both dilutions, *Brucella abortus* is always lysed by all phages at both dilutions while *Brucella suis* and *Brucella neotomae* are not lysed at RTD but are lysed at 10, 000 x RTD.

3.1 5 Bruce-Ladder Multiplex PCR

3.15.1 Inoculation of *Brucella* isolates to obtain template DNA

The Bruce-ladder multiplex PCR was carried out at the University of Navarra, Pamplona and Centro Investigacion Technologia Agroalimentaria (CITA), Zaragoza, Spain. The solates were inoculated onto freshly prepared Trypticase soy agar (TSA) and incubated in 5% CO_2 in a CO_2 incubator at 37^0C for 48-72 hours.

3.15.2 Extraction of genomic DNA

Genomic DNA was extracted from the pure *Brucella* cultures of all the 16 *Brucella* isolates using a standard commercial microbial DNA extraction kit known as QIAamp DNA (QIAGEN, Hamburg, Germany).

Briefly, the fresh *Brucella* cells were resuspended in 0.5 ml of sterile TE buffer (10nM Tris-HCL, 1mM EDTA, pH, 8.0). The suspension was heated at 80^oC for 15 minutes and then incubated at 37^oC for 1 hour with 0.5% sodium dodecyl sulphate (SDS) and Proteinase K (200 mg/ml). Cell wall debris, denatured proteins and polysaccharides were then removed by precipitation with 5M NaCl and CTAB-NaCl solution and incubated at 65^oC for 10 minutes. The DNA was then extracted by a standard protocol with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol and washed with ethanol. The DNA pellet was re-dissolved in 50-100µl of sterile distilled water for PCR.

3.15.3 Primers used in Bruce-ladder multiplex PCR

The PCR primers used were based on species-specific or strain-specific genetic differences as reported by Lopez-Goni *et al.* (2008) (Table 3.2).

Table 3.1: Oligonucleotides primers used in Bruce-ladder multiplex PCR

Primer	Sequence (5'-3')	Amplicon (bp)	DNATarget	Source of genetic difference
BME10998f BMEI0997r	ATC-CTA-TTG-CCC-CGA-TAA-GG GCT-TCG-CAT-TTT-CAC-TGT-AGC	1682	Glycosyltransferase wboA	IS*711* insertion in BMEI0998 in *B. abortus* RB51, and deletion of 15,079 bp in BMEI0993-BMEI1012 in *B. ovis*
BMEI0535f BMEI0536r	GCG-CAT-TCT-TCG-GTT-ATG-AA CGC-AGG-CGA-AAA-CAG-CTA-TAA	450 (1320)[b]	Immunodominant antigen, gene *bp26*	IS*711* insertion in BMEI0535-BMEI0536 in *Brucella* strains isolated from marine mammals
BMEII0843f BMEII0844r	TTT-ACA-CAG-GCA-ATC-CAG-CA GCG-TCC-AGT-TGT-TGT-TGA-TG	1071	Outer membrane protein, gene *omp31*	deletion of 25,061 bp in BMEII826-BMEII0850 in *B. abortus*
BMEI1436f BMEI1435r	ACG-CAG-ACG-ACC-TTC-GGT-AT TTT-ATC-CAT-CGC-CCT-GTC-AC	794	Polysaccharide deacetylase	deletion of 976 bp in BMEI1435 in *B. canis*
BMEII0428f BMEII0428r	GCC-GCT-ATT-ATG-TGG-ACT-GG AAT-GAC-TTC-ACG-GTC-GTT-CG	587	Erythritol catabolism, gene *eryC* (Derythrulose-1-phosphate dehydrogenase	deletion of 702 bp in BMEII0427-BMEII0428 in *B. abortus* S19
BR0953f BR0953r	GGA-ACA-CTA-CGC-CAC-CTT-GT GAT-GGA-GCA-AAC-GCT-GAA-G	272	ABC transporter binding protein	deletion of 2653 bp in BR0951-BR0955 in *B. melitensis* and *B. abortus*
BMEI0752f BMEI0752r	CAG-GCA-AAC-CCT-CAG-AAG-C GAT-GTG-GTA-ACG-CAC-ACC-AA	218	Ribosomal protein S12, gene *rpsL*	point mutation in BMEI0752 in *B. melitensis* Rev.1
BMEII0987f BMEII0987r	CGC-AGA-CAG-TGA-CCA-TCA-AA GTA-TTC-AGC-CCC-CGT-TAC-CT	152	Transcriptional regulator, CRP family	deletion of 2,203 bp in BMEII0986-BMEII0988 in *B. neotomae*

Source: Lopez-Goni *et al*. (2008)

a. Designations are based on the *B. melitensis* (BME) or *B. suis* (BR) genome sequences. f: forward; r: reverse.

b Due to a DNA insertion in the *bp26* gene, the amplicon size in *Brucella* strains isolated from marine mammals is 1320 bp

3.15.4 Preparation of primer cocktail

0.5µl of the 16 primers (8 pairs) were aliquoted into a pre-labelled 1.5 ml thin walled microfuge tube. A total of 8 µl of primer cocktail was required for each isolate. This amount was prepared for 25 test tubes. This was made up of sixteen test isolates and nine reference strains as control. The primer cocktail was briefly vortexed to ensure homogeneity.

3.15.5 Preparation of master mix

This was carried out in laminar flow hood (Telsar Bio-IIA, class II biological safety cabinat, Progen Scientific, London, UK) (in PCR Lab**)**. All pipettes, tips, tubes, PCR water needed for this work were left opened and were sterilized by exposing to UV light for 10 minutes. Reaction reagents were pipetted into a sterile 1.5ml microfuge tube in the order below (Table 3.5).

The eight pairs of primers were from http://orders.sigma-genosys.eu.com, deoxynucleotide triphosphate (dNTP) from http://btools.com/, Immobuffer or reaction buffer from www.bioline.com, MgCl2 from www.bioline.com and Immolasa DNA polymerase fom www.biotine.com and ultra pure water.

Table 3.2 Bruce-ladder multiplex PCR mix preparation (per one reaction, final volume of (25µl)

Reagents	Final conc.	1rxn	x 25rxn
PCR buffer 10 X	1x	2.5µl	62.5
dNTPs Mix (2mM)	400mM each one	5.0µl	125.0
$MgCl_2$ (50mM)	3.0mM	1.5µl	37.5
Bruce-ladder eight primer cocktail (12.5)	6.25pmol each one	7.6µl	190.0
H_2O (PCR grade)	-	7.1µl	177.5
DNA Polymerase	1.5U	0.3µl	7.5
Total Volume		**24.0µl**	**600.0µl**

Source: Lopez-Goni *et al*. (2008)

The mix was vortexed briefly to ensure homogeneity of reagents and to avoid bubbles. Then, 24µl was pipetted into 25 pre-labelled 0.2 ml thin walled microfuge tubes. One µl of template DNA was added to the 24µl PCR reaction mixture to make a total of 25µl for each sample. Ultrapure water was used as the negative control.

3.16 PCR Cycling Conditions.

Programme name: *Brucella* (Lopez-Goni *et al*., 2008)

PCR reaction mixtures were placed in a GenAmp PCR system 9700 (Applied Biosystems) and set for amplification at the following cycling conditions.

Initial denaturation:	95^0C for 7 minutes
Template denaturation:	95^0C for 35 sec
Primer annealing:	64^0C for 45 sec
Primer extension:	72^0C for 3 minutes
Final extension:	72^0C for 6 minutes
Total number of cycles:	25

Cool to 4^0C

3.17 Gel Electrophoresis and Analysis

After PCR amplification, 2 µl of PCR product and 8µl of bromophenol blue (loading buffer) were loaded into wells in 1.5% agarose gel in Tris-Hcl, boric acid and Ethylene diethyl tetracetic acid (EDTA) (TBE) buffer in a cuvette flooded with TBE slightly covering the gel. The loading of the PCR products in wells in the agarose gel must correspond with the arrangement or numbering of the isolates and always starting from the second well.

One hundred base pairs DNA ladder or 1kb plus DNA ladder (Invitrogen LTD) (www.invitrogen.com) was used as molecular marker. This was loaded in the first well of each of the gels.

Sterile ultrapure water was used as negative control and was loaded in the last well of the gel.

The gel was stained with 7% SYBR Safe DNA Gel Stain (www.lifetechnologies.com).

The cuvette was covered with the electrophoresis equipment, and the electrodes applied accordingly. The electrophoresis equipment was set to run at 130V for 50 minutes.

Note: Ethidium bromide (5µg/ml) (200µl solution in one litre of distilled water) could be used as an alternative stain.

The gel was then visualised under UV light in the computerized gel documentation equipment (Syngene, G.Box Chemi XX6, Synoptics LTD, California, USA). The bands were observed; the pictures were downloaded on to the computer, analyzed and saved appropriately.

The Bruce-ladder multiplex PCR DNA from *Brucella abortus* strains amplified five fragments of 1,682, 794, 587, 450 and 152bp in sizes; with *Brucella melitensis* DNA, an additional 1,071bp is amplified. *Brucella ovis* is distinguished by the absence of 1,682bp fragment and *Brucella suis* is distigduished by the presence of additional 272bp fragment (also present in *Brucella canis* and *Brucella neotomae*). PCR with *Brucella abortus* S19 DNA does not amplify the 587bp common to all *Brucella* strains, and *Brucella abortus* RB51 is distinguished by the absence of the 1,682bp and 1,320bp fragments and by a specific additional 2,524bp fragment. The *Brucella melitensis* Rev.1 (vaccine strain) is distinguished from other *Brucella melitensis* strains by a specific additional 218bp fragment. *Brucella canis* is distinguished by the absence of the 794bp fragment and *Brucella neotomae* by the absence of 152bp fragments. When DNA from *Brucella* strains isolated from marine mammals are tested, a specific additional 1,320bp fragment is amplified but deletion occurs at the 450bp fragment (Garcia-Yoldi *et al*., 2006; Lopez-Goni *et al*., 2008).

3.18 Data Analysis

Data for serosurvey was first entered into Microsoft Excel spreadsheets. Positive sera were represented with '1' while negative ones were represented with '0'. Prevalence of brucellosis was determined by simple percentage to give a picture of the distribution of the disease and the burden it imposes on livestock in the study areas.

Chi-Square (Snedecor and Cochran, 1980) was used to compare *Brucella* prevalence between the two study areas (Jos Plateau and KGR) and this captures the difference in prevalence of brucellosis in sedentary (Kachia) and nomadic (Jos Plateau) Fulani populations. P-Value of 0.05 was considered as the level of significance.

A computerised gel documentation system (Syngene, Synoptics Ltd, USA) was used to analyse electrophoresis bands from the gel and pictures captured onto to the computer, labelled and saved.

