BACTERIOLOGICAL AND SEROLOGICAL STUDIES OF BRUCELLOSIS IN SHEEP AND GOATS IN A RESEARCH FARM IN ZARIA, NIGERIA

BY

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DEPARTMENT OF VETERINARY MICROBIOLOGY, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

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DECLARATION

I hereby declare that the work described in this thesis was carried out by me in the Microbiology Laboratory of National Animal Production Research Institute, Zaria and Department of Veterinary Microbiology Laboratory, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervision of Professors J.O.O. Bale, H.M. Kazeem and S.B. Oladele.

The works of other researchers referred to are duly acknowledged. No part of this thesis has previously been submitted elsewhere for the award of a Degree or Diploma.

Sylvester Reuben BALA _____ _____

Signature Date

CERTIFICATION

This is to certify that this thesis titled "BACTERIOLOGICAL AND SEROLOGICAL STUDIES OF BRUCELLOSIS IN SHEEP AND GOATS IN A RESEARCH FARM IN ZARIA, NIGERIA" by BALA Sylvester Reuben meets the regulations governing the award of the Degree of Master of Science of Ahmadu Bello University, Zaria and is approved for its literary presentation and contribution to scientific knowledge.

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DEDICATION

I dedicate this research to the memories of my late mother (Mrs. Christiana Bala) and step mother (late Mrs. Sarah Bala).

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2-ME,	2-Mercaptoethanol test
c-ELISA	Competitive enzyme linked immunosorbent assay
CFT	Complement fixation test
CMI	Cell mediated immunity
CO_2	Carbon dioxide
CPE	Cytosoluble protein extract
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
EIA	Enzyme immunoassay
FAO	Food and Agriculture Organization
FPA	Fluorescence polarization assay
GD	Gel diffusion
i-ELISA	Indirect enzyme linked immunosorbent assay
IFN-γ	Interferone gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MRT	Milk ring test
NaCl	Sodium Chloride
NH	Native hapten
OIE	Office des International Epizooties
OMP	Outer membrane protein
OPS	Outer polysaccharide
PAT	Plate agglutination test
PCFIA	Particle concentration fluorescence immunoassay
PCR	Polymerase chain reaction
RBPT	Rose bengal plate test
RID	Radial immunodiffusion
SAT	Serum agglutination test
SDA	Serum dextrose agar
sLPS	Smooth lipopolysaccharide
WHO	World Health Organization

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ABSTRACT

This study was aimed at detecting the presence of *Brucella* organisms and the prevalence of *Brucella* antibodies in sheep and goats in a Research Farm in Zaria, Nigeria. A total of 580 blood samples (265 from goats and 315 from sheep) were examined for culture and serology. Blood samples with anticoagulants were cultured while serological tests such as; Rose Bengal plate test (RBPT), Serum agglutination test (SAT) and 2-Mercaptoethanol (2-ME) test were performed on sera collected. *Brucella abortus* antigens for RBPT and SAT from Veterinary Laboratories Agency, Weybridge, UK were used. There were no growth of Brucellae from blood cultures. Rose Bengal plate test detected antibodies to *Brucella* organism in 33.58% (89/265) of the goats and 33.65% (106/315) in sheep while SAT detected antibodies to *Brucella* organism in 25.66% (68/265) of goats and 6.35% (20/315) of the sheep and there was significant association between the presence of *Brucella* antibodies in goats and sheep using SAT ($P<0.05$). RBPT detected antibodies to *Brucella* in 33.62% (195/580) of all goats and sheep sampled while SAT detected antibodies in 15.17% (88/580) of all the goats and sheep sampled, there was significant association between the use of RBPT and SAT in detection of antibodies in the goats and sheep ($P<0.05$). The results of RBPT showed that females had a higher seroprevalence of 36.49% than males (22.22%) in goats and there was significant association between the presence of antibodies to *Brucella* in female and male goats as detected by RBPT ($P<0.05$) while a similar result was recorded in sheep where females had a higher seroprevalence of 34.03% than males (32.47%), though there was no significant association ($P>0.05$). The results of SAT in goats showed higher antibodies to *Brucella* organism in males (29.63%) than females (24.64%), while in sheep, males also had a higher seroprevalence of 9.09% than females (5.46%). There was no significant association between the presence of *Brucella* antibodies in

male and female goats and sheep using SAT (P>0.05). The 2-ME test revealed that 15.17% of animals had IgM. In conclusion, this study has established serological evidence of *Brucella* infection in sheep and goats in the Research Farm studied though the organism could not be isolated from blood. It is therefore recommended that attempts should be made to isolate *Brucella* from other samples like milk, vaginal secretions and semen from animals on the farm, new animals should be screened for brucellosis before introduction into the farm. Moreso, handlers of animals in the farm should be educated on the zoonotic implications of brucellosis.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Animal population was estimated at 15.2 million cattle, 23 million sheep and 28 million goats in Ngeria (FAO, 2006). The minimum daily protein requirement is 0.45 grams per kilogramme of body weight per day (WH0, 2007). About 80% of the estimated total animal population in Nigeria are still under the management of the traditional nomadic Fulani pastoralists who are accustomed to extensive system, the rest belong to Government-owned Livestock Centres and privately-owned farms (Ocholi, 2005). Several constraints to livestock productivity have been identified. These include socio-cultural problems, poor nutrition, poor herd health management, diseases, poor housing and other environmental factors directly or indirectly affecting production (Nuru, 1982). Many well-documented bacterial, viral and parasitic diseases (both directly and indirectly transmitted), occur commonly and are poorly controlled in both livestock and human populations (McDermott and Arimi, 2002). Brucellosis has been identified to be one of the most significant diseases affecting livestock leading to heavy losses in productivity and profits (Eze, 1981).

Brucellosis was first reported in Nigeria in 1927 as contained in the Annual Reports of the Veterinary Department for that year where 10 cases of contagious bovine abortion were reported (Anon, 1927). Few reports based on isolation of *Brucella* from sheep and goats have been reported (Okoh, 1980; Falade, 1981; Bale *et al.,* 2003). *Brucella* spp. are highly infectious pathogens and safety precautions must be observed during culture, serology and susceptibility testing procedures (WHO, 2004).

1.2 STATEMENT OF RESEARCH PROBLEM

Fewer studies are carried out in sheep than in goats in small ruminants' studies of brucellosis. Socio-economic and public health significance of brucellosis acquired through sheep and goats have been reported by several investigators in Nigeria for years (Falade *et al*., 1974; Falade, 1978, 1981; Okoh, 1980; Bale, 1982; Okewole *et al*., 1988; Brisibe *et al*., 1993 and Bale *et al*., 2003). The serological evidence of brucellosis in sheep and goats in Nigeria has been recognized as far back as 1934 (Eze, 1977). Falade *et al.* (1974) showed that the prevalence of brucellosis in Western Nigeria was 4.3% in goats but could be as high as 8.9% in some areas. Junaidu *et al*. (2006) reported a prevalence of 23.61% in sheep while Cadmus *et al.* (2006) reported 0.86% in goats in Ibadan. Junaidu *et al*. (2010) reported a prevalence of 22.93% in goats in Sokoto, Bertu *et al*. (2010) reported 14.5% and 16.1% prevalence rates in sheep and goats, respectively in Plateau State. These demonstrated how brucellosis had been identified as an endemic and problematic disease in Nigeria. These few studies are an indication that brucellosis in sheep and goats is wide-spread in Nigeria and can be far greater than imagined; thus the need to add to the existing literature as well as conduct more research on brucellosis in sheep in Nigeria.

1.3 JUSTIFICATION FOR THE STUDY

Small ruminants make up the bulk of the population of animals in Nigeria totalling about 51 million (FAO, 2006). This work was carried out in a Livestock Research farm in Zaria. This research farm among other things serve as a breeding center for animals and the health status of some of these animals is not known. Fertility rate has been observed to be on the decline due to abortion in some pens where the goats were kept (Personal communication, 2011). There was a report of an isolation of *Brucella*

organism from an aborted foetus from a doe (Personal communication, 2011). This study is necessary, considering the zoonotic/public health significance of brucellosis so that preventive and control measures can be instituted. It is important to obtain baseline information so as to determine the health status of these sheep and goats vis-à-vis brucellosis to ensure that animals that are bred and distributed to farmers and consumers are brucella-free and to prevent infection spread to other farm animals and humans.

1.4 RESEARCH QUESTIONS

1. Are *Brucella* organisms and their antibodies present in sheep and goats from the research farm in Zaria?
2. Are the *Brucella* antibodies found more in goats than in sheep in the research farm?
3. Does age and sex influence brucellosis in sheep and goats in the research farm in Zaria, Nigeria?

1.5 AIM AND OBJECTIVES

The aim of this research was to determine the presence of brucellosis in sheep and goats in a Livestock Research farm in Zaria, Nigeria.

The objectives of the research include:

1. To isolate *Brucella* organism from blood of sheep and goats in a Livestock Research farm in Zaria, Nigeria.
2. To detect *Brucella* antibodies in sheep and goats in a Livestock Research farm in Zaria, Nigeria.

CHAPTER 2

LITERATURE REVIEW

2.1 BRIEF HISTORY OF BRUCELLOSIS

Brucellosis is an infectious disease caused by the bacteria of the Genus *Brucella*. These bacteria are primarily passed among animals, and they cause disease in many different vertebrates. Various *Brucella* species affect sheep, goats, cattle, deer, elk, pigs, dogs, and several other animals (CDC, 2010).

Brucellosis is an ancient disease that can possibly be traced back to the 5th plague of Egypt around 1600 BC. Recent examination of the ancient Egyptian bones, dating to around 750 BC, showed evidence of sacroiliitis and other osteoarticular lesions, common complications of brucellosis (Pappas and Papadimitriou, 2007). David Bruce isolated *Brucella melitensis* (*Micrococcus melitensis* at that time) in 1887 from the spleen of a British soldier who died from a febrile illness (Malta fever) common among military personnel stationed on Malta. For almost 20 years after isolation of *Micrococcus melitensis*, Malta fever remained a mystery and was thought to be a vector-borne disease until Themistocles Zammit accidentally demonstrated the zoonotic nature of the disease in 1905 by isolating *B. melitensis* from goat's milk. It was believed that goats were not the source of infection since they did not become ill when inoculated with *Brucella* cultures. The discovery that healthy goats could be carriers of the disease has been termed one of the greatest advances ever made in the study of epidemiology (Wyatt, 2005; Sriranganathan *et al.,* 2009). In 1897, a Danish veterinarian, L.F. Benhard Bang, discovered Bang's bacillus or bacillus of cattle abortion (*B. abortus*) to be the causative agent of Bang's disease. Alice Evans, an American scientist who did landmark work on pathogenic bacteria in dairy products, confirmed the relationship between Bang's disease and Malta fever and renamed the genus *Brucella* to honor

David Bruce. Her work on *Brucella* was central in gaining acceptance of the pasteurization process to prevent human brucellosis in United States of America. The discovery of the *Brucella* in marine mammals in early 1990's has changed the concept of a land-based distribution of brucellosis and associated control measures (Sriranganathan *et al.,* 2009).

2.2 AETIOLOGY OF BRUCELLOSIS

Brucella spp. are facultative, intracellular, Gram-negative, cocco-bacilli, non-spore-forming and non-capsulated organisms. Although *Brucella* spp. are described as non-motile, they carry all the genes except the chemotactic system, necessary to assemble a functional flagellum (Fretin *et al*., 2005). They belong to the alpha-2 subdivision of the Proteobacteria, along with *Ochrobactrum*, *Rhizobium*, *Rhodobacter*, *Agrobacterium*, *Bartonella*, and *Rickettsia* (Yanagi and Yamasato, 1993).

The genus currently consists of ten (10) species, eight of them affect terrestrial animals and include: *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. microti* and *B. inopinata* (Verger *et al*., 1987; Scholz *et al*., 2008; Scholz *et al.,* 2009). The first three species, i.e., *B. abortus*, *B. melitensis* and *B. suis* are called classical *Brucellae* and within these species, seven biovars are recognized for *B. abortus*, three for *B. melitensis* and five for *B. suis*. *B. microti* was isolated from the Common Vole (*Microtus arvalis*) (Scholz *et al.,* 2008) while *B. inopinata* was isolated from a human breast implant (De *et al.,* 2008; Scholz *et al.,* 2009). The other two were isolated from marine animals and include: *B. ceti* from cetaceans (whales, porpoises and dolphins) and *B. pinnipedialis* from pinnipeds (seals, sea lions and walruses) (CFSPH, 2007; Foster *et al*., 2007).

Five out of the ten known *Brucella* species can infect humans and the most pathogenic and invasive species for human is *B. melitensis*, followed in descending order by *B.suis*,

B. abortus and *B. canis* (Acha and Szyfres, 2003). The zoonotic nature of the marine brucellae (*B. ceti*) has been documented (Brew *et al*., 1999; Sohn *et al*., 2003; McDonald *et al*., 2006). The strains of brucellae were named based on the host animal preferentially infected (Verger *et al.*, 1987).

2.3 MODES OF TRANSMISSION

2. 3.1 Routes of Excretion and Contagious Material

Generally, transmission occurs in the same way in sheep and goats as in cattle, materials excreted from the female genital tract forming the main supply of organisms for transmission to other animals and man. Therefore, in most circumstances, the primary route of dissemination of *Brucella* is the placenta, foetal fluids and vaginal discharges expelled by infected ewes after abortion or full-term parturition (Anon, 2001). Very large numbers of organisms are shed at the time of parturition or abortion. In goats, excretion of the organisms from the vagina is prolonged and copious (2 to 3 months generally). In sheep excretion is generally less prolonged, usually ceasing within 3 weeks after abortion or a full-term parturition. Shedding of *Brucella* is also common in udder secretions and semen, and *Brucella* may be isolated from various tissues, such as lymph nodes from the head and those associated with reproduction, and sometimes from arthritic lesions (Alton *et al.,* 1988).

The persistent infection of the mammary glands and supramammary lymph nodes leads to a constant or intermittent shedding of the organisms in the milk in succeeding lactations. It provides an important source of infection for man and young animals (Anon, 2001).

2.3.2 Modes of Infection

The modes of infection are direct or indirect. Animals become infected directly by infected aerosols or by uptake of infected material. Another mode of infection is grazing pastures where infected animals mix with brucellosis-free animals or get in touch with contaminated premises, manure, farm tools and materials

In the male, localisation in the reproductive organs generally results in the shedding of *Brucella* in the semen. However, when used for natural mating, the risk seems low that infected males transmit the disease to susceptible females. It should also be noted that experimental work with cattle has demonstrated that embryo transfer is unlikely to be a method of transmission, while being a useful method of preserving valuable genetic lines. Provided that recommended embryo washing and other hygienic measures are taken, embryos can be moved from infected donors to healthy recipients with minimal risk (Anon, 2001).

Dogs have been shown to be mechanical and biological vectors of brucellosis (Joint FAO/WHO Expert Committee on Brucellosis, 1986). Spreading via waterways is rare, and can be effective over short distances only. Persistent infection of mammary glands is associated with constant or intermittent shedding of the organisms in the milk in succeeding lactations (Philippon *et al.,* 1971). The number of *Brucella* excreted in milk is relatively low but is sufficient to allow transmission to lambs and kids, and indirectly through the milker's hands (Anon, 2001).

2.3.3 Vertical Transmission

Similar to *Brucella abortus* infection in cattle, *B. melitensis* can be transmitted from the dams to lambs or kids. A small proportion of lambs or kids can be infected *in utero*, but the majority of infections are probably acquired by consumption of colostrum or milk

(Grilló *et al.,* 1997). These lambs or kids may have infections in the lymph nodes draining the gastro-intestinal tract and may shed *Brucella* organisms in the faeces. It is also probable that a self-cure mechanism similar to that suggested in cattle takes effect in most of the infected lambs (Grilló *et al.,* 1997). In that case, lambs and kids remain fully susceptible when they reach sexual maturity. Despite the low frequency of transmission, the existence of latent infections greatly increases the difficulty of eradicating this disease, as *Brucella melitensis* persists without having a detectable immune response because of immunotolerance. The exact mechanism of the development of latent *B. melitensis* infections remains unknown (Grilló *et al.,* 1997).

In goats, the infection may vary in duration from very short periods of slight infection which is rapidly eliminated (especially in vaccinated animals) to persistence for years, where excretion of the organism in the milk may continue for two or more lactations (Anon, 2001). The situation in sheep is very different, though this varies with the susceptibility of the breed (Alton, 1990). After recovery from infection with *B. melitensis*, sheep are very resistant to reinfection (Alton, 1990). Werschilova and Striedter (1938) demonstrated a strong resistance to reinfection, even during pregnancy, up to 8 to 9 months, after which it began to decline, although some resistance was still demonstrated after 2 years. Durán-Ferrer (1998) reported a long-lasting immunity after an experimental infection.

