BACTERIOLOGICAL AND SEROLOGICAL STUDIES OF BOVINE BRUCELLOSIS IN OBUDU CATTLE RANCH, CROSS RIVER STATE, NIGERIA

BY

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JANUARY, 2013

DECLARATION

I declare that the work in the thesis entitled "BACTERIOLOGICAL AND SEROLOGICAL STUDIES OF BOVINE BRUCELLOSIS IN OBUDU CATTLE RANCH, CROSS-RIVER STATE, NIGERIA" was carried out by me in the Department of Veterinary Microbiology under the supervision of Prof. J.O.O. Bale, Prof. I. Ajogi, and Dr. C. N. Kwanashie.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another Degree or Diploma at any University.

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Signature Date

CERTIFICATION

This thesis, entitled "BACTERIOLOGICAL AND SEROLOGICAL STUDIES OF BOVINE BRUCELLOSIS IN OBUDU CATTLE RANCH, CROSS RIVER, NIGERIA", by Nanven Abraham MAURICE meets the regulations governing the award of Master of Science Degree of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my Parents WO11 Maurice Ceader Nimmak (rtd) and Mrs Yilkon Naomi Maurice and my beloved wife Dr. (Mrs) Nanven, Magdalene B. C. Above all Almighty God.

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ABSTRACT

Brucellosis is one of the most important zoonotic diseases worldwide particularly in developing countries and it is endemic in Nigeria. The study is to detect *Brucella* organism by serology and cultural methods in Obudu Cattle Ranch. Fourteen vaginal swab from cows that aborted and a hygroma fluid from a bull were collected and cultured for *Brucella*. A total of 354 cattle sera were examined for *Brucella* antibodies using Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), 2-Mercaptoethanol (2-ME) and Competitive Enzyme Linked Immunosorbent Assay (c-ELISA). Out of the 354 sera examined 149(42.09%), 142(40.11%), 132(37.29%) and 100(28.25%) were positive for *Brucella* antibodies using RBPT, SAT, 2-ME and c-ELISA respectively. Of the 113 males examined 40(35.40%), 39(34.51%), 36(31.86%) and 27(23.89%) of the males were positive while 109(45.23%), 142(58.92%), 85(35.27%) and 73(30.29%) of the 241 females were positive to *Brucella* antibodies using RRBPT, SAT, 2-ME, and c-ELISA respectively. There was however, no statistical significant association ($P>0.05$) between sexes. A total of 194 of the cattle examined were >5 years of age, 110 were of 2-5 years and 50 were of < 2 years. The cattle < 2 years of age had 10(20.00%), 9(18.00%), 6(12.00%) and 5(10.00%), while age group 2-5 years of age had 44(40.00%), 42(38.18%), 28(25.42%) and 24(21.82%), and the age groups >5 years showed that 95(48.97%), 91(46.91%), 87(44.85%) and 71(36.60%) were all positive to *Brucella* antibodies using RBPT, SAT, 2-ME and c-ELISA respectively. There was a statistically significant association ($P<0.05$) in prevalence rate between age groups with animals in the age group >5years. Breed-Specific prevalence rate showed that of the 117 Kuri examined 73(62.39%), 69(58.97%), 62(52.99%) and 35(29.91%) and of the 131 Friesian examined 10(7.63%), 10(7.63%),

9(6.87%) and 9(6.87%) and of the 106 of White Fulani examined 66(62.26%), 63(59.43%), 51(48.11%) and 56(52.83%) of breeds were positive for *Brucella* antibodies using RBPT, SAT, 2-ME and c-ELISA test respectively. There was a statistically significant association ($P<0.05$) between prevalence rate in breeds examined. There was also high level of correlation (0.7589) between the results obtained using RBPT, SAT and c-ELISA test. In conclusion *Brucella abortus* biotype 1 was isolated and the serology status as it relates to brucellosis has been documented, further studies will be carried out to characterize the *Brucella* organism.

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ABBREVIATIONS

µg	Microgramme
2-ME	2-Mercaptoethanol
30UTR	Untraslated region
AMOS	Abortus melitensis ovis polymerase chain reaction
BBAT	Buffered Brucella antigen tests
BF	Basic Fuchsin
Bk_2	Berkeley Phage
BPAT	Buffered plate agglutination test
CD14	Cluster of differentiation
CDc42	Cell division control protein 42
c-ELISA	Competative Enzyme Linked Immunosobent Assay
CFT	Complement Fixation Test
CO_2	Carbon dioxide
CXCL	Chemokines
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EEA1	Early endosomal antigen 1
ELISA	Enzyme linked immunosorbent assay
F2α	Prostagladin
FC	Fragment crystallization receptors
FPA	Fluorescent polarization assay
GMP	Guanidine monophosphate
GTP	Guinidine triophophate
GTPase	Guanidine triophosphatase
H_2S	Hydrogen sulphide
HeLa	Henrietta Lacks immortal cells

HK	Hstidine kinase
IFNγ	Interferon gamma
IFNα	Interferon alpha
IgG	Immunoglubulin G
IgM	Immunoglubulin M
IL	Interleukin
IS	Insertion seguence
LAMP1	Lysosme-associated membrane glycoprotein
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
ML	Milliliter
MRT	Milk ring test
MyD88	Myeloid differentiation factor 88
NF-κβ	Nuclear factor kappa beta
NK	Natural killer cells
NRAMP1	Natural resistance associated macrophage protein 1
OIE	Office of International Epizootiology
OMP	Outer membrane protein
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PIP3	Phosphatidylinositol 3-kinase
RBPT	Rose Bengal Plate Test
RER	Rough endoplasmc reticulum
RFLP	Ristriction fragment length polymorphism
Rho	Rhodopsin
RID	Radial immunodiffusion

RR	Response Regulator
Rrna	Ribosomal ribonucleic acid
RTD	Routine test dilution
S19	Strain 19 Brucella vaccne
SAT	Serum agglutination test
SDA	Serum dextrose agar
T4SS	Type 4 secretion system
Tb	Tbilisi Phage
TCS	Two Component system
Th	Thionine
Th1	T helper cell type 1
TIR	Toll/interleukin-1 receptor
TNFα	Tumor necrosis factor-alpha
Wb	Weybridge Phage
α/β	Alpha/Beta receptor
β	Beta
γ/δ	Gamma/delta receptor

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Brucellosis is an important zoonotic disease worldwide, particularly in developing countries (Godfroid-Kasbohrer, 2002; Pappas *et al.,* 2006). It has been identified as an endemic and problematic disease in Nigeria (Cadmus *et al.,* 2006). In some of the endemic areas such as Africa, Middle East, South America and Asia brucellosis results in significant human morbidity and economic losses in animal production due to abortion and condemnation of milk (Boschiroli *et al.,* 2001).

The disease in animals is characterized by fever, anorexia and depression. Abortion during the last trimester of gestation, still-birth, weak newborn calves and infertility (Poester *et al.,* 2005). Metritis, endometritis and placentitis are also seen in infected female animals (Adams, 2002). The disease may be inapparent in infected male(Alcina *et al.,* 2010). The most significant lesion in male animals is orchitis, which is associated with seminal vesiculitis and epididymitis (Adams, 2002; Poester *et al.,* 2005; OIE, 2009; Alcina *et al.,* 2010), and infertility (Pappas *et al.,* 2005; Barquero-Calvo *et al.,* 2007; Groussaud *et al.,* 2007; OIE, 2009; Gonzalez-Barrientos *et al.,* 2010; Alcina *et al.,* 2010).

Animals become infected via consumption of contaminated pasture, through semen or during coitus and vertical transmission can occur in calves (Alcina *et al.,* 2010; Gonzalez-Barrientos *et al.,* 2010). Humans can acquire the infection by consumption of contaminated non-pasteurised milk and cheese and direct contact with uterine secretion via skin abrasion and mucus membranes (Bale, 1991; Doganay *et al.,* 2003; OIE, 2009). Occupational

exposure can occur to veterinarians, butchers or handlers of infected livestock (Pappas *et al.,* 2006; OIE, 2009; Alcina *et al.,* 2010). Accidental laboratory infection with vaccine strains or virulent *Brucella* isolates can occur (Aygen *et al.,* 2002; Doganay *et al.,* 2003; McDonald *et al.,* 2006).

Bovine brucellosis continues to be a problem for humans, livestock and a variety of wild animals throughout the world (Her *et al.,* 2010). Testing of livestock for brucellosis is done by culture, serology and molecular methods (Abdoel *et al.,* 2008). The disease has important economic, veterinary and public health consequences (Franco *et al.,* 2007). Therefore, knowledge of the spread and prevalence of the infection is essential especially if effective control measures are to be put in place.

1.2 STATEMENT OF RESEARCH PROBLEM

Studies have identified brucellosis in sub-Saharan Africa as a major priority for control and prevention through its impact on multiple livestock species including; cattle, sheep, goats, pigs, camels and poultry. In Nigeria brucellosis is recognized as an endemic disease and is widespread among animal population in ranches, livestock market, dairy farms, livestock breeding centres and nomadic Fulani herds (Bale *et al*., 1981; Ocholi *et al*., 2004a).

Nomadism is common and nomads with their cattle often cross neighbouring states and countries in search of green pasture and trade. There are no instituted control measures especially border surveillance (Kudi *et al.,* 1997). As a consequence brucellosis remains a largely neglected disease with little or no attention to control and prevention except in South Africa where a successful control and policy has been instituted (McDermott *et al.,* 2002). In the developed world bovine brucellosis has been eradicated in Australia, Canada,

Cyprus, Denmark, Finland, Norway, Sweden, Netherland, New Zealand, United Kingdom, Israel and United State of America. There protocol was based on vaccination programmes combined with test and slaughter policies (Emslie *et al.,* 2002).

There is need for more epidemiological investigation into brucellosis in bovine and other livestocks to elucidate occupational risk/ hazard in terms of animal to human transmission of *Brucella* species. So this study is aimed at addressing and filling this gap in our knowledge on brucellosis in Obudu Cattle Ranch where herds of animals are found and human traffic is on the increase as a result of tourism.

1.3 JUSTIFICATION FOR THE STUDY

Obudu Cattle Ranch is one of the ranches that have an organized ranching for cattle in this country with an estimated 30,500 cattle. There is influx of visitors to the area as tourist centre and most of them consume the milk produced by the farm as well as from the Fulani herd nearby. Since the establishment of the ranch in 1956 by the Colonial masters, there is paucity of information on brucellosis study on the ranch and this study is carried out to fill this gap as well as the zoonotic importance of the disease to the public.

1.4 AIMS OF THE STUDY

The aim of this research is to study the presence of *Brucella* organism by cultural and serological techniques in bovine from Obudu Cattle Ranch.

1.5 OBJECTIVES FOR THE STUDY

The objectives of the study include to:

1. Isolate and identify *Brucella* organism from cattle in Obudu Cattle Ranch.
2. To examine the sera of cattle for the presence of antbodies to *Brucella* using serological techniques in Obudu Cattle Ranch.
3. To determined by sex, age and breed of cattle for the presence of antibodies to *Brucella* in Obudu Cattle Ranch.

1.6 RESEARCH HYPOTHESES

Null Hypothesis (H_0)

- There is no *Brucella* organism among cattle in Obudu Cattle Ranch.
- There is no *Brucella* antibodies in the serum of cattle in Obudu Cattle Ranch.

CHAPTER 2

LITERATURE REVIEW

3.1 HISTORY

Brucella species are facultative intracellular pathogens that establish and maintain chronic infections in a wide variety of mammalian hosts. During the infection cycle, *Brucella* species rapidly adapted to the intracellular niche to resist the harsh conditions generated by the immune system (Moreno and Moriyon, 2002). The genus *Brucella* is divided into several species that are genetically highly related and differ mainly in pathogenicity and preferential host specificity (Godfroid *et al.,* 2005).

In contrast, the closely related *Ochrobactrum* species are members of the soil microbiota and potentially human pathogens unable to establish chronic infections. *Brucella* species and the type species *Ochrobactrum anthropis* present a complex genome with two chromosomes (chromosomes 1 and 11) of unequal sizes (Leal-Klevezas *et al.,* 2005). The complete genome sequences of ten *Brucella* isolates of different species have become available (Delvecchio *et al.,* 2002; Paulsen *et al.,* 2002; Chain *et al.,* 2005; Halling *et al.,* 2005; Tsolis, 2002). In gene bank comparative genomics of *Brucella* species reveal extensive similarity and conserved genetic organization.

Pathogen-host interactions during bacterial infection expose bacteria to multiple physiological and biological stresses. Two component systems (*TCS*) are the predominant signal transduction mechanism by which bacteria sense and respond to intracellular and

extracellular signal mainly, through regulation of gene expression. Typically these signaling system are composed of a sensor histidine kinase (*HK)* and a response regulator (*RR*) (Paulsen *et al.,* 2002). The total number of TCS and other signalling proteins encoded in a bacterial genome can be used as a measure of the adaptive potential of the organism (Chain *et al*., 2005).

2.2 EPIDEMIOLOGY OF THE ISOLATON OF *BRUCELLA*

In Africa, reports on the different biotypes of *Brucella* have been documented. Isolation of biotype 1, 3, 8, 9 of *Brucella abortus* has been reported in Kenya (Waghela, 1975). In Uganda *B. abortus* biotypes 3, 8, and 9 have been reported (Elliott *et al*., 1977) while *B. abortus* biotype 7 have been reported in Egypt and South Africa. *B. abortus* biotype I and 3 have been isolated in Senegal with a very strong predominance of biotype 3 (Verger *et al.,* 1979). Seventeen isolates of *B. abortus* from cattle in Ivory Coast, 9 were biotypes 1 and 8 (Pilo- Moron *et al.,* 1979). Andreani *et al.,* (1983), isolated *B. abortus* biotype 6 in milk in Somalia.

In Nigeria Eze, (1978) isolated *B. abortus* biotype 1 and 2 from Nigerian cattle. Bale and Kumi-Diaka, (1981), isolated biotypes 1, 3, and 4 from Livestock Investigation and Breeding Centre (LIBC) in Kano State. Ocholi *et al.,* (2004b; 2005), isolated *B. abortus* biotype 1 and *B. abortus* from north central and north east and aborting sheep in Bauchi State respectively. *B. abortus* biotype 1 from milk of sheep and *B. suis* from Piggery farms in northern Nigeria have been isolated by Bale and Nuru, 1985and Bale *et al*., 2002). The fact that only one species, *B. abortus*, biotype 1 was isolated. It can be said to be representative of the area from which they were obtained. Eze, (1978), indicated that out of 20 strains of *B. abortus*, isolated from cattle 19 (95%) were of biotype 1 while only 1 (5%)

was of biotype 2. Similarly out of 8 strains of *B. abortus* obtained from cattle by Bale and Kumi-Diaka, (1981) 5 (62.59%) were of biotype 1, 25% were of biotype 3 and 12.59% were of biotype 4. It does appear from past reports that *B. abortus* biotype 1 may be the predominant strain associated with the disease problem in Nigerian livestock.

2.3 EPIDEMIOLOGY OF BRUCELLOSIS

Brucellosis is a major zoonotic disease, widely distributed in both humans and animals, especially in the developing world (Godfroid *et al.,* 2005). Food Agriculture Organisation (FAO) (2003) described brucellosis as an infectious and contagious bacterial zoonotic disease caused by *Brucella* species in human and various species of domesticated, feral (wild) animals and marine mammals.

In 1887, Sir David Bruce described a disease of long clinical duration characterized by fever, profuse perspiration, splenomegally, frequent relapses, rheumatoid or neuralgic pain, swelling of the joints and orchitis (*Pappas et al.,* 2005). He isolated the organism from the spleens of soldiers who died as a consequence of Malta disease (Nicoletti, 2002).

Health of human and animal population is pivot for economic development, prosperity and stability (WHO, 2006). The burden of infectious diseases affect health and reproduction of livestock, thereby greatly reducing its value and opportunities for trade (Smits *et al.,* 2004). Zoonotic diseases like brucellosis are not only of veterinary importance but also human health, contributing to morbidity and reduction of working capacity with concomitant loss of income (OIE, 2009). Brucellosis causes appreciable economic losses to the livestock industry in infected areas, resulting from abortion, sterility

decreased milk production, veterinary care costs and the cost of culling and replacing animals (FAO, 2003). Brucellosis has been reported from almost all countries in Africa (Refai, 2002; Perry *et al.,* 2002).

It is important to note that brucellosis is a zoonosis and the stimulus for hopeful elimination is primarily a public health responsibility. Nearly every case of human brucellosis has an animal origin (Godfroid *et al.,* 2005). Pappas *et al*., (2006) Considered brucellosis as the World most widespread of all zoonoses and apart from its toll on people, it has an enormous impact on animal industry, as the international trade in animals and animal products and movement of people increase, the risk of introduction and re-introduction of brucellosis is growing (McDermott *et al.,* 2002; Godfroid *et al.,* 2005). One of the major factors contributing to the spread of the disease is free movement of nomadic pastoralists who are accustomed to the traditional extensive system of management (Almajali *et al.,* 2007).

2.3.1 **Epidemiology of Brucellosis in Nigeria and parts of the world**

Brucellosis is endemic in Nigeria, and frank outbreaks have been reported in almost all part of the country (Cadmus *et al*., 2006). Investigation and evidence of serological prevalence rates were 6% to 63.3% (Collard, 1962; Falade, 1974; 2002; Alausa *et al.,* 1976; Brisibe *et al.,* 1993; Asanda *et al.,* 2001; Edu, 2005; Cadmus *et al.,* 2006; Ofukwu *et al.,* 2007).