Spatial analysis was carried out using ArcGIS® 9 and ArcEditor™ 9.3.1 package, 1999-2009, and ArcView GIS version 3.1, ESRI, USA. All analyses were done in collaboration with the epidemiology unit, NVRI, Vom. Further analyses were referred to the Swiss Tropical and Public Health Institute (TPHI), Switzerland and Avia-GIS, Belgium.

CHAPTER FOUR

RESULTS

4.1 Overall Seroprevalence of Brucellosis in Cattle in KGR and on the Jos Plateau by Standard RBPT

A total of 1,982 serum samples from the forty cattle herds in the six blocks in KGR were tested for *Brucella* antibodies by standard RBT. Ten (0.5%) animals were positive which represented the individual animal prevalence of brucellosis in KGR by this test. Out of a total of 40 herds, 7 (17.5%) had one or more animals positive for *Brucella* antibodies, which represented the herd prevalence of brucellosis in KGR (Table 4.1).

Similarly, a total of 2,880 serum samples from the thirty six selected cattle herds in six villages on the Jos Plateau were tested for *Brucella* antibodies by standard RBPT. Twenty nine (1.0%) animals were positive and 11(30.6%) of the 36 herds were positive. These represented the individual animal and herd prevalence rates respectively on the Jos Plateau by this test (Table 4.1).

4.2 Overall Seroprevalence and Herd Prevalence of Brucellosis in Cattle in KGR and Jos Plateau by Modified RBPT

A total of 1982 serum samples from KGR were again tested by the modified RBPT. Fifty eight (2.9%) individual animal prevalence was obtained, while 21 (52.5%) herd prevalence was obtained (Table 4.2).

When the 2880 serum samples from Jos Plateau were tested by the modified RBPT, ninety one (3.2%) were positive while 23 (63.9%) of the 36 herds were positive. The individual animal and herd prevalences were 3.2% and 63.9% respectively (Table 4.2).

The GIS maps showing the distribution of brucellosis positive and negative herds in the study areas are presented in figures 4.1 and 4.2.

Table 4.1: Overall prevalence and herd prevalence of brucellosis in cattle in KGR and on the Jos Plateau by standard RBPT (25 µl antigen: 25 µl serum)

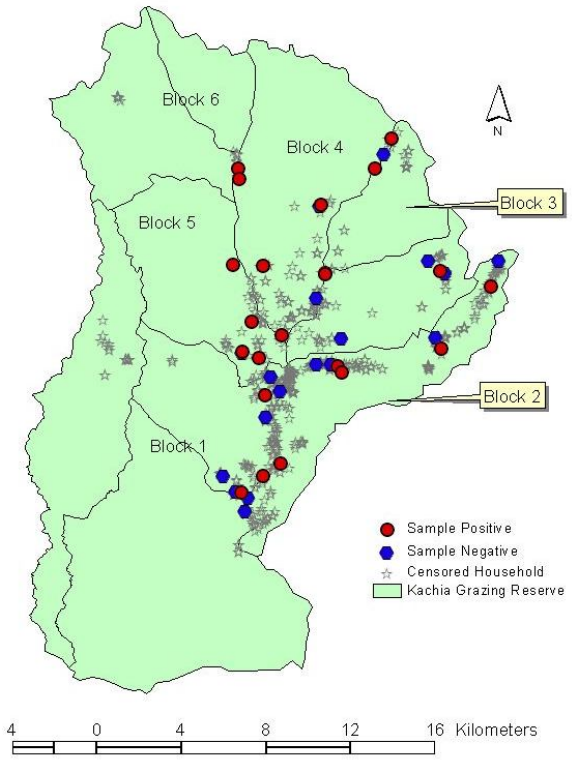
Study areas	No. samples	No.(%) positive		No. herds	Herds (%) positive	
KGR	1982	10	(0.5)	40	7	(17.5)
Jos PL	2880	29	(1.0)	36	11	(30.6)

P-Value = 0.4405, P>0.05

Table 4.2 Overall prevalence and herd prevalence of brucellosis in cattle in KGR and on the Jos Plateau by modified RBPT (25 µl antigen: 75 µl serum)

Study areas	No.tested	No. (%) positive		No.of herds	Herds (%) Positive	
KGR	1982	58	(2.9)	40	21	(52.5)
Jos PL	2880	91	(3.2)	36	23	(63.9)

P-Value = 0.7045, P > 0.05



Source: ICONZ Project (FP7/2007-2013)

Figure 4.1: A map showing spatial representation of the 40 selected herds and brucellosis prevalence in KGR



Source: ICONZ Project (FP7/2007-2013)

Figure 4.2: Spatial presentation of brucellosis prevalence in the selected villages on the Jos Plateau showing brucellosis positive and negative herds

4.3 Seroprevalence of Brucellosis in KGR by Blocks

This describes the individual animal and herd seroprevalence of brucellosis in the various blocks in KGR based on modified RBPT.

The highest individual animal prevalence rate was recorded in block five (6.0%) while the lowest was in block one (1.0%). Other prevalence rates were 5.8% in block three, 5.0% in block six, 3.4% in block four and 2,2% in block two (Table 4.3).

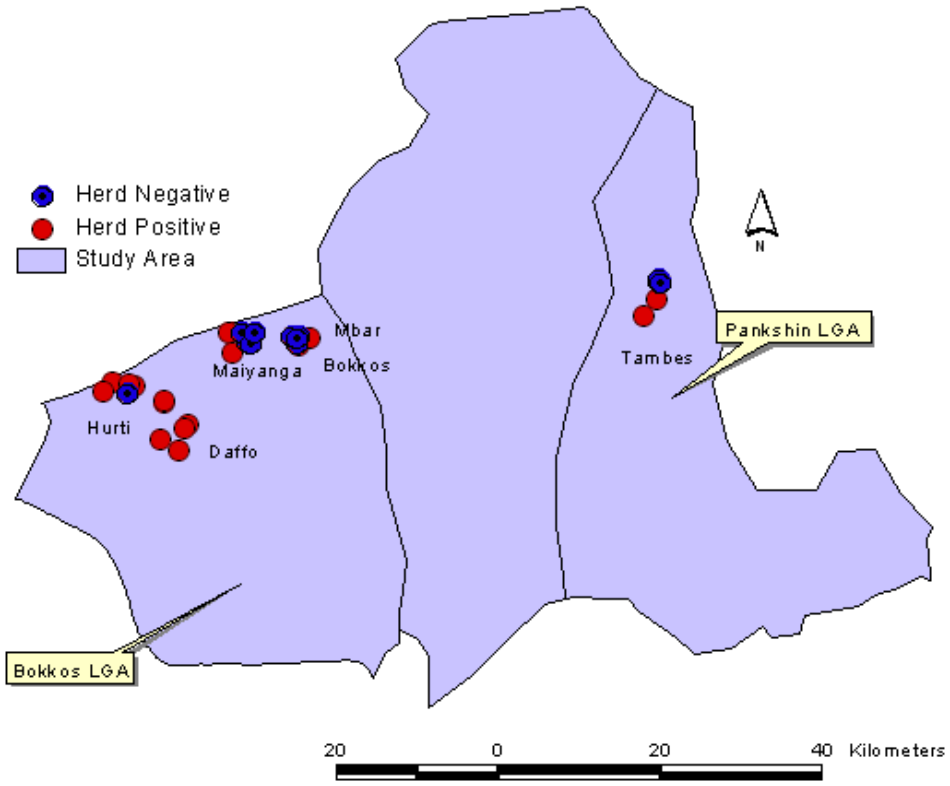
The highest herd prevalence was recorded in block 6 (100%) while the lowest was in blocks one and three, both having 33. 3%. The herd prevalence in other blocks that were between the highest and the lowest prevalence, in descending order were block five (77. 8%), block four (75. 0%) and block two (50.0%).

An enlarged map of the three LGAs that make up the Jos Plateau study area is presented showing the villages and brucellosis positive and negative hreds (Figure 4.3).

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Table 4.3 Individual animal and herd prevalence of brucellosis in cattle in KGR based on blocks by modified RBPT (25µl antigen :75 µl serum)

Block	No.tested	No(%) positive	No. herds	No. hred positive
1	459	5 (1.0)	6	2 (33.3)
2	673	15 (2.2)	14	7 (50.0)
3	223	13 (5.8)	6	3 (33.3)
4	499	17 (3.4	9	7 (77.8)
5	84	5 (6.0)	4	3 (75.0)
6	60	3 (5.0)	1	1 (100.0)



Source: ICONZ Project (FP7/2007-2013)

Figure 4.3: Spatial map of the three selected LGAs and six villages where study was carried out on the Jos Plateau showing brucellosis positive and negative herds

4.4 Seroprevalence of Brucellosis in Cattle on the Jos Plateau According to Villages by Modified RBPT

Considering brucellosis seroprevalence in the various selected villages on the Plateau, the highest individual animal prevalence was recorded in Daffo (7.5%) while the lowest was in Maiyanga (0.4%). Prevalence in other villages between these two values in descending order were Hurti (6.0%), Mbar (2.3%), Bokkos (1.7%) and Tambes (0.8%) respectively (Table 4.4).

The herd prevalence was also highest in Daffo (100.0%) and the lowest was in Maiyanga (33.3%). The herd prevalence in other villages fell between these two in descending order are Hurti (83.3%), Bokkos (66.7%) and Mbar and Tambes both (50.0%) each (Table 4.4).

Table 4.4: Individual animal and herd prevalence of brucellosis on the Jos Plateau according to villages by modified RBPT ((25 µl antigen: 75 µl serum)

Village	No.tested	No. (%) positive	No. Herd tested	No. (%) Herd positive
Hurti	480	29 (6.0)	6	5 (83.3)
Bokkos	480	8 (1.7)	6	4 (66.7)
Mbar	480	11 (2.3)	6	3 (50.0)
Maiyanga	480	3 (0.4)	6	2 (33.3)
Daffo	480	36 (7.5)	6	6 (100.0)
Tambes	460	4 (0.8)	6	3 (50.0)

4.5 Brucellosis Seroprevalence in Cattle in KGR and Jos Plateau Based on Herd Size by Modified RBPT

The relationship between brucellosis seropositivity and herd sizes in both KGR and on the Jos Plateau was determined (Table 4.5).

Seropositivity was found to increase proportionately with herd size as the prevalence was higher in the larger herds but lower in herds with fewer animals.

In KGR, seroprevalence in herds less than 25 animals was 36.4% while the prevalence was 80.0% in herds that had 76 animals and above (Table 4.5).

