PHENOTYPIC AND BIOLOGICAL CHARACTERIZATION OF *BRUCELLA* STRAINS ISOLATED FROM NIGERIAN LIVESTOCK

BY

REUBEN ADAMA OCHOLI

May, 2005

PHENOTYPIC AND BIOLOGICAL CHARACTERIZATION OF *BRUCELLA* STRAINS ISOLATED FROM NIGERIAN LIVESTOCK

BY

REUBEN ADAMA OCHOLI

Doctor of Veterinary Medicine (DVM), Ahmadu Bello University, Zaria, 1984

Master of Science (MSc.) Ahmadu Bello University, Zaria, 1991

A Dissertation Submitted to the

Ahmadu Bello University, Zaria

in Partial Fulfillment for the Degree of

Doctor of Philosophy (Ph. D)

Department of Veterinary Public Health and Preventive Medicine,

Faculty of Veterinary Medicine,

Ahmadu Bello University,

Zaria, Nigeria.

May, 2005

DECLARATION

I hereby declare that the work described in this thesis was carried out by me in the Brucellosis Research Unit, Bacterial Research Department, National Veterinary Research Institute, Vom, and in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervision of Professor J. K. P. Kwaga, Dr. I. Ajogi and Dr. J. O. O. Bale.

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration. The work of other investigators is duly acknowledged and referred to in the text.

The thesis has not been submitted in part or in full to any other University for a Degree or Diploma

_____ _____

Ahmadu Bello University Reuben Adama Ocholi
May, 2005

CERTIFICATION

This dissertation entitled "Phenotypic and Biological Characterization of *Brucella* Strains Isolated from Nigerian Livestock" by Reuben Adama Ocholi meets the regulations governing the award of the Degree of Doctor of Philosophy of Ahmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

_____ Date: _____
Professor J. K. P. Kwaga
Chairman, Supervisory Committee.
Department of Veterinary Public Health
and Preventive Medicine,
Ahmadu Bello University, Zaria.

_____ Date: _____
Dr. I. Ajogi
Member, Supervisory Committee.
Department of Veterinary Public
Health and Preventive Medicine,
Ahmadu Bello University, Zaria.

_____ Date: _____
Dr. J. O. O. Bale.
Member, Supervisory Committee.
Animal Production Research Project
National Animal Production Research
Institute, Shika,
Ahmadu Bello University, Zaria.

_____ Date: _____
Dr. I. Ajogi
Head of Department
Veterinary Public Health and Preventive Medicicne
Ahmadu Bello University, Zaria

_____ Date: _____
Prof. J. U. Umoh
The Dean,
Post-Graduate School
Ahmadu Bello University, Zaria

ACKNOWLEDGMENT

First and foremost, I wish to thank God the Almighty for giving me life, ability, guidance and protection to endure and complete this project.

I am highly grateful to my Supervisors, Professor J. K. P. Kwaga, Dr. I. Ajogi and Dr. J. O. O. Bale for their inspiration, continuous encouragement and persistent push that made it possible for me to bring this study to a successful completion.

I wish to thank the Director, National Veterinary Research Institute (NVRI), Vom, for the financial support provided for this study programme. My sincere appreciation goes to Dr. (Mrs.) L. H. Lombin, the Project Officer of the NVRI- National Agricultural Research Project (NARP) activities for her support.

The biotyping of some of the isolates of *Brucella* was carried out at the FAO/WHO collaborating Centre for Brucellosis Reference and Research, Veterinary Laboratories Agency, Weybridge, UK. I obtained valuable current literature on brucellosis from the Library and familiarized myself with most recent brucellosis techniques. My stay was under the supervision of Mrs. J. A. Stack and Mrs. L. L. Perrett. To them I am grateful. I also appreciate them greatly for supplying the monospecific antisera and brucellaphages used for the biotyping of the rest of *Brucella* isolates. Iam highly indebted to the Head of the Centre, Dr. A. P. Macmillan, for his understanding and cooperation, and for approving the materials and reagents given me. The cooperation and support received from the entire Staff of the Centre and the Library during my attachment period there is highly appreciated. The funding for this aspect of the work was through an International Atomic Energy Agency (IAEA) training Fellowship in the use of Nuclear Techniques in the Diagnosis of Animal Diseases. I am grateful to the IAEA for the support and to Dr. K. A. Majiyagbe, the Project Contract Officer who assisted in securing the funding.

I wish to express my profound gratitude to Dr. C. Nwosuh, Head of the Viral Vaccine Production Department of NVRI for providing the necessary chemicals and reagents, and for permitting me the use of the facilities for Freeze- drying the isolates of *Brucella*. To the staff of the Freeze-Drying Section, I am highly grateful. Iam highly grateful to Dr. T. M. Joannis for providing some of the antibiotics used for the work.

I am grateful to a friend, Dr. D. A. Y. Adawa and his wife for their encouragement and support, and their hospitality any time I was in Zaria.

I most sincerely acknowledge the moral and encouraging support of my darling

wife, Helen and Children, Joshua and Emmanuel. Their patience particularly at the time I was away from home in the process of completing this work, is tremendous and worthy of note.

I wish to express my top appreciation to the Staff of the Brucellosis Research Unit, Dr. W. J. Bertu, Mr. L. Badung and Mrs. M. Dalyop for their technical support in carrying out the biological study aspect of the work.

To all who have contributed in the various ways towards the success of this study and have not been specifically mentioned, please accept my sincere gratitude.

ABSTRACT

In this study, bacteriological examination for the isolation of brucellae was carried out in order to obtain the species and biovars. A total of 1,020 clinical samples consisting of 283 milk, 10 hygroma, 581 vaginal swabs, 20 aborted fetuses, and 126 blood samples obtained from cattle, sheep, goats, pigs, dogs and horses drawn from Plateau, Kaduna, Kano, Borno, Kogi, Nassarawa, Adamawa, Taraba, Bauchi and Enugu States of Nigeria were investigated. The culturing of the samples, and identification and biotyping of the isolates were done according to standard bacteriological techniques.

A total of 32 stored cultures of *Brucella* isolated from Nigerian cattle in previous works between 1975 and 1982 whose identity were unknown were reactivated and re-biotyped.

To determine if the field strains of *Brucella* isolated in the present work were virulent or attenuated in animals, the immune and pathological responses of BALB/c mice inoculated with *B. abortus* strains MK5, SH_3 and HS_2 were compared with that of *B. abortus* S19 (vaccine strain) and *B. abortus* strain w.544 (WHO pathogenic reference strain).

Twenty five strains of *Brucella* were isolated, identified and biotyped. The isolates were designated according to the laboratory identification number of the samples from which the isolates were obtained. All the isolates belonged to one species and biovar, *Brucella abortus* biovar 1. Most of the isolates did not require carbon dioxide (CO_2) for growth even on initial isolation. The isolates had identical reactions in the various tests. All the isolates produced colonies with smooth shiny surface. Of the tests used to differentiate species and biovars, all the isolates showed the following common characteristics: produced hydrogen sulphide (H_2S), grew in the presence of basic fuchsin

(20ug ml^{-1}), but not in the presence of thionin (20ug ml^{-1}). In slide agglutination test, all the isolates were agglutinated by the monospecific antisera A, and all the isolates were lysed by Wb, Tb and Bk_2 phages.

Successful isolation of *Brucella* was made only from clinical samples obtained from cattle, sheep and horses. Out of the 25 isolates, 17 (68%) were from cattle, 5 (20%) from sheep and 3 (12%) from horses. Of the isolation from clinical samples, 12 (48%) were from milk samples, 6 (24%) from hygroma fluids, 5 (20%) from vaginal swabs and 2 (8%) from aborted fetuses. Isolations were made from samples from Plateau, Taraba, Adamawa, Bauchi, Nassarawa and Sokoto States.

All the stored isolates belonged to one species, *Brucella abortus*. Out of the 32 strains, 31(96.9%) were of biovar 1 while only 1 (3.1%) was of biovar 2.

Following intraperitoneal injection of mice, *B. abortus* S19, and w.544, MK5, SH_3 and HS_2 could be detected in the blood stream of infected mice as from 4 days post inoculation (p.i.) to 21^{st}, 24^{th}, 70^{th}, 42^{nd} and 42^{nd} days p.i. for the different strains respectively. The splenic colony forming units (CFU) of *B. abortus* peaked at 7days p. i. for S19, 14 days for w.544 and HS_2 and 21days for MK5 and SH_3. The *B. abortus* S19 was cleared from the spleen as at 12 weeks p.i. while the w.544, MK5, SH_3 and HS_2 persisted in the spleen for at least 20 weeks p.i. The splenic/body weight ratio peaked at 7 days p.i. for S19 and SH3, 14 days for w.544 and HS_2, and 21 days for MK5. Sera obtained from mice infected with all strains of *B. abortus* contained detectable antibodies to *Brucella* as from 3 weeks p.i. Mice infected with the field strains of *B. abortus* induced significant production of antibodies comparable to S19 and w.544-infected mice. All the strains of *B. abortus* induced production and persistence of antibodies for at least 20 weeks p.i. Small germinal centers and lymphoid depletion occurred in spleens of

mice during the first week of infection with all strains of *B. abortus*. Large germinal centers and disappearance of the macrophage accumulations in the spleen did not appear until 10 weeks after infection of mice with S19 and until 20 weeks after infection of mice with w.544, MK5, SH_3 and HS_2.

The epidemiological significance of these findings is discussed. Some observations on the zoonotic and public health implications of *Brucella* infections in Nigerian livestock are presented. A control programme involving improved management, animal movement restrictions, public health education and mass vaccination of animals is suggested.

These results indicate that the field strains of *B. abortus* are virulent and cannot be used for testing as vaccine candidates.

More strains would have been obtained if molecular techniques for detection and typing were used.

TABLE OF CONTENTS

TITLE	PAGE
TITLE PAGE	ii
DECLARATION	iii
CERTIFICATION	iv
ACKNOWLEDGEMENT	v
ABSTRACT	vii
TABLE OF CONTENTS	x
LIST OF TABLES	xvii
LIST OF FIGURES	xviii
CHAPTER ONE: INTRODUCTION	1
1.1 Statement of the Problem	2
1.2 Justification	3
1.3 Objectives of the Study	4
CHAPTER TWO: LITERATURE REVIEW	6
2.1 Historical Notes and Nomenclature	6
2.2 Description of the Genus *Brucella*	7
2.2.1 Nutritional Requirements	7
2.2.2 Gaseous Requirements	8
2.2.3 pH Requirements	8
2.2.4 Temperature Requirements	8
2.2.5 Colonial Morphology	9
2.2.6 Staining Reactions	9
2.2.7 Dissociation Property	9
2.2.8 Biochemical Reactions	10

2.2.9 Antigenic Characteristics 10
2.2.10 Susceptibility to Phages 11
2.2.11 Susceptibility to Dyes 11
2.2.12 Susceptibility to Antibiotics 12
2.2.13 *Brucella* Evolution 12
2.2.14 *Brucella* Taxonomy 14
2.2.15 *Brucella* Genome Sequencing 17
2.3 Epidemiology of Brucellosis 18
2.3.1 Geographical Distribution 18
2.3.2 Host Preference 19
2.3.3 Dissemination of *Brucella* 19
2.3.4 Modes of Infection 20
2.3.5 Survival of *Brucella* in the Environment 21
2.3.6 Decontamination of Environment/Materials Exposed to *Brucella* 23
2.3.7 Resistance to Infection 23
2.4 Pathogenesis 24
2.5 Immune Response 26
2.5.1 Humoral Immunity 27
2.5.2 Cell-Mediated Immunity 28
2.6 Clinical Signs 29
2.7 Pathology 30
2.7.1 Gross Pathology 30
2.7.2 Microscopic Lesions 30
2.8 Diagnosis 31
2.8.1 Clinical Signs 31

2.8.2 Direct Smear 31
2.8.3 Cultural Isolation, Identification and Typing 31
2.8.4 Molecular Detection and Typing 34
2.8.5 Animal Inoculation 36
2.8.6 Serological Diagnosis 36
2.8.6.1 Milk Ring Test 37
2.8.6.2 Rose Bengal Plate Test 37
2.8.6.3 Serum Agglutination Test 38
2.8.6.4 Complement Fixation Test 38
2.8.6.5 Anti-globulin (Combs) Test 39
2.8.6.6 Other Conventional Tests 39
2.8.6.7 Enzyme-Immunoassay (EIA) Methods 39
2.8.6.8 Skin-Delayed-Type Hypersensisitivity Test (SDTH) 40
2.9 Public Health Importance 40
2.10 Economic Importance 42
2.11 Prevention and Control 44
2.11.1 Use of Vaccines 46
2.11.1.1 *B. abortus* Strain 19: 47
2.11.1.2 *B. melitensis* Rev. 1: 48
2.11.1.3 *B. abortus* Strain 45/20 49
2.11.1.4 *B. abortus* Strain RB51 50
2.11.1.5 *B. suis* Strain 2 51
2.11.1.6 *B. melitensis* H38 51
2.11.1.7 *B. melitensis* Strain M111 51
2.11.1.8 *B. abortus* Strain VTRA1, *B. melitensis* Strain VTRM1 and *B. suis* Strain VTRS1 52

2.11.1.9 *B. abortus* Strain *rfb*K 53
2.11.1.10 Novel Vaccines 53
2.11.2 Test-and-Slaughter 53
2.11.3 Prevention of Spread between Animals 54
2.11.4 Prevention of Human Brucellosis 55
2.12 Treatment 56
2.13 Brucellosis in Nigeria 57
2.13.1 Overview 57
2.13.2 Epidemiology 58
2.13.2.1 Bovine Brucellosis 58
2.13.2.2 Ovine and Caprine Brucellosis 59
2.13.2.3 Canine Brucellosis 59
2.13.2.4 Porcine Brucellosis 60
2.13.2.5 Equine Brucellosis 60
2.13.2.6 Brucellosis in Camels 61
2.13.2.7 Human Brucellosis 61
2.13.3 Problems of Brucellosis Control 61
2.13.4 Prospects of Brucellosis Control 63
CHAPTER THREE: MATERIALS AND METHODS 66
3.1 Isolation of Brucellae from the Nigerian Livestock 66
3.1.1 Purpose 66
3.1.2 Vaginal Swabs Sticks: 66
3.1.3 Collection of Samples 66
3.1.3.1 Aborted Fetuses 66
3.1.3.2 Milk Samples 66

3.1.3.3 Hygroma Fluids 67
3.1.3.4 Vaginal Swabs 67
3.1.3.5 Blood 67
3.1.4 Culturing of the Collected Samples for Brucellae 67
3.1.4.1 Aborted Fetuses and Aborted Materials 67
3.1.4.2 Milk Samples 68
3.1.4.3 Hygroma Fluids 68
3.1.4.4 Vaginal Swabs 68
3.1.4.5 Blood 68
3.1.5 Incubation of Culture Plates 68
3.1.6 Direct Observation of Colonies 69
3.1.7 Identification of Brucellae 69
3.2 Biotyping of the *Brucella* Strains Isolated From Nigerian Livestock 69
3.2.1 Purpose 69
3.2.2 Chemical Reagents 69
3.2.3 *Brucella* Reference Strains 69
3.2.4 Brucellaphages 69
3.2.5 *Brucella* Monospecific Antisera A and M 70
3.2.6 *Brucella* Culture Suspension 70
3.2.7 Test for Requirement of Added CO_2 for Growth 70
3.2.8 Slide Agglutination Test 70
3.2.9 Production of Hydrogen Sulphide 70
3.2.10 Production of Urease 71
3.2.11 Susceptibility to Lysis by Phages 71
3.2.12 Growth in the Presence of Dyes 71

3.2.13 Growth in the Presence of Penicillin 71
3.3 Testing Isolated Strains of *Brucella abortus* for Virulence in Mice ... 72
3.3.1 Purpose 72
3.3.2 *Brucella* Strains 72
3.3.3 Experimental Animals: 72
3.3.4 *B. abortus* Cultures 73
3.3.5 Infection of mice with *B. abortus* and collection of tissues. 73
3.3.6 Blood Culture 73
3.3.7 Measurement of *B. abortus* CFU in Spleens 73
3.3.8 Collection of Serum Samples 74
3.3.9 Serologic Testing 74
3.3.9.1 Rose Bengal Plate Test 74
3.3.9.2 Serum Agglutination Test 74
3.3.10 Histopathology 75
CHAPTER FOUR: RESULTS 77
4.1 Isolation of *Brucella* from the Nigerian Livestock 77
4.2 Biotyping of the Brucella Isolated From Nigerian Livestock 78
4.3 Testing Isolated Strains of *Brucella abortus* for Virulence in Mice ... 79
CHAPTER FIVE: DISCUSSION AND CONCLUSIONS 92
5.1 Discussion 92
5.2 Summary and Conclusions 104
REFERENCES 106
APPENDICES .....

LIST OF TABLES

TABLE **PAGE**

2.1: Studies of *Brucella* Survival Time in the Environment 22

2.2: Differential Characteristics of Species of the Genus *Brucella* and the Biovars 33

3.1: Samples Collected from Animals for the Isolation of Brucellae from Various States of Nigeria 76

4.1: Isolation of Brucellae from Culture Samples Obtained From Livestock in Various States of Nigeria 81

4.2: Growth Characteristics of Brucellae Isolated From Livestock in Nigeria 82

4.3: Growth Characteristics of stored cultures of Brucellae Isolated from Cattle in Nigeria 83

4.4: Differential Characteristics of *B. abortus* Strain 19 and Biovar 1 Field Isolates of *Brucella* 84

4.5: Spleen/ Body Weight Ratio on Days Post-Inoculation of Mice With Different *B. abortus* Strains 85

4.6: Presence of *B. abortus* in the Blood of Mice on Days Post-Inoculation with Different *Brucella* Strains 86

4.7: Spleen Colony- Forming Units of *B. abortus* on Days Post-Inoculation of Mice with Different *Brucella* Strains 87

4.8: Serological Findings on Serum of Mice on Days Post-Inoculation with Different Strains of *B. abortus* 88

LIST OF FIGURES

FIGURE **PAGE**

4.1: Spleen/Body Weight Ratio of BALB/c Mice during Infection with *Brucella abortus* 89

4.2: Persistence of *B. abortus* in Spleen of Infected Mice 98

4.3: Bar Chart of Antibody Production in Mice infected with *B. abortus* S19, w.544, MK5, SH_3 or HS_2 99

CHAPTER ONE

INTRODUCTION

1.1 Statement of the Problems

There is an increasing demand on the livestock industry in Nigeria for more beef and dairy production to enhance the availability of adequate supply of animal protein for the ever increasing population. Maximum production efficiency is therefore required of the livestock population, if it is to meet the high protein requirement of the Nigeria population (Eze, 1981).

Nigeria has an estimated livestock population of 18.4 million cattle, 14.5 million sheep, 22.5 million goats, 6.9 million pigs, 18,000 camels and 0.24 million horses (Anon, 1995). About 80% of the estimated total animal population in Nigeria is still under the management of the traditional nomadic Fulani pastoralists who are accustomed to extensive system of production. The rest belong to Government-owned Livestock Centres and privately-owned farms. Several constrains 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). The greatest obstacle to increased animal production is diseases among which ectoparasites, helminths, blood parasites, and mycotic, bacterial and viral diseases are the most prominent. High ranking among these diseases are rinderpest which is under control and contagious bovine pleuro-pneumonia of which there are efforts in the control through vaccination. The next most significant disease is brucellosis (Eze, 1981).

There is considerable reproductive wastage among Nigerian livestock in terms of high age at first conception, low conception rates, low calving rates, low calf survival

rates, abortion and reduced fertility (Osori, 1976; Oyedipe *et al.*, 1981). Several disease agents such as *Brucella* (Esuruso, 1979; Eze, 1981), *Leptospira* (Ezeh, 1988), *Listeria* (Oni *et al.*, 1989), *Campylobacter* (Elegbe *et al.*, 1987 and *Mycoplasma* (Chima, 1988) have been incriminated, but there are no recent information to confirm these. Brucellosis caused by *Brucella* is a chronic intracellular bacterial infection which can result in abortion and infertility in animals (Enright, 1990) and undulant fever, endocarditis, osteomyelitis and occasionally meningitis in humans (Madkour, 2001). The disease continues to be a major problem recognized world wide from both economic and public health aspects (WHO, 1986).