2.4 DESCRIPTION OF THE GENUS BRUCELLA

2.4.1 Nutritional Requirements

The first successful cultivation of *Brucella* on chemically defined media was by Zobell and Meyer (1932). Attempts for the definition of the specific nutritional requirements of *Brucella* led to the development and modification of media over the years with addition

of meat infusion, vitamins, and/or antibiotics. *Brucella* requires biotin, thiamine and nicotinamide. The growth is improved by serum or blood (Anon, 2001). Minerals are supplied by the addition of organic or inorganic salts of the minerals e.g., NaCl, to the medium to make it physiological. Nitrogen sources are usually from amino acids. Glucose is included as carbon and energy source at concentration of 0.1-0.4%. Erythritol, L-glutamate and asparagines are other sources of carbon and energy (McCullough and Dick, 1942). Living animal cells and body fluids are very efficient media for brucellae, hence embryonated chicken eggs, mammalian leukocytes and tissue culture proved especially useful in the *in-vivo* and *in-vitro* growth of the organism (Gay and Damons, 1955).

2.4.2 Gaseous Requirements

Brucellae members are aerobic, but some strains require an atmosphere containing 5-10% carbon dioxide (CO_2) added for growth, especially on primary isolation. Many strains of *B. abortus* and *B. ovis* require an atmosphere, containing 5-10% CO_2 (Alton *et al.,* 1988). On repeated culture, isolates of *B. abortus* frequently lose the requirement of added CO_2 for growth and may grow in air alone (Eze, 1981).

2.4.3 pH Requirements

The optimum hydrogen ion concentration (pH) for growth of brucellae varies from 6.6-7.4 and culture media should be adequately buffered near pH 6.8 for optimum growth.

2.4.4 Temperature Requirements

Brucella spp like most other bacteria are incubated at 37^0C, although the optimum temperature is 36-38^0C and most strains can grow between 20^0C and 40^0C (Anon, 2001).

2.4.5 Colonial Morphology

On suitable media, *Brucella* colonies are visible after 2 days incubation in air or under 5-10% CO_2. *Brucella* isolates grow on Serum Dextrose Agar (SDA) to produce circular, convex colonies, 1-3 mm in diameter with a smooth glistening surface. The colonies are transparent, honey colour in transmitted light and have a bluish translucent appearance in reflected light. Rough *Brucella* isolates produce colonies of a similar size and shape but of a more opaque off-white colour and often with a rather granular surface. Mucoid colonies are similar in size and shape to rough colonies but have an obviously slimy appearance and may be darker in colour (Corbel *et al.,* 1987). On blood agar, growth is slower than on SDA, with the production of non-haemolytic greyish-white glistening colonies 0.5-1 mm in diameter after 48-72 hours incubation. Little or no growth is produced by *Brucella* strains on MacConkey agar even after 5 days at 37^0C (Corbel *et al.,* 1979).

2.4.6 Staining Reactions

Brucellae are Gram negative coccobacilli or short rods 0.5-1.5µm in length and 0.5-0.7µm in breadth. They occur singly and less frequently in pairs or small groups. Brucellae are non-motile, non-sporing with no flagella, pili or capsules and do not show bipolar staining. They are not truly acid-fast but resist discoloration by weak acids, thus staining red with bluish background by Stamp's modification of Ziehl-Neelsen method (Alton *et al.,* 1988; Anon, 2001).

2.5 EPIDEMIOLOGY OF BRUCELLOSIS

2.5.1 Geographic Distribution

The distribution of brucellosis is worldwide; yet it is more common in countries with poorly standardized animal and public health programme (Capasso, 2002). The routes of infection are multiple i.e., food-borne, occupational or recreational, linked to travel and even to bioterrorism. New *Brucella* strains or species may emerge and existing *Brucella* species adapt to changing social, cultural, travel and agricultural environment (Godfroid *et al.,* 2005). Infection in sheep appears to occur endemically in the Mediterranean region, especially along its northern and eastern shores, stretching through Central Asia as far south as the Arabian Peninsula and as far east as Mongolia. Parts of Latin America are also seriously affected, especially Mexico, Peru and northern Argentina. The disease also occurs in Africa and India. However, North America (except Mexico) is believed to be free, as are Northern Europe (except for sporadic incursions from the south), Southeast Asia, Australia and New Zealand (FAO/OIE/WHO, 1997).

The incidence of reactors in newly established cattle farms may be more than 30%, however, the highest rate (72.9%) of infection has been reported in the Palestinian Authority (Shuaibi, 1999). It is interesting to note that the second highest prevalence (71.42%) of brucellosis has been reported in mules from Egypt (Anonymous, 2011a). Invariably, all domestic animals suffer from this disease. Brucellosis in buffaloes has been reported from Egypt (10.0%) and Pakistan (5.05%). Since cattle are found throughout the world, prevalence of brucellosis (0.85 to 23.3%) in cattle has been reported from a wide range of countries.

In camels, brucellosis has been reported from Arabian and African countries (0.0-17.20%), where the disease also occurs in buffaloes, equines and swine. Variable

prevalence of this disease has been reported in sheep and goats. Bio-varieties of *Brucella* vary with respect to geographic region. *B. melitensis* biovar 1 from Libya, Oman and Israel and *B. melitensis* biovar 2 from Turkey and Saudi Arabia have been isolated. *B. melitensis* biovar 3 is the most commonly isolated species from animals in Egypt, Jordan, Israel, Tunisia and Turkey (Refai, 2002). *B. abortus* biovar 1 in Egypt, biovar 2 in Iran, biovar 3 in Iran and Turkey and biovar 6 in Sudan have been reported (Halling and Boyle, 2002). The countries with the highest incidence of human brucellosis include, Saudi Arabia, Iran, Palestinian Authority, Syria, Jordan and Oman. Bahrain is reported to have no incidence (Refai, 2002). The per cent prevalence of ovine brucellosis has been reported to decrease in Ireland and Italy during the year 1999-2000 but there had been a trend towards a significant increase in Azores (Jacques and Kasbohrer, 2002).

2.5.2 Seasonal prevalence

In general, brucellosis can be found in any season of the year. The epidemic peak occurs from February to July and is closely related to the months associated with parturition and abortion in animals (Shang *et al.,* 2002). In humans, prevalence of the disease is high (39.5%) in summer season (Salari *et al.,* 2003). Notifications of human brucellosis, which are mandatory in Italy, reach a peak between April and June. However, considering the standard incubation period of 2-4 weeks, and the fact that lamb slaughter is traditionally at a peak during the Easter period, it might be expected that occupational exposure would result in a peak of human cases between March and May. The observed peak between April and June could be related to the production and consumption of fresh cheese, starting just after lamb slaughter (De-Massis *et al.,* 2005).

2.5.3 Sex and Age Prevalence

There are controversial reports regarding the prevalence of brucellosis in relation to sex of animals, as some of the research workers reported significantly higher prevalence in females than in males (Hussein *et al.,* 2005), whereas MacMillan *et al*. (1982) were of the view that *B. abortus* causes intermittent bactaeremia in the mares but not in the stallions. The relatively higher incidence reported among human females than males might be due to more involvement of females in handling of livestock. These females may be highly exposed to the risk of infection through direct contact with animals, consumption of raw milk and milk products. Moreover, risky practices in rural areas, such as skinning of stillborn lambs and kids, as well as crushing the umbilical cord of newborn lambs and kids with teeth can also be contributing factors (Hussein *et al.,* 2005). However, some reports indicated that *Brucella* antibody titers are not associated with sex (Muma *et al.,* 2006).

The antibody titer against *B. abortus* appears to be associated with age, as low prevalence in young stock has been reported than the adults (Ahmed and Munir, 1995). Kazi *et al.* (2005) reported higher prevalence of infection in animals more than 4 years of age compared to younger animals. It appears that the high prevalence of brucellosis among older cows might be related to maturity with the advancing age. Thereby, the organism may have propagated to remain either as latent infection or it may cause clinical manifestation of the disease (Kazi *et al.,* 2005).

Brucellosis is essentially a disease of the sexually mature animals, the predilection site being the reproductive tract, especially the gravid uterus. Allantoic factors including, erythritol, possibly steroid hormones and other substances stimulate the growth of most of the *Brucellae* (Radolf, 1994). The tropism of *Brucella* to the male or female reproductive tract was thought to be by erythritol, which stimulates the growth of the

organism, but *Brucella* has also been found in the reproductive tract of animals with no detectable level of erythritol (Anon, 2011b). Erythritol, a sugar alcohol synthesized in the ungulate placenta and stimulates the growth of virulent strains of *B. abortus*, has been credited with the preferential localization of this bacterium within the placenta of ruminants (Smith *et al.*, 1962).

2.5.4 Test-Based Prevalence

The main serological test used for diagnosis of brucellosis is the Rose Bengal Plate Test (RBPT), which has very high (>99%) sensitivity but low specificity (Barroso *et al.,* 2002). As a result, the positive predictive value of this test is low and a positive result is required to be confirmed by some other more specific test like serum agglutination test (SAT) and ELISA. However, the negative predictive value of RBPT is high as it excludes active brucellosis with a high degree of certainty.

Prevalence of brucellosis on the basis of SAT and RBPT in various species of animals and humans varies very widely. Equine showed a wide variation of brucellosis occurrence (0.24-37.50%), followed by bovine (0.58-35.90%), caprine (0.40-33.3%), ovine (0.28-16.70%) and camelidae (1.8-7.48%) while humans had the least prevalence (0.89-4.10%) (Gul and Khan, 2007).

Brucella has also been isolated from a variety of wildlife species, such as bison, elk, African buffalo, reindeer, caribou, feral swine, wild boars, foxes and hares (Davis, 1990). Anti-*Brucella* spp. antibodies were detected by tube agglutination test, ELISA and immunoblotting in 53% serum samples of Pacific bottlenose dolphins (*Tursipa aduncus*) from the Solomon Islands (Tachibana *et al*., 2006). Ribeiro *et al.* (2003) tested fistulus withers secretions from three horses by the plate agglutination test (PAT), SAT, buffered RBPT and 2-mercaptoethanol test (2-ME), and compared the results with

standard agglutination test. Titers were higher in the PAT, SAT and 2-ME and positive reaction was observed in RBPT. *B. abortus* was isolated from the secretion of fistulous withers, collected from one animal. These results suggest that the modified tests may be used as alternative test to diagnose brucellosis in horses with fistulous withers.

Bale and Kumi-Diaka (1981) reported the first case of *Brucella abortus* biotypes in Nigeria. They isolated 11 (15.5%) *Brucella abortus* from 71 samples, comprising of cattle semen, abomasal contents, testicular exudates and foetal heart blood. Eight (8) strains of the isolates belonged to biotypes 1, 3 and 4. Five (5) strains were biotype 1 (three from semen, one from abomasal contents and one from, testicular exudates) while two (2) strains were biotype 3 (one from abomasal content and one from foetal blood) and *Brucella abortus* biotype 4 was isolated from another semen sample.

Ocholi *et al.* (2004a) isolated *Brucella* from aborted fetuses, hygroma fluid, milk and vaginal swabs obtained from aborting cattle, sheep, goats, pigs and horses. A total of 25 isolates, obtained mainly from cattle, sheep and horses, were biotyped. All strains belonged to one species, *B. abortus* biovar 1. Ocholi *et al.* (2004b) isolated *B. abortus* from a horse which had carpal bursitis. In a subsequent study, Ocholi *et al.* (2005) examined serum and milk samples from ewes for *Brucella,* a total of seven isolates of *Brucella* were obtained from milk samples and vaginal swabs collected from aborting ewes. All isolates were identified and bio-typed as *B. abortus* biovar 1.

2.6 BRUCELLOSIS IN MAN

Brucellosis is one of the most important zoonotic diseases worldwide, particularly in developing countries and Central Asia (Godfroid and Kasbohrer, 2002; Pappas *et al.,* 2006). In some of the endemic areas such as Africa, Middle East and Latin America, brucellosis results in significant human morbidity (Boschiroli *et al.,* 2001). The

incubation period of brucellosis normally is 1–3 weeks, but it can be several months before showing signs of infection. *B. melitensis* is associated with acute infection whereas the infections with other species are usually subacute and prolonged (Mantur *et al*., 2007). Most common symptoms of brucellosis include undulant fever in which the temperature can vary from 37^0C in the morning to 40^0C in the afternoon; night sweats with peculiar odor, chills and weakness. Common symptoms also include; malaise, insomnia, anorexia, headache, arthralgia, constipation, sexual impotence, nervousness and depression (Acha and Szyfres, 2003). Human brucellosis is also known for complications and involvement of internal organs and its symptoms can be very diverse depending on the site of infection and include encephalitis, meningitis, spondylitis, arthritis, endocarditis, orchitis, and prostatitis (Acha and Szyfres, 2003). Spontaneous abortions, mostly in the first and second trimesters of pregnancy, are seen in pregnant women infected with *Brucella* (Khan *et al*., 2001). Although a rare complication, Brucella endocarditis (<2% of cases) is most commonly associated with *B. melitensis* infection and is the most severe complication. It accounts for at least 80% of deaths due to brucellosis (Peery and Belter, 1960; Reguera *et al*., 2003). Symptoms and signs of brucellosis usually referred to as fever or pyrexia of unknown origin (PUO) can be confused with other diseases including enteric fever, malaria, rheumatic fever, tuberculosis, cholecystitis, thrombophlebitis, fungal infection, autoimmune disease and tumors (Mantur *et al*., 2007). Live animal vaccines *B. melitensis* Rev. 1 and *B. abortus* strain 19 are known to cause disease in humans if one accidentally injected self. The course of the disease with vaccine strains is usually shorter and more benign (Acha and Szyfres, 2003). Direct person-to-person spread of brucellosis is extremely rare. Mothers who are breast-feeding may transmit the infection to their infants and sexual transmission has also been reported (Arroyo Carrera *et al*., 2006; Kato *et al*., 2007).

2.8 BRUCELLOSIS IN ANIMALS

2.8.1 Brucellosis in cattle

In livestock species (cattle, sheep, goats, swine, and camel), the most frequent clinical sign following infection with *Brucella* is abortion (Acha and Szyfres, 2003). The principal strain that infects cattle is *B. abortus*, cattle can also become transiently infected by *B. suis* and more commonly by *B. melitensis* when they share pasture or facilities with infected pigs, goats and sheep. *B. melitensis* and *B. suis* can be transmitted by cow's milk and cause a serious public health threat (Ewalt *et al*., 1997; Kahler, 2000; Acha and Szyfres, 2003). The main symptom in pregnant females is abortion (premature or full-term birth of dead or weak calves) usually in the second half of gestation with retention of placenta and metritis (Acha and Szyfres, 2003). There is an estimated 25% reduction in milk production in infected cows (Acha and Szyfres, 2003). The brucellae localize in the supra-mammary lymph nodes and mammary glands of 80% of the infected animals and thus continue to secrete the pathogen in milk throughout their lives (Hamdy and Amin, 2002). Most infected cows abort only once although the placenta will be heavily infected at subsequent apparently normal calvings (Morgan, 1969).

2.8.2 Brucellosis in sheep and goats

The main etiologic agent of brucellosis in goats is *B. melitensis*. In Brazil, where there is no *B. melitensis*, goats can get infected with *B. abortus* (Lilenbaum *et al*., 2007). As in cattle, brucellosis in goats is characterized by late abortion, stillbirths, decreased fertility and low milk production (Lilenbaum *et al*., 2007). Sheep brucellosis can be divided into classical brucellosis and ram epididymitis. Ram epididymitis is caused by non-zoonotic agent *B. ovis*, while classical brucellosis is caused by *B. melitensis* and

constitutes a major public health threat equal to goat brucellosis (Acha and Szyfres, 2003). Besides the abortion, swine may also develop orchitis, lameness, hind limb paralysis, or spondylitis; occasionally, metritis or abscesses (Glynn and Lynn, 2008).

2.8.3 Brucellosis in camels

Camels can be infected by *B. abortus* and *B. melitensis* when they are pastured together with infected sheep, goats and cattle. Milk from infected camels represents a major source of infection that is underestimated in the Middle East and Nigeria (Musa *et al.*, 2008).

2.8.4 Brucellosis in dogs

The main etiologic agent for dog brucellosis is *B. canis*, but sporadic cases of brucellosis in dogs caused by *B. abortus*, *B. suis* and *B. melitensis* have been reported (Acha and Szyfres, 2003). Dogs infected with *B. canis* may have reproductive related conditions (abortions during the last third of a pregnancy, stillbirths, or conception failures) and/or non-reproductive tract related conditions (including ocular, musculoskeletal, or dermatologic lesions) (Acha and Szyfres, 2003; Wanke, 2004). Humans are susceptible to *B. canis*, although less than classic brucellae, several cases have been reported in the U.S.A, Mexico, Brazil, and Argentina (Acha and Szyfres, 2003).