Table 4.5: Brucellosis seropositivity in cattle in KGR and Jos Plateau based on herd size by modified RBPT (25 µl antigen: 75 µl serum)

Study area	Herd size	No.herds	No. (%) Pos	
KGR	<25	11	4	(36.4)
	26-50	11	5	(45.5)
	51-75	8	5	(62.5)
	>76	10	8	(80.0)
JOS Plateau	<100	8	3	(37.5)
	101-125	7	3	(42.9)
	126-150	7	4	(57.1)
	151-175	6	5	(83.3)
	>175	8	7	(87.5)

P-Value = 0.2166; P<0.05

4.6 Brucellosis Seroprevalence in KGR and Jos Plateau Based on Sex by Modified RBPT

Seroprevalence of brucellosis based on sex and age of cattle in KGR and Jos Plateau were determined. Brucellosis seroprevalence was higher in the female animals compared to the males (Table 4.6).

A prevalence of 3.5% was recorded for female animals in the two study areas while 1.6% and 2.0% prevalence were recorded for the males in KGR and Jos Plateau respectively (Table 4.6).

4.7 Brucellosis Seroprevalence in KGR and Jos Plateau Based on Age by Modified RBPT

On the basis of age, the prevalence was higher in adults compared to young animals in both KGR and Jos Plateau (Table 4.7).

Aprevalence of 1.2% was recorded in young animals while 3.5% was recorded in adult animals in KGR. Similarly, 2.2% prevalence was recorde in young animals while 4.2% prevalence was recorded in adults animals on the Jos animals (table 4.7).

Table 4.6: Brucellosis seropositivity in cattle in KGR and Jos Plateau based on sex bymodified RBPT (25 µl antigen: 75 µl serum)

Variable	Study area	Category	Total no.	No (%) pos.
Sex	KGR	Male	564	7 (1.6)
		Female	1418	49 (3.5)
	Jos Plateau	Male	640	13 (2.0)
		Female	2240	78 (3.5)

P-Value = 0.0130; P < 0.05

Table 4.7: Brucellosis seropositivity in cattle in KGR and Jos Plateau based on age by modified RBPT (25 µl antigen: 75 µl serum)

Variable	Study area	Age Category	Total no.	No (%) pos.
Age	KGR	<1yr	279	0 (0)
		1-3yr	492	6 (1.2)
		3-5yr	744	24 (3.2)
		>5yr	558	22 (3.9)
	Jos Plateau	<1yr	364	0 (0)
		1-3yr	719	16 (2.2)
		3-5yr	966	40 (4.1)
		>5yrs	831	35 (4.2)

P- Value = 0.0000; P < 0.05

4.8 Isolation of *Brucella* from Cattle in KGR

A total of one hundred and twenty eight samples, made up of 70 vaginal swabs, 55 milk, 2 hygroma fluids and 1 placenta were collected from the 40 selected cattle herds in KGR. They were collected only from animals with history of abortion or repeat breeding.The samples were cultured for *Brucella* isolation according to standard method. *Brucella was* isolated in one each of vaginal swabs, milk and hygroma samples. A total of three *Brucella abortus* isolates were therefore obtained from this study area (Table 4.8).

4.9 Isolation of *Brucella abortus* from Cattle on the Jos Plateau

A total of 63 vaginal swabs, 36 milk and 2 hygroma fluid were collected from among the 36 selected cattle herds in the selected villages on the Jos Plateau. These samples were collected only from animals with history of abortion or repeat breeding (Table 4.9).

From these samples, *Brucella abortus* was isolated from 1 vaginal swab, 2 milk samples and 1 hygroma fluid sample (Table 4.9).

Table 4.8 ***Brucella abortus*** **isolated from various sample sources from Kachia Grazing Reserve**

Sample type	**Total No.**	**Isolates**	***Brucella*** **species**
Vaginal swab	70	1	*B.abortus*
Milk	55	1	*B.abortus*
Hygroma	2	1	*B.abortus*
Placenta	1	0	0
Total samples	**128**	**3**	**3**

Table 4.9 *Brucella abortus* isolated from various sample sources on Jos Plateau

Sample type	Total no.	Isolates	*Brucella species*
Vaginal swab	63	1	*B.abortus*
Milk	36	2	*B.abortus*
Hygroma	2	1	*B.abortus*
Total	101	4	4

4.10: Characterization of *Brucella abortus* Isolates from this Study

The isolates from this study were characterized using various biochemical tests and the results were observed and recorded. They grew both in the presence and in the absence of CO_2 after 3 days incubation at 37^0C in presence 10% CO_2 and without CO_2. The colonies were tiny (1-2mm), smooth, with pale honey colour on transmitted light and bluish in reflected light. They were all Gram negative, very tiny cocobacilli, arranged mostly in singles but rarely in pairs and in groups. They produced hydrogen sulphide (H_2S), they were urease positive, oxidase positive, positive with positive *Brucella* sera but negative with negative *Brucella* sera. They produced agglutination with *Brucella* monospecific antisera A but negative with *Brucella* monospecific antisera M. They produced agglutination in *Staphylococcus aureus* Cowan 1 co-agglutination test. All the isolates were lysed by Weybridge (Wb), Tbilisi (Tb), Izadnagar (Iz), smooth *Brucella* phages at routine test dilution (RTD) but were not lysed by rough culture (R/C) (Table 4.10).

Table 4.10: Identification and *Brucella* phage typing of *Brucella abortus* isolated from this study

Isolates / Reference strains	Other confirmatory tests				Monospecific Antisera		Sensitivity to *Brucella* phages			
	Staph Cowan 1	CO2 Req.	Oxidase test	Urease test	A	M	Tb	Wb	Iz	R/C
S19*		-					+	+	+	-
S1330*		-					-	+	+	-
16M*	+	-					-	-	+	-
O63/290*		+					-	-	-	+
Rev.1*		-					-	-	+-	-
KGR3*	+	-	+	+	+	-	+	+	+	-
PLM1**	+	-	+	+	+	-	+	+	+	-
PLB2**	+	-	+	+	+	-	+	+	+	-
KGR2**	+	-	+	+	+	-	+	+	+	-
KGR1**	+	-	+	+	+	-	+	+	+	-

Key: ***Brucella* Controls strians**
****Brucella* isolates from this study**

4.11: Characterization of *Brucella abortus* Isolates from Field Submissions and those from the Archive

The isolates from from field submissions and those from the archive were characterized using various tests and the results were observed and recorded. The isolates grew both in the presence and in the absence CO_2 after 3 days incubation at 37^0C in presence 10% CO_2 and without CO_2. The colonies were tiny (1-2mm), smooth, with pale honey colour on transmitted light and bluish in reflected light. They were all Gram negative, very tiny cocobacilli, arranged mostly in singles but rarely in pairs and in groups. They produced hydrogen sulphide (H_2S), they were urease positive, oxidase positive, positive with positive *Brucella* sera but negative with negative *Brucella* sera. They produced agglutination with *Brucella* monospecific antisera A but negative with *Brucella* monospecific antisera M, except one isolate from the archive (MKD) which produced a doubtful reaction. They Produced agglutination in *Staphylococcus aureus* Cowan 1 co-agglutination test. All the isolates were lysed by Weybridge (Wb), Tbilisi (Tb), Izadnagar (Iz) smooth *Brucella* phages at routine test dilution (RTD), except the isolate (MKD) which was negative with Weybridge. They were all negative (not lysed by rough culture) (R/C) (Table 4.11).

Table 4.11: Identification and *Brucella* phage typing of *Brucella* isolates from field submissions and archive

Isolate/ Reference strains	Other confirmatory tests				Monospecific Antisera reaction		Sensitivity to *Brucella* phages			
	Staph Cowan 1 agglu	CO2 Req.	Oxidase test	Urease test	A	M	Tb	Wb	Iz	R/C
S19*		-					+	+	+	-
S1330*		-					-	+	+	-
16M*		-					-	-	+	-
O63/290*		+					-	-	-	+
Rev.1*		-					-	-	+-	-
MBL**	+	-	+	+	+	-	+	+	+	-
HZ16***	+	-	+	+	+	-	+	+	+	-
MKD**	+	-	+	+	+-	-	+	-	+	-
VSM5***	+	-	+	+	+	-	-	+	+	-
HK15***	+	-	+	+	+	-	+	+	+	-
VSM1***	+	-	+	+	+	-	+	+	+	-
HM14***	+	-	+	+	+	-	+	+	+	-
HP19***	+	-	+	+	+	-	+	+	+	-
HO18***	+	-	+	+	+	-	+	+	+	-
JOS**	+	-	+	+	+	- -	+	+	+	-
HG13***	+	-	+	+	+	-	+	+	+	-

Key: *** *Brucella* control strains**
****Archived isolates**
*****Field submission isolates**

4.12 Biotyping of all the *Brucella abortus* Isolates from this Study

All the *Brucella abortus* isolates from this study grew on agar plates containing 10µg/ml concentration of thionin dye but did not grow on media containing 20µg/ml and 40µg/ml concentrations of thionin. They grew on media containing 20µg/ml and 40µg/ml basic Fuchsin dye. However, *Brucella abortus* S19 which is biotype 1 did not grow on agar plates containing thionin dye at 10µg/ml. All the *Brucella abortus* isolates were therefore confirmed as *Brucella abortus* biotype 3 (Table 4.11).

4.13 Biotyping of all the *Brucella abortus* Isolates from Field Submissions and from Archive.

All the *Brucella abortus* isolates from field submissions and archive were biotyped based on their growth in ager plates containing thionin and basic Fuchsin dyes at various concentrations. The isolates grew on media containing 10µg/ml concentration of thionin dye but did not grow on media containing 20µg/ml and 40µg/ml concentrations of thionin. They grew on media containing 20µg/ml and 40µg/ml basic Fuchsin dye. However, *Brucella abortus* S19 which is biotype 1 did not grow on agar plates containing thionin dye at 10µg/ml. Therefore, on the bases of their growth on agar plates containing thionin at 10 µg/ml, all the *Brucella abortus* isolates were confirmed as *Brucella abortus* biotype 3 (Table 4.12).