The antibodies to *Brucella* has been reported in various animal species including; cattle (Esuruoso, 1974; Eze, 1977; Bale *et al.,* 1981; Ajogi, 1997), horses (Bale and Kwanashie, 1984; Ocholi *et al.,* 2004a), chickens (Bale *et al.,* 1982; Abdu *et al.,* 1984; Junaidu *et al.,* 2006; Gugong *et al*., 2012). Camels (Okoh, 1979; Kudi *et al.,* 1997; Adamu *et al.,*1999),

and sheep and goats (Falade *et al.,* 1975; 1978; Bale *et al.,* 1982; Okewale *et al*., 1988; Chukwu, 1987; Brisibe *et al.,* 1993; Ocholi *et al.,* 2005; Junaidu *et al.,* 2010; Bertu *et al.,* 2010). Swine (Bale and Nuru, 1985; Adamu *et al*., 2008). In north central Wungak *et al.,* (2011) reported prevalence rate of 3.8% in Jos-South area of Plateau State, Gusi *et al.,* (2010) reported 36.93% in a diary farm in North Central, Nigeria. In north west Mbuk *et al.,* (2011) reported 7.1% prevalence rate in migratory cattle in Kaduna State, Junaidu *et al.,* (2011) reported 19.71% in breeding herds in Sokoto. while in north east Adamu, (2009) reported 6.9% in Maiduguri, Hamza, (2011) reported 14.8% prevalence in Taraba State. In south-west Cadmus *et al.,* (2006, 2009) reported 5.82% and 8.6% in Ibadan and Lagos respectively. Shola *et al.,* (2001) reported 6.28% in Ibadan, Ogundipe *et al.,* (1994) reported 6.79% prevalence rate in Ibadan

In other parts of the world Shuaibi, (1999) reported 72.9% prevalence in Palestinian Authority which is the highest so far recorded. Teoman *et al.,* (2007) reported 3.2% prevalence in Turkey, Ahmad *et al.,* (2009) reported 6.5% prevalence in Jordan, Baris *et al.,* (2010) reported 3% prevalence in Ankara, Turkey. Nakaune *et al.,* (2004) reported 3.3% prevalence in cattle in Central Africa Republic.

2.4 AETIOLOGY OF BRUCELLOSIS

Ten (10) species are recognized within the genus *Brucella*: *Brucella abortus* biovars 1-9 (Cattle), *Brucella melitensis* biovar 1-3 (Goats and Sheep), *Brucella suis* biovar 1-5 (Swine), *Brucella ovis* (Sheep), *Brucella canis* (Dogs), *Brucella neotomae* (Desert woodrat), and *Brucella ceti* (Cetaceans*), Brucella pinnipedialis* (Pinnipeds), are species isolated from marine mammals and occasionally cause infection in man (Sohn *et al.,* 2003;

Osterman *et al.,* 2006; McDonald *et al.,* 2006; Foster *et al.,* 2007; Anne *et al.,* 2010; Javier, 2010; Alcina *et al.,* 2010). In the last four to five years, a novel specie *Brucella microti* was isolated from the Vole (*Microtus arvalis*) and has been confirmed as a cause of brucellosis (Scholz *et al.,* 2008). So also, another new species has been described from a human breast inplant named *Brucella inopinata* (De *et al.,* 2008; Scholz *et al.,* 2009).

2.5 GENERAL CHARACTERISTIC OF THE GENUS *BRUCELLA*

The Genus *Brucella* is a small Gram negative, facultative, intracellular, coccobacilli pathogens that belong to the alpha 2-proteobactericeae Family. They are short rod measuring from 0.6-1.5µm long and 0.5-0.7µm wide (OIE, 2009). They are usually arranged singly, and less frequently in pairs or small groups (EC, 2001). The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident, *Brucella* are non-motile, they do not form spores, no flagella, pili or true capsules and usually does not show bipolar staining. (OIE, 2009). *Brucella* organism replicated within the host macrophages (Gorvel and Moreno, 2002; Celli *et al.,* 2003).

2.6 HOST RANGE AND SUSCEPTIBILITY TO *BRUCELLA* ORGANISM

Brucella organisms affect a range of different mammals including: man, cattle, sheep, pigs, goats, rodents, cetaceans, pennipeds and Vole (Cutler *et al.,* 2005). The classification of the genus *Brucella* is primarily based on difference in pathogenicity and host preference (OIE, 2009). Thus, the principal species are *Brucella abortus* affecting primarily cattle, man and other bovidae, Cervidae and Camelids. *B. melitensis* affects primarily goats, man, sheep, and camelids (Abbas *et al*., 2002). *B. suis* affects swine and man (FAO, 2003). All the above *Brucella* species are not host specific, and may transmit to other animal species

under appropriate and suitable conditions (Ocholi *et al.,* 1993; FAO, 2003; Ocholi *et al*., 2004b; Godfroid *et al.,* 2005). Brucellosis has also been reported in the one-humped camel (*Camelus dromedarius*) and in the two-humped Camel *(Camelus bacitrianus)*, *Brucella* species is related to contact with large and small ruminants infected with *B. abortus* or *B. melitensis* respectively (Abbas *et al*., 2002).

Brucellosis has been observed in the domestic buffalo (*Bubalus bubalus),* American and European bison (*Bison bison, Bison bonasus*), Yak (*Bos grunniens*), Elk/Wapiti (*Cervus elaphus*) and also occurs in the African buffalo (*Syncerus caffer*) and various African antelope species (Godfroid, 2002). Rhan (2000) and Godfroid (2002) reported that *Brucella* strains have also been isolated from a great variety of wildlife species such as elk, feral swine, wild boar (*Sus scrofa*), fox (*Vulpes vulpes*), hare (*Lepus capensis*), reindeer (*Rangifer tarandus tarandus*), caribon (*Rangifer tarandus groelandicus*), Chamois (*Rupicapra rubcapra*) and Ibex (*Capra ibex*). Wildlife is therefore, considered as a reservoir for zoonotic brucellosis (Avong, 2000). The broad spectrum of *Brucella* isolates has recently been enlarged to marine mammals. A number of recent reports described the isolation and characterization of *Brucella* strains from marine mammals such as Whales (Sohn *et al.,* 2003; Osterman *et al.,* 2006; McDonald *et al.,* 2006; Foster *et al.,* 2007; Anne *et al.,* 2010; Javier., 2010; Alcina *et al.,* 2010).

2.7 TRANSMISSION AND SURVIVABILITY OF *BRUCELLA*

Among animals, *Brucella* is usually transmitted by contact with the placenta, foetus, foetal fluids and vaginal discharges from infected animals (WHO, 2006). Animals can contract the disease from licking the placenta from an infected aborted foetus; coming into contact

with other infected animals at livestock shows or fairs; and the calves can become infected by drinking milk from an infected dam (Bale *et al.,* 2001; OIE, 2009). Cross- transmission of this agent to other domestic species is believed to be the most likely source of the infection since ruminants are traditionally herded together with other species such as cattle, sheep, goats, camel, donkey and chicken (Kudi *et al.,* 1997). The ruminant might have become infected through close contact between infected and susceptible animals and cross transmission between species. Animal transmission can also occur through the alimentary tract following ingestion of contaminated feed or water and also through the respiratory system via contaminated dust or droplets. Reproductive system can also be affected during coitus (Bale. 1991). *Brucella* organism can also be found in the blood, urine, milk and semen (Smith *et al.,* 2004; WHO, 2006). Infection also occurs by ingestion and through mucus membrane, broken skin and possibly intact skin (Pappas *et al.,* 2005).

Transmission of *Brucella* organism to human occur through the consumption of infected meat, unpasteurized animal milk and milk products, through direct contact with infected animal parts such as the placenta by inoculation through rupture of skin and mucus membrane, and the inhalation of infected aerosolized particles (Shehata *et al*., 2001; Refai., 2002; McDermott *et al.,* 2002). The social characteristics of shepherds, the occupational group most at risk for brucellosis both through direct contact with herds and consumption of raw milk is interesting to consider (Shehata *et al.,* 2001; Cadmus *et al.,* 2006; Ofukwu *et al.,* 2007). Animal meat and milk are increasingly important as a source of protein for the human populace, this highlights the potential importance of brucellosis for public health. According to Bale (1991), the danger lies in the fact that the western way of eating

undercooked meat and improperly roasted meat, Suya and Kilishi is gaining ground in Nigeria, although no study has isolated the organism in such meat.

Brucellosis can be spread through fomites (Lucero *et al.,* 2005). In conditions of high humidity, low temperature and no sunlight, these organism can remain viable for several months in water, aborted foetus, manure, wool, hay, equipment and clothes. *Brucella* is destroyed by several hours of exposure to direct sunlight (WHO, 2006). Brucellosis is an occupational disease in herdsmen, abattoir workers, veterinarians, dairy-industry, professionals and personel in microbiology laboratories (Cadmus *et al.,* 2006; Ofukwu *et al.,* 2007; Alcina *et al.,* 2010 Anne *et al.,* 2010). Consumption of unpasteurized dairy products especially raw milk, soft cheese, butter and ice cream is the most common means of transmission (Cadmus *et al.,* 2006; Ofukwu *et al.,* 2007; Moon *et al.,* 2010; Alcina *et al.,* 2010). cow, sheep, goat, or camels milk contaminated with *B. melitensis* is particularly hazardous as it is drunk in fairly large volume and may contain large numbers of organism. Butter, cream, or ice cream prepared from such milk also present a high risk (WHO, 2006). *Brucella* species persist for several days in milk even when it turns sour and is known to flourish in soft, fresh, small ruminants cheese (WHO, 2006; FAO/WHO, 1986).

Survival is prolonged at low temperature, especially below 0^0C, contaminated equipment can be sterilized by autoclaving (121^0C for a minimum of 15 minutes) or dry heat (160-170^0C for a minimum of an hour). Chemical treatment is recommended to destroy *Brucella* in contaminated premises. xylene (1m/litre) and calcium cyanamide (20kg/m^3) have been found to be effective in liquid manure after 2-4 weeks. One hour treatment with 2.5% sodium hypochlorite, 2-3% caustic soda and 20% freshly slaked lime suspension or 2% formaldehyde solution will destroy *Brucella* on contaminated surfaces, *Brucella* is also

susceptible to 1% sodium hypochlorite, 70% ethanol, iodine/alcohol solutions, gluteraldehyde and formaldehyde (WHO, 2006; OIE, 2009).

2.8 PATHOGENESIS OF BOVINE BRUCELLOSIS

Most common route of infection in cattle is the gastrointestinal tract (Crowford *et al.,* 1990) from where infection spreads to local lymph nodes where *Brucella* replicates intracellularly in phagocytes (Anderson *et al.,* 1986a). Invasion of lymphatic vessels is followed by bacteraemia leading to systemic infection, favouring colonization of the pregnant uterus, male genital organs and mammary glands (Ko *et al.,* 2003).

B. abortus has a strong tropism for the uterus during the last trimester of gestation (7-9 months), which is thought to be due to high concentration of erythritol and steroid hormones. Erythritol favours bacteria survival since it can be metabolized by *B.abortus* as a source of carbon and energy (Samartino *et al.,* 1996). Erythrophagocytic trophoblastic cells located at the base of chorionic villi of ruminants are considered the primary site of invasion of fetal placental tissues from where *B. abortus* disseminates to intercotyledonary trophoblasts (Anderson *et al.,* 1986a; Santos *et al.,* 1996). *Brucella* multiplication induces infiltration of inflammatory cells, trophoblastic necrosis, vasculitis, and ulceration of the allantochorion, consequently, fetal maternal metabolic exchanges are compromised resulting in abortion (Anderson *et al.,*1986a).

2.9 INVASION OF HOST CELLS BY *B. abortus*

A key step in the pathogenesis of *B. abortus* is its ability to invade phagocytic and non-phagocytic host cells. *B. abortus* is capable of invading the intestinal mucosa preferentially through the cells. Intra-epithelial phagocytes may also favour transepithelial migration and

transport of *B. abortus* to the lamina propria and submucosa (Ackermann *et al.,* 1988). Opsonised *B. abortus* is internalized in phagocytes through complement or fragment crystallization (Fc) receptors, whereas non-opsonised organisms apparently invade by interacting with lectin and fibronectin receptors (Campbell *et al.,* 1994). Opsonised bacteria phagocytosed by activated macrophages are usually killed within the phagolysosome before they reach the sites of intracellular replication. Conversely, non-opsonised organisms are more likely to survive and replicate intracellularly (Gorvel *et al.,* 2002). Attenuated strains adhere and invade host cells, but they do not survive intracellularly (Pizarro-Cerdá *et al.,* 2001; Gorvel and Moreno, 2002). Although *B. abortus* is capable of invading epithelial cell lines and bovine trophoblastic cells it is much less invasive than other facultative intracellular bacterial pathogens such as *Salmonella enterica* (Detilleux *et al.,* 1990a,b; Guzmán - Verri *et al.,* 2001; Santos *et al.,* 2004; Carvalho Neta *et al.,* 2008),). No specific receptors for *B. abortus* have been identified in non-phagocytic cells, but there is evidence of specific bacterial molecules involved in adhesion (Gorvel *et al.,* 2002). A surface protein 41Kd of *Brucella* (SP41) is associated with adherence and invasion in host cells (Castañeda-Roldán *et al.,* 2006). *In vitro* studies have demonstrated that invasion of epithelial cells by *B. abortus* is associated with recruitment of actin filaments to the site of interaction of the bacterium with the host cell membrane (Pizarro- Cerdá *et al.,* 1999; Guzmán-Verri *et al.,* 2001). The internalisation of *B. abortus* by non-phagocytic cells involves activation of small guanidine triophosphatase (GTPases) of rhodopsin (Rho) subfamily such as Rho, and cell division control protein 42 (Cdc42) (Guzmán-Verri *et al.,* 2001), which are known to be regulators of cytoskeleton. Apparently activation of Cdc42 is directly due to the contact of *B. abortus* with the host cell, whereas activation Rho appears to be indirect, in addition, molecular mediators of signalling pathways, including cyclic

guanidine monophosphate (GMP), phosphatidylinositol 3-kinase (PIP3-kinase), tyrosine kinase, and mitogen-activated protein (MAP) kinases are also involved in the internalisation process of *B. abortus* as second messengers of signal from GTPases, since the inhibition of these molecules impairs bacteria invasion (Guzmán-Verri *et al.,* 2001; Gorvel *et al.,* 2002).

2.10 INTRACELLULAR SURVIVAL OF *B. arbortus*

B. abortus intracellular survival is dependent upon its ability to resist the acidified intra phagosomal environment and to inhibit phagosome–lysosome fusion (Pizarro-Cerdá *et al.,* 1998a; Porte *et al.,* 1999; Wang *et al.,* 2001). These processes are triggered by the internalisation of *B. abortus* that redirects intracellular trafficking, changing the normal maturation process of the phagosome and blocking fusion of *Brucella*-containing phagosomes with lysosomes (Gorvel *et al.,* 2002). Intracellular trafficking of *B. abortus* and its capability of surviving intracellularly is not identical in all cell types. For instance, neutrophils are not permissive to intracellular growth of *B. abortus*, whereas the organism survives well in other phagocytes such as macrophages in which the intracellular trafficking of the pathogen is similar to non-phagocytic cells (Jiang *et al.,* 1993; Gorvel *et al.,* 2002).

Soon after internalisation of *B. abortus*, the early phagosomal complex is formed, which expresses the small GTP-binding protein Rab 5 and early endosomal antigen 1 (EEA1) (Pizarro-Cerdá *et al.,* 1998b; Chaves-Olarte *et al.,* 2002). GTPases are also activated during intracellular trafficking of *B. abortus* but they are different from those activated during the invasion process. Thus, invasion and intracellular trafficking appears to be independent (Pizarro-Cerdá *et al.,* 1998a,b; Guzmán-Verri *et al.,* 2001; Gorvel *et al.,*2002).

Acidification of the *Brucella*-containing vacuole during early steps of infection is also required for intracellular survival. This acidified environment induces changes in the profile of bacterial gene expression favouring intracellular survival (Porte *et al.,* 1999).

In cultured Henrietta Lacks immortal cells (HeLa cells), at 2 hour post-inoculation, *B. abortus* is located in multi-membranous intracellular compartments that localise with the lysosome-associated membrane glycoprotein 1 (LAMP1), a marker for autophagosomes (Pizarro-Cerdá *et al.,*1998b, 2000). The *Brucella*-containing vacuole then acquires markers of the rough endoplasmic reticulum (RER), such as calreticulin and sec61β. These findings demonstrate that *B. abortus* traffics from a phagosome compartment (Pizarro-Cerdá *et al.,* 1998b) towards the RER of the host cell, where the organism has an optimal environment for replication (Anderson *et al.,* 1986b; Pizarro-Cerdá *et al.,* 2000). Early ultrastructural analysis of *Brucella*-infected cells has also identified the RER as the site of replication (Anderson *et al.,* 1986b; Meador *et al.,* 1989; Detilleux *et al.,* 1990a).

Brucella has limited replication in early compartments, and the RER is the only compartment that sustains optimal bacterial replication (Comerci *et al.,* 2001; Chaves-Olarte *et al.,* 2002; Gorvel *et al.,* 2002).