2.9 SUSCEPTIBILITY IN SHEEP AND GOATS

2.9.1 Age

Brucella melitensis infection causes disease only in adult (sexually mature) females and males. Young animals may be infected but do not show any clinical sign and generally

show only a weak and transient serological response. However, susceptibility increases after sexual maturity and especially with pregnancy (Anon, 2001).

2.9.2 Species and breed

The goat was originally considered the principal host of *Brucella melitensis*, notably in Latin America, where sheep are not significantly infected even when kept in close contact with goats. In many other areas the disease is more important in sheep. There are several reasons for this difference. First, whereas most breeds of goat are fully susceptible to infection, a great variation in the susceptibility of different breeds of sheep has been reported. Thus, sheep-milking breeds appear more susceptible than those kept for meat production (Corbel and Brinley-Morgan, 1984). Maltese and South American sheep breeds appear very resistant, whereas the fat-tailed sheep of Southwest Asia and Mediterranean breeds are very susceptible and form a reservoir of infection that gives rise to widespread infections of humans. Therefore, in most countries bordering the Mediterranean Sea and in Southwest Asia, the brucellosis problem largely centres on sheep, while in Latin America goats are chiefly involved. Second, in the Mediterranean sheep are the predominant species, being often kept in large flocks, in conditions that favour the spread of infection. Species behaviour is also considered as a favouring factor. Ewes generally gather together at lambing or at night, while goats do not (Anon, 2001).

Excretion from the vagina in goats is more copious and prolonged than in cows and lasts for at least 2-3 months. In this animal, about two thirds of acute infections acquired naturally during pregnancy lead to infection of the udder and excretion of the organisms in the milk during the next lactation. Excretion may cease during lactation. Infection in

goats results in a greater reduction in milk production than is the case in cattle (Alton, 1985).

2.9.3 Management practice

The system of husbandry as well as the environmental conditions greatly influences the spread of infection. Thus lambing/kidding in dark, crowded enclosures is more favourable to spread than lambing/kidding in the open air in a dry environment. The spread of infection between flocks generally follows the movement or gathering of infected animals. The main risk for introducing the disease into a previously non-infected area is by purchase of infected animals. In several countries there is a strong correlation between the prevalence of brucellosis in small ruminants and the practice of transhumance. Intermingling of flocks may occur under nomadic or semi-nomadic conditions of husbandry and also in static village flocks where animals are taken daily for grazing on common pastures (Anon, 2001).

2.10 PATHOGENESIS

The establishment of infection is influenced by the size of infective dose, virulence of the bacteria and the resistance, age, sex and reproductive status of the animal. *B. abortus* and *B. melitensis* penetrate mucous membranes of the pharynx and alimentary tract and survive and multiply particularly in cells of the mononuclear phagocytic system (Herr, 1994; Godfroid *et al*., 2004b). After penetration, the organisms are phagocytosed by neutrophils and macrophages which carry them to the regional lymph nodes where they multiply and induce lymphadenitis which may persist for months. Multiplication of organisms in lymph nodes may be followed by bacteraemia which may persist for several months, resolve itself or be recurrent for at least two years in 5

to 10 per cent of animals. Recurrence occurs particularly during pregnancy (Radostits *et al*., 1994a; Godfroid *et al*., 2004a). During the bacteraemic phase, organisms are carried intracellularly in neutrophils and macrophages or free in the plasma and localize in various organs, especially the pregnant uterus, udder and supramammary lymph nodes and the spleen, and in males in the testes and male accessory sex glands (Godfroid *et al*., 2004a). Occasionally, bacterial localization occurs in synovial structures, causing a purulent tendovaginitis, arthritis or bursitis.

Localization of the infection in the endometrium of the pregnant uterus and in foetal membranes appears to be the result of the special affinity of the organism for erythritol present in the placenta and male genital tract of cattle, sheep and goats (Sangari *et al*., 2000; Godfroid *et al*., 2004a; 2004b). When invasion of the gravid uterus occurs, the initial lesion is in the wall of the uterus but the organism quickly spreads to the lumen, leading to a severe ulcerative endometritis of the intercotyledonary spaces. The allantochorion, foetal fluids and placental cotyledons are invaded next and the villi destroyed (Radostits *et al*., 1994). Depending on the severity of the placentitis, abortion especially in the last trimester, premature birth or the birth of a viable or non-viable offspring may occur. The presence of erythritol in the pregnant uterus results in massive multiplication of *Brucella* organisms in this organ although its role as a virulence factor remains questionable. The growth of most *B. abortus* strain 19 organisms is generally inhibited by the presence of erythritol, but tolerance to erythritol by some strain 19 variants may be the cause of occasional persistent infections and abortions (Sangari *et al*., 2000; Godfroid *et al*., 2004a; 2004b).

Animals generally abort once, during the mid-third of gestation, but re-invasion of the uterus occurs in subsequent pregnancies with shedding in fluids and membranes. The pregnancy can also continue to full-term. The proportion of newly infected females that

abort varies with the circumstances. The percentage of infected females lambing/kidding in a flock may reach 40%. Females that are born into an infected environment and subsequently infected, generally abort less than others. This could explain the high level of abortions in newly infected flocks and their relatively low frequency in flocks where infection is enzootic (Grillo, 1997).

The udder is a very important predilection site for *Brucella melitensis*. Infection in lactating non-pregnant goats is likely to lead to colonisation of the udder with excretion of *Brucella melitensis* in the milk (Renoux *et al.,* 1953). In goats, about two thirds of acute infections acquired naturally during pregnancy lead to infection of the udder and excretion of the bacteria in the milk during the subsequent lactation (Alton, 1985). In some goats excretion may cease during this lactation, but in many it persists and often continues during the next (Alton, 1962). Greatly reduced milk yield follows abortion, and infection of the udder following a normal birth also leads to a considerable reduction in yield. In spite of this, clinical signs of mastitis are seldom detectable in naturally infected goats (Alton, 1990).

Sheep that abort often excrete the bacteria in the milk, but generally for not more than two months (Alton, 1990). However, exceptionally, excretion may continue for 140 days (Itabashi *et al.,* 1938) and even 180 days (Biggi, 1956).

2.11 IMMUNE RESPONSE

Infection with *Brucella melitensis* usually results in the induction of both humoral and cell-mediated immune responses, but the magnitude and duration of these responses is affected by various factors including the virulence of the infecting strain, the size of infecting inoculum, pregnancy, sexual and immune status of the host (Joint FAO/WHO, 1986).

2.11.1 Humoral immunity

Following infection by natural exposure, a serological response can be expected within 2 to 4 weeks, but the response is variable and may be absent altogether. Invasion of the pregnant uterus can be expected to produce a large and persistent rise of antibodies, but this may be delayed until after abortion or parturition at the normal time. Invasion of the lactating udder causes a lesser serological response, and localisation confined to a small number of lymph nodes may fail to stimulate any response at all, or only a minimal one. The pattern of the serological response in terms of immunoglobulin production has not been extensively studied in sheep and goats, but available information suggests close similarity to that in cattle, i.e. production of IgM followed within a week or two by a predominance of IgG, with both isotypes falling to a low level in the more chronic stage of infection but with IgG predominating.

The serological response is transient and sometimes missing in young sexually immature animals. The *Brucella melitensis* Rev.1 vaccine strain when applied under standard conditions (full dose via the subcutaneous route in young replacement animals) may induce a long lasting serological response to the agglutination test that seriously interferes with serological screening for infected animals (Alton and Elberg, 1967; Elberg, 1981, 1996; Alton, 1990; MacMillan, 1990). No differences have been found between the diagnostic antigens from field strains of *Brucella melitensis* and those from the Rev.1 vaccine (Anon, 2001).

2.11.2 Cell mediated immunity (CMI)

After gaining entrance into the body, the organisms encounter the cellular defences of the host and the fate of invading bacteria is mainly determined by the cellular defences of the host, in particular macrophages and T lymphocytes. The most widely used

correlates of CMI are lymphocyte stimulation, macrophage inhibition, delayed-type hypersensitivity and γ–interferon induction. Like the humoral response and depending on the method used to measure the CMI, the response can be expected as rapidly as a few weeks, but the response is variable and may not be detected (Anon, 2001).

2.13 PATHOLOGY

Irrespective of the role of infection, the organism provokes a regional lymphadenitis which is characterized by reticuloendothelial cell and lymphoid hyperplasia as well as infiltration of large numbers of mononuclear cells and some neutrophils, few eosinophils and plasma cells. Other lymph nodes in the body and the spleen may be affected later in the course of the infection but to a lesser degree (Bishop *et al.*, 1994).

There is considerable variation in the severity of the uterine lesions at abortion. As the disease progresses, lesions advance from acute to chronic endometritis. Microscopically, the endometrium is infiltrated by lymphocytes and plasma cells and some neutrophils. Microgranulomas may be scattered in the endometrium. The chorion is not uniformly affected and large parts may appear quite normal. The lesions in and at the periphery of the cotyledons as well as those in the intercotyledonary area vary in extent, appearing to be most severe adjacent to cotyledons. The affected cotyledons or parts of them are covered by sticky, odourless, brownish exudates and are yellowish-grey as a result of necrosis. Parts of the intercotyledonary placenta are thickened, oedematous and yellowish-grey and may contain exudates on the surface. The udder in infected ruminant does not show any gross lesions, although supramammary lymph nodes may be somewhat enlarged (Godfroid *et al.*, 2004 a;b). Some aborted foetuses have varying degrees of subcutaneous oedema and blood-tinged fluid in the thoracic and abdominal cavities, while the abdominal content is sometimes turbid, bright yellow

and flaky (Nielsen and Duncan, 1990). Microscopically, most aborted foetuses reveal a multifocal bronchopneumonia, small foci of necrosis or microgranulomas in the liver, which may also occur in the lymph nodes, spleen and kidneys. In most aborted foetuses, it is not possible, or it is very difficult to demonstrate organisms in tissue sections, notwithstanding that they may have been specifically stained for brucellae (Godfroid *et al*., 2004a).

In bulls, acute to chronic, uni- or bilateral orchitis, epididymitis and seminal vesiculitis occasionally occur. Orchitis is characterized by multifocal or diffuse necrosis of the testicular parenchyma and a focal necrotizing epididymitis may occur. The scrotal circumference in these animals may be normal or severely increased (Bishop *et al*., 1994).

2.13.1 Acute Brucellosis

Although a variety of clinical signs has been described in artificially infected animals, the main clinical manifestations of brucellosis in sheep and goats are, as in all female ruminants, reproductive failure, i.e. abortion and birth of weak offspring. Abortion generally occurs during the last 2 months of pregnancy and is followed in some cases by retention of foetal membranes. In the male, localisation in the testis, epididymis and accessory sex organs is common, and bacteria may be shed in the semen. This may result in acute orchitis and epididymitis and later in infertility. Arthritis is also observed occasionally in both sexes. There is no evidence that the clinical features of *Brucella melitensis* infection in sheep and goats vary according to the biovar involved (Fensterbank, 1987).

2.13.2 Chronic Brucellosis

Animals generally abort once, although reinvasion of the uterus occurs in subsequent pregnancies and *Brucella* organisms are shed with the membranes and fluids. Non-pregnant animals exposed to small numbers of organisms may develop self-limiting, immunising infections or they may become latent carriers. Persistent infection of the mammary glands and supramammary lymph nodes is common in goats with constant or intermittent shedding of the organisms in the milk in succeeding lactations, while the self-limiting nature of the disease in sheep, which is seldom accompanied by prolonged excretion of the bacteria, has been observed (Alton, 1990, Durán-Ferrer, 1998). The inflammatory changes in the infected mammary gland reduce milk production by an estimated minimum of 10% (Anon, 2001). Orchitis and epididymitis generally lead to a chronic infection (Anon, 2001).

2.14 DIAGNOSIS

2.14.1 Clinical Signs

Several factors play a role in the epidemiology of brucellosis, therefore, it may take sometime before clinical symptoms occur. Though the existence of the disease may be suspected from herd history indicating occurrence of abortion at the 3rd trimester of pregnanacy, infertility, orchitis, epididymitis and hygroma; there are no pathognomic signs of brucellosis (Bercovich, 1998).

2.14.2 Direct Diagnosis: Agent Detection

2.14.2.1 *Isolation of bacteria*

The only unequivocal method for the diagnosis of brucellosis in small ruminants is based on the isolation of *Brucella* organisms (Alton *et al.,* 1988). Microscopic demonstration of characteristic clumps of brucella organisms in stained smears of foetal

membranes, vaginal swabs, milk and semen by modified Köster staining, modified Ziehl-Neelsen staining and Fluorescent Antibody Techniques (FAT) may provide the tentative diagnosis (Alton *et al.,* 1988; Bercovich, 1998). Accordingly, the isolation of *B. melitensis* on appropriate culture media is recommended for an accurate diagnosis. Vaginal swabs and milk samples are the best samples to use for isolation of the organisms from sheep and goats. The spleen and lymph nodes (iliac, mammary and prefemoral) are the most reliable samples for isolation purposes in necropsied animals (Marín *et al.,* 1996a).

B. melitensis does not require serum or CO_2 for growth and can be isolated on ordinary solid media under aerobic conditions at 37^0C. However, the use of non-selective media cannot be recommended because of the overgrowing contaminants usually present in field samples, and selective media are needed for isolation purposes. The Farrell's selective medium, developed for the isolation of *B. abortus* from milk (Farrell, 1974), is also recommended for the isolation of *B. melitensis* (Alton *et al.,* 1988). However, nalidixic acid and bacitracin, at the concentration used in this medium, may have inhibitory effects on some *B. melitensis* strains (Marín *et al.,* 1996b). Thus, its sensitivity for the isolation of *B. melitensis* from naturally infected sheep is sometimes lower than that obtained with the less selective Thayer-Martin's modified medium (Marín *et al.,* 1996a). The sensitivity of bacteriological diagnosis is significantly increased by the simultaneous use of both the Farrell's and the modified Thayer-Martin's media (Marín *et al.,* 1996b).

2.14.2.2 *Other methods for agent detection*

Culturing is a specific method and its sensitivity depends on the viability and numbers of *Brucella* within the sample, the nature of sample (foetal organs, foetal membranes, lymph nodes, etc.) and the number of specimens tested from the same animal (Hornitzky and Searson, 1986). The time required for culturing field specimens can be long and tissues or fluids that are only contaminated with a low number of *Brucella* may not be detected. Thus, in the case of tissues or fluids contaminated with non-viable or a low number of *Brucella*, PCR could be a potentially useful method for the diagnosis of brucellosis. Several authors reported a good sensitivity of PCR for detecting of *Brucella* DNA on pure cultures (Fekete *et al.,* 1990a, 1990b; Baily *et al.,* 1992; Herman and De Ridder, 1992; Romero *et al.,* 1995a; Da Costa *et al.,* 1996). Others showed that PCR could be a potentially useful tool when used alone (PCR, AP-PCR, rep-PCR, ERIC-PCR) or in combination with labelled probes to differentiate some *Brucella* species and biovars (Fekete *et al.,* 1992b, Bricker and Halling, 1994, 1995; Cloeckaert *et al.,* 1995; Mercier *et al.,* 1996; Ouahrani-Bettache *et al.,* 1996; Tcherneva *et al.,* 1996). The possibility of PCR techniques to detect the DNA of dead bacteria, or in paucibacillary samples and even in samples highly contaminated with other micro-organisms, could potentially increase the rate of detecting animals infected with *Brucella.* However few studies have been performed with clinical or field samples (Fekete *et al.,* 1992a; Leal-Klevezas *et al.,* 1995; Romero *et al.,* 1995b; Matar *et al.,* 1996; Rijpens *et al.,* 1996) and up till now, no technique has been demonstrated to be sensitive enough to replace classical bacteriology on all kinds of biological samples.