Table 4.12: Biotyping of *Brucella abortus* isolates from this study based on their Growth on agar plates containing various concentrations of thionin and basic fuchsin dyes

Isolates / Ref. Strains	Growth on Thionin dye			Growth on basic Fuchsin dye		*Brucella* Species and Biovars
	10µg/ml	20µg/ml	40µg/ml	10µg/ml	20µg/ml	
S19*	-	-	-	+	+	
1330*	+	+	+	-		
16M*	+	+	-	+	+	
Ovis63/290*	+	+	-	-	-	
Rev.1*	+	+	-	-	-	
KGR1**	+	-	-	+	+	*B.abortus 3*
KGR2**	+	-	-	+	+	*B.abortus 3*
KGR3**	+	-	-	+	+	*B.abortus 3*
PLM1**	+	-	-	+	+	*B.abortus 3*
PLB2**	+	-	-	+	+	*B.abortus 3*

Key: * *Brucella* control strains
** *Brucella* isolates from this study

Table 4.13 Biotyping of *Brucella abortus* isolates from field submissions and archive based on their Growth on various concentrations of Thionin and Basic Fuchsin dyes

Isolates / Ref. Strains	Growth on Thionin dye			Growth on basic Fuchsin dye		*Brucella* spp / biovar
	10µg/ml	20µg/ml	40µg/ml	10µg/ml	20µg/ml	
S19*	-	-	-	+	+	
1330*	+	+	+	-		
16M*	+	+	-	+	+	
Ovis63/290*	+	+	-	-	-	
Rev.1*	+	+	-	-	-	
MBL**	+	-	-	+	+	*B.abortus 3*
HZ16***	+	-	-	+	+	*B.abortus 3*
MKD**	+	-	-	+	+	*B.abortus 3*
VSM5***	+	-	-	+	+	*B.abortus 3*
HK15***	+	-	-	+	+	*B.abortus 3*
VSM1***	+	-	-	+	+	*B.abortus 3*
HM14***	+	-	-	+	+	*B.abortus 3*
HP19***	+	-	-	+	+	*B.abortus 3*
HO18***	+	-	-	+	+	*B.abortus 3*
JOS**	+	-	-	+	+	*B.abortus 3*
HG13***	+	-	-	+	+	*B.abortus 3*

Key: * Controls
**Archived isolates
***Field submissions isolates

4.14 Bruce-ladder Multiplex PCR of all the *Brucella* Isolates from this Study, Field Submissions and Archive

The results of Bruce-ladder multiplex PCR for all the *Brucella* isolates obtained in this study, field submissions and those from the archive are presented (Figures 4.1 and 4.2). The amplified amplicons of the isolates were compared with those of the reference *Brucella* strains. The Bruce-ladder multiplex PCR using DNA from *Brucella abortus* reference strains (S19) amplified five fragments of 1,682, 794, 587, 450 and 152bp in sizes. With *Brucella melitensis* DNA, an additional 1,071bp was amplified. *Brucella ovis* was distinguished by the absence of 1,682bp fragment and *Brucella suis* was distinguished by the presence of additional 272bp fragment (also present in *Brucella canis* and *Brucella neotomae*). PCR with *Brucella abortus* S19 DNA did not amplify the 587bp common to all *Brucella* strains, and *Brucella abortus* RB51 was distinguished by the absence of the 1,682bp and 1,320bp fragments and by a specific additional 2,524bp fragment. The *Brucella melitensis* Rev.1 (vaccine strain) was distinguished from other *Brucella melitensis* strains by a specific additional 218bp fragment. *Brucella canis* was distinguished by the absence of the 794bp fragment and *Brucella neotomae* by the absence of 152bp fragments. When DNA from *Brucella* strains isolated from marine mammals are used, a specific additional 1,320bp fragment was amplified but deletion occurs at the 450bp fragment (Figures 4.1 and 4.2).

The Bruce-ladder multiplex PCR results for all the five isolates from this study, nine from field submissions and three from the archive yielded five amplicons of 1,682bp, 794bp, 587bp, 450bp and 152bp bands which exactly corresponded to the bands

amplified by the reference *Brucella abortus* reference strain used as control (Lane 1), (Figures 4.1 and 4.2).

All the 16 *Brucella abortus* isolates were therefore confirmed as *Brucella abortus* by the Bruce-ladder multiplex PCR.

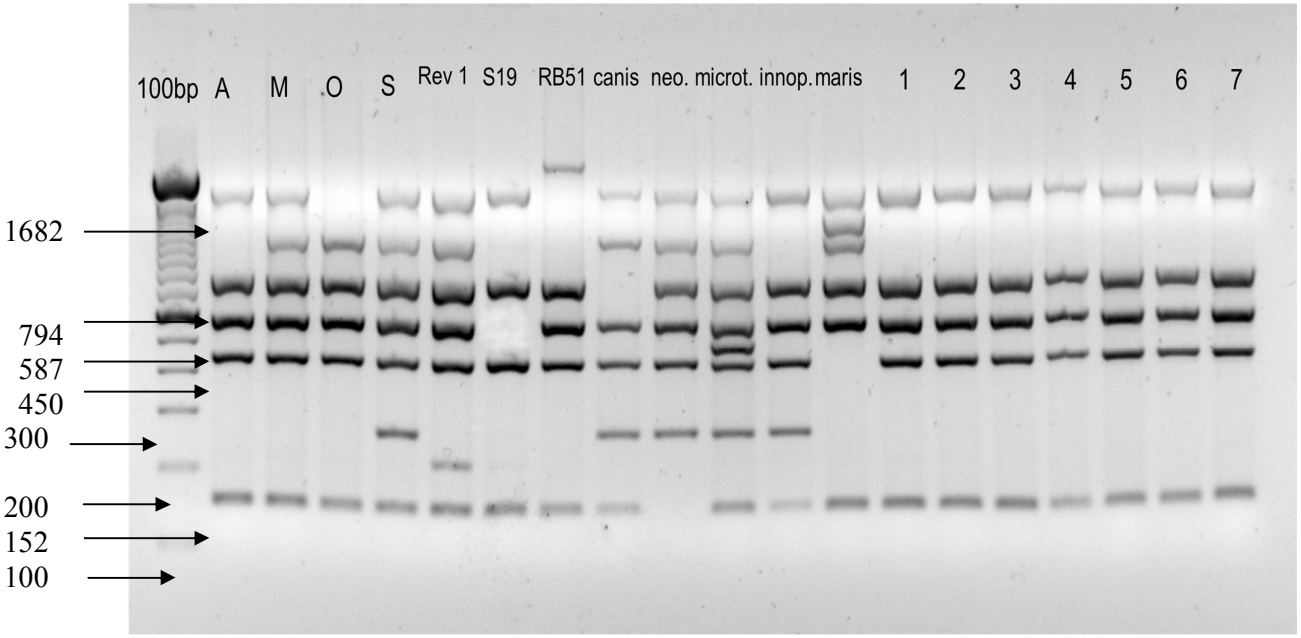


Figure 4.1 Gel photograph of Bruce-ladder multiplex PCR for *Brucella* isolates 1-7 and the 12 *Brucella* reference control strains

Molecular marker (100bp); lane 1, *B. abortus* 2308; lane 2, *B.melitensis* 16M; lane 3, *B. ovis;* lane 4, *B. suis* 1330*;* lane 5, *B. melitensis* Rev1vaccine 6, *B. abortus* S19 vaccine; lane 7, *B. abortus* RB51 vaccine; lane 8, *B. canis*; lane 9, *B. neotomae*; lane 10, *B. microti*; lane 11, *B. inopinata;* lane 12, *B. ceti*; lane 13 (1), MBL; lane 14(2), KGR2; lane15(3), PLM1; lane 16(4), PLB2; lane 17(5), KGR2; lane 18(6),KGR1, lane19(7), HZ16.

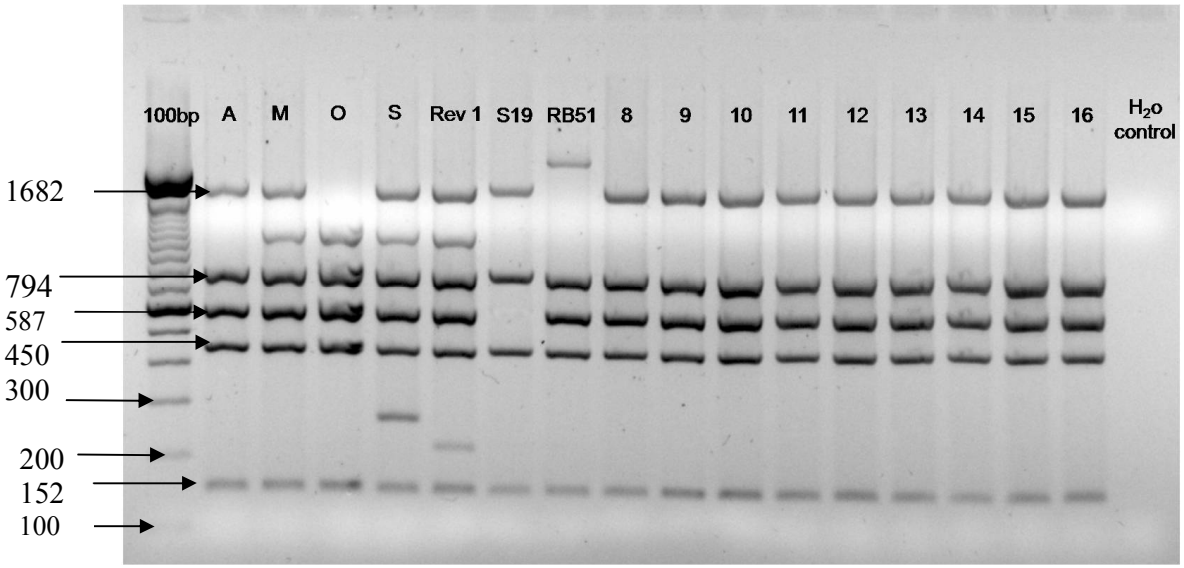


Figure 4. 2 Gel photograph of Bruce-ladder multiplex PCR for *Brucella* isolates 8-16 and 7 *Brucella* reference control strains

Molecular marker (100bp); lane 1, *B. abortus* 2308; lane 2, *B. melitensis* 16M; lane 3, *B.ovis;* lane 4, *B. suis* 1330*;* lane 5, *B. melitensis* Rev1vaccine, lane 6, *B. abortus* S19 vaccine; lane 7, *B. abortus* RB51 vaccine; lanes 8(8), MKD; lane 9(9), VSM5; lane 10(10), HK15; lane 11(11), VSM1; lane 12(12), HM14; lane 13(13), HP19; lane 14(14), HO18; lane 15(15), Jos; lane 16(16), HG13; lane 17, H_2O (negative control).

CHAPTER FIVE

DISCUSSION

The overall individual cattle prevalence of brucellosis in KGR and on the Jos Plateau based on the modified RBPT (mRBPT) were 2.9% and 3.2% respectively while the herd prevalence rates were 52.5% and 63.9% respectively**.** The overall individual animal and herd prevalence of brucellosis in KGR based on standard RBPT were 0.5% and 17.5% respectively while the overall individual animal prevalence and herd prevalence of brucellosis on the Jos Plateau were 1.0% and 30.0% respectively. Although the overall prevalence and herd prevalence of brucellosis were higher on the Jos Plateau compared to KGR, the differences were not statistically significant (P>0.05). The significance of these findings is that more animals as well as humans in these study areas are at great risk of becoming infected.