Intracellular replication of *B. abortus* in trophoblastic cells is strongly influenced by the stage of gestation, with higher replication rates within trophoblasts in late gestation when the cells actively secrete steroid hormones (Samartino *et al.,* 1994). In trophoblasts, *B. abortus* induces steroid synthesis and modulates the metabolism of postaglandin precursors, favouring bacterial growth (Anderson *et al.,* 1986b). In addition, hormonal changes take place in infected placentas, with increase in the levels of postaglandin (F2α), a decrease in progesterone, and an increase in oestrogen and cortisol. These changes mimic to some

extent what happens during parturition (Gorvel *et al.,* 2002), and are likely to contribute to the abortion.

2.11 MOLECULAR MECHANISM OF *B. abortus* PATHOGENESIS

Molecular mechanisms of *B. abortus* pathogenesis *Brucella* does not have classical virulence factors such as exotoxins, cytolysins, capsule, fimbria, flagellum, plasmids, lysogenic phages, antigenic variation, endotoxic lipopolysaccharide (LPS), and inducers of host cell apoptosis (Moreno and Moriyón, 2001).

The mechanisms of *Brucella* spp. virulence are factors that are required for invasion and intracellular survival which allow the organism to reach its intracellular replication site (Detilleux *et al.,* 1990a,b; Pizarro-Cerdá *et al.,* 1998b, 1999 Guzmán-Verri *et al.,* 2001 Moreno and Moriyón, 2001), Smooth strains of *Brucella* generally invade host cells more efficiently than rough strains, suggesting that the LPS O chain plays a role in virulence, although some rough strains are naturally virulent (Sola-Landa *et al.,* 1998; Ko and Splitter, 2003). *Brucella* LPS was originally recognised as a virulence factor due to its relatively low immunogenicity, which prevented activation of the alternative complement pathway (Ko *et al*., 2003). The role of LPS was confirmed by mutagenesis of the O chain that rendered *Brucella* more susceptible to complement-mediated bacterial lysis (Moreno *et al*., 2001) and to bactericidal cationic peptides such as defensins and lactoferrins (Lapaque *et al.,* 2005). Furthermore, *Brucella* LPS has long been recognised to be a weaker inducer of the immune response compared to enterobacterial endotoxins (Keleti *et al.,*., 1974). The LPS O chain inhibits cellular apoptosis avoiding immune response activation (Pei *et al.,* 2004; 2006). It is noteworthy that *Brucella* LPS plays a more relevant role in virulence while the organism is in the extracellular environment prior to invading host cells (Ko *et al*., 2003). Nevertheless, *B. abortus* rough mutant strains have a lower ability to survive intracellularly

than smooth strains since the LPS O chain is essential for entry and early intracellular phase of *Brucella* in macrophages (Porte *et al.,* 2003; Lapaque *et al.,* 2005).

During internalisation, *B. abortus* relies on a two-component regulatory system named BvrR/BvrS, which is required for recruitment of GTPases as described above, and maintenance of the outer membrane. Thus, BvrS–BvrR mutants are impaired for invasion of non-phagocytic cells and intracellular survival (López-Goñi *et al.,* 2002). The two components of this system are BvrS, a sensor protein member of the histidine-kinase super family, and BvrR, which is a regulator protein. This system regulates expression of outer membrane proteins (Omp) involved in invasion of host cells (López-Goñi *et al.,* 2002; Guzmán-Verri *et al.,* 2001). *B. abortus* strains with mutated bvrR and especially bvrS lack the ability to recruit GTPases of the Rho subfamily, particularly Cdc42, which is required for actin polymerisation and invasion of host cells (López-Goñi *et al.,* 2002).

Additionally, when invasion of the host cells by these mutants is stimulated artificially by enzymatic treatments, the mutants are more susceptible to host cell killing mechanisms. Attenuation in intracellular survival in this case is caused by the incapacity of the mutants to prevent phagosome–lysosome fusion (Sola-Landa *et al.,* 1998; López-Goñi *et al.,* 2002). Depletion of *Brucella* cyclic β-1,2-glucans synthetase results in lack of cyclic β-1,2-glucans, which are constituents of outer membrane that are required to the survival of *B. abortus* in mice and intracellular replication in HeLa cells. Cyclic β-1, 2-glucans are essential during early intracellular phase of *Brucella* infection since they prevent phagosome–lysosome fusion although they are not important for the trafficking of *Brucella* to the RER.

Brucella type 4 secretion system (T4SS) is encoded by the virB operon (Comerci *et al.,* 2001; Delrue *et al.,* 2001), which is composed of 12 genes, namely virB1 through virB12.

An orthologue T4SS was originally identified in the plant pathogen *Agrobacterium tumefaciens*, and later was recognised as an essential virulence mechanism of *B. abortus* that is required for intracellular multiplication of the organism (O'Callaghan *et al.,* 1999; Hong *et al.,* 2000; Sieira *et al.,* 2000). The virB encoded T4SS is required for intracellular growth of *B. abortus* in both phagocytic and non-phagocytic cells such as HeLa cells (O'Callaghan *et al.,* 1999; Sieira *et al.,* 2000; Comerci *et al.,* 2001; Delrue *et al.,* 2001).

Although the molecular mechanisms by which the T4SS system influences intracellular trafficking of *B. abortus* is not clear, apparently secreted effectors play a role in the biogenesis and maturation of the *B. abortus*-containing vacuole, and transport of *B. abortus* to its intracellular site of replication (Delrue *et al.,* 2001; Comerci *et al.,* 2001; Boschiroli *et al.,* 2002), with evidence that the system is required for fusion of the autophagosome-like vacuole with the RER. Experimental infection of mice and cultured cells with strains of *B. abortus* with a defective T4SS results in intracellular killing since the mutant strains fail to reach the RER (O'Callaghan *et al.,* 1999; Hong *et al.,* 2000; Sieira *et al.,* 2000; Comerci *et al.,* 2001; Delrue *et al.,* 2001; Sun *et al.,* 2002; Watarai *et al.,* 2002; Den Hartigh *et al.,* 2004, 2008; Kim *et al.,* 2004; Celli, 2006). Although the T4SS is absolutely required for intracellular survival and replication of *B. abortus* (Hong *et al.,* 2000; Boschiroli *et al.,* 2002; Celli, 2006), apparently the system does not play any role during invasion and the initial steps of infection of host cells (Celli, 2006).

Brucella T4SS is also required for persistent infection in mice and to induce the host immune response (Rolán and Tsolis, 2002; Roux *et al.,* 2007). It is also essential to elicit inflammatory and immune responses during *Brucella* infection in mice. *B. abortus* without functional T4SS is unable to stimulate the expression of pro-inflammatory genes and types

I and II interferon response as induced by the wild type strain in the spleen (Roux *et al.,* 2007).

In addition, a virB mutant strain persists longer in B- and T-cells knockout mice than in control mice (Rolán and Tsolis, 2007), whereas the T4SS is required for early response of cytokines such as interleukin (IL)-12 and interferon (IFN)γ that contributes to T helper cell type 1 (Th1) polarisation of the immune response (Rolán and Tsolis, 2008)

2.12 IMMUNE HOST RESPONSE AGAINST *B.* abortus

Innate immunity plays an important role during *B. abortus* infection since it decreases the initial number of bacteria and may influence the development of a protective adaptive immunity. The initial recognition of *Brucella* by neutrophils, macrophages and dendritic cells (DCs) involves Toll like receptors (TLRs) (Campos *et al.,* 2004; Dueñas *et al.,* 2004; Weiss *et al.,* 2005). Mammalian TLRs, similar to Toll from *Drosophila melanogaster* that has an antimicrobial function are membrane receptors located either in the plasma membrane or in the phagolysosome membrane (Rock *et al.,* 1998; Kopp and Medzhitov, 1999). TLRs are stimulated by conserved components of microorganisms known as pathogen-associated molecular patterns (PAMPs). PAMPs from bacteria such as lipoproteins, LPS, flagellin, and DNA are recognised by TLR2, TLR4, TLR5, and TLR9, respectively (Takeda *et al*., 2005). *B. abortus* LPS is recognised by cluster of differentiation 14 (CD14) that connects to molecules with transmembrane domains essential for signalling, particularly the TLR4. Although *Brucella* LPS stimulates TLR4, it has lower immunostimulatory activity compared to other Gram negative bacteria such as *Salmonella enterica* serotype *Typhimurium* (Ritting *et al.,* 2003; Dueñas *et al.,* 2004), which has the ability to induce an intense and acute inflammatory response (Santos *et al.,* 2003).

Nevertheless, experiments with TLR4-/- mice have demonstrated the role of this molecule in inducing an immune response and resistance to *B. abortus* (Campos *et al.,* 2004). TLR2 and TLR9 also have been shown to recognise components of *B. abortus* and to stimulate an immune response (Huang *et al.,* 2003, 2005; Macedo *et al.,* 2008).

A downstream adaptor molecule named myeloid differentiation factor 88 (MyD88) also participates in macrophage activation upon TLR-mediated recognition of PAMPs. MyD88 is required for TLR-mediated induction of pro-inflammatory cytokines via nuclear factor kappa beta (NF-κβ), and has a critical function in mounting an immune response against *B. abortus* (Weiss *et al.,* 2005). Dendritic cells (DC) activation during *B. abortus* infection results in an important regulatory pathway by inducing IFNα production by T-cells. DC activation and maturation requires MyD88, whose signalling appears to be required for development of IFNγ production by the T-cell, and control *B. abortus* infection in mice (Copin *et al.,* 2007; Macedo *et al.,* 2008). Importantly, smooth *Brucella* LPS stimulates DCs to produce interleukin 12 (IL-12), so stimulating CD4+ T-cells (Billard *et al.,* 2007).

Oxidative bursts and production of nitric oxide are efficient mechanisms by which macrophages kill bacteria and are stimulated by cytokines of innate immunity such as IFNγ and tumour necrosis factor (TNF)α (Jones *et al*., 1992; Jiang *et al.,* 1993). An *in vitro* study demonstrated that reactive oxygen intermediates are required for the intracellular control of *B. abortus* during early phases of infection in macrophages (Jiang *et al.,* 1993; Ko *et al.,* 2002).

Natural resistance against brucellosis in cattle is another important element of innate immunity (Adams *et al*., 1998). The resistant phenotype is associated with the ability of bovine macrophages to inhibit the intracellular growth of *B. abortus* (Campbell *et al*., 1992; Campbell *et al.,* 1994; Qureshi *et al.,* 1996). Studies correlate this resistant phenotype with

polymorphisms of gene NRAMP1 (Natural resistance associated macrophage protein 1) (Adams *et al*., 1998; Barthel *et al.,* 2001). Polymorphisms at 30 untraslated region (30UTR) of bovine NRAMP1 were thought to be associated with the ability of macrophages to control intracellular multiplication of *B. abortus* (Barthel *et al.,* 2001).

A thorough study that demonstrated no correlation between polymorphisms at the NRAMP1 30UTR and natural resistance against bovine brucellosis (Paixão *et al.,* 2006, 2007). Studies are underway to verify the possible association of recently identified polymorphisms of the bovine NRAMP1 with natural resistance (Martínez *et al.,* 2008). Cytotoxic activity of natural killer cells (NK) is also part of innate immunity against *B. abortus* (Salmeron *et al.,* 1992). Bovine NK cells may act directly through the secretion of IFNγ (Golding *et al.,* 2002) which is a cytokine that stimulates bactericidal activity of macrophages (Oliveira *et al.,* 2002; Wyckoff, 2002).

Proteins of the complement system contribute to the innate immune response primarily through their role in opsonisation and immediate elimination of extracellular *Brucella* or by interacting with bacteria neutralized by antibody (Corbeil *et al.,* 1988). In contrast to the abundant studies on the interaction of *Brucella* and macrophages, DCs, and non-phagocytic cells, there is very little information about its interaction with trophoblastic cells, which are key target cells in bovine brucellosis. This lack of information is due to the absence of suitable bovine trophoblastic cell lines. Experimental *in vivo* and *in vitro* studies of bovine placenta have demonstrated the ability of *B. abortus* to infect trophoblastic cells (Anderson *et al.,* 1986a; Samartino *et al.,* 1994; Samartino *et al*., 1996).

The profile of bovine trophoblast gene expression during the early stages of infection with *B. abortus* using a chorioallantoic membrane explant model (Carvalho Neta *et al.,* 2008). Interestingly, *B. abortus* suppressed pro-inflammatory genes during the early stages of

infection, which was followed by a delayed and mild expression of pro-inflammatory chemokines, particularly CXCL6 (GCP-2) and CXCL8 (IL-8) in trophoblastic cells *in vitro*. These *in vitro* results correlated well with chemokine expression in the placenta *in vivo* (Carvalho Neta *et al.,* 2008).

It has been recently demonstrated that *B. abortus* does not induce significant pro-inflammatory responses during infection in mice, and has low immune stimulatory activity and toxicity for host cells (Barquero-Calvo *et al.,* 2007). Apparently, this ability to inhibit, avoid or delay the host immune response may be a strategy of the organism to cause persistent infection in the host. This notion was further supported by the recent identification of a *Brucella* TIR (Toll/Interleukin-1 receptor) domain containing protein, which interferes with TLR signalling, inhibiting DC maturation, compromising DC cytokine secretion and antigen presentation (Salcedo *et al.,*2008; Cirl *et al.,* 2008).

An effective adaptive immune response against *B. abortus* requires a cell-mediated immunity promoted by specific T-cell activation (Oliveira *et al.,* 1995). T-cells recognise *B. abortus* through α/β receptors associated with co-receptor molecules CD4+ in T-helper cells or CD8+ in T cytotoxic cells, after which bacterial antigens can be processed and presented to antigen-specific major histocompactability complex (MHC) class II or I (Janeway, 1992; Wyckoff, 2002).

A T-cell subset population with gamma/delta (γ/δ) receptors is probably important in infected calves since a high number of γ/δ T-cells was observed in young calves (Ko *et al*., 2003). This T-cell subset population did not express CD4+ nor CD8+, so its antigen presentation pathway is not well defined (Janeway, 1992). However, γ/δ T-cells have lytic activity *in vitro*, produce cytokines such as TNFα and IFNγ so they may activate macrophages and modulate alpha/beta (α/β) T-cell activation (Ottones *et al.,* 2000).

CD4+ T-cell activation is stimulated by IL-12 secreted by infected macrophages (Jones *et al*., 1992; Jiang *et al*., 1993). IL-12 promotes T helper cell (Th0) differentiation into Th1 that is the most significant cell type in host immune response against *B. abortus.* IL-12 is also involved in adaptive immunity to direct activation of CD8+ T-cells (Zhan *et al.,* 1993). Other cytokines secreted by active phagocytes are TNFα and IL-1. Although TNFα does not seem to be essential in controlling intracellular replication of *B. abortus*, it is required for activation of proinflammatory effector cells that limit bacterial multiplication (Zhan *et al.,* 1996). IL-1 may stimulate and/or increase the number of neutrophils and macrophages in the spleen during *Brucella* infection (Zhan *et al.,* 1991, 1993).

The chief cytokine produced by Th1 cells is IFNγ, which is responsible for macrophage activation and restriction of *Brucella* infection *in vitro* and *in vivo* (Jones *et al*., 1992; Jiang *et al*., 1993; Stevens *et al.,* 1992; Golding *et al.,* 2001). Th1 cells also secrete IL-2 that promotes T-cell clonal expansion and contributes to control of *B. abortus* multiplication in macrophages (Jiang *et al*., 1993). Conversely, cytokines associated with a Th2 response such as IL-10 may act during *B. abortus* infection limiting the inflammatory response and favouring the establishment of persistent infection in mice (Golding *et al.,* 2001). CD8+ T-cells also play an important role in protection against *B. abortus* (Oliveira *et al.,* 1995; Wyckoff, 2002). Indeed, CD8+ knockout mice are more susceptible to *Brucella* infection (Oliveira *et al.,* 1995). In addition, CD8+ T-cells secrete IFNγ and increase the cytotoxic ability of *B. abortus* infected macrophages (Oliveira *et al.,* 1995; 2002). Although the protective effect of the humoral immune response in brucellosis is mild or questionable, it may have some significance in controlling *B. abortus* infection, since passive transfer of specific IgG_2 a decreases bacterial load in mice (Phillips *et al.,* 1989). However, the role of IgG during active *B. abortus* infection in cattle remains controversial (Hoffmann *et al.,*

1995). Importantly, humoral immune response is the basis for serological diagnosis of bovine brucellosis (Nielsen, 2002).

2.13 CLINICAL SIGNS OF BOVINE BRUCELLOSIS

The clinical signs of bovine brucellosis include aborton, stillbirth, and weak calves, abortion occur during the last trimester of gestation reduced milk production and an increase in number of somatic cells in the milk which impaired reproductive efficiency (Meador *et al.,*1989). Infected bulls may develop systemic signs of infection including fever, anorexia and depression, although infection is often inapparent (Campero *et al.,* 1990) most significant lesion induced by *B. abortus* in bulls is orchitis (Trichard *et al.,* 1982) which is often associated with seminal vesiculitis and epididymitis. As a result of chronic orchitis a fibrosis of the testicular parenchyma, affected bulls may developed permanent infertility (Campero *et al.,*1990).