In Nigeria, bovine brucellosis is prevalent throughout the country (Eze, 1981). It is recognized to be a major cause of serious economic losses to the livestock industry resulting from abortions, infertility, neonatal mortality, decreased milk and meat production, and decreased sales of hides and skins (Esuruoso and Hill, 1971; Rikin, 1988b). Brucellosis has been diagnosed wherever there are cattle in Nigeria and all breeds of cattle have been shown to be affected (Eze, 1985). Studies in various parts of the country indicate that the disease is widespread particularly in ranches, livestock breeding centers and diary farms where prevalence is ranging between 3.7 – 48.8% (Esuruoso and Hill, 1971; Esuruoso and Van Blake, 1972; Esuruoso, 1974a; b) and between 0.4 - 26% in nomadic herds (Nuru and Dennis, 1975; Ocholi *et al.*, 1996). There are also reports based on surveys at abattoirs and slaughter slabs where prevalences of between 0.4 - 8.6% were obtained (Nuru and Schurrenberger, 1975; Chukwu, 1987a). The incidences of the disease in other animal species such as sheep, goats, pigs, camels, horses and dogs, and human have been indicated, even though less frequent. Details of such reports are as contained in reviews by Ocholi *et al.* (1993) and Halle and Ajogi (1997). All these investigations were based on serological surveys. There are few reports

based on isolation of *Brucella* from cattle (Eze, 1978; 1981; Bale and Kumi-Diaka, 1981), sheep and goats (Okoh, 1980; Falade, 1981; Bale *et al*., 2002), pigs (Bale and Nuru, 1985) and dogs (Okoh *et al.,* 1979).

1.2 Justification

More works on isolation of brucellae from the Nigerian livestock is required in order to elucidate the epidemiology of this disease in the country. Though there have been successful attempts to isolate *Brucella* from the Nigeria livestock, the prevailing species infecting the animal population in the country have not been extensively established. An important step to this is to undertake a country-wide isolation work on *Brucella* from the various animal species.

Reports from the field indicate that abortion is a common feature among Nigerian livestock and causes have not always been subjected to detailed laboratory investigation (Okoh, 1980). The involvement of *Brucella* in such cases of abortion is not extensively known. Isolation work on *Brucella* from aborting animals to establish its involvement in some of the cases of abortion in animals is therefore required.

The role played by brucellosis in limiting livestock production and its economic impact on the livestock industry in Nigeria is widely recognized (Eze, 1985). It is estimated that annual losses due to bovine brucellosis alone is about 224 million US Dollars (Esuruoso, 1979), this may be a conservative estimate. Since it is a zoonotic disease, the additional loss in human productivity was not taken into consideration. In view of these huge economic losses to the livestock industry, there is a need for this disease to be controlled if the unrealized economic potentials are to be derived from the Nigerian livestock.

Because of the zoonotic nature of brucellosis and its envisaged negative effect on

milk production in Nigeria, early attempts were made to control the disease in government-owned farms, using *Brucella abortus* S.19 calf hood vaccination and the culling of reactors (Eze, 1985). However, no significant progress was made as such an attempt was too localized leading to the suspension of this effort (Mettam, 1947). The problems associated with the use of *B. abortus* S.19 for immunizing cattle against brucellosis constitute serious impediments to brucellosis control (Plommet, 1991b). This has renewed interests in the search for local *Brucella* isolates that can be used as vaccine candidate in replacement of the vaccine currently in use.

In view of the Federal Government of Nigeria's intention to increase animal production in the country, there is a need to institute control and or subsequent eradication of this disease. It is important to obtain basic understanding on the epidemiology of the disease before any meaningful strategy for control and or eradication programme can be established. The bacteriological isolation and identification of *Brucella* species and biotypes is crucial to obtaining accurate evaluation of the epidemiology of brucellosis among Nigerian livestock.

1.3 Objectives of the Study

The objectives of this work are to:

1. Isolate and identify Brucellae from the Nigerian livestock
2. Undertake phenotypic characterization of the Brucellae isolated from the Nigerian livestock;
3. Investigate the virulence of the local *Brucella* isolates with a view to using them for vaccine production;
4. Investigate the immune and pathological responses of mice inoculated with local *Brucella* organisms.

It is hoped that the results obtained from these investigations will lead to a better understanding of the epidemiology of brucellosis in Nigeria and provide the necessary information crucial for the formulation of strategies for the control and/or eradication of the disease in the country.

CHAPTER TWO

LITERATURE REVIEW

2.1 HISTORICAL PERSPECTIVES

Brucellosis is a bacterial disease occurring in many species of animals that is transmittable to humans, caused by Gram-negative, non-sporing, and non-motile cocco-bacillary organism belonging to the genus *Brucella*. Brucellosis as a febrile disease of humans was described by Hippocrates in 450 A.D. (Seifert, 1996). However, the first published accurate description of brucellosis as "Mediterranean gastric remittent fever" was made by Martson, a British Army physician in Malta in 1861 (Martson, 1861). During the 19th Century, brucellosis was known as Malta fever, Mediterranean fever, Gibraltar fever, gastric fever, remittent fever and undulant fever. The causative agent remained unknown until David Bruce isolated a microorganism he termed *Micrococcus melitensis* from the post-mortem spleen of a British Soldier with Malta fever (Bruce, 1887). Ten years later, Bang and Stribolt (1897) working in Denmark isolated *Bacillus abortus* from aborted bovine fetus and fetal membranes.

The relationship of the disease in man and animals was not well understood until in 1905 when Zammit (1905), the only Maltese member of the British Mediterranean Fever Commission demonstrated *Brucella* agglutinins and the organism in the blood of goats. Horrocks (1905), in the same year isolated the organism from the milk of apparently healthy goats. In the United States of America (USA), a *Brucella species* was isolated from an aborted pig fetus by Traum (1914) and he named the organism *B. suis*. Evans (1918) showed that these organisms were related morphologically, biochemically and serologically. The organisms were named *Brucella* by Meyer and Shaw (1920) in honour of David Bruce. Of further Veterinary interests, Buddles and Boyes (1955) isolated *Brucella ovis*, as the cause of epidydimitis and abortion in sheep in New Zealand

and Australia. *Brucella neotomae* was isolated from a desert wood rat (*Neotoma lepida*) by Stoener and Lackman (1957) in Utah, U.S.A. Later Carmichael and Kenny (1968) isolated *Brucella canis* from cases of canine epizootic abortion and epidydimitis, in Beagle dogs in the USA.

Recently, within the last decade, *Brucella* species that differ from the six previously recognized members of the genus (Corbel and Brinley-Morgan, 1984) have been reported from animals living in the marine environment. The first isolates were cultured from free-ranging seals and cetaceans from around the coast of Scotland (Ross *et al*., 1994) and a captive dolphin in the USA (Ewalt *et al*., 1994). Further isolation has been made from mink whales (Clavareau *et al*., 1998). The isolation of these strains and the tissues involved have been described (Foster *et al*., 1996). Results from their characterization shows that they could not be assigned to recognized nomen species of the genus *Brucella* and it was suggested that they comprise a new nomen species to be called *Brucella maris* (Jahans *et al*., 1997). However, based on molecular studies which indicate differences among the strains, which also have seals and cetaceans as their preferred hosts, two new species, *B. cetaceae* and *B. pinnipediae* have been proposed corresponding to cetacean and seal isolates respectively (Cloeckaert *et al*., 2001).

2.2 DESCRIPTION OF THE GENUS *BRUCELLA*

2.2.1 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, thiamin 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. sodium chloride 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 asparagine 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 organisms (Gay and Damons, 1955). Guinea-pig inoculation is a very important method for the isolation of brucellae from contaminated scanty source (Alton *et al*., 1988).

2.2.2 Gaseous Requirements

Brucellae are aerobic micro-organisms and do not grow under anaerobic conditions. Many strains of *Brucella abortus* and *Brucella ovis* require an atmosphere containing 5-10% carbon dioxide (CO_2) especially on primary isolations (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).

2.2.3 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.

2.2.4 Temperature Requirements

Although *Brucella* species like most other bacteria are incubated at 37^{o}C, the optimum growth temperature is 36 – 38°C and most strains can grow between 20^{o}C and 40°C (Anon, 2001).

2.2.5 Colonial Morphology

On suitable solid media, *Brucella* colonies are visible after 2 days incubation in air or under 5–10% CO_2 atmosphere. *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 0.5-1mm in diameter after 48-72 hours incubation. Little or no growth is produced by many *Brucella* strains on MacConkey agar, even after five days at 37°C (Corbel *et al*., 1979).

2.2.6 Staining Reactions

Brucellae are gram-negative cocobacilli or short rods 0.5 - 1.5Φm in length and 0.5 - 0.7Φm in breadth. They occur singly and less frequently in pairs or small groups. Brucellae are non-motile. Brucellae does not form spores, flagella, pilli 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).

2.2.7 Dissociation Property

Smooth *Brucella* cultures have a tendency to undergo variation or mutation to rough forms (R) and sometimes mucoid (M) forms during growth. Colonies are then much less transparent with a more granular, dull surface (R) or a sticky glutinous texture

(M) and range in colour from malt white to brown in reflected or transmitted light. These changes are generally associated with changes in virulence, serological properties and phage sensitivity (Anon, 2001).

2.2.8 Biochemical Reactions

Metabolism of *Brucella* is oxidative not fermentative. *Brucella* cultures show no ability to produce acids on carbohydrate media in conventional tests. The *Brucella* species are catalase positive and usually oxidase positive. Urease activity by hydrolyzation of urea varies from fast to very slow. They reduce nitrates to nitrite (except *B. ovis* and *B. canis* strains). The production of hydrogen sulphide also varies. Indole is not produced and citrate is not utilized (Anon, 2001). Methyl red and Voges - Proskauer tests are negative and brucellae neither liquefy gelatin nor lyse red blood cells (Alton *et al*., 1988).

2.2.9 Antigenic Characteristics

All smooth *Brucella* strains (*B. abortus, B. melitensis* and *B. suis*) show complete cross-reaction with each other in agglutination tests with unabsorbed polyclonal antisera, a cross reaction which does not extend to non-smooth variants. Cross-reactions between non-smooth strains can be demonstrated by agglutination tests with unabsorbed anti-rough sera. Lipopolysaccharide (LPS) comprises the major surface antigens of the corresponding colonial phase involved in agglutination. The LPS molecule carries the A and M antigens, which have different quantitative distribution among the smooth *Brucella* strains. This is of value in differentiating biovars of the major species using absorbed monospecific A and M antisera (Anon, 2001). Renoux and Mahaffey (1955) described a further antigen Z in *B. ovis*, which is also found in *B. canis* and in the rough phase of *B. melitensis* and *B. abortus*.

Serological cross-reactions have been reported between smooth *Brucella* and various other gram-negative bacteria e.g. *Escherichia coli* O: 116 and O:157, *Salmonella* group N (O:30) of Kaufmann - White, *Pseudomonas multophilia*, *Vibrio cholerae* and especially *Yersinia enterocolitica* O:9. These organisms can induce significant levels of antibodies which cross-react with S-LPS *Brucella* antigens in diagnostic tests (Anon, 2001; Corbel *et al*., 1984).

2.2.10 Susceptibility to Phages

Over 40 phages have been reported to be lytic for *Brucella* species. All phages are specific for the genus *Brucella*, and are not known to be active against any other bacteria that have been tested. Thus, lysis by *Brucella* phages is a useful test to confirm the identity of *Brucella* species and for speciation within the genus (Anon, 2001). The *Brucella* phages currently used for *Brucella* typing are: Tbilisi (Tb), Weybridge (Wb), Izatnagar 1 (Iz_1), Firenze (Fi), Berkeley 2 (Bk_2) and rough (R/C). The five former phages are used for differentiation of smooth *Brucella species* (Corbel and Brinley-Morgan, 1984). The R/C phage is lytic for *B. ovis* and *B. canis* (Corbel *et al*., 1987).

2.2.11 Susceptibility to Dyes

Susceptibility to dyes such as thionin, basic fucshin, methyl violet, pyronin and safranin O at standard concentrations (20µg/ml) is used as routine typing tests of *Brucella* (Alton *et al*., 1988). Susceptibility of brucellae to these dyes varies between species and biovars (Anon, 2001).

2.2.12 Susceptibility to Antibiotics

On primary isolation, brucellae are usually susceptible *in vitro* to gentamicin, tetracycline and rifampicin. Most strains are also susceptible to the following antibiotics: ampicillin, chloramphenicol, cotrimoxazole, erythromycin, kanamycin, novobiocin,

spectinomycin and streptomycin. Variation in susceptibility may occur between species, biovars and strains. Most strains are resistant to β-lactamins, cephalosporins, polymyxin, nalidixic acid, amphotericin B, bacitracin, cycloheximide, clindamycin, lincomycin, nystatin and vancomycin at therapeutic concentrations (Anon, 2001). Efforts to ease the primary isolation of brucellae from potentially contaminated clinical veterinary specimens due to high number of overgrowing organisms led to the development of a selective medium described by Kuzdas and Morse (1953). Several modifications of this selective medium containing antibiotics and/or bacteriostatic dyes either in combination or alone added to different basal media have been described for *B. abortus* (Ryan, 1967; Farrell and Robinson, 1972; Farrell, 1974) and Thayer-Martin medium for *B. ovis* (Thayer and Martin, 1964). The inhibitory effects of these media on the growth of *B. ovis* led to the development of a selective media, the modified Thayer-Martin medium with nitrofurantoin for the isolation of *B. ovis* (Brown *et al.*, 1971; Alton *et al.*, 1988). Recently it has been shown that the nalidixic acid and bacitracin contained in Farrell's selective medium were responsible for the inhibitory effect observed (Marin *et al.*, 1996a; b). Penicillin is used for the routine differentiation of the vaccinal strain, *B. abortus* stain 19 and streptomycin for the differentiation of *B. melitensis* Rev.1 from field strains by virtue of their sensitivity to these antibiotics (Alton *et al.*, 1988).

2.2.13 *Brucella* Evolution

Brucella organisms are alpha-*Proteobacteria* phylogenetically related to plant pathogens and symbionts such as *Rhizobium* and *Agrobacterium*, facultative, intracellular animal parasites such as *Bartonella* and *Rickettsia* and to opportunistic and free living bacteria like *Ochrobactrum* and *Caulobacter* (Velasco *et al*., 1998) as well as symbionts of both animals and plants, *Wolbachia* and *Sinorhizobium* (Tsolis, 2002). By observing the evolutionary traits, according to the ancestor descendant rules of

characters, it can be predicted that the ancestor of *Brucella* was an aerobic heterotroph free living motile bacteria inhabiting the rhizosphere of plants (Moreno *et al*., 2002). Complete chromosomal sequences of species belonging to these genera of alpha-*Proteobacteria* have been released recently (www.tigr.org/tdb/mbd/), allowing genetic comparisons with *Brucella* (Moreno and Moriyon, 2002; DelVecchio *et al*., 2002). It is accepted that the *Brucella* ancestor was a bacterium that evolved into an animal intracellular parasite by loosing structures, modifying pre-existing ones and generating or acquiring new traits. These characteristics are reflected in the genome as well as in the phenotype and life cycle of the extant *Brucella* (Moreno *et al*., 2002). Commensurate with these features are the genome size and the presence of 2 chromosomes with the same G + C content of *Brucella* which is almost identical in comparison with its free-living plant and obligate intracellular alpha-*Proteobacteria* relatives e.g. the free-living and opportunistic pathogen, *Ochrobactrum*. This suggests that the ancestors of these 2 genera already exhibited two megareplicons (Moreno, 1998).

Some of the orthologous characteristics identified as or predicted to be relevant for virulence did not probably undergo substantial modifications, as they are shared by the phylogenetic neighbours. Many are concentrated at the cell envelope, and include orninthine containing lipids, phosphatidylcholine, long chain fatty acids, perisplasmic glucans, catalase and superoxide dismutase (Moreno and Moriyon, 2002). Other modifications that are in all likelihood the result of the adaptive evolution to pathogenicity include several changes in the outer membrane features. These include changes in the lipopolysaccharide (LPS) lipid A structure in *Brucella* which was not substantially modified with respect to that of the phylogenetic neighbours (Velasco *et al*., 2000). Similarly, the high conservation of the two component BvrR/BvrS sensory regulatory system within the brucellae examined and the striking difference in its

perisplasmic sensing region with those of the phylogenetic neighbours (Lopez-Goni *et al*., 2002) also supports the idea that this adaptation has preceded speciation. In the same line of thinking, the type IV secretion system in plant endosymbionts functions in the establishment of the bacteroid condition, whereas in *Brucella* it may be devoted to transference of molecules inside phagosomes for controlling intracellular trafficking (Moreno *et al*., 2002). Further evolution of the LPS was achieved by horizontal acquisition of O-polysaccharide (OPS) genes. After this, speciation events must have occurred including those leading to the different surface characteristics of the *Brucella* species that are attached to the LPS, and possibly some quantitative changes in the outer membrane proteins (OMPs) (Santos *et al*., 1984) and fatty acids (Tanaka *et al*., 1977).

By comparing dendograms and phylogenies it seems feasible to hypothesize that *B. abortus* and *B. melitensis* shared the same ancestor while *B. canis* and *B. suis* shared a common ancestor (Moreno and Moriyon, 2001). It has been hypothesized on the basis of nutritional requirements that *B. suis* is the closest species to the *Brucella* ancestor (Plommet, 1991a). This postulation is sustained by several observations. For instance, *B. suis* is the most diverse of the classical brucellae in genomic structure and host preferences (Moreno and Moriyon, 2001) and because this species has a wider range of biochemical alternatives, its metabolic capability comes closest to heterotrophy (Moreno *et al*., 2002). *B. suis* strains are the brucellae most closely related to *Ochrobactrum* (Velasco *et al*., 1998) and is the most diverse among the *Brucella* species that parasitize terrestrial mammals, and the most closely related to the heterotrophic ancestor (Moreno *et al*., 2002).

2.2.14 *Brucella* Taxonomy

Through the years, *Brucella* taxonomists developed a classification system based

on six species, *B. melitensis, B. abortus, B. suis, B. neotomae, B. ovis* and *B. canis* (Moreno *et al*., 2002) subdivided into biovars (or biotypes). *Brucella* species constitute genetically committed groups evolutionarily linked to their preferred hosts, and for which biologically meaningful species can be defined. Thus, classical *Brucella* species display a marked although not strict host range, *B. melitensis* for sheep and goats, *B. abortus* for cattle, *B. canis* for dogs, *B. neotomae* for desert wood rats, and *B. ovis* for sheep (Corbel and Bringley-Morgan, 1984; Moreno *et al*., 2002;). This is shown not only as a preference for a given animal species but also by the observation that, when cross-infections between different animal species occur, the bacterium is seldom perpetuated in non-reservoir, non-preferential hosts once the primary host is removed (Alton, 1990; Blasco, 1990; Carmichael, 1990; Crawford *et al*., 1990). Also significant is that no naturally-occurring genetic exchange (or laboratory manipulation) has been described that converts one of the classical species to another (Moreno *et al*., 2002). Species identification is routinely based on lysis by phages and some simple biochemical tests such as oxidase, catalase, and urease tests (Alton *et al*., 1988) for *B. melitensis*, *B. abortus* and *B. suis*. The identification at the biovar level is currently performed by carbon dioxide (CO_2) requirement, production of hydrogen sulphide (H_2S), dye (thionin and basic fuchsin) sensitivity and agglutination with monospecific A and M anti-sera (Corbel *et al*., 1987). Moreover, a recently developed co-agglutination test using latex beads coated with a pair of monoclonal antibodies directed against the rough LPS and the 25kDa outer membrane protein (OMP25), makes it possible to accurately differentiate *B. ovis* from *B. canis* (Bowden *et al*., 1997).