These herds were reared under traditional management system where free movement and regular congregation of animals occured at the very few earth dams serving as watering points as well as communal grazing habits practised in these study areas. The modified RBPT is a suitable test for brucellosis in endemic but low prevalence areas where animals could be chronically infected but show very low detectable antibodies due to intracellular settling of the organisms. The RBPT performs better when the cell concentration of the antigen is low (Blasco *et al*., 1994; Fareira, 2003). In the modified RBPT, the volume of serum used was three times the volume used in the standard test. This increased volume of serum from 25 µl to 75 µl leads to dilution of the RBPT antigen, thereby increasing the sensitivity of the test without affecting the specificity (Blasco *et al.* 1994; Diaz *et al*., 2011). This was clearly manifested in this study where both the overall prevalence and herd prevalence rates for the two study areas were

higher with the modified RBPT compared to the standard RBPT. Therefore all other serological results were based on the modified RBPT.

Although the overall individual cattle prevalence rates were low (2.9% and 3.2%) in KGR and Jos Plateau respectively, the herd prevalence rates were both high (52.5% and 63.9%), which make the findings very significant and worrisome. These are very important because they show that a significant number of herds have infected animals. According to Smits (2013), not all animals in chronically infected herds show an immunological response detectable in immunological tests because of latency. However, even though the individual animal prevalence detected in immunological tests can be relatively low, the herd remains infected and is a constant source of animal and human infection. Accordingly, what matters in brucellosis is not the individual but the collective (herd) prevalence.

The prevalence of brucellosis in KGR based on blocks varied from low (blocks 1 and 2) to moderately high (blocks 3-6). However, the herd prevalence rates were very high in all the other blocks, indicating the presence of one or more *Brucella* seropositive herds in every block in the grazing reserve.

Similarly, brucellosis seroprevalence was found to be very high in all the six villages on the Jos Plateau. Except for Maiyanga and Tambes where the prevalences were below one percent (0.4% and 0.8%), the individual animal prevalence in the other four villages were above one percent (1.7- 7.5%). This showed a progressive increase from low to high prevalence in the four villages. The herd prevalence ranged from 33.3% to 100% which indicated very high prevalence rates in all the villages, with majority of the herds recording seropositivity. These results are in agreement with the findings of Mai *et al*. (2012).

The high herd prevalences (52.5% and 63.9%) in both KGR and Jos Plateau have serious implication for control. This is because the two areas in their entirety must be taken into consideration if the disease is to be controlled, which means huge resources would be required to achieve control. On the other hand, if herd prevalence was low but individual animal prevalence was high, then the few herds with the infected animals could easily be quarantined or cordoned off and control effected with minimum resources. It also has very serious public health implication because since many herds are positive, many people also may be at risk of being infected either through consumption of raw fresh milk or by direct contact with infected animals in their herds.

Brucellosis seropositivity was found to be higher in larger herds sizes (with 75 animals and above) compared to herds with few number of aninal ($P<0.05$).

In KGR, the lowest prevalence of 36.4% was recorded in herds with less than or equal to twenty five animals while the highest prevalence of 85.7% was in herds that had between fifty one and seventy five animals. This was however, higher than the 70.0% obtained in herds with seventy six to one hundred animals.

On the Jos Plateau, the lowest prevalence of 37.5% was in herds with animals less than or equal to one hundred, while the highest prevalence of 87.5% was in herds with one hundred and seventy five or more animals. It should be noted that the cattle herd sizes on the Jos plateau were much larger compared to the herd sizes in the KGR, therefore the categorization levels for the two study areas varied accordingly. Generally, the prevalence of brucellosis in herds on the Jos Plateau was also higher compared to those from KGR. The lowest and the highest herd prevalence in cattle on the Jos Plateau were correspondingly higher compared to those in KGR.

The low individual animal prevalence of brucellosis recorded in both KGR and Jos Plateau may be as a result of increased awareness about the clinical signs of brucellosis

among the herdsmen as it is well recognized by them and known in their local dialect as 'bakale'. They are able to associate the disease with hygroma, abortion and repeat breeding, usually observed among their herds. Therefore, they often sell off these animals with 'bakale' due to frequent abortions and infertility in order to buy young replacement animals.

The prevalence rates were almost similar for the two study areas, but they were slightly higher on the Jos Plateau compared to KGR. This difference was, however, not statistically significant ($P > 0.05$). One of the reasons for the higher prevalence on the Jos Plateau might be the large sample size obtained in that area compared to KGR. The study design on the Jos Plateau had been done by a project group that required the selection of larger herds for adequate groupings. Therefore, larger herd sizes were sampled in preference to smaller herd sizes. The same study design was used in this study as the field work was carried out simultaneously. On the other hand, all herd sizes were eligible for sampling in KGR where majority of households had small herd sizes. The reason for the association of large herd sizes with the risk of exposure to *Brucella* infection may be due to high cattle density on pasture or at watering points and consequently an increased chance of contact with infected animals or infected materials discharged by an infected animal. According to McDermott and Arimi (2002), the incidence of brucellosis decreases when the herd size decreases in pastoral production systems. Large herd sizes have been reported to be associated with high brucellosis seropositivity (Coelho *et al*., 2007; Sanogo *et al.,* 2012; Zinsstag *et al*., 2013). Secondly, another reason for the higher prevalence on the Jos Plateau may be as a result of the fact that the herdsmen in this area are fully nomadic and therefore they have higher chances of contact with other nomadic herds from different geographical areas as they travel over long distances in search of pasture, unlike those from KGR which are

more or less sedentary with less migration. Thirdly, the difference in weather condition (diurnal temperature) between these two study areas is likely to contribute to the slightly higher prevalence on the Jos plateau compared to KGR. This is because *Brucella* may survive for a longer period in the environment on the Jos plateau due to its colder weather, unlike in KGR which is a hot and dry lowland area. It has been reported that dry and hot environment has adverse effect on the survival of *Brucella* organisms (Davies and Casey, 1973; FAO/OIE/WHO, 2006). At low temperature, *Brucella* may persist for longer period in the environment, leading to the risk of direct transmission and persistence in the livestock (European Commision, 2001; Zinsstag *et al*., 2013). The presence or introduction of *Brucella* infected animal in a herd is an indication of potential danger for transmission of brucellosis in that herd and in the entire area as that animal remains a chronic carrier of the organism (Smits, 2013). This is made possible because of the free movement of cattle and communal grazing habits practised by pastoralists and farmers in these areas.

These individual animal prevalence rates of 2.9% and 3.2% in this study were lower than the prevalence reported by Atsanda *et al*. (1999) for south western and north eastern states of Nigeria respectively in two separate brucellosis surveys. It also differed from the findings of Bawa *et al*. (1987) and Ocholi *et al*. (1996) both carried out in Fulani herds in Kaduna State where they recorded higher prevalence rates. The differences could be due to time interval between their study and the present study. Another reason may be the possible increase in the number of herdsmen becoming aware of the disease and adopting certain traditional livestock production practices that reduce the transmission of the disease. Other studies that reported higher prevalence rates compared to the present study were those by Nuru and Dennis (1975), Shehu *et al*.

(1999) Gusi *et al*. (2010) and Maurice *et al*. (2013), all in northern Nigeria. Bovine brucellosis has recently been reported in both trade and resident cattle in a large study covering some central and northern states of Nigeria (Bertu *et al*., 2012). These reports were also higher compared to the findings in this study. Higher prevalence rates compared to these findings were also reported in trade and slaughtered cattle in Ibadan abattoir, south western Nigeria (Cadmus *et al*., 2010) and in cattle herds in three states in northern Nigeria (Mai *et al*., 2012). In two studies conducted on marketed and fresh milk from cattle herds in Jos and environs by Bertu *et al*. (2010) and in pooled market milk and fresh milk in Jigawa State by Farouk *et al*. (2013), higher prevalence rates compared to the present findings were reported. In Eastern Nigeria, Chukwu (1987) in a study conducted in abattoirs in Enugu and Nsukka reported higher prevalence than the findings in this study.

The findings in this study were however comparable to those reported by Bello (1992), Nuru and Dennis (1975) and Wungak *et al*. (2011). Elsewhere, Tolosa *et al*. (2008), Haileselassie *et al*. (2010), Asmare *et al*. (2010) reported lower prevalence rates compared to the findings in this study.

The low prevalence in this study compared to previous reports may be as a result of the very large sample size used in the study. Most brucellosis studies in Nigeria that reported high prevalence rates were based on small sample sizes except the report by Mai *et al*. (2012). Other studies were usually a single farm report, (Ajogi *et al*., 2002; Ojo *et al.,* 2007; Junaidu *et al*., 2008), an abattoir survey (Tijjani *et al.,* 2009; Gusi *et al.,* 2010) or a study over a large area but very small sample size (Wungak *et al*., 2011).

Our findings show that brucellosis prevalence varies with age and sex of the animals. The prevalence was lower in animals below the age of three years and higher in animals above three years of age. None of the animals below the age of one year was

seropositive for brucellosis in both KGR and on the Jos Plateau. However, the seropositivity increased in older animals. The prevalence was higher in animals aged between three and five compared to those below three years. Similarly, the prevalence was also higher in the animals that were above five years. Generally, brucellosis seroprevalence was higher in animals that were three years and above compared to those that were below three years old. This was observed in the animals in both KGR and on the Jos Plateau. The higher prevalence of brucellosis in old animals compared to the young animals was statistically significant ($P<0.05$). Age has been known to be one of the intrinsic factors influencing brucellosis seropositivity (Magersa *et al*., 2011). The influence of age on brucellosis seropositivity has been reported in previous studies (Kubuafor *et al*., 2000; Faye *et al.,* 2005; Muma *et al*., 2005; Chimana *et al*., 2010; Mai *et al*., 2012; Sanogo *et al*., 2012) which are in agreement with the current findings. This is to be expected because brucellosis manifests mainly at the age of sexual maturity of the animals due largely to increased activity of erythritol, a reproductive hormone that supports the growth of *Brucella* organisms in pregnant animals (Godfroid *et al*., 2004). The result is abortion in pregnant animals but infection remains latent in young animals. Another reason for age related seropositivity is that the older an animal, the higher the likelihood of contact with infected animals and therefore an increased accumulated chance of becoming exposed to the risk of *Brucella* infection.

The prevalence of brucellosis in this study was higher in females compared to males. This difference was statistically significant ($P<0.05$). The reason for the higher prevalence in females may be due to the fact that herds usually consist of more females since males are often sold out leaving only one or a few mature bulls to avoid fighting among the males during mating when cows are on heat. Therefore, there were higher number of females per herd than males; thus, more females were sampled than the

males. This finding agrees with the report of El Sherbini *et al.* (2007), Junaidu *et al*. (2011) and Sanogo *et al*. (2012). However it differs from the report by Mai *et al.* (2012) who reported higher seroprevalence in males compared to female cattle in their study.