2.14.3 Indirect Diagnosis: Serological diagnosis

There is no scientific agreement on what should be the nature and characteristics of a universal antigen for diagnosing brucellosis due to smooth *Brucella* (*B. abortus, B. melitensis* and *B. suis*). One of the most controversial points concerning the serological diagnosis of *B. melitensis* infection in small ruminants is related to which *Brucella* species and biovars are used in the production of the diagnostic antigens. The Rose Bengal test (RBPT) and the complement fixation test (CFT) are the most widely used tests for the serological diagnosis of sheep brucellosis (Farina, 1985; MacMillan, 1990). The antigen suspensions (whole cells) used in both tests are made with *B. abortus* biovar 1 (an A dominant strain) (Alton *et al.,* 1988) which means that, theoretically, infections due to M-dominant strains (*B. melitensis* biovar 1; *B. abortus* 4, 5 and 9; *B. suis* biovar 5) could be misdiagnosed (Alton *et al.,* 1988; MacMillan, 1990). However, results showed that the sensitivity of the classical RBPT antigen prepared with *B. abortus* biovar 1 (A-dominant) was adequate for diagnosing ovine populations infected with the M dominant *B. melitensis* biovar 1 (Blasco *et al.,* 1994b).

The outer membrane of the bacteria contains the main antigens involved in the humoral response against *Brucella* (Díaz *et al.,* 1968a). As in other Gram-negative bacteria, the outer membrane of smooth *Brucella* is composed of phospholipids, proteins and lipopolysaccharide (smooth lipopolysaccharide, S-LPS). The S-LPS is the immunodominant antigen. Most serological tests, particularly those using whole-cell suspensions as antigen (such as RBPT, CFT), as well as ELISA, have been developed to detect antibodies to this antigen (Díaz *et al.,* 1968a). The S-LPS of smooth *Brucella* is composed of an inner glycolipidic moiety (the core oligosaccharide plus the lipid A) and an outer polysaccharide chain (O-chain). This O-chain is the relevant antigenic moiety and is chemically composed of a perosamin homopolymer showing α-1,2 and α-

1,3 linkages (Cherwonogrodzky *et al.,* 1990). The O-chain polysaccharide of *B. abortus* biovar 1 (A-dominant) possesses a fine structure with only a low-frequency (FD2%) of α-1,3 linked 4,6-dideoxy-4-formamido-D-mannopyranoside residues.

In contrast, the O-chain polysaccharide of *B. melitensis* biovar 1 (M-dominant) contains repeated pentasaccharide units with one α-1,3 and four α-1,2 linkages. As a result, the A and M antigenic characteristics depend on the O- polysaccharides in which the frequency of α-1,3 linked residues varies. Studies with monoclonal antibodies (Douglas and Palmer, 1988) have shown that the A epitope is related to portions of at least five sugars with α-1,2 linkages and that the M epitope includes sugars with α-1,3 linkages (thus its relevance in the O-chain of *B. abortus* biovar should not be important).

Therefore, all biovars assigned as A-dominant should express few or no α-1,3 linked residues, while M-dominant strains possess a unique M epitope as well as a di-, tri- or tetrasaccharide with α-1,2 linkages, and can thus be considered to be contained within the A epitope structure (Bundle *et al.,* 1989; Meikle *et al.,* 1989; Cherwonogrodzky *et al.,* 1990). The presence of common oligosaccharides of four or less sugars is consistent with the existence of a common (C) epitope. Indeed, this C epitope has been detected with the appropriate monoclonal antibodies (Douglas and Palmer, 1988) and can account for the high sensitivity of the antigens made from A-dominant strains (*B. abortus* biovar 1) at detecting M-dominant *B. melitensis* biovar 1 infections and vice-versa (MacMillan, 1990; Díaz-Aparicio *et al.,* 1993). Infact, crude LPS extracts from either *B. melitensis* 16M (biovar 1, M-dominant) or *B. abortus* 2308 (biovar 1, A-dominant) are equally sensitive in an indirect ELISA (i-ELISA) for diagnosing brucellosis in sheep infected by *B. melitensis* biovar 1 (Anon, 2001). However, the native hapten and the S-LPS hydrolytic polysaccharides containing the O-chain and core sugars from *B. abortus* biovar 1 failed to react in precipitation tests with a large

proportion of *B. melitensis* infected sheep, goats and cattle under conditions in which the same antigens obtained from *B. melitensis* biovar 1 detected most of those animals (Díaz-Aparicio *et al.,* 1993).

The immunoelectrophoretical patterns of cytoplasmic proteins show little differences between *Brucella* species when assayed with polyclonal sera (Díaz *et al.,* 1967, 1968b). These inner antigens are considered specific for the genus, being useful to differentiate infections due to *Brucella* from those due to bacteria whose LPS cross-reacts with the *Brucella* S-LPS, as is the case with *Yersinia enterocolitica* O:9 (Díaz and Bosseray, 1974). However, a cross-reactivity among cytosolic proteins of *B. melitensis* and those obtained from *Ochrobactrum anthropi*, an opportunistic human pathogen, has been reported recently (Velasco *et al.,* 1997).

The *Brucella* cytoplasmic antigens have been used successfully for the allergic diagnosis of brucellosis in sheep and goats (Fensterbank, 1982, 1985; Ebadi and Zowghi, 1983; Loquerie and Durand, 1984; Blasco *et al.,* 1994b). Moreover, these cytoplasmic antigens have been reported to be sensitive and specific enough for the diagnosis of brucellosis in sheep and goats when used in precipitation tests (Muhammed *et al.,* 1980; Trap and Gaumont 1982; Díaz-Aparicio *et al.,* 1994). In contrast, when these cytoplasmic antigens are used in the i-ELISA, the sensitivity obtained is not adequate due to the high background IgG reactivities with sera from *Brucella* free animals (Díaz-Aparicio *et al.,* 1994; Debbarh *et al.,* 1996). An important drawback of diagnostic tests using uncharacterised cytosolic proteins is the lack of specificity when testing Rev.1 vaccinated sheep and goats. But a partially purified cytosoluble protein of 28 kDa (CP28) from the cytosoluble protein extract (CPE) of *B. melitensis* has been reported as being able to differentiate Rev.1 vaccinated from *B. melitensis* infected ewes when used in i-ELISA (Debbarh *et al.,* 1995). However, this test is less sensitive than

both the RBP and CF tests for diagnosing *B. melitensis*- infected ewes (Debbarh *et al.,* 1996). The corresponding *B. melitensis* 16M *bp26* gene was expressed in *Escherichia coli* and monoclonal antibodies were produced (Cloeckaert *et al.,* 1996a, 1996b). Sequence analysis of the cloned gene revealed that it was nearly identical to the published *B. abortus bp26* gene, coding for a periplasmic protein (Rossetti *et al.,* 1996). A competitive ELISA (c-ELISA) using CPE as antigen and some of these monoclonal antibodies showed improved sensitivity for diagnosing infected sheep, and no antibody response was detected in Rev.1 vaccinated sheep (Debbarh *et al.,* 1996).

Several authors have attempted to identify the main polypeptide specificities of the antibody response to outer-membrane protein (OMP) extracts of *B. melitensis* by using either immunoblotting or c-ELISAs with specific monoclonal antibodies (Zygmunt *et al.,* 1994a, 1994b, Debbarh *et al.,* 1995, Hemmen *et al.,* 1995, Tibor *et al.,* 1996). While OMPs of 10, 17, 19, 25-27 and 31-34 kDa were found as potential antigens for the diagnosis of brucellosis in sheep by immunoblotting or ELISA, the antibody response to them was very low and heterogeneous in *B. melitensis* infected sheep (Zygmunt *et al.,* 1994a, 1994b).

2.14.4 Serological Tests

Serological tests are widely used for the detection of infected animals. However, no serological test is appropriate in each and every epidemiological situation. Consideration should therefore be given to all factors that impact on the relevance of the test method and the test results for a specific diagnostic interpretation or application (Dohoo *et al.,* 1986). It is widely assumed that the serological tests used for *B. abortus* infection in cattle are also adequate for the diagnosis of *B. melitensis* infection in small ruminants. Most of the serological tests, particularly those using whole cell suspensions

as antigen, such as Rose Bengal plate test (RBPT), Complement fixation test (CFT) and milk ring test (MRT) have been developed against the O-polysaccharide (OPS) moiety of the smooth lipopolysaccharide (SLPS) (Nielsen, 2002).

2.14.4.1 *Rose bengal plate test (RBPT)*

The RBPT test was developed for the diagnosis of bovine brucellosis. Despite the scanty and sometimes conflicting information available (Trap and Gaumont, 1975; Fensterbank and Maquere, 1978; Farina, 1985; MacMillan, 1990; Alton, 1990; Blasco *et al.,* 1994a, 1994b), this test is internationally recommended for the screening of brucellosis in small ruminants (Joint FAO/WHO, 1986; Garin-Bastuji and Blasco, 1997). An important problem affecting the sensitivity of the RBP test concerns the standardisation of the antigen. The European Union regulations require antigen suspensions in lactate buffer at pH 3.65 ± 0.05 that are able to agglutinate at a dilution of 1:47.5 (21 IU/ml) of the International Standard anti-*B. abortus* serum (ISaBS) but give a negative reaction at a dilution of 1:55 (18.2 IU/ml) of the same serum (Council Directive, 1964). These standardisation conditions, which seem to be suitable for the diagnosis of *B. abortus* infection in cattle (MacMillan, 1990), limit the sensitivity of the test resulting in reduced performance for the diagnosis of *B. melitensis* infection in sheep (Blasco *et al.,* 1994a, 1994b). This accounts for the relatively low sensitivity of some commercial RBPT antigens when diagnosing brucellosis in sheep and goats (Falade, 1978, 1983; Blasco *et al.,* 1994a) and for the fact that a high proportion of sheep and goats belonging to *B. melitensis*-infected areas give negative results in the RBPT but positive ones in the CF test (Blasco *et al.,* 1994a). These phenomena have raised serious questions over the efficacy of using the RB as an individual test in small ruminants. However, if the antigen is standardised differently to give a higher analytical

sensitivity, the diagnostic sensitivity is much improved (MacMillan, 1997). Some workers claimed that, at least for sheep, the sensitivity of the RBPT can be improved significantly when the antigens are standardised against a panel of sera from several *B. melitensis* culture positive and *brucella*-free sheep, respectively, or when the volume tested is increased from 25µl to 75µl (Blasco *et al.,* 1994a).

2.14.4.2 *Serum agglutination test (SAT)*

Serum agglutination test is an important test to which every eligible animal is subjected. It has been the basis of eradication schemes in many countries (Bale, 2008). Zammit (1905) probably performed the first serum agglutination test on goats and servicemen on the Island of Malta. 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 (Bale, 2008). Serum agglutination test gives negative results on sera from some animals from which *Brucella abortus* can be isolated (Alton *et al.,* 1975), yet false positive can be caused by non-specific agglutinins in serum (Hess, 1953).

Haemolysed serum samples are not good for slow agglutination in tube because of the interference of phenol with free haemoglobin, which may cause "false agglutinations". For testing sheep and goats sera, the use of 5% sodium chloride with 0.5% phenol is recommended both for the dilution of the sera and the concentrated antigen to prevent prozone effects. The result of SAT is also affected by vaccination of animals and as such the results of test should be based on the vaccination status of herd or flock. Low titres cannot rule out the possibility of brucellosis in male animals, so other

supplementary tests and test of seminal plasma should therefore be conducted to establish proper diagnosis (Bale, 2008).

2.14.4.3 *Complement fixation test (CFT)*

The CF test is the most widely used test for the serological confirmation of brucellosis in animals. As in cattle brucellosis, despite its complexity and the heterogeneity of the techniques used in different countries, there is agreement that this test is effective for the serological diagnosis of brucellosis in sheep and goats (Farina, 1985; MacMillan, 1990; Alton, 1990). When testing a limited number of sera obtained from *B.melitensis* culture positive and *Brucella*-free goats, the CF test provided the same sensitivity as the RBPT and i-ELISA (Díaz-Aparicio *et al.,* 1994). However, under field conditions, the sensitivity of the CF test has been reported to be somewhat lower (88.6%) than those of the RBPT (92.1%) and i-ELISA (100%) for diagnosing *B. melitensis* infection in sheep (Blasco *et al.,* 1994a, b). Nielsen *et al.* (2000), in a Pan-American and European comparative study, the results on sensitivity for the different tests were: c-ELISA (76.0 %), buffered plate agglutination test (77.5 %), CFT (83.1 %), i-ELISA (90.1 %) and fluorescence polarisation assay (FPA) (91. 5%).

On the other hand, the CF test has many drawbacks such as complexity, variability of reagents, prozones, anticomplementary activity of sera, difficulty to perform with hemolysed sera, and subjectivity of the interpretation of low titres. Therefore, while the sensitivity of RBPT is sufficient for the surveillance of free areas at the flock level, RBPT and CFT should be used together in infected flocks to obtain accurate individual sensitivity in test-and-slaughter programmes. Moreover, an important drawback of both RBPT and CF test is their low specificity when testing sera from sheep and goats vaccinated subcutaneously with Rev.1 (Fensterbank *et al.,* 1982; Jiménez de Bagüés *et*

al., 1992; Díaz-Aparicio *et al.,* 1994). However, when the Rev.1 vaccine is applied conjunctivally (Fensterbank *et al.,* 1982), the interference problem is significantly reduced in all serological tests (Jiménez de Bagüés *et al.,* 1992; Díaz-Aparicio *et al.,* 1994).

2.14.4.4 *Enzyme linked immunosorbent-assay methods (ELISA)*

The large majority of Enzyme immuno-assays (EIAs) in use in brucellosis diagnosis are indirect ELISAs (iELISA). ELISAs are methods that involve the immobilisation of one of the active components on a solid phase, and iELISAs are those in which the antigen is bound to a solid phase, usually a polystyrene microtitre plate so that antibody, if present in a sample, binds to the immobilized antigen and may be detected by an appropriate anti-globulin-enzyme conjugate which in combination with a chromogenic substrate gives a coloured reaction indicative of the presence of antibody in the sample. It is this method that is now familiar to most diagnosticians.

Another method which is gaining prominence in the publications on brucellosis diagnosis is the competitive ELISA (cELISA) (Gorrell et al., 1984; Rylatt *et al.,* 1985; Sutherland *et al.,* 1986; Macmillan *et al.,* 1990; Greiser-Wilke *et al.,* 1991; Nielsen *et al.,* 1991; Marín *et al.,* 1999; Nielsen *et al.,* 2000). In this test, *Brucella* antigen is immobilised on the plate as with the indirect ELISA. Following that, the serum under test and a monoclonal antibody directed against an epitope on the antigen are coincubated. This anti-*brucella* monoclonal antibody is conjugated to an enzyme, the presence of which is detected if it binds to the antigen. This will only occur if there is no antibody in the serum sample which is bound preferentially.

The Particle Concentration Fluorescence Immunoassay (PCFIA) is a commercially produced immunofluorescence assay which has been used in the U.S.A (Reynolds 1987; Nicoletti and Tanya, 1993; Greenlee *et al.,* 1994).

The technique has much in common with the competitive ELISA in that antigen is immobilised onto polystyrene, except that in this case it is initially in the form of sub-micron particles held in suspension. Serum is coincubated with an anti-*brucella* conjugate in the presence of the antigen coated particles, and they compete for binding. In this case, the antibody is labelled with a fluorescent probe. The unreacted reagents are separated from the polystyrene particles which are themselves concentrated by filtration through a membrane at the bottom of each well. The commercial producer claims the ability to analyse 550 samples per hour with that system (Greenlee *et al.,* 1994).

The term ELISA covers a variety of possible reagent combinations which have a direct and significant effect on performance. A wide variety of antigen preparations has been used in the iELISA ranging from whole cells to crude and semi-purified smooth lipopolysaccharide (sLPS) preparations to polysaccharides and proteins. The antigens of sLPS are the most immunodominant and, due to the fact that they are very strongly adsorbed to polystyrene, have become the antigens most widely used for routine diagnosis. Even in relatively crude extracts or when whole cells are used, it is the sLPS antigens which are most active (Nielsen *et al.,* 2000).

The diagnostically most relevant epitopes reside on the chain component rather than the lipid A or core polysaccharides. The O-chain structure of all species of *Brucella* has been elucidated and has been shown to be a linear homopolymer of 4,6-dideoxy-4-formamido-α-D-mannose (Cherwonogrodzky *et al.,* 1984; Bundle *et al.,* 1987; Meikle *et al.,* 1989; Perry *et al.,* 1990).

In practice, the use of an sLPS antigen derived from either *B. abortus* or *B. melitensis* is adequate for the diagnosis of either, but there is some evidence that it is preferable to use a homologous antigen. There is little evidence for any significant difference between antigens prepared from *B. abortus* or *B. melitensis*, whether LPS, O-polysaccharide or native hapten is used for the detection of brucellosis in vaccinated or non-vaccinated cattle (Nielsen *et al.,* 1983, Devi *et al.,* 1987).