The insight of the early *Brucella* researchers that the host range is a likely biological characteristic in the definition of *Brucella species* is also consistent with the results of molecular analysis. The first work that supported the division of the genus into

the classical species was the numerical analysis of the cellular fatty acids performed by Tanaka *et al*. (1977), but only partial conclusions could be drawn on the internal diversity of the species. Considering the high degree of DNA homology (>90%) for all species, based on DNA-DNA hybridization, *Brucella* has been proposed as a monospecific genus in which all six classical species should be regarded as biovars of *B. melitensis* (Hoyer and McCullough, 1968a; b; Verger *et al*., 1985; 1987). Studies with a limited number of strains, including in some cases the reference strains of the classical species and biovars, show that they can be discriminated by restriction fragment length polymorphism (RFLP) analysis of whole DNA (Allardet-Servent *et al*., 1988; Jensen *et al*., 1999), the Bru-RS1 and Bru-RS2 elements (Halling and Bricker, 1994), the insertion sites of IS 711 (Bricker and Halling, 1994; Ouahrani-Bettache, *et al*., 1996; Clavareau *et al*., 1998; Bricker, 1999), the REP and ERIC sequences (Mercier *et al*, 1996; Tcherneva *et al*., 2000) and other DNA sites (Cloeckaert *et al*., 1996). These were met with total or partial success depending on the particular method, DNA region, and the level of discrimination, i.e. a single species, several or all species, or even most but not all biovars using a single method (Moreno *et al*., 2002). From a taxonomic standpoint, the species-specific localization of IS711 is particularly meaningful since these elements are in a relatively high number in *Brucella* genomes and represents a possible source of internal diversity for a group where there are little chances for genetic exchange.

Moreover, molecular markers for *B. melitensis, B. suis, B. abortus, B. ovis, B. canis* and *B. neotomae* (all which display characteristic host ranges) have been identified in *omp*25 (*omp* 31a), *omp*31, *omp*2a and *omp*2b in studies that followed earlier work that suggested that the genes of the outer membrane proteins (OMP) contain taxonomically relevant information (Fitch *et al*., 1990; 1996).

The emergence of *Brucella* strains isolated from marine animals raises new questions with regard to taxonomy of the genus *Brucella*. The isolation of brucellae from a variety of marine animals including seals, dolphins, harbour porpoises and mink whales (Ewalt *et al*., 1994; Jahans *et al*., 1997; Ross *et al*., 1996; Foster *et al*., 1996) or serological evidence of *Brucella* infections (Jepson *et al*., 1997; Tryland, *et al*., 1999; Nielsen *et al*., 2001) have been reported. As a group, the isolates obtained so far can be differentiated from the brucellae that parasitize the terrestrial species by the electrophoretic profiles of Xba I DNA digests (Jensen *et al*., 1999), IS711 - based DNA fingerprinting (Bricker *et al*., 2000; Cloeckaert *et al*., 2000), ribotyping with Hind III (Verger *et al*., 2000), and by a group-specific marker in the *omp*2b gene (Cloekaert *et al*., 2001). However, the electrophoretic profiles of the DNA digests (Jensen *et al*., 1999), the IS 711 fingerprints (Bricker *et al*., 2000) and polymorphism at the omp2 genes (Cloeckaert *et al*., 2001) show that they are a more heterogenous group than the terrestrial species. Molecular analyses show that it is inappropriate to group them into a single species, "*B. maris*" (Jahans *et al*., 1997). The strains isolated from dolphins, porpoises and mink whales on one hand, and the strains isolated from seals on the other, can be classified into two different groups by the polymorphism at the *omp*2 locus (Cloeckaert *et al*., 2001), suggesting a broad host range distribution corresponding to cetaceans and pinnipeds. Therefore, based on *omp*2 DNA polymorphism and with respect to the current classification of *Brucella species* according to the preferential host, two new species names were proposed for marine mammal *Brucella* isolates: *B. cetaceae and B. pinnipediae* for cetacean and seal isolates respectively (Cloeckaert *et al*., 2001).

2.2.15 *Brucella* Genome Sequencing

The genome of *B.melitensis* strain 16M was sequenced and was found to contain 3,294,935 base pairs (bp) distributed over two circular chromosomes of 2,117,144 and

1,177,787 bp encoding 3,197 open reading frames (ORFs). Housekeeping genes are distributed on both chromosomes (Del Vecchio *et al.,* 2002). Analysis of the *B. suis* 1330 genome shows a high level of similarity and gene synteney with that of 16M throughout the whole genome length with a small number of isolated rearrangenments or inversion events. A definite set of differences that could be responsible for the differences in virulence and host preference between these organisms could be defined (Paulsen *et al.,* 2002). Preliminary sequencing data from 1899 genomic clones from *B. abortus* S2308 confirm a high degree of sequence identity (Sanchez *at al.,* 2001; Michaux-Charanchon *et al.,* 2002). Several *Brucella* virulence and pathogenesis genes homologous to known bacterial virulence factors have been identified. The genome sequence of these pathogens epmphasizes the similarity between these animal pathogens, and plant pathogens and symbionts (Shanchez *et al*., 2001; Paulsen *et al.,* 2002; Del Vecchio *et al.*, 2002).

2.3 THE EPIDEMIOLOGY OF BRUCELLOSIS

2.3.1 Geographical Distribution

Brucellosis occurs worldwide in domestic and wild animals; the disease creates a serious problem for the intensive and extensive animal production systems of the tropics (Seifert, 1996). While the disease has been eradicated in most developed countries especially in Europe and the USA through intensive schemes of control and eradication, its occurrence is increasing in developing countries (Warner, 2001; Ragan, 2002). This is as a result of policy of importation of high production breeds without having the required veterinary infrastructure and the appropriate level of development of the socio-economic situation of the animal holder. Furthermore, the increasing international animal trade with increasing movement of animals and the trend towards intensification of animal production favour the spread and transmission of the infection. Brucellosis has been reported wherever animals are raised all over the world (Seifert, 1996).

2.3.2 Host Preference

Epidemiological evidence shows that *Brucella* usually has definitive host preferences (Bercovich, 1998). Even though the *Brucella* species are relatively host specific, interspecies transmission does occur. *B. abortus* is recognized as the main cause of contagious abortion in cattle, but sheep, goats, dogs, horses, camels, buffalos as well as wild animals that have been in contact with infected cattle may also contract infection (FAO, 2004) *Brucella melitensis* is highly contagious to sheep and goats, however, cattle, dogs, camels and pigs may also become infected with *B. melitensis* (Zowghi and Ebadi, 1988). *Brucella suis* which usually infects swine may also infect cattle, horses, dogs and wild animals. *Brucella ovis* infects mainly sheep while *B. canis* infects mainly dogs.

2.3.3 Dissemination of *Brucella*

The primary route of dissemination of *Brucella* is the placenta, fetal stomach fluids and vaginal discharges expelled by infected animal after abortion or full-term parturition. Very large numbers of organisms are shed at the time of parturition or abortion (Anon, 2001). Excretion of *B. abortus* in the vaginal discharge of infected cattle not only occurs but may also start as early as 39 days after exposure (Phillipon *et al*., 1970). Contamination of a cowshed or pasture takes place only when infected cattle abort or have full-term parturition. The massive excretion of brucellae may continue for 15 days. Although the infectious materials from the genital tract usually clear after 2-3 months, the cattle become carriers of *Brucella* and excrete it intermittently for many years (Herr *et al*., 1990). 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 at full-term parturition (Okoh, 1980). Shedding of *Brucella* is also common in udder secretions and semen, and

Brucella may be isolated from various tissues, such as lymph nodes especially those associated with reproductive organs, and sometimes from arthritic lesions (Alton *et al*., 1988). The persistent infection of the mammary glands and supra-mammary 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).

2.3.4 Modes of Infection

The only significant sources of infection with *Brucella* are animals that excrete the organism. Animals become infected directly by infected aerosols or uptake of infected materials. Transmission of *Brucella* is very likely to occur via the oral route because cattle tend to lick aborted fetuses or the genital discharges of an aborting cow (Bercovich, 1998).

Exposure to *Brucella* organisms is also likely to occur *in utero* or when animals born to healthy dams are fed on colostrum or milk from infected dams (Grillo *et al*., 1997, Catlin and Sheehan, 1986). Another mode of infection is grazing pastures where infected animals mix with brucellosis free animals or get in touch with contaminated premises, manure, materials etc. (Anon, 2001). It has been established that brucellosis in the male animals does not usually result in infertility although semen quality may be affected. Localization in the reproductive organs generally results in the shedding of *Brucella* in the semen especially during the acute phase of the disease. However, when used for natural mating, the risk of infected males transmitting the disease to susceptible females is high. If semen from an infected bull is used for artificial insemination, it may introduce brucellosis into a *Brucella*-free herd. Dogs have been shown to be mechanical and biological vectors of brucellosis (WHO, 1986). Persistent infection of mammary

glands is associated with constant or intermittent shedding of the organisms in the milk in succeeding lactation (Phillipon *et al*., 1970).

2.3.5 Survival of *Brucella* in the Environment

The ability of *Brucella* to persist outside mammalian hosts is relatively high compared with most other non-sporing pathogenic bacteria, under suitable conditions (Anon, 2001). The survival of the organism in the environment may play a role in the epidemiology of the disease (Bercovich, 1998). Numerous studies have shown that when pH, temperature and light conditions are favourable, i.e. pH > 4, high humidity, low temperature and absence of direct sunlight, *Brucella* may retain infectivity for several months in water, aborted fetuses, and fetal membranes, feaces and liquid manure, wool, hay, on buildings, equipment and clothes (Table 2.1). Brucellae are able to withstand drying particularly in the presence of extraneous organic material and will remain viable in dust and soil. Survival is prolonged at low temperatures, especially below 0^{o}C (Alton, 1985; Ho and Cheers, 1982; Nicoletti, 1980). *Brucella* organisms are sensitive to direct sunlight, disinfection, or pasteurization, and under dry conditions they survive only if embedded in protein (Davies and Casey, 1973). In tap water brucellae can survive for several months at 4-8°C, 2.5 years at 0°C, and several years in frozen tissues or medium. Brucellae can survive 30 days in urine, at least 75 days in aborted fetuses, and more than 200 days in uterine exudates (Nicoletti, 1980). The survival of *Brucella* in milk and dairy products is related to a variety of factors including the type and age of product, humidity level, temperature, changes in pH, moisture content, biological action of other bacteria present and conditions of storage (Nicoletti, 1989; Plommet *et al*., 1988). *Brucella* does not persist for a long time in ripened fermented cheese (Nicoletti, 1989).

Table 2.1: Studies of *Brucella* Survival Time in the Environment (Nicoletti, 1980; WHO, 1986)

Environment	Conditions	Survival time
Direct sunlight	< 31°C	4h 30min
Water	- 4°C	4months
Water (laboratory)	20°C	2.5months
Water (lake)	37°C, pH = 7.2	< 24h
	8°C, pH = 6.5	> 2months
Soil	Dried in laboratory	< 4days
	Dried at 18°C	69 – 72days
	Wet	< 7days
	Humid atmosphere	> 2months
	Autumn (90% humidity)	48 – 73days
	February (rapid drying)	72days
Urine	37°C, pH = 8.5	16hours
	8°C	48hours
Raw milk	25 – 37°C	24hours
	8°C	48hours
	- 40°C	2.5years
Whey	17 – 24°C	< 5days
	5°C	> 6days
Manure/dung	Summer	24hours
	25°C	1month
	Winter	2months
	8°C	1year
	- 3°C	3months
Liquid manure	Summer	3months
	Winter	6months
	25°C	1month
	8°C	1year
	- 3°C	3months
Wool	In warehouse	4months
Hay		Several days to months
Street dust		3 – 44days
Wooden walls or floors or pens		4months
Pasture	Sunlight	< 5days
	Shade	> 6days

In contrast to dairy products, the survival time of *Brucella* in meat seems extremely short, except in frozen carcasses where the organism can survive for years. The number of organisms per gram of muscle is small and rapidly decreases with the pH drop of the meat (Anon, 2001).

2.3.6 Decontamination of Environment/Materials Exposed to *Brucella*

Direct contamination of abattoir workers is prevented by a proper and hygienic removal and disposal of mammary glands, reproductive organs and lymph nodes which are most heavily contaminated. Contaminated equipment can be sterilized by autoclaving (121°C). Chemical treatment is recommended to destroy *Brucella* in contaminated premises. Xylene (1ml/litre) and calcium cyanamide (20kg/m^3) have been found to be effective in liquid manure after 2-4 weeks. A 1 hour treatment with 2.5% sodium hypochlorite, 2–3% caustic soda, 20% freshly slaked lime suspension or 2% formaldehyde solution will suffice to destroy brucellae on contaminated surfaces (Anon, 2001).

Most commonly available disinfectants readily kill *Brucella* in aqueous suspensions at normally recommended concentration (phenol 10g/l, formaldehyde, xylene, 1ml/l) except in the presence of organic matter or at low temperature, which drastically reduces the efficacy. Diluted hypochlorite solutions, ethanol, isopropanol or iodophores and substituted phenols are effective for decontamination of the exposed skin (WHO, 1986).

2.3.7 Resistance to Infection

It is well documented that age, sex and breed of animal influence the progression of *Brucella* infection (Anon, 2001). There is a suggestion that young cattle are less

susceptible to infection than sexually mature cattle. Data also suggests that pregnant females are more likely to become infected than males because gravid uterus sustains the organism and thus, influences the progression of the disease (Crawford *et al*., 1990). It seems that the younger the fetus is at the time of infection, the longer the incubation period. Data from cattle with experimentally induced infection and from cattle with naturally occurring brucellosis suggest that natural resistance to *Brucella* infection also influences the course and incidence of the disease (Harmon *et al*., 1989; Ho and Cheers, 1982).

2.4 PATHOGENESIS

The susceptibility of the animal depends on their natural resistance, age, level of immunity, environmental stress and on the reproductive status (Seifert, 1996). The virulence of *Brucella* varies considerably according to species, strain and the size of infecting inoculum (Anon, 2001). Brucellae are facultative intracellular parasites of the endothelial system with the ability to survive and multiply within phagocytic cells.

Brucellae can enter mammalian hosts through skin abrasions or cuts, the conjunctiva and through the mucosa of the respiratory and gastrointestinal tract (Brinley-Morgan and Corbel, 1990). Organisms are rapidly ingested by polymorphonuclear leukocytes, which generally fail to kill them and are also phagocytosed by macrophages (Canning *et al*., 1985; 1986). The organisms are transported either free or within phagocytic cells to regional lymph nodes. Bacteraemia then occurs with the localization in other lymphoid tissues, liver, spleen, mammary gland, joints, kidneys and bone marrow. Spread via lymph and blood to other organs may occur, predilection sites being uterus, udder and associated lymph nodes (Thoen and Enright, 1986). In pregnant animals, the uterus is a preferred site of infection where it leads a necrotizing placentitis

that results in abortion (Seifert, 1996). After abortion, there is persistent infection of mammary glands where it is generally maintained from one gestation period to the next, and in the supramammary and genital lymph nodes leading to constant or intermittent shedding of the organisms in the milk and genital secretions (Fensterbank, 1987). In non-pregnant animals, the first infection occurs in the udder followed by the infection of the uterus later after the onset of pregnancy. In cattle, the uterus is the central site of multiplication of the pathogen. The enhanced virulence of the brucellae inside the reproductive system is supposed to be the consequence of the increased level of the sugar, erythritol (Seifert, 1996).

Brucella can be isolated from hygromas of knee joints, epididymis and testes of animals (Thoen and Enright, 1986). Numerous animals may develop self-limiting infections or they become asymptomatic latent carriers and potential excretors (Fensterbank, 1987).

The variable facets of clinical symptoms which are typical for brucellosis are the consequence of the individual level of host defense which is specific for each breed, but also for each individual and which is the result of the sum of influences of genetically determined resistance, level of immunity, age of the animal, productivity, condition, environmental influences as well as virulence of the pathogen (Seifert, 1996).

To be a successful infectious agent, *Brucella* requires four steps, like other pathogenic intracellular bacteria: adherence, invasion, establishment, and dissemination within the host (Findlay and Falkow, 1989). Opsonized or nonopsonized *Brucella* organism can infect macrophages, suggesting that antibody or complement mediated phagocytosis as well as the direct *Brucella*-host cell contact allows adherence and invasion (Ko and Splitter, 2003).

Brucellae organism not only resist killing by neutrophils following phagocytosis (Caning *et al*., 1986) but also replicate inside macrophages (Jones and Winter, 1992) and nonprofessional phagocytes (Detilleux *et al*., 1990).

In macrophage phagosomes, *Brucella* cells survive and multiple by inhibiting phagosome-lysosome fusion through rapid acidification of the phagosome following uptake (Porte *et al*., 1999). Thus *Brucella* organisms have ability to avoid the killing mechanisms and proliferate within macrophages, and also escape the extracellular mechanisms of the host defense such as complement and antibodies (Ko and Splitter, 2003). The survival of *Brucella* in macrophages is considered to be responsible for the establishment of chronic infections, and this determines the virulence of *Brucella*. The recent availability of genetic techniques to manipulate the *Brucella* genome permits identification of virulence factors of *Brucella* at the molecular level. Previous studies to identify attenuation relied on culture passage of isolated strains or collection of diverse field strains following phenotypic characterization based on LPS structure (rough or smooth form) or injection of animal models (Winter *et al*., 1996). Since the early 1990s, molecular manipulation of the *Brucella* genome has permitted the characterization and identification of virulence genes by producing specific or random mutants searching for avirulent phenotypes (Halling *et al*., 1991; Ko and Splitter, 2003; Hong *et al*., 2000). Recently, the complete genome sequence of some *Brucella* species has helped in the identification of virulence genes (Sanchez *et al*., 2001; Del Vecchio *et al*., 2002; Paulsen *et al*., 2002).

2.5 IMMUNE RESPONSE

Mouse models have recently been used in clarifying immune mechanisms in brucellosis. It has been shown that both humoral and cell-mediated immune responses do

contribute to protective immunity against brucellosis (Plommet, 1987; Phillips *et al*., 1989; Winter, 1990). Active or passive immunization indicated that humoral responses may be effective in eliminating the organism in the early stages of infection, but require other immune mechanisms for protection in the later stages of infection.

2.5.1 Humoral Immunity

Following infection by natural exposure, a serological response can normally be expected within 2 to 4 weeks (Bercovich, 1998). 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. The pattern of serological response in terms of immunoglobulin production is that of IgM production 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 (Anon, 2001).

Despite numerous reports regarding a role for humoral immunity in resistance to brucellosis, the ability of antibody to protect the host appears controversial, and there is no correlation between antibody titers and resistance to disease (Hoffmann and Houle, 1995). In cattle, protective immunity is not elicited by *Brucella* LPS while in mice, passive transfer of sera containing anti-LPS antibodies could protect against challenge with virulent *B. abortus* (Montaraz and Winter, 1986). Also, passive transfer of *B. abortus* O-antigen-specific monoclonal antibody (IgG2a) could reduce bacterial infection in mice or protect mice from *B. abortus* infection (Montaraz and Winter, 1986). In addition to antibodies to *Brucella* LPS, antibodies have been demonstrated against a variety of specific bacterial proteins. This suggests extracellular lysis of bacteria for antigen recognition by antigen specific B cells, as well as major histocompatibility complex (MHC) class II antigen presenting cells. IgG2a and IgG3 are dominant antibody

isotypes detected from the infected mice and cattle suggesting that a Th 1 immune response to *Brucella* infection occurred (Elzer *et al*., 1994).

The ability of passive transfer of specific antibodies to protect mice but not cattle appears as another host difference in the resolution of disease in different animal species. The mechanism(s) for this difference in antibody protection in various species remain unknown. Opsonization, probably coupled with enhancement of intracellular killing, is regarded as the principal protective role of antibody against *Brucella* infection (Ko and Splitter, 2003).