2.14 POST-MORTEM FINDING/ GROSS PATHOLOGY

Pregnant cows develop a local lymphadenopathy that progresses to acute lymphadenitis (Schlafer *et al.,* 2007). The infected uterus contains variable amount of a fetid yellow to brownish exudates containing fibrin and necrotic debris, Placentitis, uterus containing multifocal fibrinous exudates on the caruncular surface, fibrinous necrotic exudates and multifocal haemorrhage (necrotizing placentitis), metritis, endometritis (Schlafer *et al.,* 2007). Aborted fetuses may undergo autolysis within the uterus, although not seen in all cases, pleuropneumonia is the common fetal lesion (Lopez *et al.,* 1984). Grossly the pleural are thickened, oedematous, haemorrhagic, and recovered by fibrin, characterizing a fibrinous pleuritis. The lung may have areas of pneumonia, which are firm and have

thickened interlobular septa (Gorham *et al.,* 1986) fibrinous inflammation may also be seen in other body cavities so aborted fetuses may have, in decreasing order of frequency, fibrinous pleuritis, peritonitis and or pericarditis (Schlafer *et al.,* 2007). In addition, aborted fetuses may develop enlargement of internal iliac, bronchial and hepatic lymphnodes (Enright *et al.,* 1984, Gorham *et al.,*1986), enlargement of the adrenal glands, and hypotrophy of the thymus, which may be associated with petechial haemorrhages (Enright *et al.,*1984).

2.15 HISTOLOGY

Trophoblastic cells, which are the primary target cells in the placenta, are swollen and filled with coccobacilli. There is usually a neutrophilic and histiocytic inflammatory infiltrate, associated with necrosis, oedema, fibrin deposition and in some cases vasculitis. The endometrium has a severe infiltration of neutrophils, lymphocytes, plasma cells and a few eosinophils. The inflammatory reaction is associated with multifocal erosion or superficial ulceration of the luminal epithelium (Meador *et al.,* 1988). Necrotic debris in the caruncular crypts and endometrial surface contains large number of intra- and extra-cellular *B. abortus*. Contaminated milk. *B. abortus* induces a multifocal interstitial mastitis with interstitial accumulation of macrophages and intra-acinar infiltrate of neutrophils (Meador *et al.,* 1989; Alcina *et al.,*2010), associated moderate numbers of predominantly with intracellular organism.

Histopathological changes also include lymphoid hyperplasia in lymph nodes that may be associated with a neutrophilic infiltrate that may progress to a granulomatous lymphadenitis (Meador *et al.,* 1988, 1989; Alcina *et al.,* 2010).

Histologically, fibrinous pleuritis may be associated with bronchointerstitial pneumonia characterized by the accumulation of macrophages and neutrophils, variable amounts of fibrin and haemorrhages and the presence of the bacteria associated with the lesions. In some cases, necrotizing vasculitis is observed in the pulmonary parenchyma (Enright *et al.,* 1984; Lopez., 1984; Meador *et al.,*1988) fibrinous pericarditis may also occur in fetuses, there is lymphoid hyperplasia in lymph nodes and spleen whereas lymphoid depletion occurs in the thymus (Enright *et al.,* 1984). A granulomatous inflammatory process may be eventully seen in liver, spleen, and kidney (Meador *et al.,* 1988; Hong *et al.,* 2000) and rarely the central nervous system may be affected with multifocal or diffuse histiocytic meningitis (Hong *et al.,* 2000).

2.16 CLINICAL MANIFESTATION OF HUMAN BRUCELLOSIS

Clinical symptoms in human brucellosis include fever, anorexia, polyarthritis, meningitis, pneumonia, endocarditis (Sauret *et al.,* 2002). The main causes of morbidity in spinal brucellosis are epidural abscess, rediculoneuritis (Emine *et al.,* 2008). Most common complaint are night sweating, malaise, back pain (Doganay *et al.,* 2003; Young *et al.,* 2005). However, localized symptoms may be observed due to spinal cord or nerve root compression, furthermore, neurobrucellosis may developed as a complication of spinal brucellosis and clinical manifestation are generally meningitis or meningoencephalitis (Aygen *et al.,*2002; Doganay *et al.,* 2003).

2.17 DIAGNOSIS OF BOVINE BRUCELLOSIS

Diagnostic test for brucellosis fall into two categories: those that demonstrate the presence of the organism and those that detect an immune response to its antigens (OIE Manual,

2009). All abortion in cattle in late gestation, starting from the fifth month, should be treated as suspected brucellosis and should be investigated (Nielsen *et al*., 1979). The clinical picture is not pathognomonic, although the herd history may be helpful. Unequivocal diagnosis of *Brucella* infections can be made by isolation and identification of *Brucella*, but in situations where bacteriological examination is not practicable, diagnosis must be based on serological methods. There is no single test by which a bacterium can be identified as *Brucella*. A combination of growth characteristics, serological, bacteriological and/or molecular methods are usually needed (Bricker *et al*., 2002).

2.18 IDENTIFICATION OF THE AGENT BY STAINING METHODS

Brucella species are coccobacilli or short rod measuring from 0.6 to 1.5µm long and from 0.5 0.7µm wide. They are usually arranged singly, and less freguently in pairs or small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. *Brucella* are non-motile. They do not form spores, and flagella, pilli, or true capsules are not produced. *Brucella* are Gram negative and usually do not show bipolar staining. They are not truly acid-fast, but are resistant to decolorisation by weak acids and thus stain red by the Stamps modification of the Ziehl-Neelsen method. This is the usual procedure for the examination of smears of organs or biological fluids that have been previously fixed with heat or ethanol, and by this method, *Brucella* organisms stain red against a blue back ground (Roop *et al.,* 1987; OIE, 2009).

2.19 SEROLOGICAL DIAGNOSIS OF BRUCELLOSIS

No single serological test is appropriate in all epidemiological situations; all have limitations especially when it comes to screening individual animals (Godfroid *et al.,* 2002;

Nielsen *et al.,* 1979)). Consideration should be given to all factors that impact on the relevance of the test method and test results to a specific diagnostic interpretation or application. In epidemiological units where vaccination with smooth *Brucella* is practised, false-positive reactions may be expected among the vaccinated animals because of antibodies cross-reacting with wild strain infection.

The serological methods described represent standardised and validated methods with suitable performance characteristics to be designated as either prescribed or alternative tests for international trade. This does not preclude the use of modified or similar test methods or the use of different biological reagents. However, the methods and reagents described in this work represent a standard of comparison with respect to expected diagnostic performance.

It should be stressed that the Serum Agglutination Test (SAT) is generally regarded as being unsatisfactory for the purposes of international trade. The Complement Fixation Test (CFT) is diagnostically more specific than the SAT, and also has a standardised system of unitage. The diagnostic performance characteristics of some Enzyme Linked Immunosorbent Assays (ELISAs) and the Fluorescence Polarisation Assay (FPA) are comparable with or better than that of the CFT, and as they are technically simpler to perform and more robust, their use may be preferred (Nielsen *et al.,* 1995; Wright *et al.,* 1997). The performances of several of these tests have been compared. For the control of brucellosis at the national or local level, the buffered *Brucella* antigen tests (BBATs), i.e. the Rose Bengal Test (RBT) and the Buffered Plate Agglutination Test (BPAT), as well as the ELISA and the FPA, are suitable screening tests. Positive reactions should be retested using a suitable confirmatory and/or complementary strategy.

2.19.1 Rose Bengal Plate Test (RBPT)

Rose Bengal Plate Test (RBPT) is a quick qualitative test of macroscopic agglutination performed with only one dilution, and which mainly detects IgG (Alton *et al.,* 1988).

Procedure for RBPT

The test is performed by placing 0.03 ml of antigen on each square of white tile and 0.03 ml of the serum sample alongside (but not into) the antigen. The antigen and serum is thoroughly mixed with tooth pick or sterile applicator stick and the mixture test rocked for four minutes. The test read by examining for agglutination.

A known positive serum and negative serum will be included in each test.

Interpretation

The results of the RBPT were interpreted as either: negative (-) or no agglutination; positive (+) for any degree of agglutination. Positive reaction is also considered as either "weak" or "strong" according to the degree of agglutination.

2.19.2 Serum Agglutination Test (SAT)

The SAT is an important test to which every eligible animal is subjected. It has been the basis of eradication schemes in many countries. Agglutination is the clumping of cells by an agglutinating antibody. Multivalent antibody molecules link with multivalent antigen on the particle surface to form a three dimensional aggregate. When sufficient antigen particles are linked together the aggregate is clearly visible as a floccular precipitate which sinks to the bottom of the tube.

Procedure for SAT

0.5 ml of phenol saline was added to five test tubes. And 0.3 ml mark with serum sample was transfered to the first test tube. It was mixed gently without frothing. 0.5 ml was transferred to the next test tube and carry on to the last test tube and discard 0.5 ml. A mixed contents of antigen was added to each tube and mixed well again. Incubate at 37^0C for 20 ± 1 hours.

Standards, positive and negative controls will also be run concurrently.

Interpretation: Any agglutination is taken as a positive reaction.

The titre of the serum is the highest dilution giving a definite reading of at least 1+, recorded as the degree of agglutination (numerator) and dilution (denominator). Thus a titre of 3/80 is 3+ agglutination at a dilution of 1:80.

2.19.3 Test Using 2-Mercaptoethanol (2-ME)

2-Mercapto-ethanol breaks down the IgM molecule of the immunoglobulin (Bale, 2008) by reducing the disulphide bonds, thus destroying its antibody activity. It has no effect on the IgG molecule because the disulphide bonds are not sensitive to 2-ME. The test can therefore be used to detect the presence of IgG in a serum sample, by comparing the titre before and after treatment with 2-ME.

Procedure for 2-ME

The procedure was carried out as described by Bale, (2008) as follows: Five test tubes were set up. Serum (0.1ml) under test was placed in the first test tube with 0.4 ml of 0.85% saline plus 0.5 ml of 0.2 molar 2-ME while 0.5 ml of 0.5% saline solution was dispensed

into the remaining tubes to the last tube. The tubes were incubated for an hour at 37^0C. Serial dilution was done by transferring 0.5 ml from the first tube into the second, mixed and 0.5 ml was transferred to the next tube and the serial dilution was continued up to the last test tube and 0.5 ml from the last test tube was discarded. Then 0.5 ml of *Brucella abortus* antigen diluted at 1: 10 with normal saline was added to all the tubes and incubated at 37^0C for 20$\pm$ 2 hours. A titre of 1/80 before and 1/10 after 2-ME treatment is evidence that the only immunoglobulin (Ig) present is IgM. If the titre remains unchanged or increased after treatment, this indicates that the immunoglobulin present is IgG and indicative of infection (Bale, 2008).

2.19.4 Competitive Enzyme Linked Immunosorbent Assay

Competitive Enzyme Linked Immunosorbent Assay (c-ELISA) for brucellosis diagnosis was used. The plate was prepared by adding 100µl of the diluting buffer to each well, followed by

1. 20µl of negative control sera into wells A11, A12, B11, B12, C11 and C12.
2. 20µl of positive control sera was placed into wells F11, F12, G11, G12, H11 and H12 while , D11, D12, E11, and E12, served as conjugate controls.
3. 20µl of each of the test sera was then added to the remaining giving a serum dilution of 1/6.
4. 100µl of the prepared conjugate was immediately dispensed into each well.
5. The plate was shaken with hand for 2 minutes, in order to mix the serum and conjugate solution. The microtitre plate was covered with lid and incubated at room temperature for 30minutes.

6. The microtitre plate was again shaken for 30 seconds, followed by 10seconds hand shaking at 10minutes interval for an hour. This was done carefully to keep the liquid within their respective wells.
7. The contents of the plate was then discarded and rinsed 5 times with washing solution and then dried by tapping on an absorbent tissue paper. The substrate chromogen solution was then immediately prepared and mixed thoroughly; 100µl of the solutions then added to each well.
8. The plate was left at room temperature for 10 minutes; the reaction can be slowed down by adding 100µl of the stopping solution to each well.

Interpretation

Positive (+) result showed a very weak or no colour development in wells which indicated presence of *Brucella* antibodies in the serum.

Negative (-) result gave a strong colour development (orange) in wells which indicated absence of *Brucella* antibodies.

c-ELISA has the advantages of eliminating false- positive serological reaction due to cross-reacting bacteria (Nielson, 2002; Bale, 2008), residual antibody produce in response to vaccination with S19 and prozone effects.

2.20 NATIVE HAPTEN AND CYTOSOL PROTEIN-BASED TESTS

Native hapten tests are highly specific in S19 vaccination contexts, and have been used successfully in an eradication programme in combination with the Rose Bengal Test (RBT) as a screening test (Asarta, 1989). The optimal sensitivity (close to that of CFT but lower

than that of RBT and sLPS-based I-ELISAs) is obtained in a Reverse Radial Immunodiffusion (RID) system in which the serum diffuses into a hypertonic gel containing the polysaccharide (Diaz *et al.,* 1979; Jones *et al.,* 1980). However, the double gel diffusion procedure is also useful (Lopez-Goni *et al.,* 2008; Lord *et al.,* 1992). Calves vaccinated subcutaneously with the standard dose of S19 at 3–5 months of age are negative 2 months after vaccination, and adult cattle vaccinated subcutaneously 4–5 months previously with the reduced dose of S19 do not give positive reactions unless the animals become infected and shed the vaccine in their milk (Jones *et al.,* 1980). The conjunctival vaccination (both in young and adults) reduces the time to obtain a negative response in native hapten tests.

A remarkable characteristic of the Reverse Radial Immunodiffusion (RID) test is that a positive result correlates with *Brucella* shedding as shown in experimentally infected cattle and in naturally infected cattle undergoing antibiotic treatment (FAO/WHO, 1986). Precipitin tests using native hapten or *Brucella* cytosol proteins have also been shown to eliminate false positive reactions in most cases (Munoz *et al.,* 2005).

2.21 MILK TESTS

2.21.1 Milk I-ELISA

As with the serum Indirect Enzyme Linked Immunosorbent Assay (I-ELISA) numerous variations of the milk I-ELISA are in use. Several commercial I-ELISAs are available that have been validated in extensive field trials and are in wide use. In the interests of international harmonisation, the three OIE ELISA Standard Sera should be used by National Reference Laboratories to check or calibrate the particular test method in question. The I-ELISA should be standardized such that the OIE ELISA strong positive standard

when diluted 1/125 in negative serum and further diluted 1/10 in negative milk consistently tests positive. Bulk milk samples are generally tested at much lower dilutions than sera, i.e. undiluted to 1/2 to 1/10 in diluent buffer, with the remainder of the assay being similar to that described for serum. The c-ELISA should not be used to test whole milk but may be used with whey samples. (Nielsen *et al.,* 2004, 2006)

2.21.2 **Milk ring test (MRT)**

In lactating animals, the MRT can be used for screening herds for brucellosis. In large herds (> 100 lactating cows), the sensitivity of the test becomes less reliable. The MRT may be adjusted to compensate for the dilution factor from bulk milk samples from large herds. The samples are adjusted according to the following formula: herd size < 150 animals use 1 ml bulk milk, 150–450 use 2 mls milk sample, 451–700 use 3 ml milk sample. False-positive reactions may occur in cattle vaccinated less than 4 months prior to testing, in samples containing abnormal milk (such as colostrum) or in cases of mastitis. Therefore, it is not recommended to use this test in very small farms where these problems have a greater impact on the test results (OIE, 2009).

Test procedure

The test is performed on bulk tank milk samples. If necessary, samples could be pretreated with preservative (0.1% formalin or 0.02% bronopol) for 2–3 days at 4^0C prior to use.

i) Bring the milk samples and antigen to room temperature (20 ± 3^0C); only sufficient antigen for the day's tests should be removed from the refrigerator.

ii) Gently shake the antigen bottle well.

iii) The test is performed by adding 30–50 µl of antigen to a 1–2 ml volume of whole milk (the volume of milk may be increased for bulk samples from larger herds).

iv) The height of the milk column in the tube must be at least 25 mm. The milk samples must not have been frozen, heated, subjected to violent shaking or stored for more that 72 hours.

v) The milk/antigen mixtures are normally incubated at 37^0C for 1 hour, together with positive and negative working standards. However, overnight incubation at 4^0C increases the sensitivity of the test and allows for easier reading.

vi) A strongly positive reaction is indicated by formation of a dark blue ring above a white milk column. Any blue layer at the interface of milk and cream should be considered to be positive as it might be significant, especially in large herds.

vii) The test is considered to be negative if the colour of the underlying milk is white which exceeds that of the cream layer.

viii) When the MRT is adjusted for large herd sizes (2 or 3 ml of milk used), 0.1 ml of pooled negative cream is added to the test tube and is followed by 30–50 µl of the ring test antigen. After mixing, the test is incubated and read in the same manner as the unadjusted MRT. The negative pooled cream is collected from the separation of composite, unpasteurised milk from a brucellosis negative herd of 25 or more cows

2.22 NUCLEIC ACID RECOGNITION METHOD

The recently developed Polymerase Chain Reaction (PCR) technique provides an additional means of detection and identification of *Brucella* spp. (Bricker *et al.,* 2002, 2003a; 2003b). PCR is fast, can be performed on any body tissue, and can yield positive results as soon as 10 days after inoculation (Pappas *et al.,* 2005). It was first developed for brucellosis in 1990, using a 635-bp fragment of *B. abortus* strain 19.72 subsequently, two major gene sequences have been used as target: The 16S rRNA gene sequence, which

presents total genus-specific homology and has been satisfactory in clinical settings, and the BCSP31 gene, which encodes an immunogenic protein of the external membrane of *B. abortus* and has been extensively studied in clinical practice (Romero *et al.,* 1995; Nimri, 2003).