Although it is recommended that purified sLPS antigen should be used in the iELISA, there is little doubt that less purified preparations such as autoclaved or sonicated cell extracts are adequate, at least for screening purposes (Cherwonogrodzky *et al.,* 1986; Nielsen *et al.,* 1988). There is also consensus that the purity of the antigen has little effect on the sensitivity of the assay, but as diagnostic specificity becomes increasingly important with decreasing disease prevalence, it can be expected that less pure antigens give rise to a slightly higher number of false positive reactions. On the other hand, it is suspected that extremely pure sLPS has the tendency to form micelles in aqueous solution causing it to adsorb irregularly to polystyrene. This results in assay variation and high negative backgrounds. The hot water/phenol extraction method appears to be one of the best methods currently in use (Anon, 2001).

All four IgG isotypes are present in serum and milk although IgG_1 predominates. All isotypes are theoretically detectable in the iELISA depending on the immunological specificity of the anti-globulin used, and a wide variety of conjugates have been evaluated ranging from those of broad specificity to reagents specific for a single isotype. The use of anti-γ globulin reagents introduces a bias in favour of IgM, a class of antibody often associated with non-specific reactions in other assays. This bias can be reduced by using an anti-IgG (heavy and light chain) which would still be capable of detecting IgM as a result of its light chain specificity but would favour the detection of

IgG isotypes which are likely to be more specific (Lamb *et al.,* 1979; Nielsen *et al.,* 1983, 1984; Wright and Nielsen, 1986; Huang, 1987; Nielsen *et al.,* 1988).

The diversity and multiplicity of epitopes on sLPS make it likely that the immune response to this antigen will be very heterogeneous in terms of relative specificities and affinities. This has significance in that there will be competition for a limited number of binding sites between antibodies of different isotypes. There is undoubtedly competition both directly at a single epitope, but also as a result of the structure of the O-chain, by steric hindrance between antibodies binding to adjacent or overlapping sites (Anon, 2001). Thus a conjugate of high specificity for a single isotype may fail to detect the presence of antibody if this isotype is prevented from binding to the antigen due to competition with other isotypes. This would lead to a false negative test result (Anon, 2001).

The vast majority of ELISAs involve a common methodology utilising 96 well polystyrene plates. In the context of discussions on mass screening as part of eradication campaigns this is a progress as it is suitable for cost effective automation.

Relatively little information is available on the value of the ELISA for the diagnosis of *B. melitensis* in small ruminants. The indirect ELISA, using more or less purified S-LPS of *B. melitensis* as antigen and polyclonal conjugate (anti-IgG H+L), has been reported to be sensitive enough for the diagnosis of infection in sheep and goats (Jiménez de Bagüés *et al.,* 1992; Díaz-Aparicio *et al.,* 1994; Blasco *et al.,* 1994b; Delgado *et al.,* 1995). A similar technique has been proposed for diagnosing sheep brucellosis in individual or pooled milk samples (Biancifiori *et al.,* 1996), but due to the low rate and frequency of *brucella* antibodies in milk, the test lacks sensitivity compared with tests performed on serum.

One of the problems of the i-ELISAs performed on serum is the high background reactivity obtained when testing sera from *brucella*-free animals (Jiménez de Bagüés *et al.,* 1992). The use of protein G as conjugate significantly reduces this problem, increasing the ELISA specificity (Díaz-Aparicio *et al.,* 1994; Ficapal *et al.,* 1995). This increased specificity is also obtained when testing *brucella*-free sheep in the above i-ELISA but using a monoclonal anti-ruminant IgG_1 conjugate.

2.14.4.5 *Gel precipitation test*

As with the RBPT and CFT, the specificity of the i-ELISA is quite low when testing sera from sheep and goats subcutaneously vaccinated with the live *B. melitensis* Rev.1 vaccine (Jiménez de Bagués *et al.,* 1992; Díaz-Aparicio *et al.,* 1994; Marín *et al.,* 1999). Under these conditions, only the Gel Diffusion (GD) or the Radial immunodiffussion (RID) tests with Native Hapten (NH) as antigen (Díaz and Morityon, 1989) are specific enough to discriminate the immune responses of, respectively, sheep (Marín *et al.,* 1999) and goats (Díaz-Aparicio *et al.,* 1994) infected with *B. melitensis* from those due to Rev.1 vaccination.

As demonstrated by the contrasting results of the i-ELISA and the c-ELISA, the superior diagnostic specificity of the latter in vaccinated sheep (Marín *et al.,* 1999) can be due to the elimination by the competing anti-C monoclonal antibody of the low avidity antibodies, supposed to be dominant in the sera from vaccinated animals. This antibody avidity, rather than epitopic differences in the antigens used, is also likely to account for the high specificity of the NH precipitation tests. The higher specificity of the precipitation tests with NH may result from the higher threshold avidity required in precipitation tests as compared to that of i-ELISAs (Anon, 2001). This could explain why NH fails to react with sera from vaccinated animals in the former but not in the

latter assay, if low avidity antibodies are predominant at a given time after vaccination. Obviously, comparison of the results of the indirect and competitive ELISAs indicates that the sera of Rev.1 vaccinated sheep, at a given time after vaccination, contain mostly antibodies of lower avidity than those from infected sheep, and this is consistent with the hypothesis proposed.

Many serological responses occurring in healthy animals (particularly in the Rev.1 immunised ones) in endemic areas may be the consequence of mere secondary antigenic contacts and do not correspond always to established infections in the host. In fact, under these conditions, the RBPT, CFT and i-ELISA tests have been reported to be highly unspecific to detect exclusively the bacteriologically positive animals (Blasco *et al.,* 1994b). Having in mind that more additional research and field studies are needed for a proper confirmation, the NH precipitation test (Díaz and Moryion, 1989; Díaz-Aparicio *et al.,* 1994; Marín *et al.,* 1999) again appears to be the most specific serological test to detect exclusively the epidemiologically relevant animals, particularly after Rev.1 mass vaccination programmes in endemic situations.

2.14.4.6 *Antiglobulin Test (Coomb's Test)*

The antiglobulin test or Coombs' test (Coombs *et al.,* 1945) was developed to detect antibodies which, although they combine with cellular antigens of *Brucella* do not give rise to agglutination. The presence of these so-called "incomplete agglutinins" can be detected by using an antibody directed against the IgG fraction of the animal species being tested (MacMillan, 1990). Early in 1955, the test was described as the most effective immunological method for the detection of brucellosis in goats (Esteban, 1959). The classical time-consuming methodology of the test in man has been considerably improved by its adaptation to a microtiter plate format (Otero *et al.,* 1982).

Farina (1985) reported that the Coombs' test could be useful to check sera from animals that give negative, suspicious or non-conclusive responses to SAT, due to the presence of incomplete antibodies in these sera. There is evidence (Alton, 1990; Unel *et al.,* 1969) suggesting that the antiglobulin test is effective in diagnosing brucellosis in sheep and also in goats, but because of the complexity of the technique its use may be restricted to special situations, e.g. for the detection of antibodies in anticomplementary sera. Its use is not recommended in bovines vaccinated with strain S19 (MacMillan, 1990) or in small ruminants immunised with Rev.1 vaccine (Farina, 1985), because of its low specificity as compared to the CFT**.**

2.14.4.7 *Immunocapture Test*

The immunocapture test is a one-step technique, very easy to perform, able to detect antibodies of medium to high affinity against *Brucella*, and suitable for simple standardisation and automation. The test is based on a blue-coloured cellular antigen of *B. melitensis* (strain 16M) and on anti-total species immunoglulin coated polystyrene microtiter plates of 96 U-wells. The test was initially evaluated for the serodiagnosis of human brucellosis and has shown high sensitivity and specificity both in the first stages of the disease and, in particular, in chronic cases as well as in relapses and reinfections (Gómez *et al.,* 1999; Orduña *et al.,* 2000). Moreover, the immunocapture test and Coombs' test have a similar performance in the diagnosis of human brucellosis but the immunocapture test is more sensitive than the Coombs' test and usually shows higher titers (Orduña *et al.,* 2000).

The usefulness of the immunocapture test for the recognition of *B. melitensis* infection in sheep has been evaluated in experimental models of vaccination and infection (Duran *et al.,* 2011). The test revealed an optimal sensitivity in the detection of animals with

active brucellosis (abortion and excretion) and adequate specificity in Rev.1 immunised animals that were protected against challenge with the standard virulent strain 53H38 of *B. melitensis*. Moreover, the specificity of the immunocapture test was higher than RBT and CFT either in lambs or in adult animals vaccinated with Rev.1 by conjunctival route.

2.14.4.8 *Fluorescence Polarization Assay (FPA)*

The principle of fluorescence polarisation was described by Perrin in 1926. It is a simple technique for measuring antigen/antibody interaction and may be performed in a laboratory setting or in the field. It is a homogeneous assay in which analytes are not separated and it is therefore very rapid (Corbel and MacMillan, 2000).

The premise for the Fluorescence Polarization Assay (FPA) is that a small molecule in solution randomly rotates at a rate inversely proportional to its size. Plane polarized light allows measurement, using an attached fluorochrome, of the rate of rotation through a given angle. Thus a small molecule rotating at a high rate will revolve through the angle rapidly, resulting in a low polarization value. If an antibody is attached to the small molecule, the increased size will cause a decrease in the rate of rotation, resulting in a higher polarization value. The FPA is a homogeneous assay, requiring no steps to remove unreacted reagents, and can therefore be performed in minutes, even outside the laboratory, and is very cost effective.

Based on this, Nielsen *et al.* (1996) developed the FPA for the detection of antibodies to *B. abortus* in cattle, using as antigen a small molecular weight fragment of O-polysaccharide prepared from *B. abortus* lipopolysaccharide (average 22 kDa) conjugated with fluorescein isothiocyanate. This antigen is added to diluted serum or

whole blood and a measure of the antibody content is obtained in about 2 minutes using a fluorescence polarisation analyser.

The sensitivity and specificity values of the FPA for bovine brucellosis are almost identical to those of the cELISA (Corbel and MacMillan, 2000). The diagnostic sensitivity has been determined to be over 99 %, while the diagnostic specificity approaches 100%. The diagnostic specificity for cattle recently vaccinated with *B. abortus* S 19 is over 99% (Nielsen *et al.,* 1996). The test has been evaluated with positive outcomes under field conditions in different epidemiological situations (Nielsen *et al.,* 1996). The technique has also been reported to be a valuable asset to the diagnosis of porcine brucellosis (Nielsen *et al.,* 1999); the sensitivity (93.5%) was estimated much higher than for cELISA (90.8 %) and also the specificity (97.2 % versus 96.6 %).

Little information is available on the usefulness of the FPA for the serodiagnosis of ovine and caprine brucellosis. In a preliminary study comparing different serological tests for ovine brucellosis (Nielsen *et al.,* 2000), the sensitivity of the FPA (91.5 %) was estimated as higher than other tests (BPAT, 77.5 %; CFT, 83.1%; iELISA 90.1 %, and cELISA 76.1%). The specificity of FPA (99%) was similar to that of the other tests. Residual vaccinal antibodies would not cause reactivity in most cases in the cELISA and in the FPA. Based on these data, the FPA appears to be as efficient as other commonly used serological tests and may prove to be a useful tool for the presumptive diagnosis of *B. melitensis* infection in small ruminants (Nielsen *et al.,* 2000).

2.14.5 Tests Based on Cell Mediated Immunity

2.14.5.1 *Brucellin Test*

Brucella cytoplasmic antigens, known also as brucellin (Jones *et al.,* 1973) have been used with variable success for the allergic diagnosis of brucellosis in sheep and goats (Fensterbank, 1982, 1985; Ebadi and Zowghi, 1983; Loquerie and Durand, 1984; Blasco *et al.,* 1994b). Cold saline protein extracts of the rough *B. melitensis* 115 a strain devoid of the O-chain polysaccharide were used as allergen (Jones *et al.,* 1973).

Most authors consider that the site and route of allergen inoculation is not important for Delayed type hypersensitivity (DTH) (Alton, 1990; Fensterbank, 1985). The method considered efficient for sheep is the subcutaneous inoculation in the lower eyelid of 50 µg of allergen suspended in a volume of 0.1 ml saline, with readings taken 48 hours after inoculation (Jones *et al.,* 1973; Fensterbank, 1985; Alton *et al.,* 1988). However, the intensity of the DTH responses peaks 72 hours after inoculation (Blasco *et al.,* 1994b). As mixed DTH-antibody mediated intradermal reactions can occasionally be observed (Blasco *et al.,* 1994b), the antibody-mediated responses should be more apparent at shorter times and, accordingly, reading time longer than 48 hours seems to be advisable for a better assessment of true DTH reactions. A relatively important anergic state can be induced by repeated skin testing and this should be taken into account if the DTH test to *Brucella* has to be used as a diagnostic test in the field. The DTH sensitivity is generally higher than that of the classical RBP and CF serological tests (Trap and Gaumont, 1982; Ebadi and Zowghi, 1983; Blasco *et al.,* 1994b).

Although DTH has a higher sensitivity than RBPT for diagnosing culture positive animals, and its use in some circumstances as a screening test in the field could be more practical than that of a serological test, a high proportion of culture negative animals belonging to infected flocks have been reported to be DTH positive (Blasco *et al.,*

1994b). With respect to specificity, it is generally acknowledged that DTH tests show no positive reactions (100% specificity) when testing *Brucella*-free flocks (Dubray, 1985; Fensterbank, 1985; Alton, 1990). However, the *B. melitensis* 115 allergen also causes DTH reactions in rams infected with *B. melitensis* Moreover, it cannot distinguish whether an animal is infected by *B. melitensis* or vaccinated with Rev.1. Accordingly, the DTH results should be carefully interpreted in countries where animals are infected by *B. ovis*. This test is not suitable in countries in which vaccination programmes with Rev.1 are being applied.

2.12.5.2 *Interferone-Gamma Test*

Tests for the *in-vitro* detection of Cell Mediated Immunity (lymphocyte transformation and proliferation assays) showed a lack of acceptable efficacy in order to be applied for the large scale routine diagnosis of *Brucella* infection (Nicoletti and Winter, 1990). However, the recognition of the role of some cytokines, such as interferon (IFN) gamma, in immunity against intra-cellular agents has enabled the development of an *in-vitro* test with useful diagnostic applications. The test was first designed for the diagnosis of bovine tuberculosis (Rothel *et al.,* 1990, Wood *et al.,* 1990, 1991).

Brucella are facultative intracellular bacteria that survive and replicate in both phagocytic and non-phagocytic cells (Dubray, 1985). Phagocytes play a key role in initiating T-cell responses by processing and presenting antigens. In this path, IFN-gamma is one of the most important T-cell stimulated cytokines in the course of an infection. It is a potent activator of macrophages and monocytes and up-regulates their metabolic activities to produce oxidative metabolites and other microbicidal molecules. Based on these data, the usefulness of IFN-gamma assay in the diagnosis ovine

brucellosis (Garrido, 1992; Durán-Ferrer, 1998) and bovine brucellosis (Weynants *et al.,* 1995) has been explored.

The performance of the IFN-gamma test is divided in two phases: firstly, cultures of whole blood samples from donors are stimulated with specific antigen, and then IFN-gamma released is measured by immunodetection with a sandwich ELISA. In principle, such versatile design could lead to the use of the test for different purposes by using different stimulating antigens (S-LPS, cytosolic proteins).

Studies of Weynants *et al.* (1995) pointed to IFN-gamma with cytosolic proteins as a candidate stimulant tool for the detection of false positive reactions in serological tests, which have become a major problem in the surveillance of bovine brucellosis.

With regard to ovine brucellosis caused by *B. melitensis*, the test has been evaluated in experimental models of conjunctival vaccination (De Frutos *et al.,* 1994) and in experimental infection (Durán-Ferrer, 1998) of ewes, either with surface antigens or cytosol proteins. The experiments revealed that this test may be superior to serological tests (RBPT, CFT and iELISA) for the detection of acute infection in vaccinated ewes, but show similar specificity (Anon, 2001)**.**

2.15 VACCINES

Vaccination is often the first step in the control of infectious diseases. There is agreement that vaccination of sheep and goats is the only practical and effective procedure to reduce the incidence of brucellosis in many countries. Vaccines commonly used in the control of brucellosis in sheep and goats are discussed below.

2.15.1 *Brucella melitensis* Rev.1 vaccine

The live attenuated *B. melitensis* Rev.1 strain is presently recognised as the best available vaccine for the prophylaxis of brucellosis in sheep and goats. Numerous independent field and controlled experiments confirm its value for this purpose (Alton, 1990; Elberg, 1981 and 1996; Blasco, 1984; 1997).

The ability of the vaccine (Rev.1 strain) to produce a high level of immunity against both artificial and natural challenge has been convincingly demonstrated both for sheep and goats (Alton, 1990). It has been well established that a large proportion of vaccinated animals is protected against infection (Garrido, 1992), and in those vaccinated animals where infection occurred, it is often transitory.