2.5.2 Cell-Mediated Immunity

After gaining entry into the body, the *Brucella* organisms encounter the cellular defenses of the host, in particular neutrophils (Canning *et al*., 1985), Natural killer (NK) cells (Yabu e*t al*., 1991), macrophages (Jiang *et al*., 1993) and T– lymphocytes (Smith, 1990). Studies have shown that protective immunity against *Brucella* infection is due mainly to cell mediated immunity (CMI) mediated by T cells of both $CD4^+$, $CD8^+$ and $\gamma\partial$ subsets (Schurig *et al*, 1991; Ko and Splitter, 2003). The critical function of the CMI is the production of cytokines. The cytokines regarded as key players in brucellosis, are interleukin 1 (IL-1), IL-2. IL-12, IFN-γ and tumour necrosis factor (TNF-α) and IL-6 (Ko and Splitter, 2003). IL – 12 is a key cytokine produced by B cells and macrophages and leads Th 1 immune responses in the host that will ultimately induce the secretion of IFN– γ from T Cells (Zhan and Cheers, 1995; 1998). The major role of T- cells in *Brucella* immunity is secretion of IFN-γ for the activation of bactericidal function in macrophages and cytotoxic T-lymphocyte activity as well as IgG2a and IgG3 antibody isotype switching. Functionally, this can be classified into 3 mechanisms. First, IFN-γ produced by $CD4^+$, $CD8^+$, and T cells activate the bactericidal function in macrophages

to hamper the intracellular survival of *Brucella*. Secondly, cytotoxicity of $CD8^+$ and T cells kills the infected macrophages. Thirdly, Th1– type antibody isotypes such as IgG2a and IgG3 opsonize the pathogen to facilitate phagocytosis (Ko and Splitter, 2003).

2.6 CLINICAL SIGNS

The incubation period varies from few days to several weeks and this is often tied up with the stage of pregnancy at the time of exposure (Seifert, 1996). The main clinical signs are reproductive failure, i.e. abortion, stillbirth, birth of weak offspring, infertility and or sterility.

Abortion generally occurs during the last trimester of pregnancy and is followed in some cases by retention of fetal membranes (Anon, 2001). If an infection appears in a herd which has been immunologically naive, all pregnant animals will abort in what is known as "abortion storm" (Seifert, 1996). In the male, orchitis, epidydimitis seminal vesiculitis, and later infertility are the major signs. Additionally, in both sexes hygromas in particular on the carpal joint, and catarrhal or purulent endometritis are characteristic features of chronic infections (Fensterbank, 1987).

In small ruminants, consequences of brucellosis are high mortality rate in lambs/kids, mastitis and reduced milk production. Persistent infection of the mammary glands and supramammary lymph nodes is common in goats (Anon, 2001).

In horses the common association of *Brucella* infection is with chronic bursal enlargements of the neck, poll evil and fistulous withers, and chronic lesions of the joint bursae and cervical membranes causing intermittent lameness (Radostits *et al*., 1997). In the mare abortion and infertility may occur and the organism has been isolated from mares which have aborted (Ray *et al*., 1988). Some horses appear to suffer generalized infection with clinical signs including general stiffness, fluctuating temperature and

lethargy (Macmillan, 1985).

2.7 PATHOLOGY

2.7.1 Gross Pathology

A few days before abortion, a slimy pus-like, grey-whitish to reddish secretions appear in the vagina. The aborted fetuses are covered by a yellowish, slimy layer. The fetuses may be macerated (Seifert, 1996). Some aborted fetuses may have an excess of blood-tinged fluids in the body cavities, with enlarged spleen and liver (Anon, 2001).

The placenta is edematous, slushy, the affected cotyledons or parts of them are covered by a sticky, odourless, brownish exudate and are yellowish-grey as a result of necrosis. Parts of the intercotyledonary placenta are thickened, edematous, and yellowish-grey, and may contain exudates on the surface (Seifert, 1996). Though many fetuses may show no gross changes, petechia are often to be found in the abomasums and on the mucosa of the bladder of the fetus. Spleen, liver and lymph nodes are enlarged. Pneumonia with greyish-white foci may be present, sometimes accompanied by pleuritis (Radostits *et al*., 1997).

Gross lesions are not evident on the udder, though the supramammary lymph nodes may be enlarged. Palpable nodules and the production of clotted and watery milk may occur (Anon, 2001).

In the male, the scrotum may become heavily swollen and painful. The testes and epididymis are enlarged.

2.7.2 Microscopic Lesions

Necrotizing placentitis with the infiltration of the stroma of the chorion by numerous mononuclear cells and some neutrophils may be observed. Many of the

chorionic epithelial cells are packed with numerous intracytoplasmic bacteria (Seifert, 1996).

Multifocal bronchopneumonia, bronchitis and bronchiolitis and necrotic or microgranulomas in the liver, the lymph nodes, spleen and kidneys of aborted fetus may be seen (Radostits *et al*., 1997).

In the udder, lymphoplasmacytic and histiocytic interstitial mastitis is evident.

The seminal epithelial cells are necrotic and desquamative, and large numbers of organisms are present. There is focal, necrotizing epididymutis (Seifert, 1996).

2.8 DIAGNOSIS

2.8.1 Clinical Signs

Because several factors play a role in the epidemiology of brucellosis, it may take some time 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 pregnancy, infertility, orchitis, epidydimitis and hygroma, there are no pathognomonic signs of brucellosis (Bercovich, 1998).

2.8.2 Direct Smear

Microscopic demonstration of characteristic clumps of *Brucella* organisms in stained smears of fetal membranes, vaginal swabs, milk and semen by modified Köster staining, Stamp's modified Ziehl-Neelsen staining, and flourescent antibody techniques

(FAT) may provide the tentative diagnosis (Alton *et al*., 1988; Bercovich, 1998). However, morphologically related micro-organisms such as *Chlamydia psittaci* and *Coxiella burnetti* can mislead the diagnosis (Radostitis *et al*., 1997).

2.8.3 Cultural Isolation, Identification and Typing

The only confirmatory and unequivocal method for the diagnosis of brucellosis is based on the isolation of *Brucella* from clinical specimens such as, milk, vaginal swabs, aborted fetuses, placentas, hygroma fluid, blood and semen. The spleen and lymph nodes are the most reliable samples for isolation purposes in necropsied animal (Alton *et al*., 1988). *Brucella* can be isolated on ordinary solid media. However, the use of non-selective media is not usually recommended because of overgrowing contaminants usually present in field samples. Therefore selective media are needed for isolation purposes (Corbel *et al*., 1987). The Farrell's selective medium, developed for the isolation of *B. abortus* from milk (Farrell, 1974), is recommended for the isolation of *Brucella* species (Alton *et al*., 1988). However, nalidixic acid and bacitracin, at the concentration used in this medium, may have inhibitory effects on some strains of *B. abortus, B. melitensis* and *B. ovis* (Marin *et al*., 1996b). Thus, its sensitivity for the isolation of *Brucella species* from naturally infected sheep is sometimes lower than that obtained with less selective Thayer-Martin's medium (Marin *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 (Marin *et al*., 1996b).

The identification of *Brucella* involves colonial and cellular morphology, Gram's reaction, agglutination with monospecific antisera A, M or R antigens, and results from routine biochemical tests (Corbel *et al*., 1987). Species are separated on the basis of lysis by bacteriophage and oxidative profiles on selective amino acid and carbohydrate substrates. Typing of isolates of *Brucella* into biovars involves conventional procedures for cultural characteristics that include: CO_2 requirement, sensitivity to dyes (thionin, basic fuchsin), H_2S and urease production (Table 2.2), sensitivity to erythritol and susceptibility to antibiotics (Alton *et al*., 1988).

Table 2.2 Differential characteristics of species of the genus *Brucella* and their biotypes

Species	Bio-types	CO_2 dependence	H_2S Production	Biochemical tests						Agglutination in sera			Lysis by Tb. phage		Most common host reservoir
				Urease activity	Growth in dyes										
					Thionin			Basic Fuchsin		A	M	R	1	10^4	
					a	b	c	b	c				(RTD)		
B. melitensis	1	-	-	variable	-	+	+	+	+	-	+	-	-	-	goats
	2	-	-	variable	-	+	+	+	+	-	+	-	-	-	goats
	3	-	-	variable	-	+	+	+	+	+	+	-	-	-	goats
B. abortus	1	+ (-)	+	1 to 2 hr	-	-	-	+	+	+	-	-	+	+	cattle
	2	+	+	1 to 2 hr	-	-	-	-	-	+	-	-	+	+	cattle
	3	+ (-)	+	1 to 2 hr	+	+	+	+	+	+	-	-	+	+	cattle
	4	+ (-)	+	1 to 2 hr	-	-	-	+	+	-	+	-	+	+	cattle
	5	-	-	1 to 2 hr	-	+	+	+	+	-	+	-	+	+	cattle
	6	- (+)	- or +	1 to 2 hr	-	+	+	+	+	+	-	-	+	+	cattle
	7	-	- or +	1 to 2 hr	-	+	+	+	+	+	+	-	+	+	cattle
	8	+	-	1 to 2 hr	-	+	+	+	+	-	+	-	+	+	cattle
	9	- (+)	+	1 to 2 hr	-	+	+	+	+	-	+	-	+	+	cattle
B. suis	1	-	++	0 to 30min	+	+	+	-	-	+	-	-	-	+	pigs
	2	-	-	-ditto-	-	+	+	-	-	+	-	-	-	+	pigs
	3	-	-	-ditto-	+	+	+	+	+	+	-	-	-	+	pigs
	4	-	-	-ditto-	+	+	+	+	+	+	+	-	-	+	reindeer
B. neotomae	1	-	+	-ditto-	-	-	+	-	-	+	-	-	-	+	desert wood rat
B. ovis	1	+	-	-	+	+	+	+	+	-	-	+	-	-	sheep
B. canis	1	-	-	1 to 30min	+	+	+	+	+	-	-	+	-	-	dogs

Dye concentrations in agar: a = 1/25,000,; b = 1/50,000; c = 1/100,000; Tb. = Tbilisi, RTD = routine test dilution, + (-) = usually positive but may be negative.

Though the full-proof method for the diagnosis of brucellosis is based on the isolation of *Brucella* organism, the procedure is laborious, time-consuming, costly, and hazardous. Furthermore, the probability of successful recovery of *Brucella* is strongly reduced when the material is heavily contaminated or when only a few organisms are present. The sensitivity of the isolation method also depends on the viability of *Brucella* within the sample, the nature of sample and the number of specimens tested from the sampled animal (Hornitzky and Searson, 1986). Moreover, negative culture results do not exclude infection (Bercovich, 1998).

The development of colony blot Enzyme-linked immunosorbent Assay (ELISA) presents an interesting application of monoclonal antibodies in rapid *Brucella* identification. Colony blot ELISA exploits monoclonal antibodies to smooth *Brucella* O-chain to visualize colonies on agar media even in the presence of contaminants (Roop *et al*., 1987).

2.8.4 Molecular Detection and Typing

While culturing is a specific method, the time required for culturing field specimens can be long and tissues or fluids that are only contaminated with nonviable or a low number of *Brucella* may not be detected (Anon, 2001).

Therefore, the identification of a stable DNA-specific marker is considered a high priority for diagnostic, epidemiological and taxonomic purposes. Detection by Polymerase Chain Reaction (PCR) for routine diagnosis was investigated by Fekete *et al*. (1990; 1992). However, since brucellae share a high degree of DNA homology (>90% for all species) on the basis of a DNA - DNA hybridization, which did not yield the necessary clues to explain the phenotypic differences among extant *Brucella* species and biovars, *Brucella* has been proposed as a monospecific genus (Verger *et al.,* 1985; 1987).

Several molecular techniques have been employed to find DNA polymorphisms which would enable the molecular typing of *Brucella species* and their different biovars. These include enterobacterial repetitive intergenic consensus (ERIC) sequence-PCR, and repetitive intergenic palindromic (REP) sequence-PCR (Mercier *et al.*, 1996; Tcherneva *et al.*, 1996), randomly amplified polymorphic DNA-PCR or arbitrary primed -PCR (Fekete *et al.*, 1992; Tcherneva *et al.*, 2000). Several investigators reported a good sensitivity of PCR for detecting *Brucella* DNA on pure culture (Baily *et al.*, 1992; Herman and De Ridder, 1992; Romero *et al.*, 1995; Da Costa *et al.*, 1996). Others have shown that PCR could be a potentially useful tool when used in combination with labelled probes to differentiate some *Brucella* species and biovars (Grimont *et al.*, 1992; Bricker and Halling, 1994; 1995; Cloeckaert *et al.*, 1995, Ouahrani-Bettache *et al.*, 1996; Fitch *et al.*, 1990; 1996). Among these, detection of restriction fragment length polymorphisms is considered a reproducible and easily performed method within a laboratory. However, laboratory-to-laboratory reproducibility has been problematic and this makes the universal application of the methods unlikely (Bricker *et al.*, 2003). Alternatively, pulsed-field gel electrophoresis (PFGE) of total genomic DNA digested with restriction enzymes (RE) that cut infrequently has been a useful technique for discriminating among the species and among several isolates in the same species of *Brucella* (Jensen *et al.*, 1995; 1999) but a common problem is incomplete digestion by RE of the DNA caused by incomplete cell lysis or incomplete protease digestion (Farber, 1996).

Major Outer-membrane protein (OMP) genes of *Brucella* have attracted attention as they exhibit sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars (Cloeckaert *et al.*, 1996). Studies of the RELP profiles of two closely related genes, *omp* 2a and *omp*2b, encoding and potentially expressing the

Brucella spp major OMP of 36 kDa (Fitch *et al*., 1990) and for 25 kDa (Wergifosse *et al*., 1995) have been useful for the differentiation of *Brucella* species, biovars and strains and for the differentiation among isolates from marine mammal hosts (Cloeckaert *et al*., 2001). However, as a tool for the epidemiology of brucellosis in livestock, this technique is limited by the low rate of natural sequence divergence within the locus at the biovar level (Bricker *et al*., 2003). Consequently, *B. canis* remained indistinguishable from *B. suis* biovars 3 and 4 (Cloeckaert *et al*., 1995).

Recently, the publishing of complete genome sequence of *B. melitensis* (DelVecchio *et al.,* 2002), *B. suis* (Paulsen *et al*., 2002) and the completion of the genomic sequencing of *B. abortus* (Sanchez *et al*., 2001) have made it possible to search for regions of DNA sequence variability that might be useful as markers for diagnostic identification and differentiation. This led to the development of a technique known as 'HOOF' - Prints for Hyper-variable Octameric Oligonucleotide Finger-Prints (Bricker *et al*., 2003). The microsatellite fingerprinting exploits the occurrence of variable number tandem repeats (VNTRs). The technique is highly discriminatory among *Brucella species*, among previously characterized *Brucella* strains, and among unrelated field isolates that could not be differentiated by classical methods.

2.8.5 Animal Inoculation

A suspension of suspected material is injected intraperitoneally into guinea pigs or mice and the animal necropsied 3-6 weeks later. If positive, the *Brucella* organisms are recovered from spleen by culturing onto appropriate media (Alton *et al*., 1988).

2.8.6 Serological Diagnosis

Because brucellosis is a chronic disease, detection of antibodies in serum, uterine discharge, vaginal mucus, milk or semen plasma of suspected animal may be indicative

of brucellosis in a herd (Bercovich, 1998). These body fluids may contain different quantities of antibodies of the IgM, IgG_1, IgG_2 and IgA isotypes directed against *Brucella* (Beh, 1974). Since the reliability of serological tests to detect brucellosis depends on antibodies that may or may not be present at the time of examination, inevitably some infected animals may elude detection. Because infected animal may or may not produce all antibody isotypes in detectable quantities, it is important to combine several or more than a single diagnostic test in order to determine the status of an individual animal (Bercovich, 1998). The commonly used tests are the milk ring test (MRT), Rose Bengal plate test (RBPT), serum agglutination test (SAT); complement fixation test (CFT), anti-globulin (Coombs) test, 2-mercaptoethanol, rivanol and enzyme-linked immunosorbent assay (ELISA).

2.8.6.1 Milk Ring Test

The diagnostic properties of the MRT depend on the presence of lacteal anti-*Brucella* IgM and IgA bound to milk fat globules. Although the MRT is cheap, easy and quick to perform, it is only fairly sensitive and reasonably specific. Milk that contains colostrum, milk at the end of the lactation period, and milk from cows suffering a hormonal disorder or mastitis may test false-positive (Kerkhofs *et al*., 1990). Milk that contains low concentrations of lacteal IgM and IgA or which is lacking the fat-clustering factors may test false-negative (Patterson and Deyeo, 1976). MRT is not recommended for use in sheep and goats (WHO, 1986).

2.8.6.2 Rose Bengal Plate Test

The RBPT is a spot agglutination technique that uses *B. abortus* antigen stained with Rose Bengal dye, and it is usually used to screen sera for *Brucella* antibodies. The test does not need special laboratory facilities and it is simple and easy to perform.

Although the test is based on detection of specific antibodies of IgM or IgG types, it is more effective in detecting antibodies of the IgG_1 type than in detecting IgM and IgG_2 types (Levieux, 1974). The test may yield negative results in infected cattle that give positive results with CFT. Although the low pH (+3.6) of the antigen enhances the specificity of the test, the temperature of antigen and the ambient temperature at which the reaction takes place may influence sensitivity and specificity (Macmillan, 1990).

2.8.6.3 Serum Agglutination Test

The SAT has been the principal serological test used for the diagnosis of brucellosis. Historically, the SAT contributed to the international harmonization of brucellosis control and eradication although it is not the most sensitive test. The test measures agglutinating antibodies of IgM, IgG_1, IgG_2 and IgA types (Levieux, 1974). The SAT is relatively simple and easy to perform but it requires basic laboratory equipment. It can be used to detect acute infections, as antibodies of the IgM type usually appear first after infection and are more reactive in the SAT (Beh, 1974). However, because the SAT may yield both false negative or false positive results, it effectively detects brucellosis only on a herd basis (Corbel *et al.*, 1984).

2.8.6.4 Complement Fixation Test

The CFT became the most important test for the detection of cattle infected with *Brucella* because of its specificity. The CFT is based on the detection of specific antibodies of the IgM and IgG_1 types that fix complement (Levieux, 1974). The CFT is highly specific but it is laborious and requires highly trained personnel as well as suitable laboratory facilities. Although its specificity is very important for the control and eradication of brucellosis, it may test false negative when antibodies of IgG_2 type hinder complement fixation (Macmillan, 1990).

2.8.6.5 Anti-globulin (Coomb's Test) Test

Coomb's test detects incomplete *Brucella* antibodies of the IgG_2 type and it is used to confirm SAT results. It is used as anamnestic test to identify and remove latent carriers provided S.19 vaccine has not been used in animals. The test, although laborious, is particularly important when the SAT is positive and CFT results are negative or inconclusive (Kiss, 1971). The main limitation of this test is that the test results are indicative for infection only when its titers are at least two times the titres of the SAT. It has been used as an amnestic test to identify and remove latent carriers (provided Strain 19 has not been used in the animals) (Cunningham, 1977).

2.8.6.6 Other Conventional Tests

The 2-mercaptoethanol and the rivanol tests detect specific IgG (Rossi and Cantini, 1969) and are usually used to differentiate between infected and vaccinated cattle.

2.8.6.7 Enzyme-Immunoassay (EIA) Methods

The need for automation of the serological diagnosis, and the fact that the conventional serological tests fail to detect all infected animals resulted in the development of the Enzyme Immunoassay (Bercovich, 1998). The large majority of EIAs in use in brucellosis diagnosis are indirect enzyme-linked immunosorbent assays (i-ELISA). The i-ELISA methods involve the mobilization of the antigens on 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 (Anon, 2001).

Nielsen *et al*. (1988) has reviewed the i-ELISA procedures, antigens, conjugates

and substrates that have been used for antibody detection in both serum and milk.

The test can be used for screening and confirmation of brucellosis and can differentiate between vaccinated cattle and cattle with naturally occurring brucellosis (Bercovich and Taaijke, 1990). Other types of EIAs are the competitive ELISA (c-ELISA) and the particle concentration Fluorescence Immunoassay (PCFIA) described by Macmillan (1990).

The main advantage of the EIAs lies in its relative simple test procedure. The assay is very costly when only few samples are tested. Therefore, it is unsuitable for testing individual animals but it is the ideal test for large-scale screening of suspected herds (Bercovich, 1998).

2.8.6.8 Skin-Delayed-Type Hypersensitivity Test (SDTH)

The SDTH is independent of circulating antibodies. It is used to confirm serologic test results, confirm brucellosis in cattle with ambivalent serologic test results and detects latent carriers of *Brucella* (Bercovich, 1999). The test employs the use of brucellin to elicit an allergic response in infected animals (Bercovich, 1998).