Cross-reactivity with *Acrobactrum* is noticed sporadically with both techniques. A comparison of the two techniques showed superiority of the 16S Rrna target in terms of sensitivity (Lavaroni *et al.,* 2004). Despite the high degree of deoxyribonucleic acid (DNA) homology within the genus *Brucella,* several molecular methods, including PCR, PCR Restriction Fragment Length Polymorphism (RFLP) and Southern Blot, have been developed that allowed, to a certain extent, differentiation between *Brucella* species and some of their biovars (Bricker *et al.,*2003b; Moreno *et al.,* 2002). New diagnostic developments such as hand-held polymerase chain reaction machines offer promising new opportunities for the development of bed-side diagnostics and pen-side tests for brucellosis (Emmanuel *et al.,* 2003). Nested PCR has proved to have superior specificity and sensitivity, although it is more prone to contamination (Matar *et al.,* 1996; Briker, 2002a). Real-time PCR is most likely the diagnostic tool of the future, offering the possibility of results in 30 minutes (Redkar *et al.,* 2001; Probert *et al.,* 2005). PCR- ELISA is another new promising variation (Virioni *et al.,* 2004).

The first species–specific multiplex PCR assay for the differentiation of *Brucella* was described by Bricker and Halling (Bricker *et al.,* 1994). The assay, named abortus melitensis ovis suis PCR (AMOS-PCR), was based on the polymorphism arising from species-specific localization of the insertion sequence 711 (IS711) in the *Brucella* chromosomes, and comprised five oligonucleotide primers that can identify without differenting *B. abortus*, biovars 1, 2 and 4 but could not identify *B. abortus* biovars 3, 5, 6

and 9. Modification to the assay have been introduced over time to improve performance, and additional strain-specific primers were incorporated for identification of the *B. abortus* vaccines strains, and other biovars and species (Bricker *et al.,*1995, 2003; Ewalt *et al.,* 2003; Ocampo-sosa *et al.,* 2005).

A new multiplex PCR assay (Bruce-ladder) has been proposed for rapid and simple one-step identification of *Brucella* (Garcia-yoldi *et al.,* 2006). The major advantage of this assay over previously described PCRs is that it can identify and differentiate in a single step most *Brucella* species as well as the vaccine strains *B. abortus* S19, B. *abortus* RB51 and *B. melitensis* Rev.1. In contrast to other PCRs, Bruce-ladder is able to detect also DNA from *B. neotomae, B. pinnipedialis* and *B. ceti*. In addition *B. abortus* biovars 3, 5, 6, 7 ,9 and *B. suis* biovars 2, 3, 4, 5 can be identified by this new multiplex PCR. The only minor inconvenience of the Bruce-ladder is that some *B. canis* strains be identified erroneously as *B. suis*. (Marin *et al.,*1996).

Other variations of PCR exist, such as arbitrarily primed PCR, PCR with random amplification of polymorphic DNA. Although PCR is very promising, standardization of extraction methods and set-up is lacking, and a better understanding of the clinical significance of the results is still needed, Pulse-field gel electrophoresis has been develop that allow the differentiation of several *Brucella* species (Jensen *et al.,* 1999). However, none of these methods has been fully evaluated and standardized and none is widely available.

2.23 TREATMENT OF BRUCELLOSIS

Though, treatment in animals is not usually advocated due to cost and possible relapse in which the animal continue to shed the organism in the milk and during mating (Radwan *et al.,* 1995) described a successful control of brucellosis in large herd in Saudi Arabia using

antibiotic therapy. A combination of long-acting oxytetracycline at a dose of 25mg/kg IM, every two days for 16 days. In addition, milking animals were given oxytetracycline intramammary infusion at a rate of 10ml/teat every 2 days for 8 days. This regimen was found to be effective in eliminating the shedding of *Brucella* organisms by animals, with no relapse. Moreover, all treated animals became sero-negative within 16 months after treatment. Sero-negative animals were vaccinated for the first time with the *B. melitensis* Rev. 1 strains vaccine. No *Brucella* organisms were recovered from repeated udder secretion samples from all vaccinated milking animals, and no abortions were recorded among pregnant vaccinated animals (Radwan *et al.,* 1995).

The current World Health Organization recommended first-line therapy for brucellosis is the combination of tetracycline (tetracycline, 500mg every 6 hour orally or doxycycline. 100mg every 12 hours orally) for 6 weeks with an aminoglycoside (preferably, streptomycin, 1g/day intramuscularly for 2-3 weeks or gentamycin 5mg/kg/day intramuscularly for 7-10 days). The combination of rifampicin (600-900mg/day orally) and doxycycline (100mg every 12 hours orally) for 6 weeks is suggested as the principal alternative therapy. In spinal brucellosis, it is noted that the same regimens can be given, but that the duration of therapy should be for 8 weeks or more (Emine *et al.,* 2008). Other regimens, such as the combination of rifampicin or co-trimoxazole with dorxycycline, have been shown to be less effective than doxycyline-streptomycin (Emine *et al.,* 2008).

As a result, other alternative regimens have been investigated in recent years. Fluoroquinolones are the most commonly studied antibiotics because of their good bactericidal activity and high rates of sensitivity for *Brucella* species (Pappas *et al.,* 2005). Furthermore, due to the ability of fluoroquinolones to penetrate and achive increased concentrations in tissues and their proven efficacy in bone and soft tissue infections, these

may lead to better outcomes in spinal brucellosis. However, monotherapy with these antibiotics has shown high relapse rate, though combination regimens with rifampicin have been found to be as effective as doxycycline plus rifampicin in brucellosis (Pappas *et al.,* 2005).

2.24 PREVENTION AND CONTROL OF BRUCELLOSIS

The control of animal brucellosis has been approached with a combination of procedure: test and slaughter and hygienic measures (Joint FAO/WHO, 1986; Radostits *et al*., 2002). Brucellosis is just one of many infectious diseases to be controlled by national and local animal health and disease control services (McDermott *et al.,* 2002). The aim of an animal control programme is to reduce the impact of a disease on human health and the economic consequences. The elimination of the disease from the population is not the objective of a control programme, and it is implicit that some acceptable level of infection will remain in the population (WHO, 2006). Because brucellosis is a disease of major economic and zoonotic importance, a strategy for its control in ruminants is essential in endemic areas (EC, 2001). Control programmes have an indefinite duration and will need to be maintained even after the acceptable level of infection has been reached, so that the disease does not re-emerge. In many countries, methods for the control of brucellosis are backed by governmental regulation/legislation; in others, no authorities exist (Rikin, 1988a; WHO, 2006). Subsequent steps, after control, can include eradication from a region by test and slaughter with compensation, following successful eradication, measures like mass vaccination help to prevent reintroduction of the disease (EC, 2001; WHO, 2006).

One important epidemiologic step in controlling brucellosis in the community is the screening of household members of infected persons (Almuneef *et al.,*2004). Cross-transmission of this agent across other domestic species is believed to be the most likely

source of the infection since animals traditionally herded together with other species (Kudi *et al.,* 1997). Control could only be achieved when all susceptible domestic species are considered together and intergovernmental co-operation is initiated to prevent cross-border transmission (Kudi *et al.,* 1997). Some of the control measures are as follows:

2.24.1 Test and Slaughter/Isolation

The control of bovine brucellosis in most countries is based on serological surveillance of target animals, followed by slaughtering of positive animals and vaccination of heifers with full or reduced dose of B. *abortus* strain 19 (Refai, 2002). Similarly, (Songer *et al.* 2005) have advocated a well conducted comprehensive control and eradication programmes, with screening and confirmatory tests and prompt removal of reactors, in addition to biosecurity and vaccination. Therefore, serological tests are the usual method of identifying possible infected animals (WHO, 2006). Bacteriological procedures are useful for confirming test and results for epidemiology studies. The decision about slaughter of test positive animals is made after regulatory, economic and prevalence factors are considered . In most cases, test and slaughter of positive animals is only successful in reducing the incidence if the herd or flock is very low like 2% (WHO, 2006). The isolation of test positive animals is essential, especially during and after parturition. The immediate slaughter of test positive animals is expensive and requires animal owner cooperation. Compensation is usually necessary (WHO, 2006).

2.24.2 Sanitary Control or Herd Management

The goal in the application of hygiene methods to the control of brucellosis is reduction of exposure of susceptible animals to those that are infected, or to their discharges and tissues during calving (Songer *et al.,* 2005, WHO, 2006). Since most brucellosis contamination occurs following an abortion or delivery by an infected female and to reduced exposure,

appropriate disposal methods should be instituted for all placentas, birth fluids, foetal membranes and aborted fetuses. Those handling foetal membranes, fluids, placentas, and aborted fetuses should use appropriate hygienic practices; masks, gloves and protective clothing should be worn when handling infected animals, this is a classical procedure in disease control (FAO, 2003). Factors such as animal husbandry patterns like clinical signs, management system, type of facilities and the degree of dedication of animal owner, will also determine success. Owners are often poorly informed about disease transmission and recommendations, such as separaton of parturient animals, can be difficult or impossible to implement (WHO, 2006).

2.24.3 Control of Animal Movement and Quarantine

This may be regarded as an aspect of hygiene; however, it is essential in any programme to limit the spread of brucellosis (WHO, 2006). The control of brucellosis in bovine hinder by lack of reliable demographic information, uncontrolled movement of cattle across the borders and the imports of cattle from other countries for economic reasons, cattle should be individually identified by brand, tattoo or ear tag. Unauthorised sale or movement of animals from an infected area to other areas should be forbidden (OIE Manual, 2004). Similarly, importation of cattle into clean areas must be restricted to animals that originate from brucellosis free areas, that have a flock history of freedom from the disease and that have given negative reactions to recently performed diagnostic test (OIE, Manual,2004; WHO, 2006). Access to potentially infected animals should be restricted, and newly acquired animals should be quarantined, in practice, it is much more difficult to control the movement of cattle and small ruminants kept under the nomadic or semi-nomadic condition than that of beef and dairy cattle kept under intensive system, The owners of the herds and

flock may be accustomed to seasonal migrations which may cross national boundaries (Abbas *et al.,* 2002; McDermott *et al.,* 2002).

2.24.4 Vaccination

There is general agreement that the most successful methods for prevention and control of brucellosis in animals is through vaccination (McDermott *et al.,* 2002) The use of strain -19 vaccines has been recommended for use in controlling bovine brucellosis (Refai, 2002). In countries where cattle are kept extensively and where sero-prevalence of the diseases low, whole herd vaccination is recommended, proceeded by blood testing using the Rose Bengal test. Sero-positive animals could be identified by branding or earmarking and retested. In high prevalence countries with intensive management system of cattle production, test and slaughter method followed by vaccination is recommended (FAO, 2003).

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY AREA

Cross River is a coastal state in South-South Nigeria, located in the Niger Delta, which occupies an estimated land area of about 20,156 square kilometers. The State lies between Latitudes $5^0$45N and between Longitudes $8^0$30E. It shares boundary with Benue State to the North, Enugu and Abia States to the West, Akwa-Ibom and Atlantic Ocean to the south and Cameroon Republic to the East (Figure 1). Cross-River State has a human population of 2,888,966 million with a population density of 92.6/km^2 (NPC, 2006).

The economy is predominantly agriculture and their main crops are Cassava, Yam, Rice, Plantain, Banana, Cocoyam, Maize, Cocoa, Groundnut and Palm product. The major livestock in the state are cattle, goats and sheep. Rearing activities are mainly undertaken by local farmers and nomadic Fulanis, except in Obanliku, at the Obudu Cattle Ranch where organized cattle ranching take place.The people of Cross River State speak Efik, Efut and Quacs which are predominantly found in Calabar municipality.

Obudu Cattle Ranch is located in Obanliku Local Government Area. It location has an area of 1,057km^2 and a population of 110,320 (NPC, 2006). The inhabitants are mostly farmers and the language spoken are Bechebe, Becheve and Obudu on the ranch. The number of visitors depend on the time of the year, like if it is during festive period like Christmas and Ester period, the influx of visitor is usually within 300-500 in a month. The total of animal on the ranch is estimated to be upto 30000(thirty thousand) that is own by the Fulani herdsmen and the friesien cattle in the diary farm are upto 500 (five hundred) in number.



Fig. 1. Map of Cross River State showing Local Government Areas and study area been darkened (Reference. www. crs.gov.com)

3.2 SAMPLE SIZE

The sample size was calculated using the formular of Thrusfield (2005).

$$n = \frac{Z^2pq}{d^2}$$

Where:

n = sample size

p = prevalence rate (Gusi *et al*., (2010) reported 36.93%)

d = allowable error (0.05)

Z = desired precision/ confidence 95 % (1.96)

q = 1 – prevalence rate

$$\frac{1.96^2 \times 0.3693 \times (1- 0.3693)}{0.05^2} = \frac{3.84 \times 0.3693 \times 0.6307}{0.0025} =$$

$$\frac{3540.89}{0.0025} = 354 \text{ Samples}$$

Three hundred and fifty four samples were collected for the study.

3.3 SAMPLES AND SAMPLING TECHNIQUE

One hygroma fluid, fourteen vaginal swab, were obtained from cattle in the ranch and Fulani herds. Samples were obtained from the animals.

3.3.1 Collection of Samples (Blood)

The animals were properly restrained and 5ml of blood was collected from jugular vein using sterile needle and syringes and was dispensed into labelled vacutainer without anticoagulant. The tube containing blood without anticoagulant was placed in a slanting position to avoid haemolysis of blood cell and to assist in serum separation which was kept in an ice pack cooler before it was transported to the hospital. The blood sample was centrifuged at 1000 rpm for 5 minute to allow for proper separation of serum from the clotted red blood cells, Serum were decanted into serum vials and transported in an ice pack cooler to *Brucella* laboratory of National Veterinary Research Institute, Vom and stored in the refrigerator at -20^{0}C until required for testing. The Friesien breed sample are 131, Kuri breed are 117 and White Fulani are 106, given a total of 354 cattle sampled.

3.3.2 Isolation

Fourteen vaginal swabs and one hygroma fluid, were cultured. Culturing of the sample were performed according to the techniques described by Alton *et al.,* (1988). Primary isolation of *Brucella* organisms was made by culturing the samples on Farrell's Modified Serum Dextrose Agar prepared from blood agar supplemented with 5% calf serum, 1% dextrose, and ready mixed antibiotics supplemented at the following amount per ml of media: bacitracin (25 iu), polymyxin B (5iu), cycloheximide (100µg), nalidixic acid (95µg), nystatin (100iu) and vancomycin (20µg). Each sample were inoculated into petri dish plate and incubated at 37^{0}C for primary isolates and observed for 5-7 days for *Brucella*-like colonies. The plates were discarded after 7 to10 days if no growth was seen.

3.3.3 Identification Procedures

Colonies typical of *Brucella* species were sub-cultured on serum dextrose agar from which subsequent growth was examined by Gram stain, Modified Ziehl-Nelsen stain and slide agglutination with *Brucella* A and B monospecific antisera. It was observed for catalase, and oxidase production.

3.3.4 Biotyping of *Brucella*

The tests were performed according to the techniques described by Alton *et al.,* (1988): Culture of suspected *Brucella* isolates on serum dextrose agar were tested for H_2S and urease production, dye sensitivity by growth in the presence of basic fuchsin (20ug ml^{-1}), thionin (20ug $ml^{-1)}$ and susceptibility to lysis by Tb, Wb and Bk_2 brucellaphage (Corbel *et al.,* 1987). Reference strains of all species of brucellaphage (Tb, Wb and Bk_2) and *Brucella* polyvalent and monospecific antisera (A and B) was obtained from the FAO/WHO Collaborating Centre for Brucellosis Reference and Research at the Veterinary Laboratories Agency, Weybridge, United Kingdom (UK).

3.4 SEROLOGICAL TEST

Serum samples were tested for *Brucella* antibodies by Rose Bengal Plate Test (RBPT), Serum agglutination test (SAT) as described by Alton *et al.,* (1988) 2-Mercaptoethanol (2-ME) as described by Bale, (2008) and the Competitive Enzyme Linked Immunosorbent Assay (c-ELISA), according to the manufacturer's instructions. Test antigens as well as c-

ELISA Kits were obtained from Central Veterinary Laboratory Agency, Surrey, Weybridge, United Kingdom.

3.4.1 Rose Bengal Plate Test (RBPT)

Rose Bengal Plate Test (RBPT) is a quick qualitative test of macroscopic agglutination performed with only one dilution, and which mainly detects IgG (Alton *et al.,* 1988*).*

Procedure for RBPT

The test was performed by placing 0.03 ml of antigen on each square of white tile and 0.03 ml of the serum sample alongside (but not into) the antigen. The antigen and serum was thoroughly mixed with tooth pick or sterile applicator stick and the mixture rocked for four minutes.

The test was read by examining for agglutination.

A known positive serum and negative serum was included in each test.

Interpretation

The results of the RBPT was interpreted as either: negative (-) or no agglutination; positive (+) for any degree of agglutination. Positive reaction was considered as either "weak" or "strong" according to the degree of agglutination.