In this study, three *Brucella abortus* were isolated in KGR while four *Brucella abortus* were isolated on the Jos Plateau, giving a total of seven *Brucella abortus* isolates. The fact that *Brucella abortus* strains were isolated in both study areas is very significant as it is a confirmation of the endemicity of brucellosis in both KGR and on the Jos Plateau. This has far reaching implications as the infection may easily spread to other cattle herds as well as sheep and goats which are usually reared along with cattle due to shedding of the organisms on pasture. Infection could also be spread from infected cows to non infected cows and and heifers by bulls through mating and also through loaning of bulls commonly practiced by herdsman. There is also very serious effect on production as infected animal may abort and thereafter become infertile. Infection could also be spread to none infected herds by dogs are scavengers and usually carry aborted foetuses and placenta from one area to another and in the process contaminating the envrironment with *Brucella* organisms. Further spread of brucellosis from both KGR and Jos Plateau to other animals could also occur during migration as the cattle move along their various routes to distant areas in search of greener pasture and or because grazing areas have been used for crop farming.

The isolation of *Brucella abortus* from milk in both KGR and Jos Plateau is of great public health significance because *Brucella* organisms are usually shedded through the milk of any infected cow and which is freely consumed raw or as fermented milk by the Fulanis. There is a strong cultural belief among the herdsmen that favour the

consumption of raw milk against pasteurized milk. The most common means of transmission of brucellosis from animals to humans is through the consumption of unpasteurized or raw milk and milk products (Kang'ethe *et al*., 2000). The fact that their children take raw milk directly from the udder of a lactating cow, poor knowledge about the dangers of brucellosis and the lack of measures for the control of brucellosis, the risk to human health could be great.

The isolation of *Brucella abortus* from the vaginal swab is also of great public health significance. This is because the herdsmen are in the habit of putting their bare hands through the vulva of a parturient cow to correct the position of the calf in cases of dystocia to enable successful calving. Also in cases of retained placenta, the herdsmen usually use their bare hands to pull the placenta out of the vulva. These habits and practices expose them to high risk of contracting brucellosis through direct contact with the organisms through their bare hands.

It is interesting to note that the two blocks in KGR (blocks 1 and 2) where the three *Brucella abortus* isolates were obtained were contiguous. Similarly, two of the three villages on the Jos Plateau (Mbar and Maiyanga) where the *Brucella abortus* isolates were obtained were also contiguous. This could be explained by the close proximity of these blocks or villages which allows for easy and frequent movement of cattle from one block to the other or from one village to the other while grazing and at watering points. This makes constant contacts of cattle from these blocks (KGR) or villages (Jos Plateau) possible, thereby favouring the spread of brucellosis from one block or village to the other. A similar finding has been reported by Mamisashvili *et al*. (2012). The reverse is the case when the blocks or the vallages are not contiguous. The possibility of contacts between infected animals in one block or village with the susceptible

animals in the distant block or village may be remote; hence there is little or no possibility of transmission of brucellosis between the two blocks or villages. This explains the clustering of *Brucella* isolation in only few contiguous blocks in KGR and in two villages on the Jos Plateau. An exception to this situation, however, is the isolation of *Brucella abortus* from Hurti village which is far away from the other two villages. This is therefore an isolated case which might have been as a result of purchase of an infected replacement animal from the village cattle market into the herd, rather than through infected animals from the other herds.

The isolation rate of *Brucella abortus* in this study was higher in hygroma fluids (50%) compared to other samples. This was evident in the isolation of *Brucella abortus* from two out of the four hygroma fluid sampled as compared to a total of five islates obtained from two hundred and twenty four samples comprising of vaginal swabs and milk samples. *Brucella* was not isolated from the single placenta sample collected. A possible explanation to this is that hygroma fluid is comparatively less contaminated compared to vaginal swab, milk or aborted tissues which may easily become contaminated prior to or during sample collection for laboratory investigation. This is because these other samples are usually exposed to contaminations due to contact with microbes in the environment, but hygroma fluids are usually well enclosed in a sack beneath the skin of the animal and is usually collected directly into a sterile syringe through a sterile needle with no exposure to environmental contaminants.

This finding is consistent with reports of previous investigators in Nigeria where majority of the isolates were from hygroma fluids and only a few from other samples such as milk, vaginal swabs, aborted foetuses and placentae (Eze, 1978; Bale and Kumi-Diaka, 1981; Ocholi *et al*., 2004b). This further attests to the ease of isolating the

organism from hygroma fluids as argued by previous authors (Corbel, 2006; Godfroid *et al*., 2010; OIE, 2009), that although *Brucella* could be isolated from other samples and tissues, hygroma is the most valuable in terms of success rate of isolation in nomadic and semi-nomadic cattle herds in Africa compared to other samples. The isolation of *Brucella* from hygroma and vaginal swabs is of clinical significance as abortion, leading to copious excretion of *Brucella* organisms through the vagina and the presence of knee-joint hygroma are the most common clinical signs that aid clinicians in the diagnosis of brucellosis. The non-isolation of *Brucella* from the placenta sample may be as a result the fact that the sample was collected long after the the abortion occurred and had already started deteriorating due to necrosis. Secondly, the sample may have been heavily contaminated as it was collected on the ground muddled up with sand. Isolation of *Brucella* from heavily contaminated samples is very difficult, because fast-growing contaminants would usually suppress and overwhelm the growth of *Brucella* if present, since it is slow-grawing.

The isolation of *Brucella abortus* from specimens examined in this study confirmed its presence in the study areas and this agrees with reports of previous invstigators that bovine brucellosis in Nigeria is generally caused by *Brucella abortus* (Eze, 1978; Bale and Kumi-Diaka, 1981; Ocholi *et al*., 2004; Ocholi *et al*., 2005). *Brucella abortus* comprises seven classical biovars, namely, 1, 2, 3, 4, 5, 6 and 9.

The findings in this study have provided additional data and new location in Nigeria where *Brucella abortus* have been isolated. The successful isolation of *Brucella abortus* from field samples submitted from various states in Nigeria is also an attestation to the widespread distribution of brucellosis due to *Brucella abortus* in Nigeria. The fact that *Brucella* isolates which have been in the archive for decades show consistency with

those in this study and those from field submissions indicates that the species of *Brucella* found in Nigerian cattle over the years has remain the unchanged, with no other *Brucella* species isolated in cattle to date besides *Brucella abortus*.

This study exhaustively carried out all the classical biotyping procedures for the identification of all the *Brucella* isolates. Accordingly, the isolates were all identified as *Brucella abortus* biotype 3. This finding differs from those of other previous investigators (Eze, 1978; Ocholi *et al*., 2004; Ocholi *et al*., 2005) in which *Brucella abortus* biotype 1 was reported as the prevailing biotype in Nigeria. However, a similar finding had previously been reported (Bale and Kumi-Diaka, 1981). *Brucella abortus* S19, a *Brucella abortus* biotype 1 strain was used as control srain during the biotyping. Differences between biotypes 1 and 3 are not very apparent and can only be distinguished based on their sensitivity to thionin dye (Banai and Corbel, 2010). While *Brucella abortus* S19 Biotype 1 (control) did not grow in presence of thionin at concentrations of 10µg/ml, 20µg/ml and 40µg/ml, all the sixteen isolates tested in this study grew in the presence of thionin at 10µg/ml but did not grow at 20µg/ml and 40µg/ml thionin concentrations which is a typical behaviour of *Brucella abortus* biotype 3. This confirmed all the isolates as *Brucella abortus* biotype 3. All the isolates including those from the archive and those from field submissions which were earlier reported as being biotype 1, have been confirmed as biotype 3. The omission of thionin at concentration of 10µg/ml and using only 20µg/ml and 40µg/ml in previous biotyping may be the reason why the isolates were not identified as biotype 3. This is because both *Brucella abortus* biotype 1 and biotype 3 do not grow in the presence of thionin at 20µg/ml and 40µg/ml concentrations. They are, however, differentiated based on their

growth or absence of growth in the presence of thionin at 10µg/ml. While biotype 3 grows at this concentration, biotype 1 does not.

The biotyping of isolates in this study was carried out in Nigeria and later validated along with archived isolates at Department of Microbiology and Parasitology, University of Navarra, Pamplona, Spain and Centro Investigacion Technologia Agroalimentaria (CITA), Zaragoza, Spain. Secondly, representative isolates from previous studies in Nigeria that were reported as biotype 1 were also subjected to this classical biotyping and they all showed reactions consistent with *Brucella abortus* biotype 3. This new finding may be as a result of improved technology in classical biotyping of *Brucella* organisms.

Other studies have reported *B. abortus* biovar 3 as the biotype circulating in countries that border Nigeria (Sanogo *et al.,* 2013b) and the indiscrimate animal movements between these countries and Nigeria points to more likely circulation of *Brucella abortus* biotype 3 in Nigeria and not biotype 1.

This study may be the first report to use the Bruce-ladder multiplex PCR for the identification of *Brucella* isolates from Nigeria. The Bruce-ladder multiplex PCR was able to successfully identify all the sixteen phenotypically characterized *Brucella abortus* isolates from Nigeria to species level as *Brucella abortus.* This has further confirmed the accuracy of the bacteriological identification of the isolates as *Brucella abortus*. Although Bruce-ladder multiplex PCR cannot differentiate biovars from the same species, it is species specific and all the strains and biovars of the same *Brucella* species give the same profile (Lopez-Goni *et al*., 2008). A major advantage of the Bruce-ladder PCR for identification of *Brucella* species is that it is capable of

identifying and differentiating all the *Brucella* species and the vaccine strains in the same test in one reaction (Garcia-Yoldi *et al*., 2006; Lopez-Goni *et al*., 2008).

To our Knowledge, there is only one available report on the molecular detection of *Brucella* species from broth culture of clinical samples using a genus-specific primer based on IS6501 sequence (Antiabong *et al*., 2009). That technigue had limited epidemiological significance as it was unable to type the organisms to species level.

The isolation of *Brucella abortus* biotype 3 in this study is consistent with reports from other African countries, particularly in West Africa, an important area for livestock production and having the largest population of ruminants after East Africa (OECD, 2008; FAO, 2010). *Brucella abortus* appears to be the main species infecting cattle in West Africa and biotype 3 appears to be the most common biotype (Sanogo *et al*., 2013b). Except in Mali, biotype 3 was identified in 7 out of 8 countries of the Sub-region where biotyping has been carried out. *Brucella abortus* biotype 3 has been described in Senegal (Verger e*t al.,* 1979), Togo (Verger *et al.,* 1982), Niger (Akakpo *et al*., 1986), and most recently, The Gambia (Bankole *et al*., 2010) and Ivory Coast (Sanogo *et al*., 2013b). A similar trend was reported in Central Africa (Domenech *et al*., 1983) where most of the isolates were *Brucella abortus* biotype 3.