2.9 PUBLIC HEALTH IMPORTANCE

According to the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Office International des Epizooties (OIE), brucellosis is still one of the most important and widespread zoonoses in the world especially in developing countries of the world (Poester *et al*., 2002). Brucellosis in humans is commonly known as "Malta fever", "undulant fever", "relapsing fever" or "Mediterranean fever" (Young, 1995). Animal and human brucellosis are inextricably linked because of dependence of people on animals for nutrition, socio-economic development and companionship (Young and Corbel, 1989). The occurrence of the

disease in humans is largely dependent on the animal reservoir. Where brucellosis exists in sheep and goats, it causes the greatest incidence of human infection. In many developed countries, the animal disease has been brought under control, which has led to subsequent decrease in the number of human cases (WHO, 1986).

Although the disease in animals has been brought under control in several industrialized countries, it occurs sporadically in individuals who acquire the infection by ingestion of unsafe animal products and occupationally exposed groups (WHO, 1986). Out of the six species of *Brucella* currently known, *B. melitensis, B. suis B. abortus* and *B.canis* have public health implication.

Brucellosis in humans is either a food borne disease or an occupational disease. Humans usually acquire brucellosis by consumption of raw or unpasteurized milk or milk products. Soft cheeses made from unpasteurized milk are common sources of infection. Brucellosis is recognized as an occupational hazard for farmers, veterinarians, workers of the meat industry especially abattoir workers, hunters, and laboratory workers. Farmers and veterinarians are most often exposed while handling aborted fetuses or apparently healthy calves born to infected cows and by performing gynaenecological and obstetric manipulations, or rectal examination of infected cattle. Needle sticks and ocular exposure are the most common vaccine accidents (Anon, 2001). Hunters and workers in abattoir or slaughter houses may become infected by contact with infected blood. Laboratory exposures usually result from aerosols generated by improper centrifugation, mouth pippeting or other careless behaviours.

Brucellosis in humans is a multisystemic, acute to chronic disease characterized by fever, headache, joint pains, musculo-skeletal pains, sweating, malaise and body wasting. Symptoms of acute brucellosis are “flu-like” and highly nonspecific

(McDermott and Arimi, 2002).

2.10 ECONOMIC IMPORTANCE

Brucellosis is one of the most widespread and economically ravaging of zoonoses recognized worldwide, not only because of the heavy economic losses and reduction in animal protein supplies to which it is responsible but also because of its direct impact on human health (WHO, 1986). Brucellosis like other abortion causing diseases of animals, causes economic losses through temporary infertility arising from chronic metritis, actual abortion and resulting decreased milk production by dairy cows, birth of weak animals that die soon after birth, retention of the placenta, death from acute metritis, sterility, arthritis or bursitis (hygroma), and increased cost of animal replacement as well as lowered sale value of infected animals (FAO, 2004).

The economic losses due to human brucellosis, even though difficult to estimate, result from physical and psychological suffering due to infection, hospitalization, the cost of drugs and the loss of work or income due to illness, physical incapacity and reduction in productivity (WHO, 1986).

Additionally, there are general indirect economic losses for countries due to enforcement of control and eradication of brucellosis. The country incurs costs generated by prophylactic activities taken to control brucellosis, i.e. vaccination by veterinarians and their assistants, vaccine costs and compensation paid to the farmers for sanitary slaughter of infected animals (FAO, 2004).

Valuing economic impacts across animal species and humans is complicated. Economic impacts of brucellosis on livestock species can be estimated because prices can be estimated for direct losses due to morbidity and mortality and indirect losses due to treatment costs. Control programmes can then be evaluated based on the potential

benefits derived by avoiding these direct and indirect losses compared to the programme costs, in a Benefit-cost Analysis studies (Morris, 1999; McDermott and Arimi, 2002). However, for human morbidity and mortality losses, a different measure, Disability-Adjusted Life Years (DALY) is applied (Murray and Lopez, 1996). This avoids the complication of financially valuing human mortality and morbidity. In assessing the impact of control programmes on human brucellosis, cost-effectiveness analysis is used, ranking programmes based on their costs per DALY averted.

Few studies have assessed the direct or indirect losses associated with brucellosis in livestock in Nigeria (Chukwu, 1985b; Rikin, 1988a; Ajogi *et al*., 1998; Ajogi and Akinwumi, 2001). Indirect losses, particularly those that require brucellosis-free status to access regional or international livestock markets, have not been estimated in sub-Saharan Africa but could be a considerable constraint to future trade (McDermott and Arimi, 2002).

The impact of brucellosis and other zoonoses affecting livestock production are considerably magnified by their consequences in humans. On a global scale, brucellosis does not rank among the top diseases based on DALY losses. However, specific studies assessing the impact on high-risk groups such as pastoral populations and occupations working closely with livestock could provide justification for targeted control in these high risk groups. DALY calculations combine two sources of burden, Years of Life Lost (YLLs) and the years of life lived with Disability (YLDs). For brucellosis years of life lived with disability will be the major impact. Additionally, it is important to assess the costs to local health services, particularly because brucellosis is a chronic and relatively difficult-to-treat infection (McDermott and Arimi, 2002; Roth *et al.*, 2003).

2.11 PREVENTION AND CONTROL

Because brucellosis is a disease of major economic importance, a strategy for its control in animals 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 minimized. It is accepted that before a zoonosis control programme 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 optimization of expenses, and the evaluation of activities performed and achievements to be assessed (Thursfield, 1995).

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 programme 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 programme, so that they can be informed about the progress of the work (WHO/MZCP, 1998).

Different countries and even ecologically distinct areas within a country may require different strategies for the prevention and control of brucellosis in an animal population, depending on epidemiological and socio-economic conditions. For example, calfhood vaccination with regular test and slaughter was found to be economically viable control strategy for brucellosis in cattle herds in Nigeria (Ajogi, 1998). Decisions as to

the appropriate strategy for the control and/or elimination of brucellosis are usually a national responsibility, though in some large countries this may be delegated to regions or provinces or made applicable to individual Islands or communities. In deciding on the strategy to be adopted, many factors including the following must be considered: the type of animal husbandry, the geography of the area, the patterns of commerce, financial, technical and personnel resources available and, most importantly, the prevalence of disease and the acceptance of strategy by the livestock owners (WHO/MZCP, 1998).

In addition to the strategy selected, simple non-specific measures of prevention of the spread of the infection should be applied in a systematic way. These include general hygienic measures and the provision of clean accommodation that can be disinfected at parturition. All parturitions in an infected herd should be regarded as potential sources of infection and all the non-living products and contaminated materials must be incinerated or buried (Alton, 1990).

The provision of information and education concerning the disease to farmers and local communities is essential. The farmer must be informed of all the advantages of the control campaign, such as the economic benefits and the elimination of risk to health of his family and himself. Also, professional training is essential for the implementation of the strategies by the appropriate national services. The training of the different groups in society will ensure that the right actions are taken and that the necessary resources are mobilized (WHO, 1986).

The key economic information required for disease control decisions are the potential returns to different control strategies (McDermott and Arimi, 2002).

Conditions in different countries throughout the world vary so much that a single universal programme for the control and/or eradication of brucellosis is not feasible.

WHO (1986) recommended the following programme based on the above understanding:

Cases of very Mild Infection (1-2%): Surveillance procedures for detecting infected herds and the elimination of reactors with or without vaccination of heifer calves and possibly of non-reactor adults.

Cases of Moderate Infection (2 – 5%): In areas where the above eradication programme is not economically feasible, the following control programme is recommended until such a time as eradication can be initiated. These include surveillance, and testing procedures for potentially infected herds. In herds where infection rate is low, there should be elimination of reactors with or without vaccination of heifer calves and non-reactor adults. In herds with high rate of infection, testing and retention of reactors in segregated groups, vaccination of heifer calves and possibly of adult non-reactors is recommended. In non-reactor herds, vaccination of heifer calves and adults is recommended.

Cases of Heavy Infection Areas (>5%): In areas where neither of the above programme is feasible because of very high rate of infection a programme of mass vaccination of young and adult cattle with approved *Brucella* vaccines and the identification of vaccinated animals will suffice until such a time as the weight of infection has been reduced reasonably to allow for an eradication exercise.

In general, the selection of the strategies for the implementation of control of brucellosis is based on the following principles:

2.11.1 Use of Vaccines

Control of brucellosis can be achieved by using vaccination to increase the population 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). In general, mass immunization is indicated where the prevalence of infected animals is high. It should be based on knowledge of the prevalence in the flock or region. As a general rule, induction of an effective, long lasting protective immune response to facultative intracellular parasites like *Brucella* requires the use of live vaccines or in some cases the use of multiple injections of appropriate protective antigens in the presence of adjuvants which favour cell mediated immune mechanisms (WHO, 1986).The follwing are the vaccines used for the prevention of brucellosis:

2.11.1.1 *B. abortus* Strain 19 Vaccine

One of the most commonly used vaccines to prevent bovine brucellosis is *B. abortus* strain 19 (Nicoletti, 1990). Strain 19 (S19), described by Buck in 1930 (Buck, 1930) was originally isolated as a virulent strain from the milk of a Jersey Cow in 1923 and left at room temperature for a year in the laboratory, was found to have become attenuated. This strain was able to induce protective immunity in cattle. Effectiveness may fluctuate depending on a variety of variables including age at vaccination, dose, route and prevalence of brucellosis in vaccinated herds (Nicoletti, 1990). Strain 19 is an attenuated organism of smooth morphology normally unable to grow in the presence of erythritol (Jones *et al*., 1965). Although S19 is of low virulence for cattle and vaccination with 40 - 120 $\times$ 10^9 CFU (classical dose) of living *B. abortus* organisms gives a fair to good protection, it also has some disadvantages (Plommet, 1991b). Vaccination of pregnant cows can result in abortions. It induces persistent antibody response that confuses the serological diagnosis of brucellosis since they prevent easy differentiation of vaccinated from infected cattle. It is excreted in milk and may induce brucellosis in humans.

The presence of LPS with an O-chain on S19 explains the appearance and persistence of antibodies in serum following administration of this vaccine. Appearance and persistence of these antibodies depends on age, dose and route of vaccination (Nicoletti, 1990). Their production makes the continued use of the vaccine incompatible with simultaneous application of test and slaughter procedures for the control of brucellosis.

To diminish these undesirable effects of vaccination with S19, two vaccination procedures have been suggested. In one procedure, calves are vaccinated once with 3 - 10 × 10^9 CFU (reduced dose) at an age of 4 - 8 months and for a second time with 3 - 10 × 10^9 CFU as adults. The second procedure suggests a conjunctiva vaccination of calves with two drops of vaccine containing 4 - 10 × 10^9 CFU at age of 4 - 10 months and a second conjunctiva vaccination with the same dose six months later (Nicoletti, 1990).

2.11.1.2 *B. melitensis* Rev. 1 Vaccine

Rev.1 vaccine is a live, attenuated *B. melitensis* strain derived from a virulent *B. melitensis* isolate which became dependent on streptomycin for its growth, but lost this characteristic, although remaining streptomycin resistant, upon further subculture (Elberg and Faunce, 1957). It stimulates protection against infection with *B. melitensis* in sheep and goats and also protects rams against infection with *B. ovis (*Alton, 1985). This vaccine is attenuated with field strains but retain some virulence. Depending on the dose administered during pregnancy, abortions may occur with variable frequency (Alton *et al*., 1988). Apparently in rams the vaccine is avirulent (Erasmus and Bergh, 1985) or of low virulence (Lantier and Fensterbank, 1985). Rev. 1 is a smooth organism, therefore it induces positive serology which interferes with diagnosis. The use of Rev. 1 in cattle has been investigated and results indicate that it gives better protection than strain 19 (Garcia-Carrillo, 1980).

2.11.1.3 *B. abortus* Strain 45/20 Adjuvant Vaccine (Duphavac)

B. abortus smooth strain 45 was isolated from a cow in 1922 and a phenotypically rough derivative was obtained after 20 passages in guinea pigs. This derivative was called strain 45/20 and was able to protect guinea pigs and cattle from *Brucella* infection (McEwen, 1940). Unfortunately, when used as a live vaccine, strain 45/20 was not stable and tended to revert to the smooth virulent form defeating the purpose of attenuation usually associated with rough strains and also lead to the induction of positive, diagnostic serology (Taylor and McDiarmid, 1949). Recent studies confirmed the presence of substantial amounts of O-chain in phenotypically rough strain 45/20 colonies using monoclonal antibodies specific for the O-chain (Schurig *et al.,* 1991). In order to overcome the lack of stability problem of the live strain 45/20 vaccine, strain 45/20 was used as a bacterin incorporated in adjuvants usually based on water and oil emulsions. Much work has been devoted to the use of this vaccine with differing results regarding protective efficacy and induction of positive serology. Protection induced by one or two vaccinations has been described as being superior, equal or inferior to protection induced by S19. Most investigators felt that 2 vaccinations were necessary (Alton *et al*., 1983; Chukwu, 1987a). Regarding serology, it appears that strain 45/20 may or may not induce the diagnostically undesirable antibodies. The absence of O-chain in phenotypically rough organisms is most probably associated with this observation (Schurig *et al*., 1984; Chukwu, 1985c).

The variability of the reports of the findings plus the presence of severe local reactions observed with some animals at the site of vaccine inoculation eventually prompted the discontinuation of the use of the vaccine.

2.11.1.4 *B abortus* Strain RB51 Vaccine

B. abortus strain RB51 was selected by growth of *B. abortus* strain 2308 in the presence of rifampicin (Schurig *et al*., 1991). "R" standing for "rough" and "B" for *Brucella* while 51 refers to an internal laboratory nomenclature used at the time it was derived. Strain RB51 is essentially devoid of the O-chain, its roughness being very stable after multiple passages *in vitro* and *in vivo* through various species of animals (Colby, 1997). Since it lacks an O-chain, it does not induce anti-O-chain antibodies measurable by conventional serological tests or ELISA, regardless of age, dose or frequency of injections. Strain RB51 is attenuated as indicated by studies carried out in mice, guinea pigs, goats and cattle where it is cleared in a relatively short time and has no or highly reduced abortifacient characteristics (Schurig *et al.*, 1991; Palmer *et al.*, 1997). When used in single vaccination protocols, its protective effect in cattle is similar to that of S19 but no antibodies to the O-chain are induced (Cheville *et al*., 1993; 1996). Multiple applications of the vaccine do not induce anti-O-chain antibodies but probably increase the immunity of the animal. Recent experiments carried out indicate that strain RB51-induced immunity (at least one year after vaccination) is better than the immunity induced by strain 19 (Lord *et al*., 1998).

Studies in mice indicate that strain RB51 can protect against infections with *B. abortus, B. melitensis, B. suis* and *B. ovis* (Winter *et al*., 1996). Ongoing work with various animal species confirms excellent protective activity in swine against infection with *B. suis* under field conditions (Lord *et al*., 1998) and has shown that RB51 can protect up to 93% of RB51 vaccinated goats against *B. melitensis* infection. Preliminary results in sheep suggest that RB51 does not induce protective immunity in this species if used as a single vaccine. Strain RB51 has been approved for use as an official vaccine in

the USA, replacing S19 (Stevens *et al*., 1997).

2.11.1.5 *B. suis* Strain 2 Vaccine

The live attenuated *B. suis* strain 2 (S2), produced in China by serial transfer of a virulent *B. suis* biovar 1 strain of swine origin, has been widely used in that country (Xie, 1986) and in Libya (Mustafa and Abusowa, 1993), particularly for immunization of sheep and goats (in drinking water). Its virulence is approximately the same as *B. abortus* S19 and is stable. Although the S2 strain also gave a satisfactory protection rate in cattle (Nicoletti, 1990), its efficacy against experimental *B. melitensis* infection in pregnant ewes (Verger *et al*., 1995) or against *B. ovis* infection in rams (Blasco *et al*., 1993) was inferior to that of *B. melitensis* Rev. 1 vaccine.

2.11.1.6 *B. melitensis* H38 Vaccine

The H38 vaccine is composed of killed smooth, virulent cells of *B. melitensis* in adjuvant. The vaccine gives good protection and can be administered to pregnant and lactating animals. However, because it stimulates a long lasting immune response, that interferes with the serological diagnosis of brucellosis and induces a marked skin reaction on the injection site of the vaccine. It is not used very often (Alton, 1985; Plommet, 1991b).

2.11.1.7 *B. melitensis* Strain M111 Vaccine

The rough *B. melitensis* strain M111 vaccine (M111) was developed in China in the 1980s for use as a live vaccine. Strain M111 was obtained from a smooth *B. melitensis* strain by selection of non-agglutinating colonies. It has cultural and biochemical properties of *B. melitensis* biotype 1 but is rough. Its virulence is slightly less than that of *B. suis* S2. It showed no change in properties after 50 sub-cultures or after 8 passages in guinea-pigs or 4 passages in pregnant sheep. Post mortem

examinations showed that the strain did not produce lesions in sheep or goats, nor did it produce abortion in pregnant animals. Rabbits, sheep, goats and cattle vaccinated with strain M111 did not produce antibodies against smooth *Brucella* detectable in conventional serological tests compared to S2, S19 and S45/20 vaccines. It did however, stimulate antibodies to rough *Brucella* strains (Anon, 1997).

Strain M111 was able to produce effective immunity against challenge with virulent *B. melitensis* strain M28 in guinea pigs, sheep and goats. The vaccine could be applied by parenteral injection or orally. The protective efficacy was 85% for sheep and 78% in goats. In field trials, the vaccine proved to be safe for sheep and goats and was effective in stimulating immunity against wild type *B. melitensis*, (Anon, 1997).

2.11.1.8 *B. abortus* Strain VTRA1, *B. melitensis* Strain VTRM1 and *B. suis* Strain VTRS1 Vaccines

The rough Strains *B. abortus* VTRA1 (VTRA1), *B. melitensis* VTRM1 (VTRM1) and *B. suis* VTRS1 were constructed using the *Brucella rfb*U gene sequence to produce disruption mutants of *B. abortus* Strain 2308, *B. melitensis* 16M and *B. suis* 40 (biovar 4) respectively. The *rfb*U gene in *Brucella* codes for mannosyltransferase, an enzyme necessary for the assembly of the *Brucella*-O-Chain (Winter *et al*., 1996). The VTRM1 and VTRS1 were tested in mice for their virulence (spleen clearance after intraperitoneal injection) and their ability to protect mice against infection with heterologous and homologous strains of *Brucella*. The virulence of VTRM1 and VTRS1 was lower than the virulence of the parent strains but the attenuation achieved was not as great as with RB51 (Winter *et al*., 1996). This indicates that *rfb*U is involved in virulence but also suggests that other genes, most probably not involved in the synthesis of LPS components, play an important role in attenuation (Anon, 1997).

Strain VTRM1 and VTRS1 induced immunity against infection with *B. abortus*,

B. melitensis, B. suis and *B. ovis* isolated from a variety of animal species in the mouse model. In several instances, protection induced by VTRM1 and VTRS1 was superior to the protection afforded by strain RB51, probably because these strains replicate more vigorously and remain longer in the host than strain RB51. Neither strain induced antibodies to the O-chain (Winter *et al*., 1996).

2.11.1.9 *B. abortus* Strain *rfb*K Vaccine

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

2.11.1.10 Novel Vaccines

Other interesting approaches to develop new generation vaccines, such as the construction of recombinant strains deleted in relevant diagnostic proteins, or DNA based vaccines, are being currently investigated (Anon, 1997; Schurig *et al*., 2002). It is probable that some of these vaccines will become candidates to replace the available classical vaccines in the near future.

2.11.2 Test-And-Slaughter

Elimination of infected animals by test-and-slaughter policy to obtain brucellosis free herds and regions is usually an accepted programme which 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 programme it is essential that the animals are under strict surveillance and movement control. Animals must be individually identified and an efficient and well organized veterinary service for

surveillance and laboratory testing must be in place (WHO, 1986). 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. 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 essential that full cooperation of farmers be sought 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 either be voluntary or compulsory. Voluntary schemes may be useful in the early stages of campaign but may need to be supported by adequate incentives. Compulsory eradication is required in the final stages (WHO, 1986).

2.11.3 Prevention of Spread between Animals

In areas where 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 animals 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 Office International des Epizooties (OIE) which also describes essential testing of animals and quarantine measures (Schnurrenberger *et al*., 1987; WHO, 1986).