Hence, the period of *Brucella* excretion from the udder or vagina is shorter, the degree of microbial contamination of the surroundings is reduced and, consequently, disease transmission within and between herds is significantly reduced (Garrido, 1992). As with all highly-contagious diseases, the effect of vaccination increases the greater the coverage of the animal population. Erratic administration of vaccines or their use without adequate quality control is not effective. Adequate protection is only possible if the vaccine quality is good and if the vaccines are administered to at least 80 % of the animals at risk (Garrido, 1992).

The duration of immunity conferred by vaccination with Rev.1 was investigated by vaccinating Maltese goats when they were 4 to 12 months of age and challenging some at 2½ years (Alton, 1966) and others at 4½ years (Alton, 1968) after vaccination. Those challenged at 4½ years were as resistant as those challenged at shorter intervals after vaccination, and it was concluded that immunity could be considered lifelong. Similar results were observed in sheep in Iran challenged 2½ years after vaccination (Biggi, 1956; Alton, 1990).

The Rev.1 vaccine is a useful tool for the control of brucellosis in sheep and goats and to stop the infection of human beings. Its administration should be related to the epidemiological situation in order to be compatible with an eradication policy based on test-and-slaughter. Used exhaustively in whole flock vaccination programmes, the live *B. melitensis* Rev.1 vaccine greatly decreases the prevalence of brucellosis in both sheep and human population (Elberg, 1981, 1996). Once the prevalence has been diminished, a more efficient control of the disease may be achieved through the implementation of a programme based on Rev.1 vaccination of lambs combined with the test-and-slaughter of adults. Finally, it may be possible to use a test-and-slaughter programme only (Garin-Bastuji *et al.,* 1998).

2.15.2 *B. suis* S2

B. suis S2, a classically obtained *Brucella* attenuated strain with smooth LPS, was apparently successfully used in controlling brucellosis following oral administration to small ruminants in field conditions in China (Xin, 1986) and Libya (Mustafa and Abusowa, 1993). However, this vaccine showed no protective effect against *B. melitensis* in sheep in fully controlled experimental conditions (Verger *et al.,* 1995).

2.15.3 *B. abortus* RB51

Another classically obtained, live attenuated but rough (S-LPS lacking) Brucella strain is the *B. abortus* RB51 strain (Schurig *et al.,* 1991). The strain RB51 has been reported to be equally effective as S19 vaccine in protecting against *B. abortus* in cattle, without inducing anti-O chain antibodies as detected by serological tests (Palmer *et al.,* 1997). Preliminary experiments suggest that this vaccine can be effective for the prophylaxis of *B. melitensis* infection in goats (Suarez *et al.,* 1998). In contrast, it has been

demonstrated that this vaccine does not confer protection against *B. ovis* in controlled experiments in rams (Jiménez de Bagués *et al.,* 1995).

2.15.4 VTMR1

VTRM1 is a live rough strain obtained by transposon mutagenesis from smooth *B. melitensis* 16M. Mutagenesis resulted in a truncated gene for mannosyltransferase, an enzyme necessary for the synthesis of the S-LPS and accordingly, VTRM1 does not induce antibodies against the S-LPS and does not interfere with classical serological tests. However, the VTRM1 vaccine does not confer adequate protection against *B. melitensis* in goats (Elzer *et al.,* 1998).

2.15.5 RfbK STRAIN

The mutant rfbK is a live rough strain obtained by transposon mutagenesis from smooth *B. abortus* 2308 (Adams *et al.,* 1998). Mutagenesis resulted also in a truncated gene for mannosyltransferase. Preliminary experiments suggest that this mutant strain can be effective for the prophylaxis of *B. melitensis* infection in goats (Suarez *et al.,* 1998).

2.16 STRATEGIES FOR THE CONTROL, PREVENTION AND ERADICATION OF BRUCELLOSIS IN SHEEP AND GOATS

Because brucellosis is a disease of major economic and zoonotic importance, a strategy for its control in small ruminants is essential in endemic areas. The initial aim of the strategy selected will be the reduction of infection in the animal population to such a level that the impact of the disease on human health as well as on animal health and production will be minimised (Kaplan, 1966). Subsequent steps can include eradication

from a region by test and slaughter and, following successful eradication, measures to prevent reintroduction of the disease.

Control of a zoonosis is a general term that embraces all the measures designed to reduce the incidence and prevalence of a disease in a defined animal population.

The term eradication has first been used to mean the extinction of an infectious agent. Accordingly, eradication is not completed if a single infectious agent survives anywhere in nature (Cockburn, 1963). The meaning of the term has been modified with time and the most common meaning of eradication in veterinary practice refers to the regional extinction of an infectious agent.

The term elimination has been created to identify an intermediate situation. Elimination refers to the reduction in the incidence of the disease below the level achieved by control, so that either very few or no cases occur, although the infectious agent may persist (Payne, 1963; Spinu and Biberi-Moroianu, 1969).

Elimination is the task for most programs implemented for the control of brucellosis in small ruminants.

It is accepted that before a zoonosis control program is designed and implemented, a well-functioning surveillance system, which should be fed with valid data collected from the field, must be established. The main purpose of the surveillance system will be to determine the prevalence of the disease, so that appropriate measures for its control can be taken. The system must be able to detect early any change in incidence and prevalence. The surveillance system allows for the optimisation of resources, for alternative actions to be designed, for the continuous justification of expenses, and the evaluation of activities performed and achievements to be assessed (Thrusfield, 1995; WHO/MZCP, 1998).

Once an accurate surveillance system is in existence and is fed with valid data collected from the field, the progress, impact, adequacy, efficiency, and efficacy of a control program can be continuously assessed and evaluated. It is a crucial matter that the information output must be issued not only to the decision-makers but also to all the members participating in the program, so that they can be informed about the progress of their work. This is very important especially in zoonosis control programs, because the advantages of the program are not so obvious as in other diseases, whose occurrence is characterised by heavy losses in animal population. There are many cases that zoonosis control programs had failed because the people participating had lost motivation since they did not see any obvious benefit (WHO/MZCP, 1998).

A selection can be made from the following strategies, which are implemented for the control of brucellosis.

2.16.1 Immunization to Reduce the Rate of Infection in Specified Herds

Control of brucellosis can be achieved by using vaccination to increase the population's resistance to the disease. Vaccination practically eliminates the clinical signs of brucellosis and is accompanied by a reduced contamination of the environment as well as exposure of the population at risk to the infectious agent (Nicoletti, 1993).

The initial campaigns to control brucellosis in small ruminants were based on the vaccination of young animals, kept as replacements, with the Rev.1 vaccine. This approach was based on the hypothesis that the Rev.1 vaccine offered life-long immunity and that after implementing the vaccination program for 5-7 years (the productive life-span of sheep and goats) the whole population would be vaccinated and fully protected against brucellosis. This method was also recommended to minimise postvaccinal diagnostic problems and to avoid abortions (Nicoletti, 1993; Blasco, 1997).

However, in many countries, where the animals were kept under extensive conditions with nomadic or semi-nomadic husbandry, this approach was impractical and failed to reduce the incidence and prevalence of the disease, because the development of herd immunity was very slow (Kolar, 1995; Blasco, 1997). In addition, the unvaccinated adult animals remain unprotected and the infection can spread (Kolar, 1995).

Vaccination of all animals (young and adults) in a flock or region is an alternative approach for the control of brucellosis in small ruminants. Mass immunisation of small ruminants has been applied with success in many countries under different socio-economic conditions. In general, mass immunisation is indicated where the prevalence of infected animals is high. It should be based on knowledge of the prevalence in the flock or region. Mass vaccination of a flock helps to rapidly establish a relatively immune stock, and reduces the level of abortions and excretors of *Brucella*, thus reducing contamination of the environment and disease transmission (Kolar, 1995).However, this strategy has the limitation that pregnant animals cannot be vaccinated because the vaccine is not innocuous enough for pregnant animals, and the efficacy of the strategy depends on the continuous availability of the vaccine (Joint FAO/WHO, 1986).

Provided that the prevalence of disease is moderate, financial resources are available, and a well-functioning surveillance by the veterinary service is in place, vaccination of young animals can be combined with a test and slaughter policy in a long term action to control brucellosis in small ruminants (WHO/MZCP, 1998).

2.16.2 Elimination of Infected Animals by Test and Slaughter

It is usually accepted that a programme of eliminating brucellosis by test-and-slaughter policy is justified on economic grounds only when the prevalence of infected animals in an area is about 2% or less (Nicoletti, 1993).

For the implementation of such a program it is essential that the flocks are under strict surveillance and movement control. Animals must be individually identified and an efficient and well organised veterinary service for surveillance and laboratory testing must be in place (Nicoletti, 1993).

The flock sizes as well as the prevalence of brucellosis are the most important factors of this strategy which has been shown to be ineffective and unreliable when attempted in large flocks with a high prevalence of brucellosis (Kolar, 1995). The limited reliability of the diagnostic tests used which are unable to reveal all infected animals and which may give false negative results due to incubation period, latency or due to criteria used to interpret the results must also be considered. These drawbacks apply more to sheep and goat flocks than to cattle herds where a test and slaughter strategy has been more effective (Kolar, 1984; Nicoletti, 1993).

Before embarking on the implementation of such a strategy it is necessary to ensure that the epidemiological situation is favourable, the necessary facilities and financial resources are available, a pool of healthy replacement animals is available and that the resources exist for continuing surveillance for a considerable period. It is also essential that full co-operation of farmers is available as slaughter of seropositive animals can be resisted by owners because of lack of clinical signs, inadequate compensation or lack of replacement animals (Nicoletti, 1993).

A brucellosis control and eradication plan based on test and slaughter strategy can be either voluntary or compulsory. Voluntary schemes, which apply to individual flocks,

may be useful in the early stages of the campaign but may need to be supported by adequate incentives such as a bonus on the sale of milk from brucellosis-free herds or per capita payments. Compulsory eradication is required in the final stages but is often advisable from the start (Joint FAO/WHO, 1986).

2.16.3 Prevention of Spread between Animals and Monitoring of Brucellosis-Free Herds and Zones

In areas where the brucellosis-free status has been established or where such a status is suspected on epidemiological grounds, the risk of importing the disease by means of animal movement must be curtailed. Movement of potentially infected animals into such areas must be prohibited or importation permitted only from certified brucellosis-free farms or areas. This applies to the transport of animals and of certain animal products within countries as well as between countries, following the general principles and procedures as specified in the International Zoo-sanitary Code of the OIE which also describes the essential testing of animals and quarantine measures.

It should be noted that there have been incidents of spread of brucellosis by serologically negative animals originating from inadequately certified and supervised sources (Joint FAO/WHO, 1986; Schnurrenberger *et al.,* 1987).

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY AREA

The study was carried out in the Small Ruminant Research Programme of a research farm in Shika-Zaria. Zaria is located in Kaduna State, within latitudes 11^0 7′, 11^0 12′N and longitude 7^0 41′ in the Northern Guinea Savannah Zone of Nigeria. Trees and grasses characterize the vegetation of this zone with an average rainfall ranging from 1000 mm to 1250 mm and temperature of 17^0C to 33^0C (Mortimore, 1970). Zaria has an estimated population of 547,000 and a growth rate of 3.5% per annum (MED, 1996). Hausa and Fulani are the major ethnic groups. The main occupation is agriculture; approximately 40-75% of the population's livelihood is from agriculture (ABU Zaria master plan, 2000). These agricultural activities include the production of both cash and food crops and animal husbandry. Animals reared in the area include cattle, sheep, goats, poultry and pigs (ABU Zaria master plan, 2000).

3.2 MANAGEMENT SYSTEM

The sheep and goats were maintained under an intensive husbandry system and fed on concentrates like corn mash and crushed millet. The animals obtained part of their roughage by grazing on grassland or paddocks fenced with barbed wire. Animals reared in the Livetock Farm are bought from livestock markets, especially from North West, North Central and parts of Western Nigeria. The female animals were allowed to graze while males were restricted to their pens and fed roughage separately and only allowed to graze separately during shortage of roughage. Ewes and does are mated by rams and bucks in the flock and herd respectively. Sheep and goats were kept in separate pens. The sheep and goats of the research farm have no history of vaccination against

brucellosis and are routinely dipped against ectoparasites and treated against endoparasites. The population of small ruminants in the research farm was about 850 as at the time of sampling.

3.3 SAMPLE SIZE

The sample size was determined using the formula as described by Thrusfield (1997).

$$n = z^2pq/d^2$$

Where:

n - minimum sample size

z - appropriate value for the standard normal deviate set at 95% confidence interval (1.96)

p - prevalence from previous studies (14.5% for sheep and 16.1% for goats) by Bertu *et al*. (2010).

q - complementary probability (1-p)

d - desired absolute precision = 0.05

n (sheep) = 190.5

n (goat) = 207.6

The minimum number of samples (n) to be collected was, 191+208 = 399, but to reduce sampling error and improve accuracy, a total of 580 samples were collected (265 from goats and 315 from sheep).

3.4 COLLECTION OF SAMPLES

Blood samples were collected from the sheep (315) and goats (265) at the Small Ruminant Research Programme of a Livestock Research farm in Zaria. Animals that were sampled were raised intensively and allowed to graze within restricted paddocks

on an improved sown pasture. The sheep and goats were selected using simple random sample without replacement until desired numbers of samples were obtained.

Ten millilitres (10 mls) of veinous blood was collected through the jugular vein using a 10ml sterile hypodermic needles and syringes. Five millilitres each was transferred into 2 clean test tubes with one containing ethylene diamine tetra acetic acid (EDTA) for culture and the other test tubes without anticoagulant to obtain serum for serology, and all the test tubes were labelled appropriately. The blood samples in test tubes without anticoagulant were allowed to clot by laying them down in a slanting position, and then transported together with blood samples containing EDTA to the Microbiology Laboratory of National Animal Production Research Institute, Shika-Zaria, in a leak proof container with ice packs. The blood samples were then centrifuged at 2002G for 5 minutes to allow for proper separation of serum from the clotted red blood cells. Serum was decanted into 5 ml plastic tubes and stored in the refrigerator at -20^0C until required for testing (Bertu *et al*., 2010).

3.5 PREPARATION OF MEDIA AND CULTURING

Culturing of the sample was performed according to the techniques described by Alton *et al*. (1988). Commercially available *Brucella* medium base (Oxoid, CM 0169) was used. Manufacturer's instruction was strictly followed thus: 17.5g of the medium base was suspended in 500ml of distilled water and boiled to dissolve completely. This solution was sterilized by autoclaving at 121^0C for 15 minutes and cooled to 50^0C. The brucella selective supplement (Oxoid, SRA0083A) containing polymixin B (2,500 IU), bacitracin (12,500 IU), cycloheximide (50mg), nalidixic acid (2.5mg), nystatin (50,000 IU) and vancomycin (10mg) were reconstituted using methanol and distilled water (1:1) to make a 10ml solution. The reconstituted brucella selective supplement (10ml) were

added to the sterile medium (500ml) and properly mixed; then 5% v/v inactivated horse serum (Oxoid, SR0035) and 1% sterile dextrose solution were added to the medium. The medium was properly mixed and dispensed into sterile plates for storage. Each sample (blood) was inoculated into a plate and incubated at 37^0C and observed for 3-7 days for brucella colonies which are circular and 2-4 mm in diameter. The plates were discarded after 7-14 days when ever growth was absent.

3.6 SEROLOGICAL TESTS

3.6.1 Rose Bengal Plate Test (RBPT)

The procedure of Alton *et al.* (1988) modified by Bale (2008) was adopted:

A drop (about 30µl) of the antigen was placed on a clean white ceramic tile and about 30µl of the serum sample was placed by the side (but not into) the antigen. The antigen and serum was mixed thoroughly with a sterile applicator stick and the ceramic tile was rocked using the hand. Mixing was done for 4 minutes and the test was read by examining for agglutination using a good light source.

The results were interpreted as follows:

Negative or no agglutination (□)

Positive for any degree of agglutination (+)

3.6.2 Serum Agglutination Test (SAT)

The method of Alton *et al*. (1988) modified by Bale (2008) was adopted:

Five test tubes per sample were set up. The first test tube contained 0.8 ml of phenol saline, while 0.5 ml was dispensed into the second, third, fourth and fifth tubes. The test serum (0.2 ml) was added to the first tube and mixed properly and a serial dilution was carried out by transferring 0.5 ml of mixture in the first test tube to the second and so on

to the last test tube. The final 0.5 ml from the fifth test tube was discarded and 0.5 ml of *Brucella abortus* antigen diluted at 1:10 with normal saline was added to each tube and mixed properly. The tubes were then incubated at 37^0C for 22 hours before the tubes were read and recorded.