3.4.2 Serum Agglutination Test (SAT)

The SAT is an important test to which every eligible animal is subjected, When sufficient antigen particles are linked together the aggregate is clearly visible as a floccular precipitate which sinks to the bottom of the tube.

Procedure

To the five test tubes 0.5ml of phenol saline was added and draw up to the 0.3 ml mark with serum sample, It was mixed gently without frothing. 0.5 ml was transfered to the next test tube and carry on to the last test tube and discard 0.5 ml. The contents of antigen were mixed and 0.5 ml antigen added to each test-tube and mixed well again.It was incubated at 37^0C for 20 ± 1 hours.

Standards, positive and negative controls were run concurrently with the test samples.

Interpretation: Any agglutination is taken as a positive reaction. The titre of the serum is the highest dilution giving a definite reading of at least 1+, recorded as the degree of agglutination (numerator) and dilution (denominator). Thus a titre of 3/80 is 3+ agglutination at a dilution of 1:80.

3.4.3 Test Using 2-Mercaptoethanol (2-ME)

2-ME break-down the IgM molecule of the immunoglobulin by reducing the disulphide bonds, thus destroying its antibody activity. The test was used to detect the presence of IgG in a serum sample, by comparing the titre before and after treatment with 2-ME (Bale, 2008).

Procedure

The procedure was carried out as described by Bale, (2008) as follows: Five test tubes were set up. Serum (0.1ml) under test was placed in the first test tube with 0.4 ml of 0.85% saline plus 0.5 ml of 0.2 molar 2-ME while 0.5 ml of 0.5% saline solution was dispensed into the remaining tubes to the last tube. The tubes were incubated for an hour at 37^0C.

Serial dilution was done by transferring 0.5 ml from the first tube into the second, mixed and 0.5 ml was transferred to the next tube and the serial dilution was continued up to the last test tube and 0.5 ml from the last test tube was discarded. Then 0.5 ml of *Brucella abortus* antigen diluted at 1: 10 with normal saline was added to all the tubes and incubated at 37^0C for 20$\pm$ 2 hours. A titre of 1/80 before and 1/10 after 2-ME treatment is evidence that the only immunoglobulin (Ig) present is IgM. If the titre remains unchanged or increased after treatment, this indicates that the immunoglobulin present is IgG and indicative of infection (Bale, 2008).

3.4.4 Competitive Enzyme Linked Immunosorbent Assay

Competitive Enzyme Linked Immunosorbent Assay (c-ELISA) for brucellosis diagnosis was used.

The plate were prepared by adding 100µl of the diluting buffer to each well, followed by

1. 20µl of negative control sera into wells A11, A12, B11, B12, C11 and C12.
2. 20µl of positive control serum were placed into wells F11, F12, G11, G12, H11 and H12 while , D11, D12, E11, and E12, served as conjugate controls.
3. 20µl of each of the test sera was then added to the remaining giving a serum dilution of 1/6.
4. 100µl of the prepared conjugate was immediately dispensed into each well.
5. The plate was shaken with hand for 2minutes, in order to mix the serum and conjugate solution. The microtitre plate was then covered with lid and incubated at room temperature for 30minutes.

6. The microtitre plate was again shaken for 30 seconds, followed by 10seconds hand shaking at 10minutes interval for an hour. This was done carefully to keep the liquid within their respective wells.
7. The contents of the plate was discarded and rinsed 5 times with washed solution and then dried by tapping on an absorbent tissue paper. The substrate chromogen solution was immediately prepared and mixed thoroughly; 100µl of the solutions was then added to each well.
8. The plate was left at room temperature for 10minutes; the reaction can be slowed when 100µl of the stopping solution was added to each well.

Interpretation

Positive (+) Reaction is a very weak or no colour development in wells which indicated presence of *Brucella* antibodies in the serum.

Negative (-) Reacton is a strong colour development (orange) in wells which indicated absence of *Brucella* antibodies.

3.5 DATA ANALYSIS

Data obtained was presented in form of tables. Chi-square and Correlation were use to analyze the data with the aid of Statistical Package for Social Science (SPSS) Version 17.0 (SPSS Inc. Chicago IL, USA). The prevalence of bovine brucellosis was calculated by dividing the total number of seropositive animals by the total number of animals sampled and expressed as a percentage.

CHAPTER 4

4.0 RESULTS

4.1 ISOLATION RATE OF *BRUCELLA* FROM CATTLE IN OBUDU CATTLE RANCH

A total of 1(7.14%) strains of *Brucella* was obtained from 14 culture samples collected from vaginal swab of cattle that aborted before our arrival. One (100%) Isolate was obtained from hygroma fluid.

Table 4.1: Isolation rate of Brucella from cattle in Obudu Cattle Ranch.

Number of sample Collected	Culture sample	Number of samples positive
1	Hygroma	1(100%)
14	Vaginal swabs	1(7.14%)

4.2 GROWTH CHARACTERISTICS AND BIOCHEMICAL TESTS OT THE ISOLATES

All the isolates showed the following common characteristics typical of the genus *Brucella*: Non-motile, Gram-negative, cocobacilli, and oxidase and catalase positive. They grew aerobically even on primary culture and in 10% CO_2 after 5-7 days incubation at 37^0C. Colonies on serum dextrose agar (SDA) were raised, convex, with an entire edge and smooth shiny surface. They were honey colored and transparent when viewed by transmitted light, and bluish in reflected light, and in transmitted light. All isolates produced H_2S, urease and grew in the presence of basic fuchsin (20 ug ml^{-1}) but not in the presence of thionin (20 ug ml^{-1}). In slide agglutination by the A monospecific antiserum. All isolates showed clear lysis by Tb, Wb, and Bk_2 phages as shown in Table 4.2. Isolates were obtained and only one species of *Brucella abortus* was obtained and all the strains belong to biotype 1

Table 4.2: Growth characteristics and biochemical of Brucellae isolated from Obudu Cattle Ranch

				Growth on media containing		Agglut. by monospecfic antisera		Lysis by phages at RTD			Brucella spp	Biotype
Lab/no	Urease production	H_2S production	CO_2 Required	BF	TH	A	M	Wb	Tb	Bk_2	*B. abortus*	1
V_1V_1	+	++	+	+	-	+	-	+	+	+	*B. abortus*	1
V_2V_2	+	++	+	+	-	+	-	+	+	+	*B. abortus*	1

Key:

Agglut.- Agglutination

BF- Basic fuchsin 20 µg ml-1 (1/50,000)

TH- Thionine 20 µg ml-1 (1/50,000)

RTD- Routine Test Dilution

4.3 TEST-SPECIFIC PREVALENCE OF *BRUCELLA* AGGLUTININS IN OBUDU CATTLE RANCH

A total of 354 cattle sera were tested for *Brucella* antibodies using the Rose Bengal plate test (RBPT), serum agglutination test (SAT), 2-mercaptoethanol test (2-ME) and Competivive Enzyme Linked Immunosorbent Assay (c-ELISA). Out of the 354 sera tested 149 (42.09%), 142 (40.11%), 132 (37.29%) and 100 (28.25%) were positive for *Brucella* antibodies using the RBPT, SAT, 2-ME and c-ELISA respectively (Table 4.3).

Table 4.3: Test-specific prevalence of *Brucella* agglutinins in Obudu Cattle Ranch

Serological test	Total no. of samples tested	No. positive (%)
RBPT	354	149 (42.09)
SAT	354	142 (40.11)
2-ME	354	132 (37.29)
c-ELISA	354	100 (28.25)

4.4 SEX-SPECIFIC PREVALENCE RATE OF ANTIBODIES TO *BRUCELLA* IN OBUDU CATTLE RANCH

One hundred and thirteen (113) of the sera tested were males while 241 were females. Forty (35.40%), 39 (34.51%), 36 (31.86%) and 27 (23.89%) of the males were positive for *Brucella* antibodies using the RBPT, SAT, 2-ME and c-ELISA while 109 (45.23%), 142 (58.92%), 85 (35.27%) and 73 (30.29%) of the females were also positive to *Brucella* using the above tests respectively. There was no statistically significant association (*p>0.05)* between sex and serological status of the cattle (Table 4.4).

Table 4.4: Sex-specific prevalence rate of antibodies to *Brucella* in Obudu Cattle Ranch

Sex	Total no tested	No. positive to serological tests (%)			
		RBPT	SAT	2-ME	c-ELISA
Males	113	40(35.40)	39(34.51)	37(32.74)	27 (23.89)
Females	241	109(42.23)	103(42.74)	95(39.41)	73 (30.29)

($\chi^2 = 1.553; p = 0.2127; p > 0.05$)

4.5 AGE-SPECIFIC PREVALENCE RATE OF ANTIBODIES TO *BRUCELLA* IN OBUDU CATTLE RANCH

A total of 194 of the cattle tested were > 5 years of age, 110 were between 3 – 5 years while 50 were < 2 years. Among the young (<2 years of age) cattle, 10 (20.00%), 9 (18.00%), 6 (12.00%) and 5 (10.00%) were seropositive for *Brucella* antibodies using the RBPT, SAT, 2-ME and c-ELISA respectively. Also among the age group 3 – 5 years; 44 (40.00%), 42 (38.18%), 28 (25.45%) and 24 (21.82%) were positive while in the age group > 5 years 95 (48.97%), 91 (46.91%), 87 (44.85%) and 71 (36.60%) were also positive for *Brucella* antibodies using the above tests respectively. There was a statistically significant association ($p<0.05$) in serological status between the age groups with more animals in the > 5 age groups having more positive reactors (Table 4.5).

Table 4.5: Age-specific prevalence rate of antibodies to *Brucella* in Obudu Cattle Ranch

Age group	Total no tested	No. positive to serological tests (%)			
		RBPT	SAT	2-ME	c-ELISA
<2	50	10(20.00)	10(20.00)	6(12.00)	5 (10.00)
2 – 5	110	44(40.00)	42(38.18)	38(34.55)	24 (21.82)
>5	194	95(48.97)	91(46.91)	88(45.36)	71 (36.60)

($\chi^2 = 17.13$; $p = 0.0002$; $p < 0.05$)

4.6 BREED-SPECIFIC PREVALENCE OF *BRUCELLA* ANTIBODIES IN OBUDU CATTLE RANCH

Though, Friesian breed comprised majority (131) of the breed sampled compared to Kuri breed (117) and White Fulani breeds (106), Kuri breed had the highest number of cattle positive for *Brucella*. Only 73 (62.39%), 69 (58.97%), 62 (52.99%) and 35 (29.91%) Kuri breeds, 10 (7.63%), 10 (7.63%), 9 (6.87%) and 9 (6.87%) Friesians and 66 (62.26%), 63 (59.43%), 51 (48.11%) and 56 (52.83%) of the White Fulani breeds were positive for *Brucella* antibodies using the RBPT, SAT, 2-ME and c-ELISA tests respectively. There was a statistically significant association ($p<0.05$) between serological status of the cattle and the breeds tested (Table 4.6).

Table 4.6: Breed-specific prevalence of *Brucella* antibodies in Obudu Cattle Ranch

Breeds	Total no tested	No. positive to serological tests (%)			
		RBPT	SAT	2-ME	c-ELISA
Kuri	117	73(62.39)	69(58.97)	62(52.99)	35 (29.91)
Friesian	131	10(6.87)	9(7.63)	9(6.87)	14 (10.69)
White Fulani	106	66(62.26)	63(59.43)	61(57.55)	51 (48.11)

($\chi^2 = 12.53$; $p = 0.0019$; $p < 0.05$)

4.7 CORRELATION OF THE *BRUCELLA* SEROLOGICAL TESTS USED

There was also high level of correlation (*r<0.0001)* between the results obtained using the RBPT, SAT and c-ELISA test (Table 4.7). c-ELISA have more specificity when compared to RBPT, SAT, and 2-ME, but they are more sensitive than c-ELISA (OIE, 2009).

Table 4.7: Correlation of the *Brucella* test technique used

Serological test	Total no. of samples tested	No. positive
RBPT	354	149
SAT	354	142
2-ME	354	132
c-ELISA	354	100

(*r (95% CI) = 0.7589 (0.7109 – 0.7999); r < 0.0001*)

CHAPTER 5

DISCUSSION

The isolation of *Brucella* organism in this study from Obudu Cattle Ranch is an important finding. Out of the fourteen vaginal swabs collected from cows that aborted before our arrival on the ranch yielded one positive for *Brucella* isolate. The only hygroma fluid collected was also positive for *Brucella* isolate. The fact that only one species, *B. abortus* biotype 1 was encountered in this study does not indicate that this species and biotype are representative of Nigeria, it can be said to be the representative of the area from which they were obtained. It does appear from past reports and the present study that *B. abortus* biotype 1 may be the predominant strain associated with the diseased problem in Nigerian livestock. In Nigeria Eze, (1978) isolated *B. abortus* biotype 1 and 2 from Nigerian cattle. Bale and Kumi-Diaka, (1981), isolated biotypes 1, 3, and 4 from Livestock Investigation and Breeding Centre (LIBC) in Kano State. Ocholi *et al.,* (2004a; 2004b; 2005), isolated *B. abortus* biotype 1 from horses in Jos, north central and north east and aborting sheep in Bauchi State respectively. *B. abortus* biotype 1 from milk of sheep and *B. suis* from Piggery farms in northern Nigeria have been isolated by Bale and Nuru, (1985) and Bale *et al*., (2002) respectively. The fact that only one species, *B. abortus*, biotype 1 was isolated. It can be said to be representative of the area from which they were obtained. Eze, (1978), indicated that out of 20 strains of *B. abortus*, isolated from cattle 19 (95%) were of biotype 1 while only 1 (5%) was of biotype 2. Similarly out of 8 strains of *B. abortus* obtained from cattle by Bale and Kumi-Diaka, (1981) 5 (62.59%) were of biotype 1, 25% were of biotype 3 and 12.59% were of biotype 4. It does appear from past reports that *B. abortus* biotype 1 may be the predominant strain associated with the disease problem in Nigerian livestock.

The finding in this study is in agreement with previous authors across Africa, isolation of biotype 1, 3, 8, 9 of *Brucella abortus* has been reported in Kenya (Waghela, 1975). In Uganda *B. abortus* biotypes 3, 8, and 9 have been reported (Elliott *et al*., 1977) while *B. abortus* biotype 7 have been reported in Egypt and South Africa. *B. abortus* biotype I and 3 have been isolated in Senegal with a very strong predominance of biotype 3 (Verger *et al.,* 1979). Seventeen isolates of *B. abortus* from cattle in Ivory Coast, 9 were biotypes 1 and 8 (Pilo- Moron *et al.,* 1979). Andreani *et al.,* (1983), isolated *B. abortus* biotype 6 in milk in Somalia.

The isolation of *Brucella* from Obudu Cattle Ranch posed a serious health problem to the community and tourist that visit the ranch for leisure in which some of them consumed milk produced by the local Fulani cattle and the exotic breeds of cattle found on the ranch as well as the Calabar metropolis in which the milk is usually marketed.

Of the 354 sera sample analysed in this study 149(42.09%), 142(40.11%), 132(37.29%) and 100(28.25%) were positive for antibodies to *Brucella abortus* using RBPT, SAT, 2-ME and c-ELISA respectively. The prevalence rate of 42.09% obtained with RBPT is high, this is because of the sensitivity of RBPT which is used as a screening test for brucellosis, but its inability to eliminate vaccinal antibodies as well as cross reacting organism like *Salmonella* species*, Yersinia enterocolitica* which can give false positive reactions. The prevalence rate of 3.6% to 36.93% were reported in Nigeria by previous authors (Ogundipe *et al*., 1994; Cadmus *et al*., 2006, 2009; Adamu, 2009; Gusi *et al*., 2010; Wungak *et al*., 2011; Mbuk *et al*., 2011; Junaidu *et al*., 2011; Hamza, 2011). This study is also in agreement with other authors across the globe who recorded prevalence rate of 3% to 72.9% (Shuaibi, 1999; Nakaune *et al*., 2004; Teoman *et al.,* 2007; Ahmad *et*

al., 2009; Baris *et al*., 2010). The reasons for the high prevalence rate obtained from Obudu Cattle Ranch may be because of low temperature and wet environment which favour the survival and growth of the organism, another reason could be contamination of pasture by aborted fetus which persist for long in the pasture and could serve as source of infection to other susceptible host, concentration of animals on the ranch and also drinking water from the same spots could be a possible reason for the high prevalence.

The prevalence rate of 40.11% obtained with SAT, though is lower compared to the one obtained with RBPT which is higher, it has no significant association. SAT is also use for screening test and sensitivity is similar to that of RBPT. It can give false positive reaction due to vicinal antibodies. This work is in agreement with other authors which reported prevalence rate of 3.65% to 17.00% (Sharma et al., 1979; Isloor et al., 1998; Montasser et al., 1998; Adamu, 2009; Baris et al., 2010). The reasons for the high prevalence could be the same as in RBPT.

The prevalence rate of 37.29% obtained with 2-ME, Although 2-ME test are concurrently run with SAT, this is because the Ig^G *Brucella* antibodies titre can be determined by treating the serum with 2-ME to inactivate the Ig^M *Brucella* antibodies while leaving the Ig^G *Brucella* antibodies intact due to the neutralizing effect of 2-ME on Ig^M Which inhibit the serological response of false positive producers. It also picks acute or chronic stage of the infection (Bale, 2008). Baris *et al*., (2010) reported prevalence rate of 5% in turkey which is lower than the one obtained in Obudu Cattle Ranch. A major drawback to the use of 2-ME test is that 2-ME irritate the eyes and mucus membranes, it also has a very strong odour (Klein *et al*., 1981).