2.11.4 Prevention of Human Brucellosis

It is generally recognized that prevention of brucellosis in humans still depends on the eradication or control of the disease in animal hosts. These involve the exercise of hygienic precautions to limit exposure to infection through occupational activities, effective treatment of dairy products and other potentially contaminated food products, and by avoiding consumption of raw milk and raw milk products (Corbel, 1997). However, in some parts of the world this is not feasible and attempts have been made to control the disease by vaccination. Vaccination now has only a small role in the prevention of human brucellosis, although in the past, various preparations have been used.

Renewed interest in *Brucella* as a potential biological warfare agent has also drawn attention to the need for effective vaccines for humans (Anon, 2000). A derivative of strain 19, Strain 19-BA, given intradermally by scarification, was formerly used in the Asian Republics of the former USSR. It gave limited protection for a relatively short duration and re-immunization was necessary, but was accompanied by increasing frequency of hypersensitivity reactions (Kolar, 1989). Other attenuated strains, such as *B. abortus* 84-C and 105-M given intradermally or as aerosols were also used in China and the USSR (Lu and Zhang, 1989). Although apparently effective, these vaccines could provoke severe reactions if not administered correctly or if given to sensitized individuals, and they appear to be no longer in routine use.

Attempts have been made to develop sub-unit vaccines for use in humans. A sodium dedocyl sulphate (SDS) - insoluble peptidoglycan fraction of *B. melitensis* M15 was used in France (Roux and Serre, 1971) but conclusive evidence of efficacy based on double blind controlled clinical trials was not obtained (Hadjichristodolou *et al*., 1994). Similarly, an acetic acid extracted polysaccharide-protein fraction developed in the

Union of the Soviet Socialist Republic (USSR) was reported to have low reactogenicity even in previously vaccinated individuals, but evidence of protective efficacy derived from controlled clinical trial is still being awaited (Schurig *et al*., 2002).

2.12 TREATMENT

Treatment of brucellosis in domestic animals is not indicated. Prolonged treatment of infected domestic animals with a high dosage of antibiotics is not used due to the appearance of antibiotics in the human food chain and it interferes with the production of milk products. Moreover, as brucellae are facultative intracellular bacteria, relapses after treatment usually occur (FAO, 2004). However, a practical and effective treatment regimen could be used to treat valuable breeding animals and would benefit producers in countries which cannot afford to control the disease by the test and slaughter approach (Eaglesome and Garcia, 1992). Reports of unsuccessful attempts to treat bovine brucellosis involved the use of multiple injections of a combination of a long acting tetracycline and streptomycin (Milward *et al*., 1984; Nicoletti *et al*., 1985). The inadequacy of antibiotics regiments in controlling *B. abortus* may be due to the failure of the antibiotics to penetrate cell membrane barriers effectively. Therefore, the use of liposomes, small vesicles which are capable of entrapping many compounds including antibiotics, and when administered parenterally, are taken up by cells of the macrophage system, was introduced (Poste *et al*., 1984). The effectiveness of encapsulation of antibiotics in liposomes to enhance antibiotic treatment of bovine brucellosis has been investigated (Milward *et al*., 1984; Dees *et al*., 1985; Nicoletti, *et al*., 1989). It was observed that the most effective treatment regimen consisted of two intramammary infusions of streptomycin liposomes and two doses of oxytetracycline administered intramuscularly (Nicoletti *et al*., 1989). Recently, successful treatments of brucellosis using a combination of long-acting oxytetracycline and streptomycin in cows (Radwan et

al., 1993) and in sheep and goats (Radwan *et al*., 1992) have been reported.

The optimum antibiotic therapy for human brucellosis is still disputed despite extensive studies over the past 15 years (Corbel, 1997). The treatment recommended by the World Health Organization (WHO) for acute brucellosis in adults is a combination of rifampicin and doxycycline daily for a minimum of 6 weeks (WHO, 1986). Several studies (Luzzi, *et al*., 1993; Ariza *et al*., 1992; Montejo *et al*., 1993), however, suggested that treatment with a combination of streptomycin and doxycycline may result in less frequent relapse than treatment with combination of rifampicin and doxycycline. Infections with complications, such as meningoencephalitis and treatment in children may require combination therapy of rifampicin and trimethoprim/sulfamethoxazole (Lubani *et al*., 1989) while endocarditis may be treated with rifampicin, streptomycin and doxycycline for 6 weeks (Chan and Hardiman, 1993).

2.13 BRUCELLOSIS IN NIGERIA

2.13.1 Overview

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 abortion were reported (Anon, 1927). Since then, several epidemiological surveys have revealed the presence of the disease in the livestock population countrywide (Ocholi *et al.*, 1993; Halle and Ajogi, 1997). Bovine brucellosis due to *Brucella abortus* is the most prevalent *Brucella* infection in Nigeria. There is only one reported case of isolation of *Brucella suis* from pigs (Bale and Nuru, 1985).Though there have been isolation of *Brucella* from cattle, goats, sheep, horses, and dogs, cases have been associated mainly with *B. abortus* biotype 1. Isolation of *B. melitensis* from sheep and goats has been reported (Bale *et al*., 2003). There is no published report on the isolation of *B. canis, B. ovis, and B. neotomae*

from livestock in Nigeria.

Cattle, sheep, goats and pigs are the principal farm animals in this country. About 95% of the total population of these animals is managed by the traditional nomadic Fulani pastoralists who are accustomed to extensive system of management (Rikin, 1988b).

Geographical, economical and social conditions account for the existence of very different livestock production systems across the country. In the semi-arid and sub-humid climatic zones of the northern part of Nigeria, the main livestock production system is the livestock dominated and the crop–livestock systems .These include, traditional nomadic pastoral system, ranches and peri-urban systems. In the humid areas of the Southern, Western, and Eastern States, there is crop-livestock system where there are usually less livestock, particularly fewer cattle and more emphasis is on crop production. Sheep, goats and to some extent pigs are relatively more important.

2.13.2 Epidemiology

2.13.2.1 Bovine Brucellosis

Previous studies revealed that bovine brucellosis is an endemic disease in Nigeria (Eze, 1985).The disease has been diagnosed wherever there are cattle in Nigeria and all breeds of cattle have been shown to be infected. Studies in various parts of the country indicate that the disease is widespread particularly in ranches, livestock breeding centers and dairy farms where prevalence ranged between 3.7 and 48.8% (Esuruoso and Hill, 1972; Esuruoso and Van Blake, 1972; Esuruoso, 1974, Agunloye, *et al.*, 1988) and between 0.4 and 26% in the traditional nomadic Fulani herds, and in slaughtered cattle through abattoir surveys (Nuru and Dennis, 1975; Eze, 1978; Falade and Shonekan, 1981; Chukwu, 1987b; Ocholi *et al.*, 1996; Ajogi *et al.*, 1996; Ajogi, 1997). All these investigations were based on serological surveys. Very few studies (Eze, 1978; Bale and

Kumi-Diaka, 1981) have been dedicated to the investigation of the different biovars of brucellae among bovines in Nigeria. Eze (1978) isolated 20 strains of *B. abortus* from cattle in Northern States of Nigeria, out of which 19 (95%) were of biovar 1, while 1 (5%) was of biovar 2. Similarly, out of 8 strains of *B. abortus* from semen and aborted fetuses of cattle in livestock investigation and breeding centers, 5 (62.5%) were of biovar 1, 2 (25%) were of biovar 3 and 1 (12.5%) was of biovar 4 (Bale and Kumi-Diaka, 1981). It does appear from these reports that *B. abortus* biovar 1 is the prevailing species infecting livestock in Nigeria.

2.13.2.2 Ovine and Caprine Brucellosis

Brucellosis of small ruminants, sheep and goats, is considered a disease of great importance in Nigeria from the social and economic point of view. The first report of brucellosis in goats was based on serological evidence presented by Adams and Mckay (1966) and later by Kramer *et al.* (1967). Subsequent reports based on serological surveys from various parts of the country have confirmed the presence of the disease among sheep and goats (Falade *et al,* 1975; Falade, 1981; Falade and Shonekan, 1981; Bale *et al.,* 1982; Okewole *et al.*, 1988). These studies revealed that prevalence vary between 2.1 and 14.5%.There are very few reports available on the isolation of brucellae from sheep and goats. Field experiences indicate that abortion occurs frequently in sheep and goats but the causes were not subjected to detailed laboratory investigation. However, the isolation of *B. abortus* biotype 1 was reported by Falade, (1981). Okoh (1980) isolated *B. abortus* from a case of sporadic natural infection of sheep but did not specify the biotype involved.

2.13.2.3 Canine brucellosis

The social and economic importance of the disease has not been properly

assessed even though the increase in the number of commercial breeding kennels may aid in the increase in the risk factors for the transmission of the disease among dogs. Initially there were doubts as to the presence of canine brucellosis in Nigeria when serological survey by Falade (1977) found no antibodies to *Brucella* in dogs. However *B.canis* has been detected either through serology where a prevalence rate of 28.6% was obtained (Adesiyun *et al,* 1986) or through cultural recovery from a case of abortion in an imported Boxer (Okoh *et al.,* 1979).

2.13.2.4 Porcine Brucellosis

Pig production in Nigeria is limited to large scale, commercial and subsistence backyard swine keeping. Swine brucellosis has been studied since the 1940s. Early evidence of swine brucellosis in Nigeria is contained in reports by Mettam (1947) and Adams and McKay (1966). Though it is possible that the disease is present in swine raising areas of the country, there is no sufficient information to indicate the distribution and prevalence of swine brucellosis in Nigeria. In a limited study, 12 (2.7%) out of 443 pigs sampled from 6 piggeries in Northern States of Nigeria had *Brucella* antibodies and *B.suis* was isolated from 2 of the pigs (Bale and Nuru, 1985) but the biotype involved was not invetigated.

2.13.2.5 Equine brucellosis

Horses have recently gained popularity in Nigeria due to increasing interest in polo games and horse racing. The role played by horses in the epidemiology of brucellosis in Nigeria is poorly understood. There are very few reports available on the presence of the disease in horses. However, Bale and Kwanashie (1984) demonstrated *Brucella* antibodies in horses in Northern Nigeria, where 14 (8%) out of 166 were reactors. The isolation of *B. abortus* biotype 1 from an aborted equine fetus (Oladosu *et*

al., 1984) further confirmed the presence of the disease in horses in the country.

2.13.2.6 Brucellosis in Camels

The first available evidence on the presence of brucellosis in camels in Nigeria was based on serological surveys by Okoh (1978) who found prevalence of antibodies as low as 1%. However, recent serological studies by Adamu *et al*. (1997) and Adamu and Ajogi (1999) indicated higher prevalences of 27.8% and 24.3% respectively.

2.13.2.7 Human brucellosis

The first case of brucellosis in a human being reported in Nigeria was in 1962 (Collard, 1962) when *Brucella* antibodies were demonstrated in the sera obtained from healthy persons in various parts of Nigeria. Between 1962 and 1967 records obtained from University College Hospital, Ibadan indicated 9 cases of human brucellosis and *Brucella* species were isolated from 4 blood and 1 bone marrow culture (Falade, 1981). Most cases of human brucellosis were essentially an occupational hazard, occurring amongst workers in the livestock industry. Epidemiological studies revealed significantly higher prevalence of infections among occupationally exposed persons including herdsmen, abattoir workers and veterinarians than occupationally non-exposed population including blood donors and normal pregnant women (Falade, 1974; Alausa and Osoba, 1975; Alausa and Awoseyi, 1976; Alausa, 1979; Alausa, 1980). The source of infection for humans is likely and primarily from livestock reservoirs of brucellosis. Thus the risk to humans is a function of the risk in livestock and the livestock-human infection rate (Brisbie *et al*., 1993; Baba *et al*., 2001)

2.13.3 Problems of Brucellosis Control

Brucellosis is endemic in Nigeria (Eze, 1985). The information obtained from existing records indicate that brucellosis in Nigerian livestock has existed for a long time

and is still spreading despite efforts made by the government to improve the livestock industry.

Brucellosis continues to be a predominant reproductive disease in Nigeria causing abortion, infertility and sterility, resulting in heavy economic losses to the livestock industry and posing serious human health hazards (Alausa, 1983). Though there are no accurate figures available on nationwide economic losses due to brucellosis, the role played by this disease in limiting livestock production and its economic impact on the livestock industry in Nigeria is widely recognized. An annual loss due to the disease is estimated to be about 224 million US Dollars (Esuruoso, 1979) excluding losses in human productivity. Many factors have been identified to be militating against following internationally laid down procedures for the control of brucellosis leading to eradication of the disease in Nigeria:

1. One major factor contributing to the spread of the disease is free movement of animals practiced by the nomadic Fulani herdsmen who are accustomed to the traditional extensive system of management and owns about 95% of all food animal population in Nigeria (Rikin, 1988b). This aids in the increase of associated risk factors and in the dissemination of the infection.
2. Herding together various animal species like cattle, sheep and goats help in the spread of the disease among animal population where infected species of animals spread the disease to other animals within the population (Eze, 1985).
3. The Fulani cattle herd which constitutes 95% of the National animal population is migratory. As nomads, they migrate from one part of the country to another in search of grazing lands and watering points. This makes disease investigation, protective vaccination of cattle in the herd, and identification and handling of cattle within the herd difficult (Eze, 1985).

4. The primary objective of the Fulanis for rearing cattle is for prestige and enhancement of social status, rather than for financial gains and provision of more animal protein for the ever increasing human population. Therefore incentives and disease control measures aimed at improving milk and beef yield and therefore financial gains may not achieve much success with nomads as with commercial cattle producers (Eze, 1985).
5. The system of animal disease surveillance and reporting in the country is not efficient. This makes epidemiological trace-back and enforcement of control measures very difficult.
6. The importation of exotic high production breeds of cattle without having the required veterinary infrastructure and support, and the trend towards intensification of animal production favour the spread and transmission of the infection.
7. Porous international borders with free movement of infected animals across the borders into the country also allow easy transmission of the infection to the national herd.
8. At present, there is no officially coordinated control programme for brucellosis in Nigeria. A control programme for the disease should be instituted if the unrealized potentials are to be derived from the livestock industry in Nigeria.

2.13.4 Prospects for Brucellosis Control

Knowledge and understanding the prevailing species and biotypes of *Brucella* infecting the livestock population and the distribution of infection is crucial to the necessary steps required for the formulation of policies and strategies for the control of brucellosis in an animal population. A wealth of information is already available on the epidemiology of brucellosis in Nigeria, which is useful to designing a control

programme:

1. The disease is known to be endemic among livestock population in the country. Prevalence rates vary from low to medium and high in different areas of the country where pockets of infection can be identified, and also from herd to herd.

2. It appears *B. abortus* biotype 1 is the prevailing species infecting livestock in Nigeria.

3. Cattle are one of the reservoirs of *Brucella* infection in the country. Apart from epizootics occurring independently in cattle, histories of some cases of infections in other animals indicate that they had been in contact with infected cattle (Okoh, 1980; Okewole *et al*., 1988). Control measures targeted on cattle aimed at drastically reducing the incidences of the disease in this species will have tremendous impact on the National Programme of control both in animals and human beings.

4. The benefit-cost analysis for a control programme shows that all the acceptance criteria used in the analysis hold considerable promise and a favourable return from the livestock industry. In addition to the readily quantifiable benefits derived from a control programme, other more difficult to quantify benefits are the satisfaction of having a healthy national herd, increased milk and meat production, increased hides and skins for export and most of all, the reduction in human health risks (Rikin, 1988b).

5. The current drive to settle nomads in designated grazing reserves will enhance control strategies (Ajogi *et al*., 1996).

All these indicate that the prospect for the control and eradication of brucellosis

in Nigeria is high and feasible. However, the design of any brucellosis control programme for the country should take into consideration the complexity of the socio-cultural, economic and political factors of livestock owners including the biologic characteristics of the disease agent, transmission, and host responses. Additionally, the control strategies developed should be in line with extensive husbandry as well as rhyme with the practical realities of the prevailing socio-cultural set up of the Nigerian livestock industry (Rikin, 1988a).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Isolation of Brucellae from the Nigerian Livestock

3.1.1 Purpose

The purpose of this experiment was to isolate brucellae causing disease in the Nigerian livestock.

3.1.2 Vaginal Swabs Sticks:

A commercially prepared sterile vaginal swab sticks were used. Essentially the swab sticks consist of a 30 cm long plastic applicator with an absorbent cotton wool swab on one end. It is enclosed in a transparent plastic tube.

3.1.3 Collection of Samples

Samples of aborted fetuses, milk blood and vaginal swabs from cows, sheep, goats, pigs, dogs and horses and hygroma fluid from cows and horses were collected from Adamawa, Bauchi, Borno, Kaduna, Kano, Kogi, Nasarawa, Plateau, Sokoto and Taraba States (Table 3.1).

3.1.3.1 Aborted Fetuses

Aborted fetuses and aborted materials were sent in from the field in plastic bags. They were cultured immediately on arrival at the laboratory.The stomach contents were removed by searing an area of the stomach wall with heated spatula, plunging the tip of Pasteur pipette through the seared area and transferring about 3 mls into sterile bijou bottles.

3.1.3.2 Milk Samples

Teats were washed in soapy water, disinfected with a swab of alcohol and dried with a clean cloth. The milk was collected straight into sterile universal bottles which

were immediately put in ice boxes until cultured.

3.1.3.3 Hygroma Fluids

Hygroma were swabbed with cotton wool soaked in methylated spirit and allowed to dry for 2 minutes. A sterile 21 gauge needle was inserted into the hygroma. Ten ml. of fluid was then drawn into a sterile bijou bottle which was immediately put into an ice box.

3.1.3.4 Vaginal Swabs

The perineum and the vulva were washed with clean water and dried with a clean cloth. The swab was taken out of the protecting plastic tube and then inserted into the animal's vagina and rotated gently back and forth, then withdrawn back from the vagina. The swabbed end was then cut into a bijou bottle containing peptone saline using a sterile scissors. The bottles were then put into the ice box.

3.1.3.5 Blood

The skin over the jugular vein was disinfected by swabbing with cotton wool soaked in methylated spirit. After waiting 2 minutes to dry, 10 ml of blood was withdrawn into citrated vacutainer bottles.

3.1.4 Culturing of the Collected Samples for Brucellae

Most of the samples were cultured onto SDAA, TSAA and VCN the same day they were brought from the field.

3.1.4.1 Aborted Fetuses and Aborted Materials

Scrapings of fetal placenta using flamed scapel blades were spread over culture plates, which had been dried at 37°C for 24 hours. A few drops of fetal stomach contents were aseptically obtained using sterile Pasteur pipettes and were spread over the surface

of the culture plates with an inoculating wire loop. Lung and liver tissues were first sliced with sterile scissors and rubbed over the surface of the media.

3.1.4.2 Milk Samples

Five mls of whole milk samples were centrifuged at 6000g for 15 minutes in sterile Pyrex centrifuge tubes. A mixture of the floated cream and the sediment was smeared over the surface of the freshly prepared and dried culture media.

3.1.4.3 Hygroma Fluids

Fresh hygroma fluid was smeared over culture media within minutes of collection. Samples that were not transferred to the laboratory immediately were stored frozen at -20°C. They were then submitted to the laboratory for culture on ice.

3.1.4.4 Vaginal Swabs

The swabs were smeared over the surface of freshly prepared and dried culture media. The smears were then spread over the surface of the culture plates with an inoculating wire loop.

3.1.4.5 Blood

0.3ml of citrated blood was transferred onto the surface of the agar plates and was spread out using an inoculating wire loop.

3.1.5 Incubation of Culture Plates

The plates were put into anaerobic jars and 10% CO_2 was added using CO_2 generating pack (BBL) according to the method of Alton *et al*. (1988). The jars were incubated at 37°C for 3 days before the plates were examined for *Brucella*-like colonies. The plates were discarded if no growth was evident after 10 days of incubation.

3.1.6 Direct Observation of Colonies

The *Brucella* culture was streaked on SDA plates to give isolated colonies and confluent growth on the same plate. After the organisms had grown for four days, the plates were examined with obliquely transmitted light under stereo microscope as described by Corbel *et al*. (1987).