Positive and negative controls were included in the test.

Reading and interpretation of test

The degree of agglutination was read by comparing the standard against a black background with a source of light (fluorescent strip light) coming from above and behind the tubes in a constructed illuminated reading box.

The results were recorded to show the degree of agglutination (numerator) and the dilution (denominator). Sera showing less than 50 i.u were negative to the test while those showing more than 50 i.u were positive to the test. The strength of the degree of clearance in each tube was represented as follows:

i. Four plus (4+): Total agglutination
ii. Three plus (3+): 75% agglutination
iii. Two plus (2+): 50% agglutination
iv. One plus (1+): 25% agglutination

3.6.3 2-Mercaptoethanol (2-ME) Test

The procedure was carried out as described by Bale (2008):

Five test tubes were set up. Each 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 ME while 0.5 ml of 0.5% saline solution was dispensed into the remaining four tubes (i.e 2^{nd} to 5^{th}). The tubes were incubated for an hour at 37 ^{0}C. 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 ^{0}C for 22 hours.

The degree of agglutination in each tube was recorded. A titre of 1/80 before and 1/10 after ME treatment is evidence that the only immunoglobulin (Ig) present was IgM. If the titre remains unchanged or increased after treatment, this indicates that the immunoglobulin present was IgG.

Brucella abortus antigen from Veterinary Laboratories Agency, Weybridge, United Kingdom was used for all serological tests.

3.7 DATA ANALYSES

All the results obtained were put into contingency tables and Statistical Package for Social Science (SPSS), version 17.0 (SPSS Chicago Inc.) was used to determine Chi-square or Fisher's exact test where appropriate. The seroprevalence of brucellosis was determined by dividing the number of seropositive cases by the total number of animals (sheep or goat) for each category and multiplying by 100. The seroprevalence was subjected to Chi-square or Fisher's exact test for statistical significance between species (caprine and ovine), age groups (<1year, 2-3 years and >3 years), sex (male and female) and serological tests performed. P values less than 0.05 ($P<0.05$) were considered to be statistically significant at 95% confidence interval.

CHAPTER 4

RESULTS

4.1 RESULTS OF ISOLATION

Of the 580 blood samples cultured, comprising of 265 from goats and 315 from sheep, no *brucella* organism was isolated.

4.2 RESULTS OF ROSE BENGAL PLATE TEST (RBPT) AND SERUM AGGLUTINATION TEST (SAT) IN GOATS AND SHEEP

Eighty nine out of the goats had antibodies to *Brucella* as measured by RBPT representing 33.58% while 106 sheep were positive representing 33.65%. P value was greater than 0.05 ($P>0.05$) indicating that the difference was statistically insignificant. Sixty eight goats out of 265 representing 25.66% were positive while 20 (6.35%) out of 315 sheep were positive by SAT. This was found to be statistically significant ($P<0.05$) (Table 4.1).

TABLE 4.1: Results of *Brucella* antibody detection by Rose Bengal Plate Test (RBPT) and Serum Agglutination Test (SAT) in goats and sheep

	RBPT		SAT*	
Species	Positive(%)	Total	Positive(%)	Total
Caprine*	89(33.58)	265	68(25.66)	265
Ovine*	106(33.65)	315	20(6.35)	315
Total	195(33.62)	580	88(15.17)	580

Caprine*- goats

Ovine*- sheep

RBPT - Chi-square = 0.00028, P value = 0.9867

SAT*- Chi-square = 41.70, P value = 0.001

4.3 RESULTS OF ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST IN GOATS BY BREED DISTRIBUTION

Eighty nine out of 248 Red Sokoto Goats representing 35.89% were positive while none of the Sahelians had antibodies to *Brucella* as measured by RBPT. The difference between the two breeds was statistically significant ($P<0.05$).

Sixty-eight out of 248 Red Sokoto Goats representing 27.42% were positive while none of the Sahelians had antibodies to *Brucella* as measured by SAT. The difference between the two breeds was statistically significant ($P<0.05$) (Table 4.2).

TABLE 4.2: Breed distribution of *Brucella* antibodies by Rose Bengal Plate Test and Serum Agglutination Test in goats

	RBPT		SAT*	
Breed	Positive(%)	Total	Positive(%)	Total
RSG*	89(35.89)	248	68(27.42)	248
Sahelian	0(17)	17	0(17)	17
Total	195(33.62)	580	88(15.17)	580

RSG* - Red Sokoto Goat

RBPT* - P value = 0.0010

SAT* - P value = 0.0082

4.4 RESULTS OF ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST IN SHEEP BY BREED DISTRIBUTION

Two sheep of the Balami breed out of 5, representing 40% were positive, 6 (25%) out of 24 Uda sheep were positive while 98 (34.27%) out of 286 Yankasa sheep were positive by RBPT. P value was greater than 0.05 indicating that breed was not a factor in brucellosis (P>0.05). None of the Balami sheep was positive, two sheep of the Uda breed out of 24, representing 8.33% were positive while 18 (6.29%) out of 286 Yankasa sheep were positive by SAT. P value was greater than 0.05 indicating that breed was not a factor in brucellosis (P>0.05) (Table 4.3).

TABLE 4.3: Breed distribution of *Brucella* antibodies by Rose Bengal Plate Test and Serum Agglutination Test in sheep

	RBPT		SAT	
Breed	Positive(%)	Total	Positive(%)	Total
Balami	2(40)	5	0	5
Uda	6(25)	24	2(8.33)	24
Yankasa	98(34.27)	286	18(6.29)	286
Total	106(33.65)	315	20(6.35)	315

RBPT - Chi-square = 0.9432, P value = 0.6240

SAT - Chi-square = 0.4994, P value = 0.7790

4.5 RESULTS OF ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST IN GOATS BY AGE DISTRIBUTION

Twenty five (29.41%) of the 80 animals which belonged to the less than one year age group (<1 year) were positive while 40 representing 31.75% out of 126 animals between 1-3 years were positive and 24 (44.44%) out of 54 animals which were above three years (>3 years) had antibodies to *Brucella* as measured by RBPT. The differences between various age groups were statistically insignificant (P>0.05). Twenty five out of the 85 goats less than one year (<1 year) were positive representing 29.41%, 33 (26.19%) of goats between 1-3 years were positive while 10 (18.52%) goats greater than three (>3 years) had antibodies to *Brucella* as measured by SAT. This was statistically insignificant (P>0.05) (Table 4.4).

TABLE 4.4: Results of Rose Bengal Plate Test and Serum Agglutination Test in Goats by age distribution

	RBPT		SAT	
Age group	Positive(%)	Total	Positive(%)	Total
<1 year	25(29.41)	85	25(29.41)	85
1-3 years	40(31.75)	126	33(26.19)	126
>3 years	24(44.44)	54	10(18.52)	54
Total	89(33.58)	265	68(25.66)	265

RBPT - Chi-square = 0.1297, P value = 0.7187

SAT - Chi-square = 0.2642, P value = 0.6072

4.6 RESULTS OF ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST IN SHEEP BY AGE DISTRIBUTION

Twenty (44.44%) out of the 45 animals were positive, 42 (35.59%) of the sheep between 1-3 years were positive while 44 sheep greater than three years (>3 years) out of 152 representing 28.9% had antibodies to *Brucella* as measured by RBPT. This was not statistically significant (P>0.05). Two out of 45 animals belonging to the less than one year (<1 year) age group representing (4.44%) were positive, 5 (4.24%) of animals between 1-3 years while 13 (8.55%) of animals greater than three years (>3 years) had antibodies to *Brucella* as measured by SAT. This was not statistically significant (P>0.05) (Table 4.5).

TABLE 4.5: Results of Rose Bengal Plate Test and Serum Agglutination Test in Sheep by age distribution

	RBPT		SAT	
Age group	Positive(%)	Total	Positive(%)	Total
<1 year	20(44.44)	45	2(4.44)	45
1-3 years	42(35.59)	118	5(4.24)	118
>3 years	44(28.95)	152	13(8.55)	152
Total	106(33.65)	315	20(6.35)	315

RBPT - Chi-square = 1.083, P value = 0.2981

SAT - Chi-square = 2.401, P value= 0.3011

4.7 RESULTS OF ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST IN GOATS BY SEX DISTRIBUTION

Seventy seven out of the 211 female goats representing 36.49% were positive while 12 (22.22%) out of 54 male goats had antibodies to *Brucella* as measured by RBPT. The difference between the variables was statistically significant (P<0.05). Fifty two out of 211 female goats i.e 24.64% were positive while 16 (29.63%) out of 54 male goats had antibodies to *Brucella* as measured by SAT. This was statistically insignificant (P>0.05) (Table 4.6).

TABLE 4.6: Results of Rose Bengal Plate Test and Serum Agglutination Test in Goats by sex distribution

	RBPT*		SAT	
Sex	Positive(%)	Total	Positive(%)	Total
Female	77(36.49)	211	52(24.64)	211
Male	12(22.22)	54	16(29.63)	54
Total	195(33.62)	265	68(25.66)	265

RBPT - Chi-square = 3.926, P value = 0.0476*

SAT - Chi-square = 0.5601, P value = 0.4542

4.8 RESULTS OF ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST IN SHEEP BY SEX DISTRIBUTION

Eighty one out of the 238 females representing 34.03% were positive while 25 (32.47%) out of 77 male sheep had antibodies to *Brucella* as measured by RBPT. This was not statistically significant (P>0.05). Thirteen out of 238 female sheep representing 5.46% were positive while 7 (9.09%) out of 77 male sheep had antibodies to *Brucella* as measured by SAT. This was not statistically significant (P>0.05) (Table 4.7).

TABLE 4.7: Results of Rose Bengal Plate Test and Serum Agglutination Test in Sheep by sex distribution

	RBPT		SAT	
Sex	Positive(%)	Total	Positive(%)	Total
Female	81(34.03)	238	13(5.46)	238
Male	25(32.47)	77	7(9.09)	77
Total	106(33.65)	315	20(6.35)	265

RBPT - Chi-square = 0.06391, P value = 0.8004

SAT - Chi-square =1.288, P value = 0.2564

4.9 COMPARISON BETWEEN ROSE BENGAL PLATE TEST AND SERUM AGGLUTINATION TEST

One hundred and ninety five out of 580 representing 33.62% were positive by RBPT while 88 (15.17%) out of 580 were positive by SAT. This difference was statistically significant ($P<0.05$).

TABLE 4.8: Comparison between RBPT and SAT

			Test	
Positive (%)	Negative (%)	Total		
		RBPT	195	
(33.62%)	385 (66.38%)	580	SAT	88
(15.17%)	492 (84.83%)	580		

Chi-square =53.51, P value = 0.0001

4.10 RESULTS OF 2-MERCAPTOETHANOL (2-ME) TEST IN GOATS AND SHEEP SHOWING DISTRIBUTION OF IMMUNOGLOBULIN

2-Mercaptoethanol test detected immuglobulin M (IgM) in sixty eight goats representing 25.66% while twenty sheep i.e 6.35% had IgM (Table 4.9).

TABLE 4.9: Results of 2-Mercaptoethanol (2-ME) Test in goats and sheep showing distribution of Immunoglobulin

Immunoglobulin type	Goats	Sheep	Total
IgM (%)	68 (25.66)	20 (6.35)	88(15.17)

HAPTER 5

DISCUSSION

5.1 DISCUSSION

The diagnosis of brucellosis is confirmed directly by isolation of the *Brucella* organism, mostly from culture, or indirectly by the detection of immune response against its antigens. Although, 580 blood samples were cultured, there was no isolation of any *Brucella* organisms. This may be attributed to the fact that the diagnosis of brucellosis based exclusively upon *Brucella* isolation presents several drawbacks (Orduña *et al.,* 2000). The slow growth of *Brucella* in primary cultures means that diagnosis may take more than 7 days (Ariza, 1996; Yagupsky, 1999). Besides, blood culture sensitivity is often low, ranging from 50-90% depending on disease stage, *Brucella* species, culture medium, number of circulating bacteria and the culture technique employed (Gotuzzo *et al.,* 1986, Yagupsky *et al.,* 1999). *Brucella* is a facultative intracellular organism which is capable of multiplication and survives within host phagocytes. The organisms are phagocytosed by polymorphonuclear leukocytes in which some survive and multiply and are transported to lymphoid tissue and foetal placenta. The inability of the leukocytes to effectively kill virulent *Brucella* at the primary site of infection is a key factor in the dissemination to regional lymph nodes, mononuclear phagocytic system, joints and organs such as the uterus, testes and udder. The ability to survive within macrophages and leukocytes enables the organism to be protected from humoral and cellular bactericidal mechanisms during the periods of haematogenous spread (Nielsen and Duncan, 1990). The localization of brucellae in these tissues and organs reduces the number of circulating organisms in blood and thereby makes isolation of brucellae from blood very difficult. While culturing is a specific method, its sensitivity depends on the viability and numbers of *Brucella* within the sample, the nature of sample (foetal organs, foetal membranes, lymph nodes, etc.) and the number of specimens examined

from the same animal (Hornitzky and Searson, 1986). The time required for culturing field specimens can be long and tissues or fluids that are only contaminated with a low number of *Brucella* may not be detected.

Onoja *et al*. (2008) could not isolate any organisms from a flock of sheep in Zaria which had a history of 2 recent abortions even with the high serological prevalence rate of 76% by Rose Bengal Plate Test (RBPT) and Serum Agglutination Test (SAT). They collected samples from 3 cases of carpal hygroma and from vaginal swabs.

There are several reports on isolation of brucellae in sheep and goats in Nigeria by Okoh (1980), Bale *et al*. (2003) and Ocholi *et al*. (2005). Okoh (1980) isolated five brucellae organisms from 22 milk samples of ewes in a Sheep Breeding Centre in Kano State of Nigeria where there was history of abortion storm and death of neonate lambs. The *Brucella* species isolated was *Brucella abortus.* Bale *et al*. (2003) isolated ten organisms from 277 milk samples in sheep and 141 goat milk samples from government farms in different parts of northern Nigeria, four brucellae were isolated from sheep and six from goats. The isolates were biotyped and identified as rough strains of *Brucella melitensis.* Ocholi *et al*. (2005) isolated seven brucellae in a flock of Yankasa sheep in a privately owned farm in Toro near Bauchi, Nigeria where there was report of abortion. They collected milk samples and vaginal swabs and isolated three brucellae from milk samples and four from vaginal swabs from aborting ewes. All the samples were identified and biotyped as *Brucella abortus* biovar 1.

The inability to isolate brucellae organisms in this study does not rule out the presence of brucellosis in this farm as difficulties and constraints earlier stated involved in the isolation of this organism may be responsible.

The distribution of RBPT in ovine species (sheep) and caprine species (goats) showed that there was a higher prevalence in sheep (33.58%) than in goats (33.65%) though it was not statistically significant ($P>0.05$). The higher prevalence in sheep may be due to the fact that

more samples were collected from sheep (315) than in goats (265). This higher prevalence in sheep than in goats, is similar to the findings of Junaidu *et al* (2006) where they carried out a serological survey in Sokoto City Abbatoir and reported a prevalence of 23.61% by RBPT in sheep and a lower prevalence in (22.93%) goats in a separate study which was carried out in the same Sokoto Metropolitan Abbatoir (Junaidu *et al*., 2010). However, lower prevalences have been reported by other investigators. Bale *et al* (1982) reported a prevalence of 14.1% and 16.1% in sheep and goats, respectively by RBPT in a serological study of sheep and goats brucellosis in some parts of northern Nigeria. Junaidu *et al.* (2008) in their study carried out in a Prison Farm in Sokoto, Nigeria, reported a prevalence of 22.36% in sheep and 30.97% in goats using RBPT. Bertu *et al.* (2010) reported a prevalence of 9.3% in sheep and 10.1% in goats using the RBPT in a study conducted in Plateau State. In a serological survey of brucellosis in livestock animals and workers in Ibadan, Nigeria, Cadmus *et al* (2006), reported prevalence in goats to be 0.86% but none of the sheep sampled was positive to RBPT. It is pertinent to note that the margin between the differences in prevalence rates in sheep and goats using RBPT is small and this indicates that there is no much preference to infection to brucellosis in small ruminants.