Brucella typing based on detection of *Brucella* DNA (Yu and Nielsen, 2010) which are comparatively less tedious than isolation have also been carried out in other West African countries (Bankole *et al.,* 2010; Sanogo *et al.,* 2013). The present study in which Nigerian isolates of *Brucella abortus* were identified by Bruce-ladder multiplex PCR has included Nigeria among the West African countries from where *Brucella* strains have been successfully subjected to molecular identification by PCR.

The uses of geographic information system (GIS), mapping and georeferencing have become important tools in epidemiological study of animal diseases. This is because they provide pictoral presentation of disease distribution over an area and this gives a better and realistic appreciation of the situation. GPS is very useful because it can be used to obtain coordinates of study areas, even in very remote locations. Data obtained is reproducible and each location can be traced back after many years. The GPS enabled the accurate location of all the households in KGR and on the Jos using the coordinates of each household. It helps to show the accurate location of cattle herds in each of the six blocks in KGR and in each of the six villages on the Jos Plateau. It also provided the accurate location of all the selected herds where samples were collected in both KGR and on the Jos Plateau. The use of GIS in this study made it possible to obtain the maps of the study areas. It also provided the pictoral presentation of the prevalence of brucellosis in the two study areas by showing clearly, the location of brucellosis positive herds and brucellosis negative herds. This was made possible because of the availability of colour legends inherent in the GIS software. These maps show the spatial distribution of brucellosis in the study areas, which were evenly distributed. They gave very clear picture of brucellosis prevalence in the study areas which could be better appreciated and easily traced back by future researchers.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study established the serological prevalence of bovine brucellosis in KGR as shown by the individual cattle prevalence of 2.9% and herd prevalence of 52.5% and the individual cattle prevalence of 3.2% and herd prevalence of 63.9% on the Jos Plateau.

The study established that the individual animal prevalence rates were low in the two study areas.

The study revealed that the herd prevalence rates of brucellosis were very high in both KGR and on the Jos Plateau.

The study also found that the prevalence of brucellosis was widespread in all the blocks in KGR and in all the villages studied on the Jos Plateau.

It was also established that large herd sizes were associated with high prevalence of brucellosis compared to smaller herd sizes. These findings have very serious implications for the control of brucellosis as they indicated widespread brucellosis in the two study areas. The presence of seropositive animals is a good indication of infection.

The study successfully isolated 3 *Brucella abortus* from cattle in KGR and 4 *Brucella abortus* strains from cattle on the Jos Plateau, thereby confirming the endemicity of brucellosis in KGR and on the Jos Plateau.

The study established the fact that *Brucella abortus* may the species of *Brucella* circulating in cattle in the selected herds in the two study areas.

The comprehensive classical biotyping revealed that all the *Brucella abortus* strains isolated in this study were *Brucella abortus* biotype 3.

The study also established that all the *Brucella abortus* strains from field submissions were *Brucella abortus* biotype 3.

Similarly, classical biotyping of the archived isolates revealed that they were all *Brucella abortus* biotype 3.

This may be the first report to show that the prevailing *Brucella* species circulating among cattle in Nigeria is *Brucella abortus* biotype 3.

This study, to our knowledge may be the first to apply a molecular method, especially, the Bruce-ladder multiplex PCR for the identification of *Brucella* isolates from Nigeria to species level.

The Bruce-ladder multiplex PCR used in this study successfully identified all the *Brucella* strains isolated from Nigeria as *Brucella abortus*.

The findings in this study have shown that brucellosis due to *Brucella abortus* is endemic in the study areas and represents a potential threat to animal production and public health.

This study effectively produced the GIS maps of KGR showing the spatial distribution of brucellosis prevalence in all households in the reserve. Brucellosis positive and negative herds were also distinctly presented on the map.

A map of the Jos Plateau study area was also produced with all selected herds well located on the map, showing brucellosis positive and negative herds. The availability of GPS coordinates used in this study is of great importance as it will enable epidemiological trace- back of herds possible.

6.2 Recommendations

Based on the findings of this present study, the following recommendations are made.

1. More research is needed in the area of genotyping and sequencing of *Brucella* isolates from Nigeria to determine the phylogenetic position of the strains circulating in Nigeria and their relationship with those from other parts of the world that are in the data bases.

2. A similar work should be carried out in these areas to determine the prevalence of brucellosis in small ruminants and humans.

3. There is the need for government to institute a project for determining brucellosis epidemiological factors as a pre-requisite for control and eventual eradication of the disease in the study areas.

4. There is the need for educational programmes in the areas to alert, not only the livestock owners, but the general public on the danger of possible disease transmission both in animals and humans.

5. The use of Geographic information system (GIS) and goereferencing should be encouraged to effectively document the presence of brucellosis not only in these study areas but nationwide to fully understand the spatial distribution of the disease in Nigeria.

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APPENDICES

Appendix I

List of 40 selected households sampled from 6 blocks in KGR with their GPS coordinates

S/No	Block	Village	Name of Household head	Latitude.	Longitude.
1	1	Nasawara	Likita Adamu	10.17533	8.07841
2	1	Nasawara	Abubakar Muhammadu	10.15820	8.07721
3	1	Nasawara	Isa Muhammadu	10.12595	8.06539
4	1	Nasawara	Alh. Danjuma Muhammad	10.16766	8.07567
5	1	Anguwan Kofoje	Alh. Abdullahi Muhammadu	10.12643	8.06337
6	1	Nasawara	Alh. Aliyu Abdullahi	10.13298	8.05822
7	2	Wuro Nyako	Adamu Abubakar	10.12383	8.06881
8	2	Wuro Nyako	Abubakar Alh. Dare	10.11832	8.06744
9	2	Wuro Nyako	Alh. Ali Weaty	10.13328	8.07479
10	2	Wuro Nyako	Zakariya Usman	10.13855	8.08247
11	2	Tigirde	Alh. Idris Halidu Yamusa	10.18098	8.09773
12	2	Wuro Fulbe	Alh. Ahmed Sule	10.16894	8.08233
13	2	Tigirde Danladi	Jibrin Musa	10.18006	8.10638
14	2	Tigirde Danladi	Alh. Saleh Abubakar	10.18091	8.10396
15	2	Tigirde Danladi	Usman Dangana	10.17742	8.10847
16	2	Mayo Jamil	Abdullahi Ba'aso Bode	10.19234	8.14851
17	2	Mayo Jamil	Mahmud Usman	10.21386	8.17177
18	2	Mayo Jamil	Isah Ahmed	10.22467	8.17525
19	2	Maljire	Haruna Adamu	10.18744	8.15049
20	2	Wuro Fulbe	Ibrahim Saleh Dogo	10.26387	8.06442
21	3	Wuro Sale	Alh. Bako Bayero	10.21961	8.10105
22	3	Wuro Sale	Zakari Bako	10.21895	8.10141
23	3	Mayo Borno	Sambo Haruna	10.22032	8.15022
24	3	Wuro Sale	Yakabu Adamu	10.19196	8.10808
25	3	Mayo Borno	Yusuf Jibrin	10.21954	8.15249
26	3	Mayo Borno	Ismail Saleh	10.22495	8.14520
27	4	Wuro Bayero	Habubakar Baro	10.22284	8.06195
28	4	Tilde Bayero	Alh. Adamu Baba	10.24820	8.09918
29	4	Tilde Bayero	Mohammad Ibrahim	10.20885	8.09767
30	4	Mayo Amana	Ardo Saleh	10.26402	8.12256
31	4	Tudun Gorkawa	Adamu Bello	10.27697	8.1292
32	4	Wuro Azumi	Alh. Tambaya	10.27697	8.12920
33	4	Tilde Bayero	Tambaya Gagarau	10.27036	8.12641
34	4	Wuro Jatijo Yusuf	Ibrahim Yusuf	10.22276	8.07463
35	4	Tilde Bayero	Ibrahim Njaja	10.19331	8.08291
36	5	Wuro Modi	Abdullahi Adamu	10.19882	8.06977
37	5	Wuro Modi	Alh. Fadama	10.18342	8.07305
38	5	Wuro Modi	Umar Bashayi	10.18584	8.06592
39	5	Wuro Modi	Ardo Bashayi Momale	10.18579	8.06590
40	6	Wuro Gijiya	Alh. Jibrin Shaibu	10.25957	8.06486

Appendix II

List of 36 selected households from 6 villages on the Jos Plateau with their GPS coordinates

S/N	Village	Positive Households	Latitude (Degrees)	Longitude (Degrees)
1	Hurti	Alh.Gagarau	9.26388	8.79380
2	,,	Alh. Ori	9.26940	8.80288
3	,,	Chiroma	9.27431	8.77759
4	,,	Pate	9.26452	8.76647
5	,,	Monday	9.27209	8.79560
6	Bokkos	Yakubu Ibrahim	9.31637	8.98344
7	,,	Alh.Salihu Eggi	9.31579	8.98378
8	,,	Musa Ibrahim	9.32627	8.97704
9	,,	Alh.Bingel	9.32443	8.98476
10	Mbar	Alh. Abdullahi Jaoji	9.32334	8.99504
11	,,	Ibrahim Manu	9.32579	8.97696
12	,,	Abdulmumuni Ibrahim	9.32404	8.99578
13	Daffo	Alh.Shagari	9.22813	8.86219
14	,,	Alh. Jaoji	9.25265	8.83545
15	,,	Alh.Abubakar	9.21071	8.83050
16	,,	Alh.Musa	9.25344	8.83459
17	,,	Alh.Shanono	9.22340	8.85813
18	,,	Alh. Maigizo	9.19932	8.85074
19	Maiyanga	Saleh Yusuf	9.39143	8.89062
20	,,	(Alh Abdullahi) Isa	9.37247	8.90373
21	Tambes	Hon.Moses	9.34872	9.36726
22	,,	Ibrahim Doro	9.38619	9.38540
23	,,	Hamman Shaibu	9.36712	9.38167
S/N	**Village**	**Negative Households**	**LATITUDE**	**LONGITUDE**
1	Hurti	Musa Zellaini	9.26162	8.79449
1	Bokkos	Alh.Saidu	9.32466	8.98396
3	,,	Alh. Abdullahi	9.32579	8.97663
4	Mbar	Ibrahim Yusuf	9.31667	8.98290
5	,,	Ahmadu Mohammed	9.32634	8.97580
6	,,	Sule Ahmadu	9.32276	8.98264
7	Maiyanga	Adulkarim	9.38930	8.90276
8	,,	Idris Haruna	9.388924	8.90324
9	,,	Haruna Maguna	9.37783	8.90818
10	,,	Isa Idris	9.38948	8.90515
11	Tambes	Ibrahim Baba	9.38731	9.38262
12	,,	Alh.Saleh	9.38910	9.38303
13	,,	Gambo Shuaibu	9.38497	9.38486