3.1.7 Identification of Brucellae

Colonies typical of *Brucella* species were sub-cultured onto SDA from which subsequent growths were examined by Gram Stain and Slide Agglutination with *Brucella* A and M Monospecific antisera. They were also examined for catalase production. Isolates were also examined for growth on MacConkey agar and blood agar (Corbel *et al*., 1987).

3.2 Biotyping of the *Brucella* Strains Isolated From the Livestock

3.2.1 Purpose

To establish the species and biotypes of brucellae causing infection in the Nigerian Livestock.

3.2.2 Chemical Reagents

Dyes; thionin and basic fuchsin were obtained from the British Drug House (BDH).

3.2.3 *Brucella* Reference Strains

Standard reference strains of all the *Brucella* species and biotypes were stored lyophilized in the laboratory.

3.2.4 Brucellaphages

Brucella phage Tblisi (Tb), phage Weybridge (Wb) and Phage Berkeley (Bk_2) all

at Routine Test Dilution (RTD) were obtained from the World Head Organization/Food and Agricultural Organization Collaborating Centre for Brucellosis Reference and Research, Veterinary Laboratories Agency, (VLA) Weybridge United Kingdom (UK).

3.2.5 *Brucella* Monospecific Antisera A and M

The *Brucella* Monospecific antisera A and M used for the slide agglutination tests were obtained from the same source as the *Brucella* phages.

3.2.6 *Brucella* Culture Suspension

Suspensions of the *Brucella* cultures under test, stored cultures obtained from previous isolation works on *Brucella* from cattle (Eze, 1981) and the reference cultures were prepared by suspending a loopful of recently grown culture in 1.0 ml of sterile normal saline.

3.2.7 Test for Requirement of Added CO_2 for Growth

A loopful of test culture was subcultured onto SDA in duplicates. One was incubated in air and the other in an atmosphere containing an added 5 – 10% of CO_2. After 72 hrs of incubation, any growth on plates incubated in air was recorded as negative CO_2 requirement. Absence of growth on this plate but growth on plates incubated in the CO_2 supplemented atmosphere indicates a positive CO_2 requirement.

3.2.8 Slide Agglutination Test

A drop each of the Monospecific sera A and M was placed on a glass slide and a drop of *Brucella* culture suspension was added to each and mixed with a wire loop. The slide was rocked for 1 minute and presence of agglutination was read macroscopically.

3.2.9 Production of Hydrogen Sulphide

A loopful of the suspension of the Brucella culture was inoculated onto SDA

slopes and a strip of lead acetate paper was placed in the tube in such a way that it did not come in contact with the medium and held in place between the cap and the side of the tube. The slopes were incubated at 37°C aerobically or in 10% CO_2 atmosphere and were examined everyday for 3 days. Blackening of the paper strips was recorded as an indication of hydrogen sulphide production.

3.2.10 Production of Urease

Christensen's urea agar slope was inoculated with a loopful of culture and the slope incubated at 37°C and observed every hour for 5 hours and then after 24 hours. Change of colour of the medium from yellow to purple pink is an indication of positive reaction.

3.2.11 Susceptibility to Lysis by Phages

One drop of the RTD dilution of phages was placed on the freshly prepared lawn of an isolate on SDA. The plates were left undisturbed until the drops of phages have dried and then incubated at 37°C for 48 hours. A zone of lysis on the spot where phage was dropped was regarded as a positive result.

3.2.12 Growth in the Presence of Dyes

One loopful of culture suspension was used to inoculate dye plates. After drying the plates were incubated for 48 hours in air or in 10% CO_2 atmosphere. For the preparation of dye plates, see appendix.

3.2.13 Growth in the Presence of Penicillin

A loopful of culture suspensions of field strains of *Brucella* and S19 were inoculated onto dry SDA plates containing penicillin (see appendix). The plates were incubated for 72 hours in air.

3.3 Testing Isolated Strains of *Brucella abortus* for Virulence in Mice

3.3.1 Purpose

The virulence testing of the local isolates of *Brucella abortus* would determine whether they are attenuated and hence their usefulness as vaccine candidates or as virulent materials for use in the challenge studies in *Brucella* vaccine testing experiments.

3.3.2 *Brucella* Strains

The following *strains* of *Brucella abortus* were used:

B. abortus S19: -Non-virulent vaccine strain

B. abortus W.544: - (Obtained from the Veterinary Laboratories Agency, Weybridge, U.K), designated as the pathogenic type Strain by the Expert Committee on Brucellosis of the World Health Organization.

B. abortus MK5: - Local *Brucella* isolated from the milk of a White Fulani Cow No.0006 from the Livestock Investigation Department of the National Veterinary Research Institute, Vom, in 2003, stored lyophilized at -20°C.

B. abortus SH_3: - Local *Brucella* isolated from vaginal swab of Yankasa Ewe from Yankari Farms, Toro, Bauchi State in 2002, Stored lyophilized at 20°C.

B. abortus HS_2: - Local *Brucella* isolated from vaginal swab of local breed adult mare from Livestock Investigation Department of National Veterinary Research Institute, Vom in 2003, stored lyophilized at -20°C.

3.3.3 Experimental Animals:

One hundred and four (104) 8 week-old BALB/C mice obtained from Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria and National Institute of Trypanosomiasis Research, Vom, were used in the experiments when they were 10

weeks old.

3.3.4 *B. abortus* Cultures

The *B. abortus* Strain, 19, W.544, MK_5, SH_3 and HS_2 were grown on serum dextrose agar for 48 hours. The bacteria were harvested from the agar and washed twice by centrifugation using saline (0.15 M NaCl, pH 7.2).

3.3.5 Infection of mice with *B. abortus* and collection of tissues

Groups of mice (16 per treatment group) were given intraperitoneal (i.p.) injections of 0.2ml of a 0.15M NaCl saline solution (controls) or 0.2ml of saline containing approximately 5×10^8 CFU of S19 or 1.0×10^8 of strain w.544, Mk5, SH_3 and HS_2.The precise number of spleen colony forming units was determined retrospectively by viable plate counts. Blood samples and spleens were obtained aseptically from age-matched non-infected control mice and from infected mice at 4 days and 1, 2, 3, 4, 6, 10, 12, and 20 weeks after infection.

3.3.6 Blood Culture

Two ml of blood was collected from each mouse into vacutainer tubes containing sodium citrate. 0.3ml blood was spread on the surface of freshly made and dried SDA and incubated at 37°C in 10% CO_2 atmosphere for 7 days. All *Brucella abortus* growths were counted.

The following method was adopted for recovering positive results; plates showing 1 - 10 colonies were recorded as one plus (+), those with 11 - 50 colonies, two plus (++) and those with more than 50 colonies, three plus (+++).

3.3.7 Measurement of *B. abortus* CFU in Spleens

The spleens were extracted aseptically and weighed. Approximately ½ of each

spleen were excised using sterile scissors and ground in 10 ml sterile phosphate buffered saline (PBS [pH.7.2]). The spleen cells in PBS were lysed by sonication, and the number of CFU in the lysate was determined by plating dilutions on to SDA plates. The plates were incubated at 37°C in 10% CO_2 for 7 days. Results of the colony counts were expressed as mean number of CFU.

3.3.8 Collection of Serum Samples

Blood samples collected in plastic bottles were allowed to clot for 6 hours at 4°C before centrifugation. Serum samples from 2 mice from each group were put in 2.0ml plastic bottles. The serum samples were stored at -20°C and used within 1 week.

3.3.9 Serologic Testing

Thawed serum samples were measured for antibody to *B. abortus* by rose Bengal plate test (RBPT) and serum agglutination Test (SAT).

3.3.9.1 Rose Bengal Plate Test

The test was carried out using the Rose Bengal Plate test antigen obtained from Veterinary Laboratories Agency, Weybridge, UK according to the method described by Alton *et al*. (1988). Equal volumes (30Φl) of antigen and sera were mixed on clean white ceramic tile and subjected to continuous rocking for 4 minutes. Readings were done as follows: Where the clumps of agglutinated cells were separated by water-clear liquid, it was recorded as complete agglutination (+++ or ++); where there was definite agglutination of varying degrees without complete clearing in the background fluid, it was recorded as incomplete agglutination (+); and negative (-), where there was no sign of agglutination having taken place.

3.3.9.2 Serum Agglutination Test

The method of the American agglutination test as described by Alton *et al.* (1988) was adopted. A doubling dilution of test sera from 1/25 to 1/800 was done. The SAT antigen was obtained from the Bundesgesumdheitsant, Max von Pottenkoffer Institute, Abeitlung fur Veterinarmedizin, Berlin, Germany was used. For every tube of sera dilution, 2 ml of the antigen diluted in phenol saline at 1:100 dilution was added. The tubes were covered, shaken and incubated at 37°C for 20 hours. The antibody titer of a serum sample was the last dilution showing 50% agglutination. Known positive control sera of known agglutination titer and known negative control sera were incorporated into each test.

3.3.10 Histopathology

Approximately one third of spleen tissues obtained from mice in each group were fixed in 10% formol saline and processed by routine paraffin embedment before being stained with hematoxylin and eosin for light microscopy examination.

Table 3.1: Samples collected from animals for the isolation of brucellae from various States of Nigeria

State	Aborted fetuses				Vaginal swabs					Milk samples				Hygroma fluids		Blood samples	
	Cattle	Sheep	Dogs	Pigs	Cattle	Sheep	Goats	Dogs	Horses	Cattle	Sheep	Dogs	Goats	Cattle	Horses	Cattle	Dogs
Plateau	10	2	5	3	129	34	7	14	7	60	10	4	8	1	1	17	31
Bauchi	-	-	-	-	5	-	-	-	-	7	8	-	-	-	-	-	-
Adamawa	-	-	-	-	3	9	-	-	-	3	-	-	-	3	-	-	-
Taraba	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	-
Nassarawa	-	-	-	-	7	-	13	-	-	2	-	-	-	1	-	-	-
Kaduna	-	-	-	-	20	-	-	-	-	22	-	-	12	-	-	1	-
Kano	-	-	-	-	48	-	-	-	-	62	-	-	-	-	-	60	-
Bornu	-	-	-	-	48	7	-	-	-	75	-	-	-	-	-	-	-
Sokoto	-	-	-	-	72	-	-	-	-	6	-	-	-	-	-	17	-
Kogi	-	-	-	-	156	-	-	-	-	2	-	-	-	-	-	-	-
Enugu	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
Total	10	2	5	3	490	50	20	14	7	241	18	4	20	9	1	95	31

CHAPTER FOUR

RESULTS

Isolation of *Brucella* from the Livestock

Bacteriological examination for the isolation of brucellae was carried out on a total of 1,020 clinical samples consisting of 283 milk, 10 hygroma, 581 vaginal swabs, 20 aborted fetuses, and 126 blood samples. The samples were obtained from cattle, sheep, goats, pigs, dogs and horses drawn from Plateau, Kaduna, Kano, Bornu, Kogi, Nassarawa, Adamawa, Taraba, Bauchi, Enugu and Sokoto States of Nigeria (Table 3.1).

Circular, raised, convex and entire colonies typical of *Brucella* species were apparent after 3 - 5 days incubation at 37°C. Few of the isolates did not appear until after 7 days of incubation. Most isolates grew in air even at primary culture.

Brucella species were recovered from milk, vaginal swabs, aborted fetuses and hygroma fluids. Cells that were small gram-negative coco-bacilli and agglutinated by *B. abortus* antiserum were presumptively identified as *Brucella* species. All the presumptive isolates were confirmed as *Brucella* species.

Brucella species were recovered from 12 (48%) milk, 6 (24%) hygroma fluid, 2 (8%) aborted fetuses and 5 (20%) vaginal swabs. In this study a total of 25 isolates of *Brucella* were obtained. Successful isolation was made from samples obtained from Plateau (9), Adamawa (2), Bauchi (5), Nassarawa (2), Taraba (2) and Sokoto (3) States (Table 4.1).

Isolation of *Brucella* was made only from cattle, sheep and horses. Most animals involved in this study had history of abortion or infertility. For most of them, abortion had occurred between 1 week and 6 months before isolation of *Brucella* was attempted.

Most isolates grew on Farrell's medium and also on tryptone soya agar with antibiotics and Thayer-Martin medium. In few cases, however, growth was sparse (<10

colonies) while in some, growth was profuse and confluent (> 1000 colonies).

Biotyping of the Brucella Isolated From Nigerian Livestock

Each of the 25 isolates exhibited morphological and staining characteristics consistent with the bacterial genus *Brucella*. The characteristics of the isolates and their biotypes are as shown in Table 4.2.

Only one species of *Brucella* was obtained, *Brucella abortus*. All the strains of the isolates belonged to biotype I. Most isolates grew both under CO_2 and aerobically and only 1 required CO_2 (Table 4.2). Hygroma fluids often yielded pure cultures of brucellae. However, no isolation was made from few hygroma fluids that were cultured after 72 hours even though such samples were serologically positive.

Of the tests used to differentiate species and biovars, all isolates showed the following common characteristics: produced H_2S, produced urease after 24 hours, and grew in the presence of basic fuschin (20 $\mu g\ ml^{-1}$) but not in the presence of thionin (20 $\mu g\ ml^{-1}$).

In slide agglutination tests all the isolates were agglutinated by the A Monospecific antiserum. No agglutination was observed by M Monospecific antiserum.

All the isolates were lysed by Tb, Wb and Bk_2 phages at RTD (Table 4.2). All the isolates that were CO_2-independent grew on SDA containing penicillin.

The phenotypic characteristics of stored *Brucella* isolates obtained from previous works in Nigeria are indicated in Table 4.3. All the isolates belong to one species, *B. abortus*. Out of a total of 32 strains, 31 (96.9%) were of biovar 1 while only 1(3.1%) was of biovar 2.

Testing Isolated Strains of *Brucella abortus* for Virulence in Mice

The results are shown in Tables 4.5, 4.6, 4.7 and 4.8. *Brucella* organisms were detected in the blood of all infected mice as early as 4 days post-infection. At 28 days post-inoculation S.19 organisms could not be demonstrated in the blood. *Brucella abortus* w. 544, SH_3 and HS_2 were eliminated from the blood of mice by 42 days after inoculation while MK5 was eliminated from the blood by 70 days after inoculation. No *Brucella abortus* growth was detected in the blood of the non-infected control mice.

Mice infected with strain 19 exhibited a slow decline in numbers of bacteria in their spleens between 1 week and 10 weeks after infection and at 12 weeks after infection no bacteria were cultured from the spleens (Table. 4.7). In contrast to strain 19, mice infected with strain W.544 and HS_2 exhibited increase in number of bacteria in the spleen between 1 and 2 weeks. Numbers of bacteria then declined at 3 weeks and continued but bacteria were still detected in the spleens at 20 weeks after infection. While mice infected with strain MK_5 and SH_3 exhibited increase between 1 and 3 weeks. The numbers of bacteria then continued to decline sharply at 4 weeks and remained constant until 6 weeks after infection. Numbers of bacteria then declined at 12 weeks but were still present in the spleens at 20 weeks after infection. Peak numbers of *B. abortus* in the spleen of mice occurred 7 days p.i. for S19, 14-28 days for w.544 and HS_2 and 21 days p.i. for MK5 and SH_3. No *Brucella abortus* growth was obtained from the spleens of the non-infected control mice.

The spleen/body weight ratios were highest in infected mice at 7 days for S19 and SH_3, 2 weeks for W.544 and HS_2 and 3 weeks for MK_5 after inoculation. The ratios for the mice infected with strain 19, W.544, MK_5, SH_3 and HS_2 were on the average higher than those of the control mice.

The spleens of mice at one week after infection with strains 19, W.544, MK_5, SH_3 and HS_2 had profound lymphoid depletion of the white pulp; small germinal centers with few mitotic figures and multifocal accumulations of large macrophages that surrounded and sometimes covered the periarteriolar lymphoid sheaths. No significant changes were noticed in the non-infected control mice. Large germinal centers and the disappearance of the macrophage accumulations in the spleens did not occur until 10 weeks after infection of mice with strain 19 and until 20 weeks for strains W.544, MK5, SH_3 and HS_2.

Sera obtained from mice at 2 weeks after infection with strain 19, W.544, MK5, SH_3 and HS_2 contained no antibody to *Brucella abortus*.

Infected mice had measurable antibodies between 3 to 20 weeks after infection. It was highest for strain 19, W.544, SH_3 and HS_2 at 10 weeks and highest for MK_5 at 12 weeks post-infection.

Table 4.1: Isolation of brucellae from culture samples obtained from livestock in various states of Nigeria

S/No	Isolate Lab Reference	Animal Host	Culture Sample	State
1	H_1	Cattle	Hygroma Fluid	Taraba
2.	H_2	Cattle	Hygroma Fluid	Taraba
3.	Miango 1	Cattle	Milk	Plateau
4.	Miango 2	Cattle	Milk	Plateau
5.	V_1	Cattle	Aborted Fetus	Plateau
6.	H_3	Cattle	Hygroma Fluid	Adamawa
7.	H_4	Cattle	Hygroma Fluid	Adamawa
8.	Bale	Cattle	Milk	Plateau
9.	SH_1	Sheep	Milk/vaginal Swab	Bauchi
10.	SH_2	Sheep	Milk/Vaginal Swab	Bauchi
11.	NS_1	Cattle	Milk	Nassarawa
12.	H_5	Cattle	Hygroma Fluid	Nassarawa
13.	YB_2	Cattle	Milk	Bauchi
14.	YB_3	Cattle	Milk	Bauchi
15.	SB_3	Sheep	Vaginal Swab	Bauchi
16.	HS_1	Horse	Hygroma Fluid	Plateau
17.	MK5	Cattle	Milk	Plateau
18.	MK6	Cattle	Milk	Plateau
19.	HS_2	Horse	Vaginal Swab	Plateau
20.	HS_3	Horse	Vaginal Swab	Plateau
21	SK_1	Cattle	Milk	Sokoto
22	SK_2	Cattle	Milk	Sokoto
23.	SK_3	Cattle	Aborted fetus	Sokoto

Table 4.2: Growth characteristics of brucellae isolated from livestock in Nigeria.

Isolate	Urease prod'n	H_2S prod'n	CO_2 req't	Growth on media containing		Agglutination by monospecific antisera		Lysis by Phages at RTD[c]			Brucella species	Biotype
				BF[a]	TH[b]	A	M	Wb	Tb	BK_2		
H_1	+	++	+	+	_	+++	_	+	+	+	*B. abortus*	1
H_2	+	++	+	+	_	+++	_	+	+	+	*B. abortus*	1
Miango 1	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
Miango 2	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
V_1	+	++	_	+	_	++	_	+	+	+	*B. abortus*	1
H_3	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
H_4	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
Bale	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
SH_1	+	+	+	+	_	+++	_	+	+	+	*B. abortus*	1
SH_{1B}	+	+	+	+	-	+++	-	+	+	+	*B.abortus*	1
SH_2	+	+	+	+	_	+++	_	+	+	+	*B. abortus*	1
SH_{2B}	+	+	+	+	-	+++	-	+	+	+	*B.abortus*	1
NS_1	+	++	-	+	_	++	_	+	+	+	*B. abortus*	1
H_5	+	++	-	+	_	++	_	+	+	+	*B. abortus*	1
YB_1	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
YB_2	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
SH_3	+	++	+	+	_	+++	_	+	+	+	*B. abortus*	1
HS_1	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
MK5	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
MK6	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
HS_2	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
HS_3	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
SK_1	+	+	-	+	_	++	_	+	+	+	*B. abortus*	1
SK_2	+	+	-	+	_	++	_	+	+	+	*B. abortus*	1
SK_3	+	+	-	+	_	++	_	+	+	+	*B. abortus*	1

[a] BF, basic fuchsin 20ug ml^{-1} (1/50 000)

[b] TH, thionin 20ug ml^{-1} (1/50 000)

[c] RTD, routine test dilution

Table 4.3: Growth characteristics of stored cultures of brucellae isolated from cattle in Nigeria.