In c- ELISA, the prevalence rate of 28.25% was recorded in these study which acertain the specificity of c-ELISA. Because of it specificity which is capable of eliminating cross-reacting organism and vaccinal antibodies but less sensitive compared to RBPT. This study is in agreement with Adamu, (2009) reported 3.5% prevalence rate in cattle in Maiduguri and Farouk *et al*., (2011) reported 3.86 prevalence rate in cattle in Jigawa state Esuruoso (1974) in his previous study observed that the prevalence rate of brucellosis is usually low in the northern Nigeria compared with that of southern Nigeria which is as high as 20-60% which is in line with this study which recorded 42.09%, 40.11%, 37.29% and 28.25% in RBPT, SAT, 2-ME, and c- ELISA respectively.

Sex specific prevalence of bovine brucellosis in Obudu Cattle Ranch, This indicated that there is association between *Brucella abortus* and sex. The higher prevalence recorded in female than male may result from the fact that more female are been sample than male and this is because fewer male are found in most of the herds visited, it appears that the female animals are generally more susceptible to *Brucella* infection than the males because of D-erythritol, an alcohol normally found in higher concentration in fetal tissue in pregnant females than in seminal vesicles and testes of the male and is known to stimulate the growth of virulent strains of *Brucella* (Junaidu *et al.,*2011).

Age group specific prevalence, This finding is in agreement with Alton (1981) who reported that brucellosis occurs in animals of all ages but was highest in age group 2-5years and □5years. This is because older cattle are more susceptible to infection due to prolong exposure while younger cattle tended to be more resistant to infection because of maternal immunity (Alton, 1981).

In breed specific prevalence, though, Friesian breed comprised majority (131) of breed sampled compared to Kuri (117) and White Fulani(106), but Kuri and White Fulani had the highest number of positive for *Brucella* antibodies using RBPT, SAT, 2-ME and c-ELISA. Adamu (2009) reported prevalence of 23.1%, 20.9% and 13.3% in White Fulani with RBPT, SAT, and c-ELISA respectively which is in agreement with this studies. Friesian breed tend to have low prevalence this could be due to management system because the Kuri and White Fulani belong to the Fulani herds men in which the practice extensive system of management and this could exposed the animal to infection due to contamination of pasture by aborted fetus and vaginal secretion as they moved around to graze.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

This study revealed that *Brucella abortus* biotype 1 was isolated in Obudu Cattle Ranch. A total of 354 cattle sera were tested for *Brucella* antibodies using the Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT), 2-Mercaptoethanol (2-ME), and Competitive Enzyme Link Immunosorbent Assay (c-ELISA). Out of the 354 sera tested 149(42.09%), 142(40.11%), 132(37.29%) and 100(28.25%) were positive reactors to *Brucella* antibodies using the RBPT, SAT, 2-ME, and c-ELISA respectively.

There was no statistically significant association ($P>0.05$) between sexes and serological status of the cattle with more female been positive.

There was a statistically significant association ($P<0.05$) in serological status between the age groups with more animals in the >5 years age groups having more positive reactors.

Though Friesian breed comprised majority (131) of the breed sampled compared to Kuri breed (117) and White Fulani breeds (106), Kuri and White fulani breeds had the highest number of cattle positive reactors to *Brucella* antibodies, and the Friesian cattle has the lowest prevalence rate. There was a statistical significant association ($P<0.05$) between serological status of the cattle and the breeds tested. There was also high level of correlation ($r<0.001$) between the results obtained using the RBPT, SAT, 2-ME and c-ELISA test.

6.2 CONCLUSIONS

In conclusion *Brucella* biotype 1 was isolated from Obudu Cattle Ranch and the prevalence rate of antibodies due to *B. abortus* was established to be 42.09%, 40.11%, 37.29% and 28.25% for RBPT, SAT, 2-ME and c-ELISA respectively as it relates to brucellosis has been documented.

6.3 RECOMMENDATIONS

This study is an indication that bovine brucellosis is on the increase among cattle in Nigeria. In view of the strategic location of the Obudu Cattle Ranch coupled with free movement of livestock in the ranch, increases the risk of transmitting brucellosis from animal to animals or from animal to man as well as isolation of the organism from the ranch. The following recommendations are made;

1. Molecular characterization of the organism should be employed and this could assist in the development of type specific *Brucella* vaccine for ruminants
2. Brucellosis is a zoonotic disease that causes abortion in animals, for this reason there is need for proper handling of vaginal discharges, retained placenta and aborted fetuses by burning and buried deep in the ground.
3. There should be government coordinated public awareness programme on the presence of brucellosis among ruminants with emphasis on its economic impact and public health implication and mass vaccination of animal should be conducted in the ranch.
4. National brucellosis control policy programme should be initiated by the Federal government of Nigeria.

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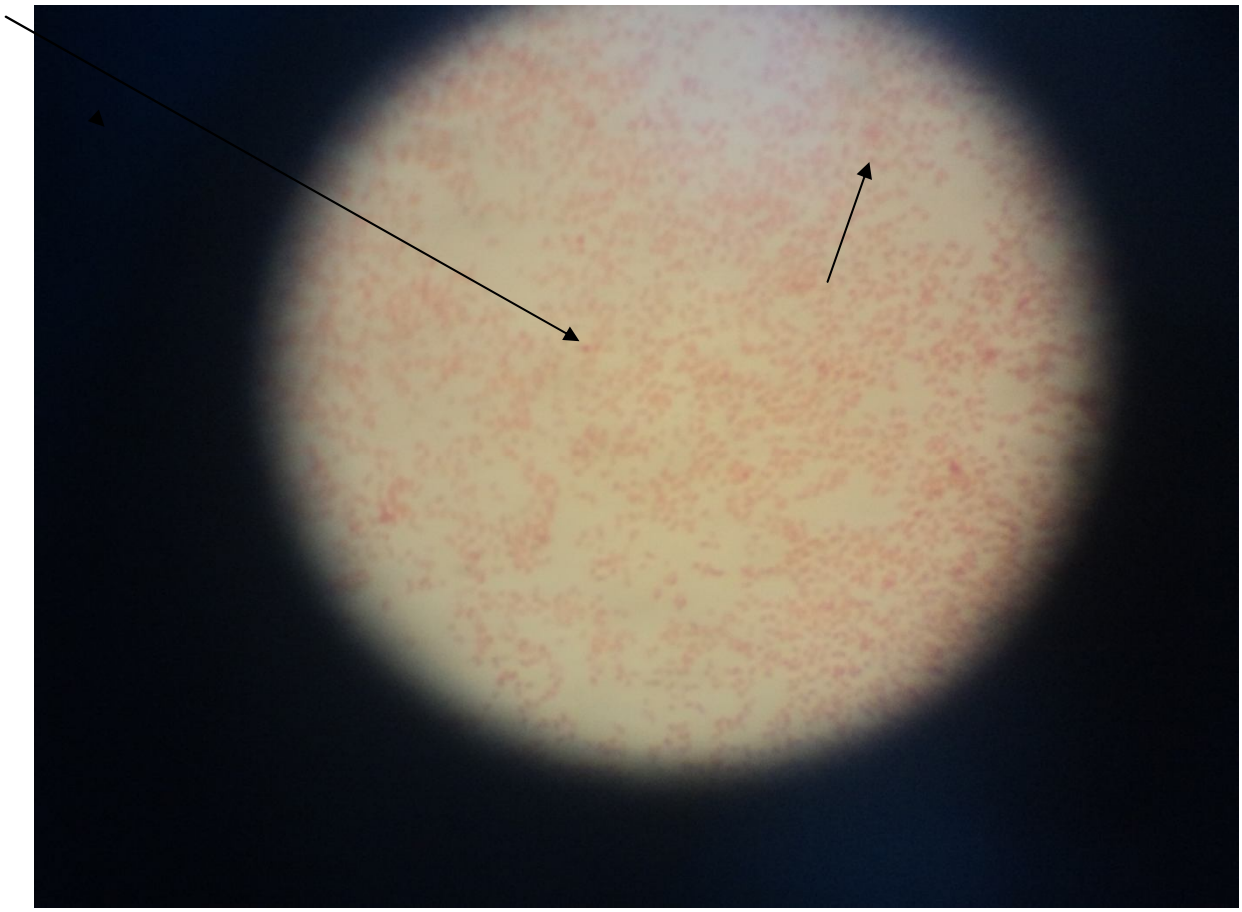
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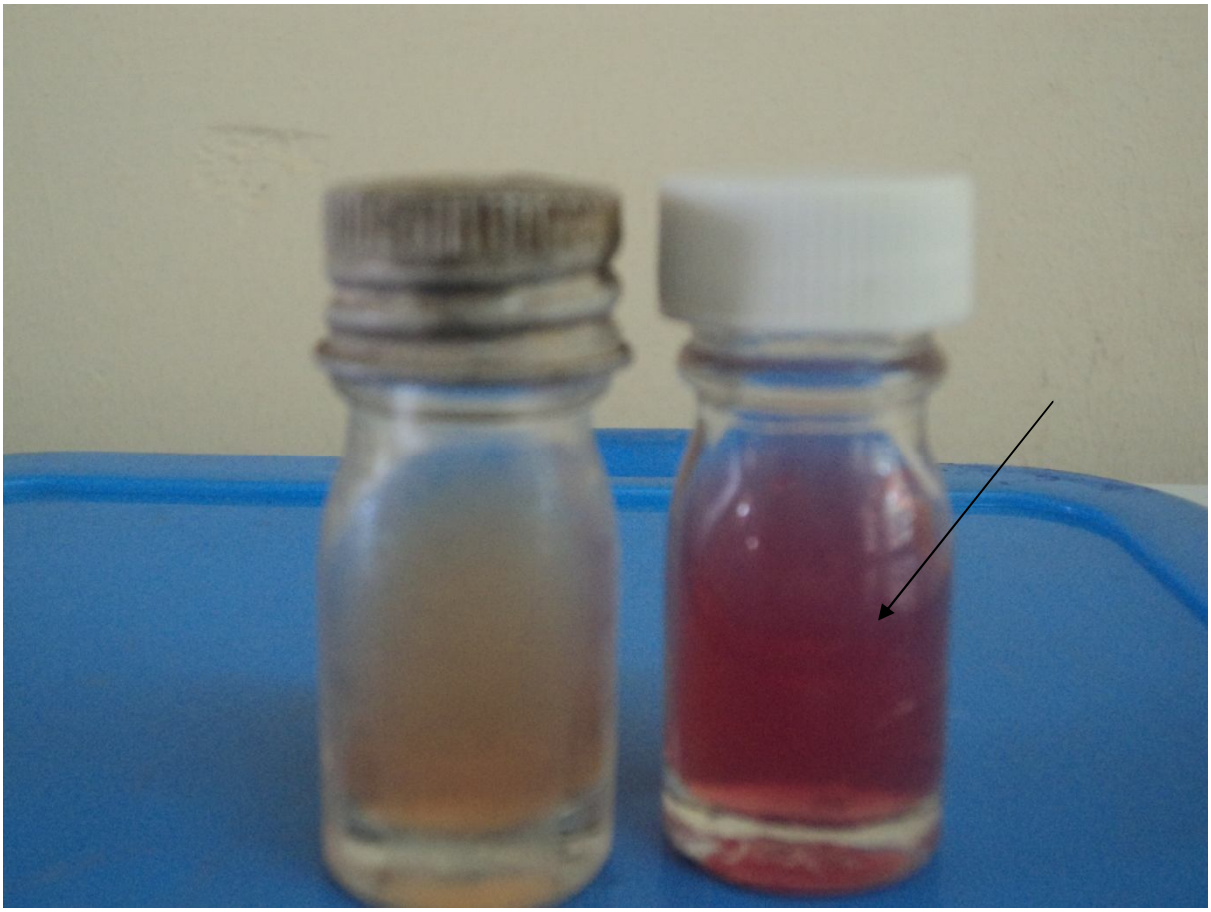
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APPENDIX I



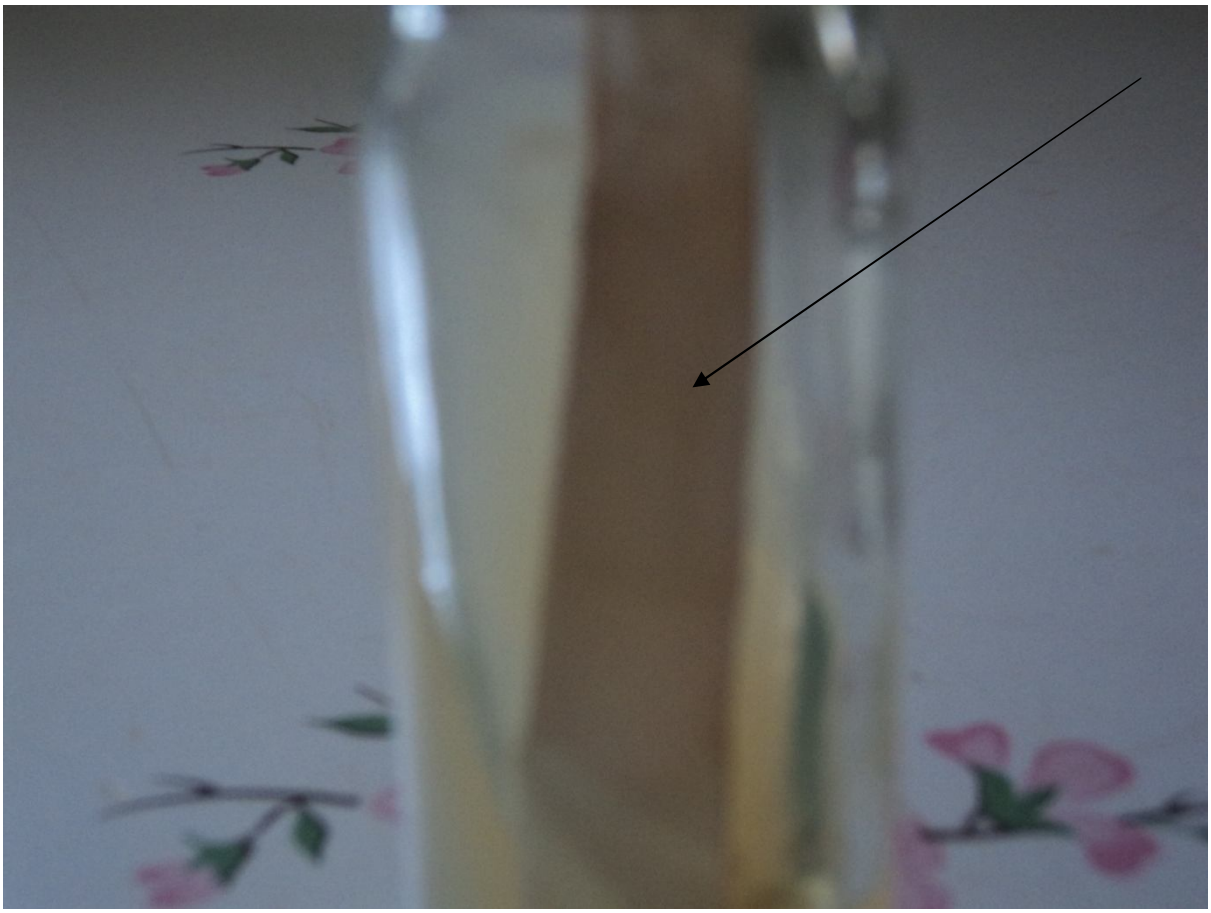
Appendix I: *Brucella* Gram negative cocobaccili (As shown by the arrow).