Appendix III

Total number of samples collected for *Brucella* isolation from KGR and Jos Plateau

Study areas	Milk	Vaginal swab	Hygroma	Placenta	Total
KGR	70	55	2	1	128
Jos Plateau	36	63	2	0	101
Total	**106**	**118**	**4**	**1**	**229**

Appendix IV

Total number of B*rucella abortus* isolates taken to Spain

Categories of isolates	S/NO	*Brucella* Strain Codes	No. of vials	State/year of isolatetion	Sample source	Animal species
Isolates from this study	1	KGR 1	2 vials	Kaduna 2011	Hygroma	Cattle
	2	KGR2	2 „	Kaduna 2011	Milk	Cattle
	3	KGR3	2 „	Kaduna 2011	Vaginal swab	Cattle
	4	PLM1	2 „	Plateau 2012	Milk	Cattle
	5	PLM2	2 „	Plateau 2012	Vaginal swab	Cattle
	6	PLB1	2 „	Plateau 2012	Milk	Cattle
	7	PLB2	2 „	Plateau 2012	Hygroma	Cattle
Isolates from field submissions	8	ISO 1	2 „	Adamawa 2007	Hygroma	Cattle
	9	Hk15	2 „	Benue 2010	Hygroma	Cattle
	10	HM14	2 „	Benue 2010	Hygroma	Cattle
	11	HP19	2 „	Benue 2010	Hygroma	Cattle
	12	HZ16	2 „	Benue 2010	Hygroma	Cattle
	13	HO18	2 „	Benue 2010	Hygroma	Cattle
	14	HG19	2 „	Benue 2010	Hygroma	Cattle
	15	VSM1	2 „	C/ River 2011	Vaginal swab	Cattle
	16	VSM5	2 „	C/ River 2011	Vaginal swab	Cattle
Isolates from archive	17	KNO 5	1Ampoule	Kano 1976	Hygroma	Cattle
	18	Butura	2 „	Plateau 1976	Hygroma	Cattle
	19	Mambilla	1 „	Adamawa 1977	Hygroma	Cattle
	20	MDG 330	1 „	Borno 1978	Hygroma	Cattle
	21	Makurdi	2 „	Benue 1978	Hygroma	Cattle

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Appendix V

Brucella abortus isolates used for classical biotyping and Bruce-ladder multiplex PCR

Categories of isolates	S/NO	*Brucella* Strain Codes	No. of vials	State/year of isolation	Sample source	Animal species
Brucella abortus isolates from this study	1	KGR 1	2 vials	Kaduna 2011	Hygroma	Cattle
	2	KGR2	2 ,,	Kaduna 2011	Milk	Cattle
	3	KGR3	2 ,,	Kaduna 2011	Vaginal swab	Cattle
	4	PLM1	2 ,,	Plateau 2012	Milk	Cattle
	5	PLB2	2 ,,	Plateau 2012	Hygroma	Cattle
Brucella abortus isolates from field submissions	6	Hk15	2 ,,	Benue 2010	Hygroma	Cattle
	7	HM14	2 ,,	Benue 2010	Hygroma	Cattle
	8	HP19	2 ,,	Benue 2010	Hygroma	Cattle
	9	HZ16	2 ,,	Benue 2010	Hygroma	Cattle
	10	HO18	2 ,,	Benue 2010	Hygroma	Cattle
	11	HG19	2 ,,	Benue 2010	Hygroma	Cattle
	12	VSM1	2 ,,	C/ River 2011	Vaginal swab	Cattle
	13	VSM5	2 ,,	C/ River 2011	Vaginal swab	Cattle
Brucella abortus isolates from the archive	14	Jos	2 ,,	Plateau 1976	Hygroma	Cattle
	15	Mambilla	1 ,,	Adamawa 1977	Hygroma	Cattle
	16	Makurdi	2 ,,	Benue 1978	Hygroma	Cattle

Appendix VI

Drug combinations and treatment reginens for human brucellosis

Clinical syndromes	Recommended	Alternative
Acute brucellosis (adults and children above 8 years of age).	Doxycycline 100 mg orally twice a day for 45 days plus either streptomycin 15 mg/kg intramuscularly daily for14–21 days,[2] or gentamicin 3–5 mg/kg intravenously daily for 7–14 days. Or Doxycycline 100 mg orally twice a day for 45 days plus rifampin 600–900 mg orally daily for 45 days.	Rifampin 600 mg orally daily for 42 days plus quinolone (ofloxacin 400 mg orally twice a day or ciprofloxacin 750 mg orally twice a day) for 42 days. Or Doxycycline 100 mg orally twice a day plus trimethoprim-sulfamethoxazole, one double strength tablet twice a day for 2 months.
Children less than 8 years old.	trimethoprim-sulfamethoxazole 5 mg/kg (of trimethoprim component) orally twice a day for 45 days plus gentamicin 5–6 mg/kg intravenously daily for 7 days. Rifampin 15 mg/ kg orally daily 45 days plus gentamicin 5–6 mg/kg intravenously daily for 7 days.	Monotherapy with doxycycline or rifampin 15mg/kg orally daily for 3- 6 weeks.
Brucellosis during pregnancy.	Rifampin 600 mg orally daily for 45 days plus trimethoprim-sulfamethoxazole one double strength tablet twice a day for 45 days.	Rifampin 600–900 mg orally daily for 45 days.
Focal infections (endocarditis, spondylitis, meningitis, paraspinous abscesses).	Doxycycline 100 mg orally twice a day and rifampin 600 mg orally daily for 6–52 weeks plus either streptomycin 1 g intramuscular daily [3]or gentamicin 3–5 mg/kg intravenously daily for 14–21 days.	Consider trimethoprim-sulfamethoxazole, ciprofloxacin 750 mg orally twice a day or ofloxacin 400 mg orally twice a day as substitute for doxycycline or rifampin. Surgery should be considered for patients with endocarditis, cerebral or epidural abscess, spleen or hepatic abscess, or other abscesses that are antibiotic resistant.

[1]Adapted from **Zinsstag *et al.* (2011)**

Appendix VII

Media, reagents and antibiotic used for culture and characterization of *Brucella* organisms

Preparation of dyes

Weigh 0.1g (0.1%) of thionin or basic fuchsin and dissolve in 100ml of distilled water to obtain a stock solution of thionin and basic fuchsin respectively and 1g in 100ml (1%) of safranin to obtain stock solution of safranin.
Heat the solutions in fowing steam for 20 minutes or in boiling water for 1 hour.
Leave for at least 48 hours before using them for the first time.
Store the stock solution of dyes at 4^0C and discard after 3 months.
Before preparing the dye media,it is advisable to remove the stock solutions of dyes from the refrigerator and allow them to rest at room temperature for 4 hours.

Preparation of dye medium

40g of Trypticase soy agar (TSA) is dissolved in 1 litre of distilled water. The suspension is autoclaved and cooled to 45-560c and 5% of calf or horse serum is added.
The appropriate amount of the dye solutions is then added.
The media is then poured into petri dishes.

Preparation of Farrell's medium.

Brucella Medium base is weighed is weighed in a 2-litre flasks, disoolved in 1 litre of distilled water and it is heated to boiling point in a magneto-stringer with a thermostat to dissolve completely. The media is divided into two flasks (500ml) each in 2-1 liter flasks.
The media is sterilized in an autoclave, and are flasks are put in bain marie at 45- 50^0C until the temperature is stabilized at 45-50^0C inside the flask.
25 ml of sterile calf serum is aseptically added and a vial of *Brucella* selective supplement is added to each flask.
The flasks are homogenized carefully to avoid making foam and gently poured into petri dishes.

Reconstitution of *Brucella* antibiotic supplement (Oxoid ref SR209A).

A septically add 10 ml (50/50) of mixture of methanol and sterile distilled water to form an even suspension.Mix thoroughly and leave for some (5-10) minutes before adding to the medium.

Preparation of CITA medium

In the absence of commercial selective antibiotic supplement, the following antibiotics are prepared in the same flask.

Bacitracin 25,000 I.U

Natamycin 50mg
Polymixin B 5, 000 I.U
Nystatin 100, 000 I. U
Vancomycin 20mg
They are dissolved in the same sterile flask in 20 ml of mixture 1:1 sterile water-methenol.

Nalidixic acid -5mg (is prepared separately and added).
To dissolve well, it is allowed to stabilized about 10 minutes.
The whole content of reconstituted antibiotics is added to each litre of medium.

Preparation of modified Thayer-martin's medium

This medium should contain the following antibiotics (per litre of medium)

Vancomycin	3mg
Colistin	7.5mg
Nystatin	100, 000 I.U
Nitrofurantoin	10mg

Preparation of urease test reagent

Prepare the urea concentrated solution by dissolving 29g of urea in 100 ml of distilled water .
Sterilize by filtration (using filter Millipore 0.22µm).
Dissolving 15g of Bacto Agar in 900 ml of distilled water and heated to dissolve the agar.
The pH is adjusted to 6.8 – 6.9.
It is sterilized in the autoclave (121 ^{0}C for 15-20 minutes).
The agar is cooled in bain marie at 45 ^{0}C.
100 ml of the concentrated urea solution is added and mix carefully.
The medium is distributed in sterile slope.

Preparation of oxidase reagent

Dissolve 1% of p-amino dimethylaniline oxalate in distilled water (100 mg in 10ml of distilled water).
Place the solution in bain marie at 50 ^{0}C
Shake periodically with vortex until dissolved.

Preparation of stock solution of crystal violet.

Dissolve 2 g of certified crystal violel dye in 20 ml of absolute ethanol.
Dissolve 0.8 g of ammonium oxalate in 80 ml of distilled water.
When both solutions have completely dissolve, mix the 2 them to form the stock solution.
Refrigerate the stock solution at 18^{0}C for up to 3 months.

Dilute it 1:40 in distilled water just before use. (once diluted, it should not be kept).

Staining colonies with crystal violet

The plate containing *Brucella* colonies (inoculated after serial dilutions of the suspension) was flooded with the diluted solution for 20 seconds.
The stain was discarded into disinfected with the Pasteur pipette used.
The plate was examined immediately with a binocular magnifying glass equipped with a system of oblique illumination.
The smooth colonies do not take up the dye.
The rough colonies are stained in various shades of red and purple and the surface may show radial cracks.