The results of SAT by species revealed that goats (caprine species) had a higher prevalence rate (25.66%) than in ovine species or sheep (6.35%). This was found to be statistically significant ($P<0.05$). This significant difference may be attributed to the fact that abortion storms and reduction in fertility rates were first observed in the goat pens after introduction of new animals; moreso there was isolation of brucella from an aborted foetus in one of the goats prior to the beginning of this study. The higher prevalence seen in goats than in sheep may also be due to the nature of vaginal secretion in goats, Anon (2001) reported that goats have a more copious vaginal discharge than sheep and the excretions from the vagina in goats is prolonged than in cows and ewes and lasts for at least 2-3 months. In sheep, excretion is

generally less prolonged, usually ceasing within 3 weeks after abortion or a full-term parturition. The above finding of a higher prevalence rate by SAT in goats than in sheep is similar to the result of Bale *et al*. (1982) where they reported a seroprevalence rate of 1.63% in goats and 0.23% in sheep.

The breed distribution of RBPT in goats showed that Red Sokoto goats had a higher prevalence (35.89%) than sahelians where all were negative and the difference was statistically significant ($P<0.05$). Brucellosis is not breed-specific (Ajogi *et al.,* 2002). The difference may be due to a lower number of sahelian goats sampled (17) compared to the Red Sokoto goat (248). This was similar to the breed distribution of SAT in goats where Red Sokoto goats had a higher prevalence (27.42%) than sahelians (0%) and the difference was statistically significant ($P<0.05$). The above finding is similar to that of Junaidu *et al.* (2010) where they recorded prevalence rates of 11.83% and 11.65% in red sokoto goats and sahelian respectively using SAT.

The results of RBPT in goats showing prevalence by age indicated that goats belonging to the oldest age group (>3 years) had the highest prevalence rate (44.44%), followed by those between 1-3 years (31.75%), while the least was recorded in animals less than one year (<1 year) with a prevalence of 29.41%. Brucellosis is particularly a disease of sexually mature animals, young animals may be infected but do not show any clinical sign and generally show a weak and transient serological response (Anon, 2001). This result is similar to the work of Junaidu *et al.* (2010) which reported the highest prevalence rate in the oldest group of animals (above 24 months) in the study in which they grouped sampled animals into three age groups (0-12 months, 13-24 months and above 24 months) respectively.

The result of SAT in goats by age showed that animals less than one year (<1 year) had the highest prevalence rate of 29.41% followed by goats between 1-3 years with 26.19%, while goats greater than three years (>3 years) had the lowest prevalence rate of 18.52%. The

differences between these values were not statistically significant (P>0.05). The detection of the highest antibodies in animals less than one year may be attributed to the uptake of antibodies in milk during suckling; also these young animals may have been exposed to other diseases of neonates whose causative agents share similar lipopolysaccharide (LPS) as that of brucellae, example, colibacillosis and salmonellosis. The LPS of *Brucella* are similar to that of *Escherichia coli, Salmonella spp, Yersinia enterocolitica 0:9* etc. This finding of the presence of highest antibody titres in the youngest group of goats (<1 year) is similar to the study by Junaidu *et al.* (2010) where they reported the highest seroprevalence of 18.45% in the youngest group of goats (0-12 months).

The age distribution of RBPT in sheep showed that the highest prevalence rate was recorded in animals less than one year (<1 year) (44.44%) followed by animals between 1 and 2 years (35.59%), while the least was recorded in animals greater than three years (>3 years). Young animals (sexually immature) do not favour the proliferation of brucellae organisms but the presence of high antibody titres suggests that these young animals might have gotten these antibodies while suckling as infants as brucellae organisms can localize in the mammary gland. The localization of brucellae organisms in the mammary gland leads to intermittent shedding of the organisms in the milk in succeeding lactations (Alton, 1990). Junaidu *et al.* (2006) reported the lowest prevalence in the oldest group (>24 months) of sheep (19.58%), they grouped the animals into three (0-12 months, 12-24 months and above 24 months respectively).

The results of SAT in sheep by age indicated the highest prevalence in animals greater than three years (>3 years) with a seroprevalence of 8.55% followed by animals less than one year (<1 year) with a prevalence of 4.4% and the least was reported in animals between 1-2 years (4.24%). The difference was statistically insignificant (P>0.05). The reason for the above finding may be due to already established findings that susceptibility to brucella infection is

more in sexually matured animals than the young. The susceptibility of livestock to *Brucella* infection is influenced by the age (young animals are less susceptible to *Brucella* than older animals) and sexually mature, pregnant animals are more susceptible to infection with the organism than sexually immature animals since their reproductive system is not active (Nicolleti, 1980).

The result of RBPT in sheep and goat by sex indicated that prevalence rates were higher in females than males in both species. The female goats (does) had a higher prevalence of 36.44% than male goats (bucks) (22.22%). This was statistically significant ($P<0.05$). Similarly, a higher prevalence rate was recorded in female sheep (34.03%) than in the males; though this was not statistically significant ($P>0.05$). This is similar to the findings of Onoja *et al.* (2008), where they reported a prevalence rate of 0.85% in rams and 69.2% in ewes in a study carried out in a flock in Zaria. Though brucellosis is known to be neither breed nor sex specific (Ajogi *et al.,* 2002), the detection of higher antibody titres in female animals than in males for both sheep and goats using the RBPT suggests the presence of suitable factors such as erythritol, which aid the growth of brucellae organisms. Erythritol, a sugar alcohol synthsesized in the ungulate placenta and stimulates the growth of virulent strains of brucellae organisms, has been credited with the preferential localization of the bacteria within the placenta of ruminants (Smith *et al.,* 1962).

The sex distribution of SAT in goats and sheep showed that males had a higher prevalence than females. The male goats had a prevalence of 29.63%, while the female goats had 24.64%, the same trend was observed in the sheep where the males had a higher prevalence of 9.09% than females (5.46%). The differences between the prevalences in males and females in both sheep and goats were not statistically significant as P values were greater than 0.05. Brucellosis is known to be neither breed nor sex specific (Ajogi *et al.,* 2002). The detection of higher prevalence rate in males may be attributed to the type of management

system. The bucks and rams are kept in separate pens and are allowed to mate the does and ewes only during breeding as they do not graze on the same pasture. It is possible that the bucks and rams were infected during mating and since they are fewer in number than the does and ewes, the possibility of repeated mating by a buck or ram increases the probability of dissemination or spread of brucellae organisms among the males than the females. The does have not been bred since the isolation of brucellae was done prior to this study. Adamu *et al.* (2012) reported a higher seroprevalence in male than female goats in a study they carried out on farmer awareness on caprine abortion and the presence of *Brucella abortus* and *Brucella melitensis* in selected flocks in an arid zone of Nigeria. Many studies and previous works share contrary reports to the above finding (Onoja *et al.,* 2008; Junaidu *et al.,* 2010).

The comparison between results of RBPT and SAT showed that a higher percentage of positive animals (33.62%) were recorded in RBPT than in SAT (15.17%). This disparity may be due to the higher sensitivity of RBPT than SAT. It is generally agreed that RBPT is a screening test which is highly sensitive but heterospecific (Olascoaga, 1976). Infection due to organisms, such as *Vibrio cholera, Yersinia enteroclitica* 0:9, *Pasteurella spp, Salmonella* or some other members of the Brucellaceae family could give false positive results than SAT and CFT (Bale, 1982). Olascoaga (1976) reported that the RBPT measures IgG_1 while SAT measures predominantly IgM and little of IgG. This probably shows that the predominant immunoglobulin is IgG because of more positive results in RBPT as SAT may be negative in chronic infections because IgM was no longer present.

CHAPTER 6

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

This study revealed that no *Brucella* species were isolated from 315 blood samples in sheep and 265 blood samples in goats in the Livestock Research farm in Zaria, Nigeria. A total of 580 (315 for sheep and 265 for goats) sera were tested for *Brucella* antibodies using Rose Bengal Plate Test (RBPT), Serum Agglutination Test (SAT) and 2-Mercaptoethanol (2-ME) test. Out of the 315 sheep sera tested, 106(33.65%), 20(6.35%) and 20(6.35%) were positive reactors to *Brucella* antibodies using RBPT, SAT and 2-ME test respectively while out of the 265 goat sera tested, 89(33.58%), 68(25.66%) and 68(25.66%) were positive reactors to *Brucella* antibodies using RBPT, SAT and 2-ME test respectively.

There was significant association ($P<0.05$) between the presence of antibodies to *Brucella* in female than male goats as detected by RBPT. Red Sokoto goats were the majority of the breed sampled for goats (248) compared to the sahelian (17) and showed significant association ($P<0.05$) using the SAT.

6.2 CONCLUSIONS

The following conclusions were drawn from this study:

1. No *Brucella* organism was isolated from blood of sheep and goats.
2. Goats had higher seroprevalence rate (25.66%) than sheep (6.35%) using serum agglutination test (SAT).
3. Female goats had higher seroprevalence (36.49%) than males (22.22%) as measured by Rose Bengal Plate test.
4. Rose bengal plate test appeared to be more sensitive than SAT as overall prevalence rates were 33.62% and 15.17% for RBPT and SAT respectively.

6.2 RECOMMENDATIONS

Recommendations drawn from this study are aimed at ensuring that safe and healthy animals are sold or distributed to other farms and breeders thereby reducing disease outbreaks in animals and humans. The following were recommended:

1. Attempts should be made to isolate *Brucella* from other samples like milk, hygroma fluids, vaginal secretions and semen from animals on the farm.
2. The Veterinarians should enlighten and educate herdsmen and other animal handlers on the zoonotic implication of brucellosis in the farm.
3. New animals should be screened for brucellosis before introduction into the herd or flock.
4. The farm should be encouraged to use Rose Bengal Plate Test as a screening test for brucellosis in the farm.

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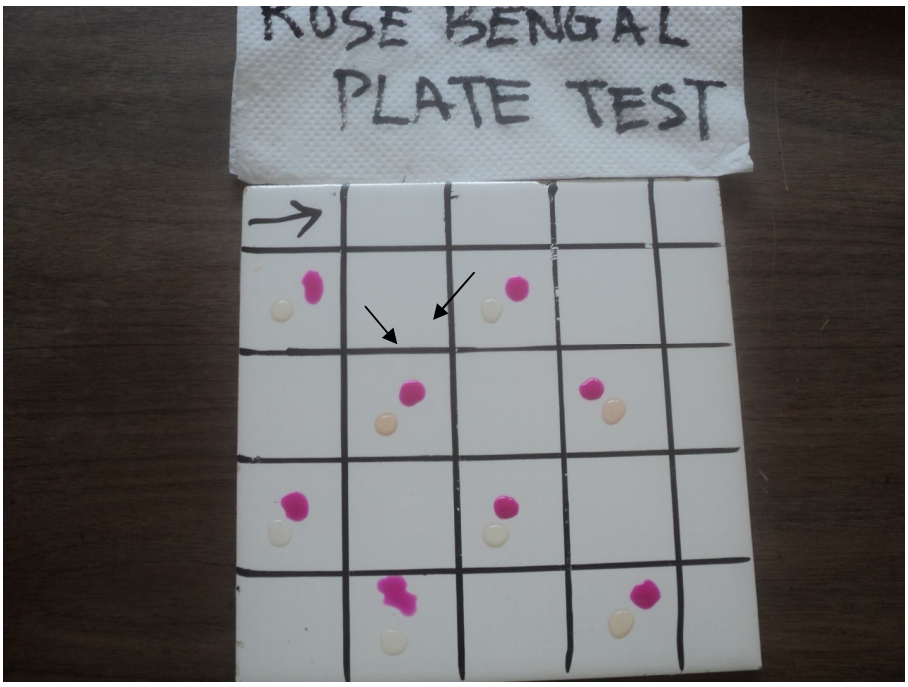
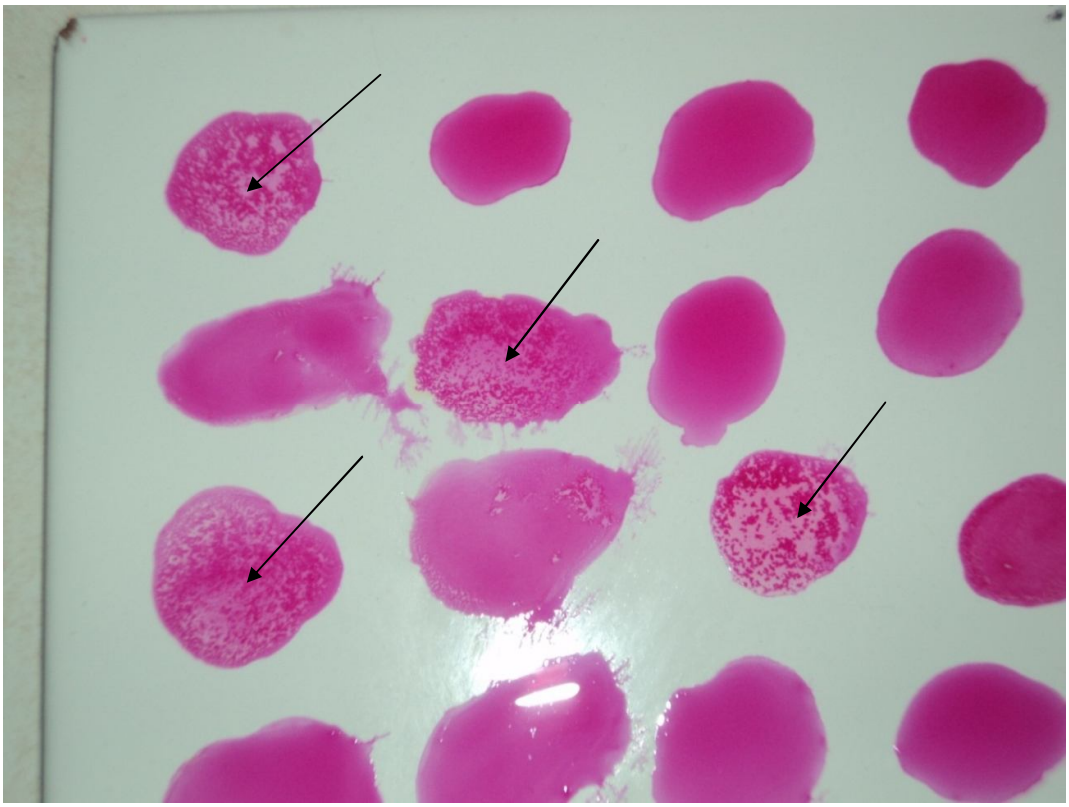
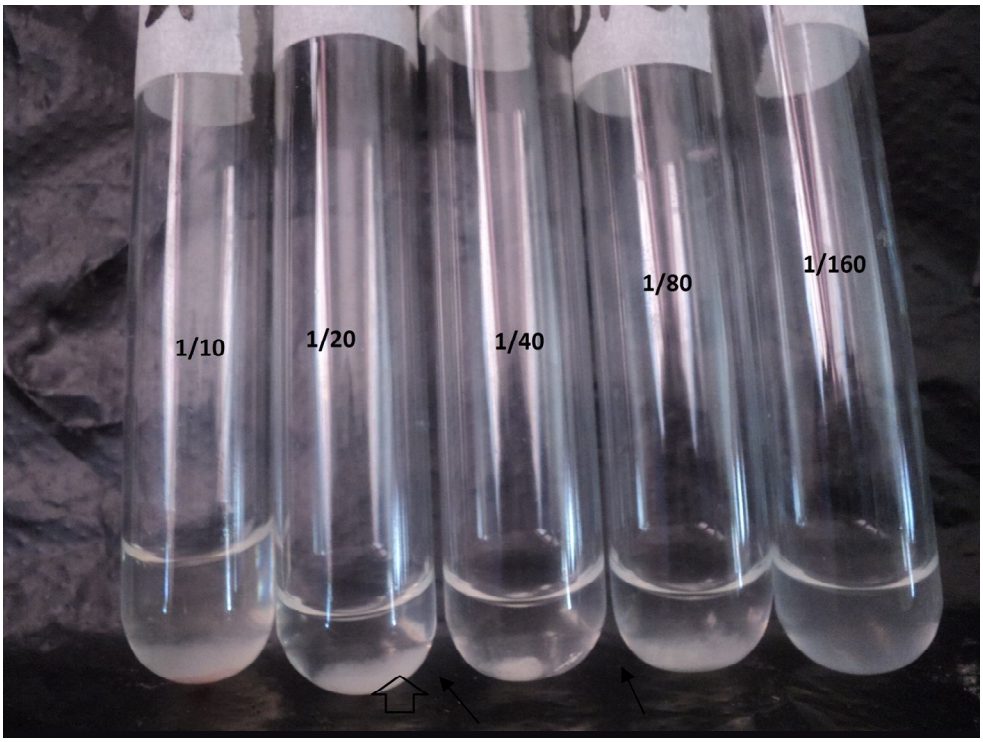
APPENDIXES



Appendix I: Rose Bengal Plate Test Before Mixing – Antigen (Pink), Test Serum (Gray)



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



Appendix III: Serum agglutination test (Agglutination shown by arrow).