APPENDIX II



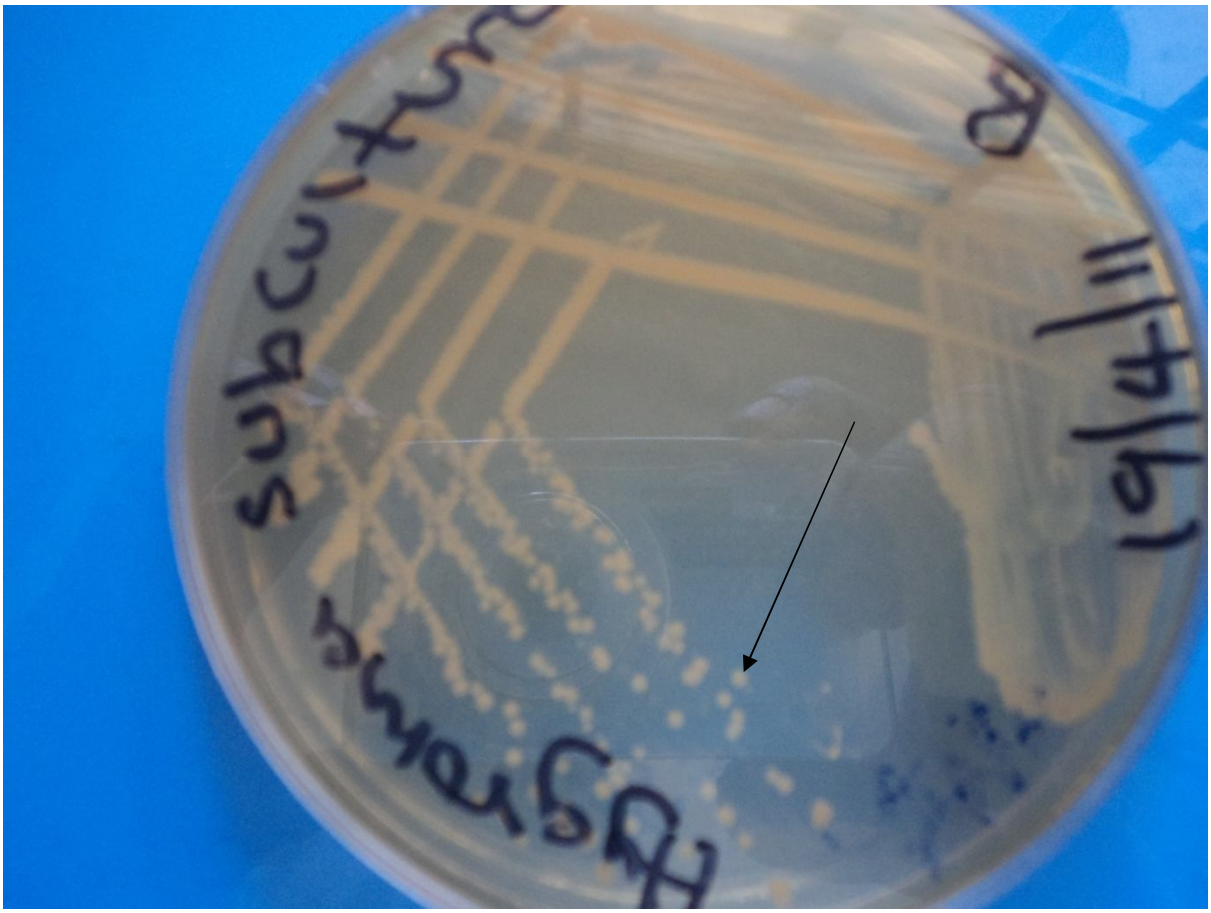
Appendix II:Positive Urease test is pink (As shown by the arrow).

APPENDIX III



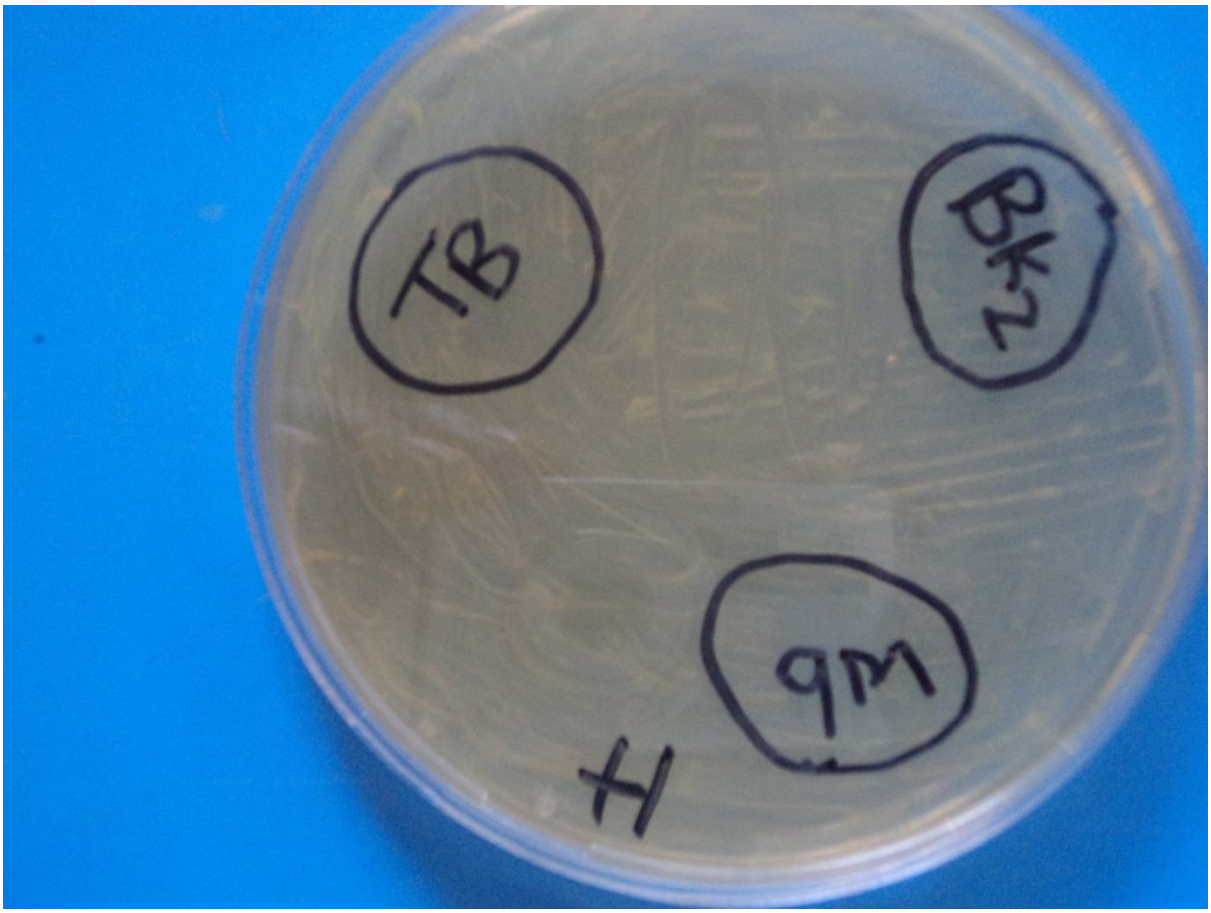
Appendix III: Hydrogen sulphide gas production. Blackening of paper (As shown by the arrow)

APPENDIX IV



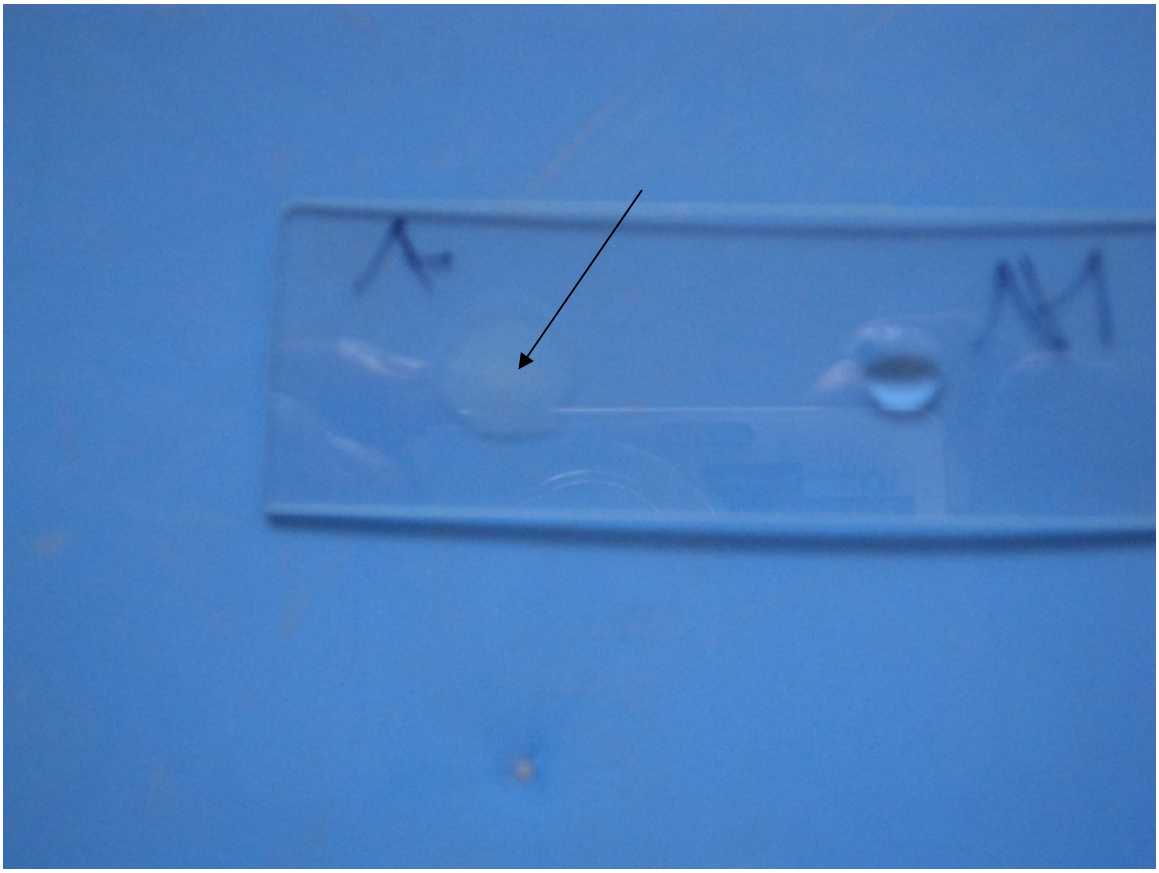
Appendix IV: Cultural isolation (*Brucella* colony as shown by the arrow)

APPENDIX V



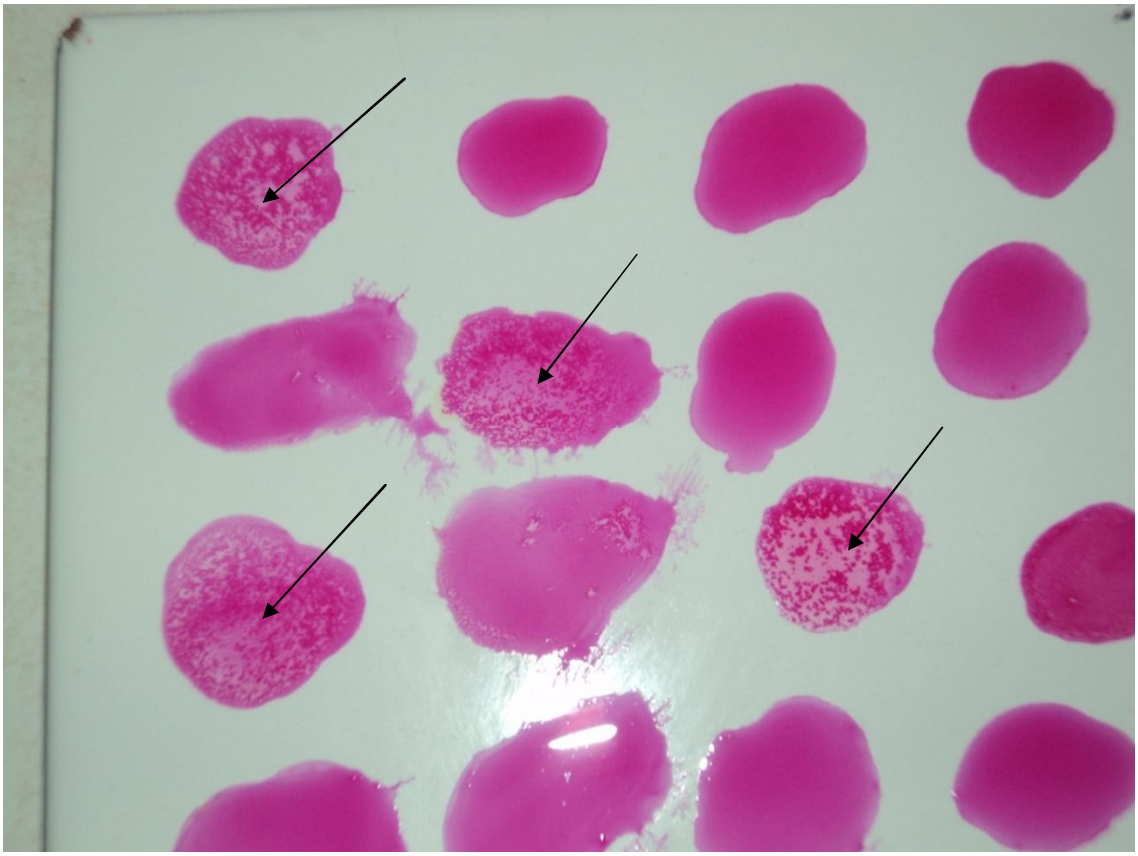
Appedix V: Lysis by phages.

APPENDIX VI



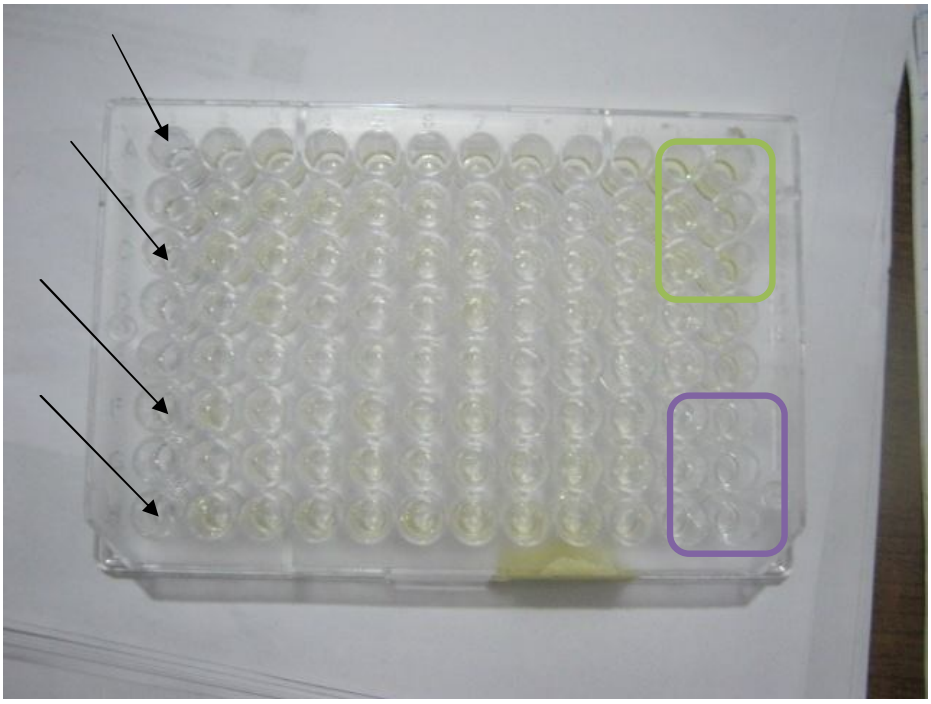
Appendix VI: Test with monospecific antisera A (As shown by the arrow).

APPENDIX VII



AppendixVII: Rose Bengal Plate Test positive agglutination (As shown by the arrow).

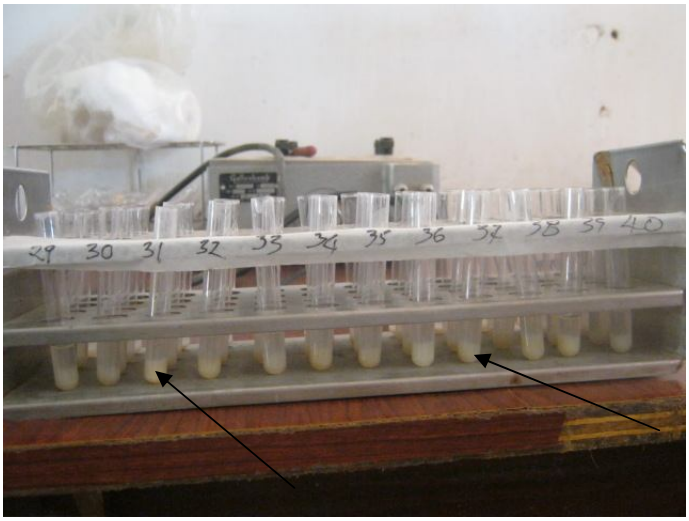
APPENDIX VIII



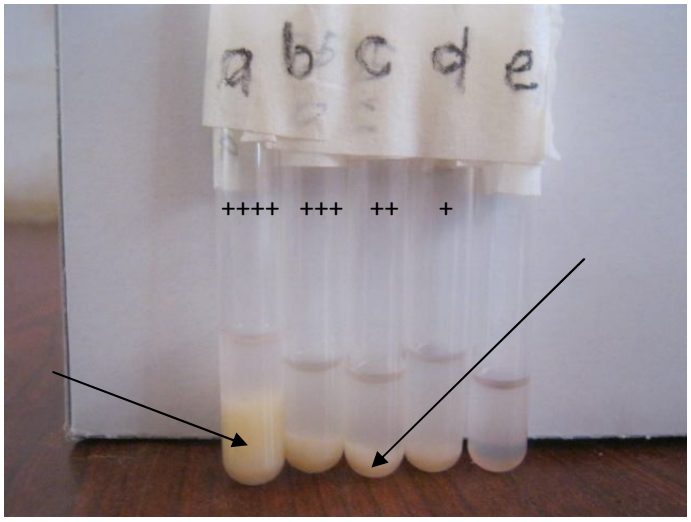
Appendix viii: Competitive Enzyme Linked Immunosorbent Asssay Kit with the test and control sera (positive sera as shown by the arrow).

Wells circled purple = Positive controls sera; Wells circled green = negative control sera.

APPENDIX IX



A



B

Appendix ix: Serum Agglutination Test A: Test samples; B: degree of clearance(As shown by the arrow).

APPENDIX X



Appendix X: 2-Mercaptoethanol tests set up. (As shown by the arrow).