Isolate	State of Origin	Urease production	H_2S production	CO_2 reqt	Growth on media containing		Agglutination by monospecific antisera		Lysis by Phages at RTD[c]			*Brucella* species	Biotype
					BF[a]	TH[b]	A	M	Wb	Tb	BK_2		
SB.3	Kano	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
SB.5	Kano	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
SD.20	Kano	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
SD. 27	Kano	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
SD. 38	Kano	+	++	_	+	_	++	_	+	+	+	*B. abortus*	1
188	Kano	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
799	Kano	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
Fula	Borno	+	++	_	+	_	+++	_	+	+	+	*B. abortus*	1
Muri	Borno	+	+	+	+	_	+++	_	+	+	+	*B. abortus*	1
Solu	Borno	+	+	+	+	_	+++	_	+	+	+	*B. abortus*	1
330 I	Borno	+	++	-	+	_	++	_	+	+	+	*B. abortus*	1
330 II	Borno	+	++	-	+	_	++	_	+	+	+	*B. abortus*	1
330 III	Borno	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
1904 I	Borno	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
1904 II	Borno	+	++	+	+	_	+++	_	+	+	+	*B. abortus*	1
TR. A	Taraba	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
TR. B	Taraba	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
TR. C	Taraba	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
TR. D	Taraba	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
TR. E	Taraba	+	++	-	+	_	+++	_	+	+	+	*B. abortus*	1
083	Niger	+	+	+-	-	_	++	_	+	+	+	*B. abortus*	2
OSB	Niger	+	+	-	+	_	++	_	+	+	+	*B. abortus*	1
12	Plateau	+	+	-	+	_	++	_	+	+	+	*B. abortus*	1
31	Plateau	+	++	+-	+	-	++	-	+	+	+	*B. abortus*	1
93	Plateau	+	++	+-	+	-	++	-	+	+	+	*B. abortus*	1
133	Plateau	+	++	-	+	-	++	-	+	+	+	*B. abortus*	1
OSB 19	Plateau	+	++	+-	+	-	++	-	+	+	+	*B abortus*	1
Mkd. 1	Benue	+	++	-	+	-	++	-	+	+	+	*B. abortus*	1
Mkd. 2	Benue	+	++	-	+	-	++	-	+	+	+	*B. abortus*	1
Mkd. 3	Benue	+	++	-	+	-	++	-	+	+	+	*B. abortus*	1
Mkd 4	Benue	+	+	-	+	-	++	-	+	+	+	*B.abortus*	1
Mkd 5	Benue	+	+	-	+	-	++	-	+	+	+	*B.abortus*	1

[a] BF, basic fuchsin 20ug ml^{-1} (1/50 000)

[b] TH, thionin 20ug ml^{-1} (1/50 000)[c] RTD, routine test dilution

Table 4.4: Differential Characteristics of *B. abortus* 19 and biovar 1 field isolates of *Brucella*

Brucella abortus strain	CO_2 Requirement	Growth in the presence of benzyl-penicillin
Strain 19	-	-
Miango 1	-	+
Miango	-	+
V_1	-	+
H_1	-	+
H4	-	+
Bale	-	+
NS_1	-	+
H_5	-	+
YB_1	-	+
YB_2	-	+
HS_1	-	+
MK5	-	+
MK6	-	+
HS_2	-	+
HS_3	-	+
SK_1	-	+
SK_2	-	+
SK_3	-	+

Table 4.5: Spleen/ body weight ratio on days post-inoculation of mice with different *B. abortus* strains

Days Post-Inoculation	Brucella Strains					Control
	S19	W.544	MK5	SH_3	HS_2	
0	0.003	0.003	0.003	0.003	0.003	0.003
4	0.004	0.005	0.008	0.007	0.003	0.004
7	0.022	0.010	0.008	0.030	0.007	0.004
14	0.016	0.021	0.009	0.019	0.015	0.005
21	0.009	0.018	0.018	0.012	0.012	0.004
28	0.007	0.012	0.012	0.014	0.008	0.004
42	0.006	0.007	0.012	0.008	0.007	0.004
70	0.005	0.008	0.010	0.008	0.007	0.004
84	0.003	0.003	0.007	0.008	0.007	0.004
140	0.003	0.008	0.008	0.013	0.013	0.004

Table 4.6: Presence of *B. abortus* in the blood of mice on days post-inoculation with different *Brucella* strains

Days Post-Inoculation	*Brucella* Strains					Control
	S19	**W.544**	**MK5**	**SH_3**	**HS_2**	
0	-	-	-	-	-	-
4	+	+	+	+	+	-
7	++	++	++	++	++	-
14	+	+	++	+	+	-
21	+	+	+	+	+	-
28	-	+	+	+	+	-
42	-	-	+++	+	+	-
70	-	-	+++	-	-	-
84	-	-	-	-	-	-
112	-	-	-	-	-	-
140	-	-	-	-	-	-

Table 4.7: Splenic colony forming units of *B. abortus* on days post-inoculation of mice with different *Brucella* strains

Days Post-Inoculation	*Brucella* Strains					Control
	S19	**W.544**	**MK5**	**SH_3**	**HS_2**	
0	-	-	-	-	-	-
4	ND	ND	ND	ND	ND	ND
7	1.0×10^4	1.0×10^3	1.0×10^4	1.0×10^4	1.2×10^3	-
14	2.0×10^2	2.0×10^3	2.0×10^4	2.0×10^4	4.0×10^3	-
21	1.0×10^2	2.0×10^3	4.0×10^4	4.0×10^4	$1.0 \text{ x}10^3$	-
28	1.5×10^2	2.0×10^3	2.0×10^3	2.0×10^3	1.0×10^3	-
42	3.0×10	1.5×10^3	2.0×10^3	2.0×10^3	6.0×10^2	-
70	1.0×10	1.0×10^3	2.0×10^3	2.0×10^3	5.0×10^2	-
84	-	1.0×10^2	1.0×10^3	1.5×10^3	1.5×10^2	-
112	-	1.0×10	1.0×10^2	1.0×10^2	1.0×10^2	-
140	-	1.0×10	1.0×10^2	1.0×10^2	1.0×10^2	-

Table 4.8: Serological findings on serum of mice at various days post-inoculated with different strains of *B. abortus*

Days Post-Inoculation	*Brucella* Strains										Control	
	S19		W.544		MK5		SH_3		HS_2			
	RBPT	SAT	RBPT	SAT	RBPT	SAT	RBPT	SAT	RBPT	SAT	RBPT	SAT
0	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
14	+	1/25	+	1/25	++	1/50	+	1/25	+	1/25	-	-
21	++	1/50	++	1/50	++	1/100	++	1/50	++	1/50	-	-
28	++	1/100	++	1/100	++	1/100	++	1/100	++	1/100	-	-
42	+++	1/200	+++	1/100	+++	1/200	+++	1/100	+++	1/100	-	-
70	+++	1/200	+++	1/200	+++	1/200	+++	1/200	+++	1/200	-	-
84	+++	1/200	+++	1/100	+++	1/800	++	1/200	+++	1/200	-	-
112	++	1/100	++	1/100	+++	1/200	++	1/100	++	1/100	-	-
140	+	1/25	+	1/25	++	1/50	+	1/25	+	1/25	-	-

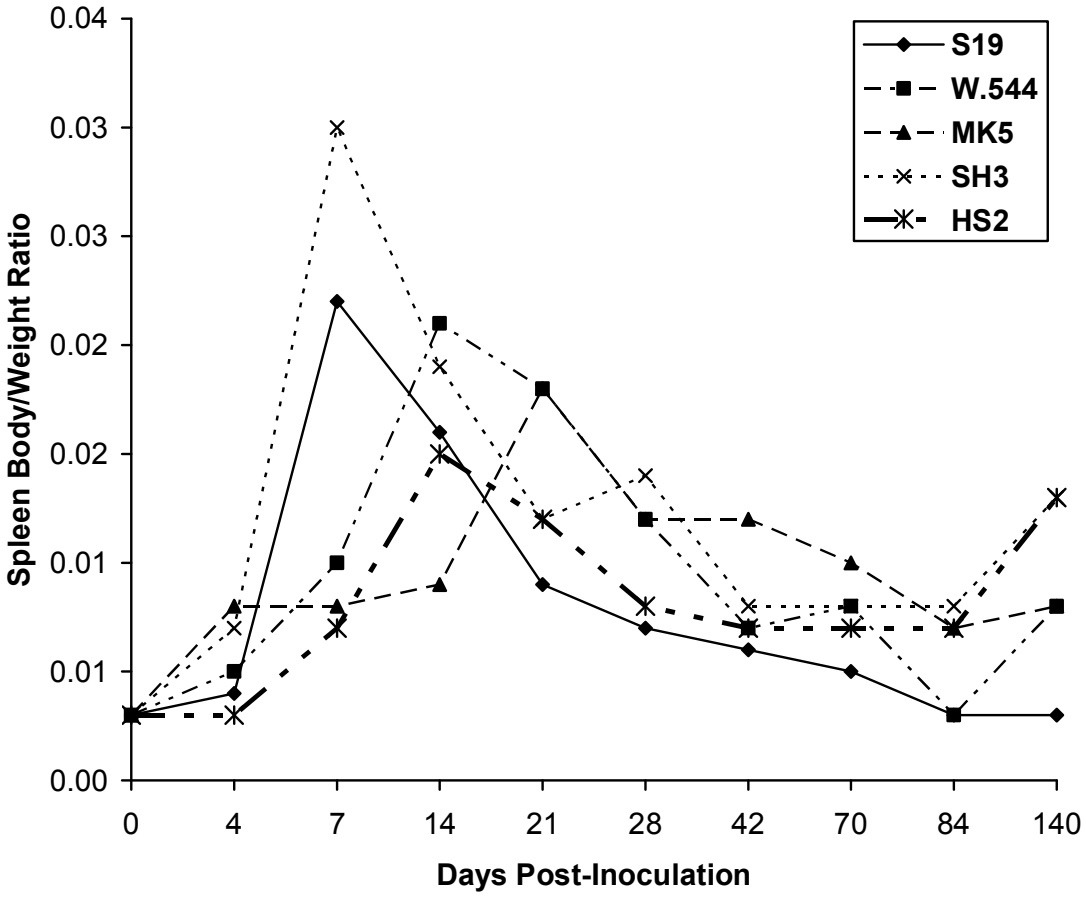


Figure 4.1: Spleen and body weight of BALB/c mice during infection with *Brucella abortus*. Mice were inoculated with S19 (5×10^8 CFU), w.544 (10^8 CFU), MK5 (10^8 CFU), SH_3 (10^8CFU) or HS_2 (10^8 CFU) by i. p. injection. Spleen weights and body weights were measured at several time points between 4 days and 20 weeks after inoculation. Results are expressed as mean ratio of spleen to body weight (n=2).

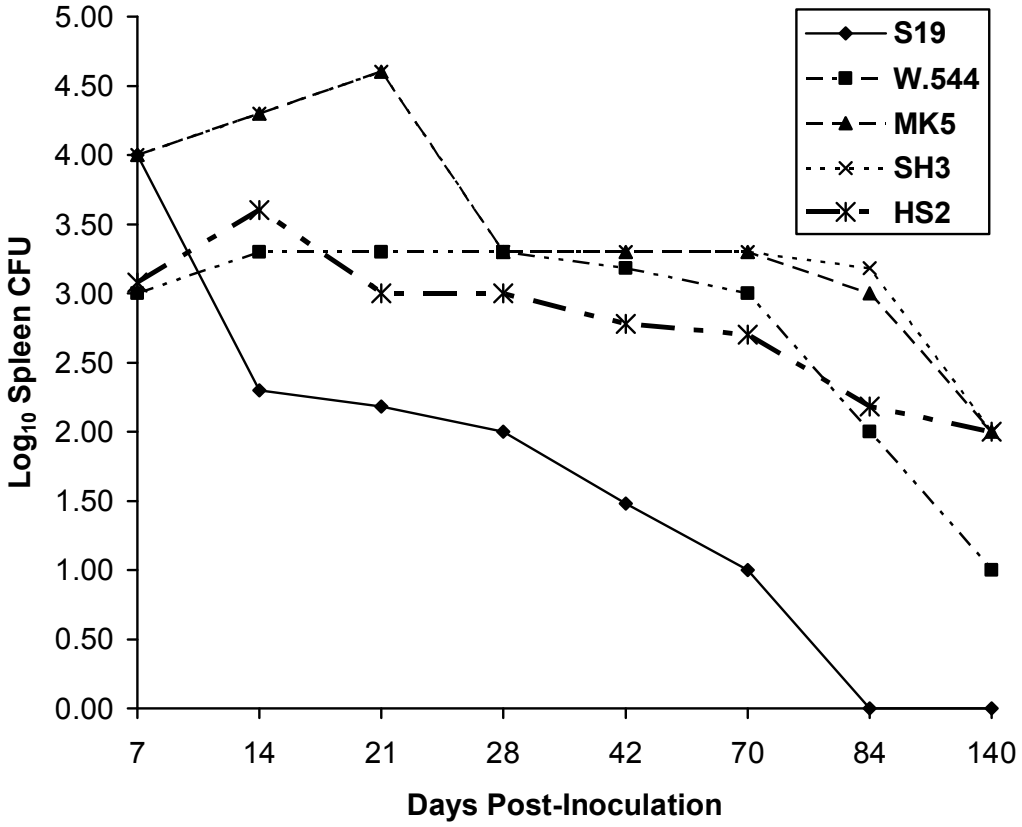


Figure 4.2: Persistence of *B. abortus* in spleen of infected mice. Mice were inoculated with S19 (5×10^8 CFU), w.544 (10^8 CFU), MK5 (10^8 CFU), SH_3 (10^8 CFU) or HS_2 (10^8 CFU) by i. p. injection. Ground spleen suspensions were plated onto serum dextrose agar plates to determine numbers of CFU at 4 days to 20 weeks after infection. Results are presented as mean CFU (n=2).

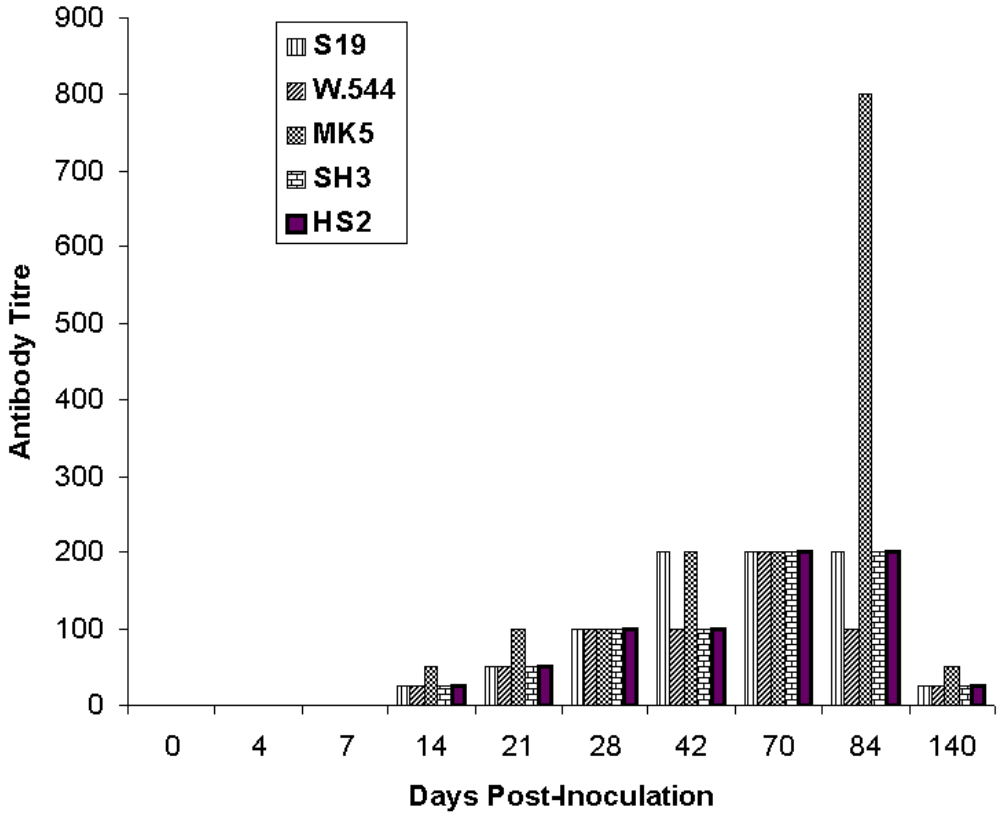


Figure 4.3: Antibody production in response to Brucella in mice infected with *B. abortus* S19, w.544, Mk5, SH_3 or HS_2. Mice were inoculated with S19 (5 $\times 10^8$ CFU), w.544 (10^8 CFU), MK5 (10^8 CFU), SH_3 (10^8CFU) or HS_2 (10^8 CFU) by i. p. injection. Serum samples obtained from mice at 4 days to 20 weeks were measured for *Brucella* antibodies by SAT. Results are presented as inverse of antibody titer.

CHAPTER FIVE

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION

Brucella from culture samples grew easily on the Farrell's medium after 3 - 5 days of incubation indicating the suitability of this medium for field isolation of *Brucella*.

Most of the field samples were not preserved under appropriate condition. Experience has shown that brucellae do not often survive the long distance from the field to the laboratory (Eze, 1981). This may explain why only 25 isolates were obtained from the culture samples. It is suggested that relevant laboratory equipments and culture media should be transported to the field, if it is possible, where inoculation of the media can be carried out on the spot.

Hygroma fluid often yielded pure isolates after 24 hours storage at -20°C. Some of the hygroma fluids obtained from the field did not yield any isolate of *Brucella*, because they did not reach the laboratory until more than 2 weeks after collection. It is suggested that hygroma fluids be cultured within 48 hours of collection after storage at 4°C or 20°C.

The recovery of *Brucella* species mainly from milk samples indicates the suitability of this sample for field isolation of *Brucella*, especially when the milk sample was obtained from animals within two weeks of abortion.

Though most animals had history of abortion with clinical signs suggestive of brucellosis, isolation of brucellae from clinical samples obtained from such animals was not possible. Culture samples were obtained from these animals between two and six

months after abortion.

The massive excretion of brucellae may continue for 15 days after abortion. Once the uterine discharge diminishes and fetal membranes are expelled, the number of *Brucella* organisms excreted decreases rapidly (Bercovich, 1998). The time after abortion at which clinical samples are collected is crucial to the recovery of *Brucella* from aborting animals.

The isolation of *Brucella* predominantly (48%) from milk samples is of particular zoonotic and public health importance. In most parts of the Northern States of Nigeria, local custom encourages the consumption of raw bovine milk. Under such conditions public health hazards due to brucellosis definitely exist.

Isolation of *Brucella* was possible only from clinical samples obtained from cattle, sheep and horses. The recovery of *Brucella* from culture samples obtained from dogs, goats and sheep was not successful. More works on isolation of *Brucella* from these species is required in order to understand their roles in the epidemiology of brucellosis in Nigeria. Such works should include camels.

Reports from various areas of Nigeria reveal that animal brucellosis is widely distributed in the country. There is a pattern of low, medium and high prevalence in specific areas of the country and prevalence also varies between herds in the same area (Nuru and Dennis, 1975; Ocholi *et al.,* 1996). Most of the reports are based on serological evidences and little effort has been made to isolate brucellae from the livestock (Bale and Kumi-Diaka, 1981; Halle and Ajogi, 1997). Relying on serological evidences alone can be potentially misleading. The confirmatory diagnosis of brucellosis is based on the isolation of the organism from the infected host. The isolation of *Brucella* strains from Nigerian livestock as reported here is therefore very significant.

Knowledge and understanding of the prevailing species and biovars of *Brucella* infecting livestock are crucial to the necessary steps required for the formulation of policies and strategies for the control of brucellosis in an animal population. It is therefore required that the genus and the biovar of all *Brucella* species isolated from livestock be established.

The biotyping of *B. abortus* isolates is generally recommended as an important epizootiological tool. Once a new infection has been confirmed in a herd, it is critical to prevent further spread of the disease to other herds. It is equally important to determine by epidemiological trace-back analysis where infection originated, how it is spread, and what measures are needed to prevent additional spread of the disease from the primary source. Wherever possible, trace-back is confirmed by comparison of the outbreak strain with isolates obtained from the primary source (Bricker *et al*., 2003). Furthermore, there is a need to differentiate between vaccine and field strains.

The fact that only one species, *Brucella abortus* and only one biovar 1 were encountered in this study does not indicate that this species and biovar are representative of Nigeria. They can be said to be representative of the area from which they were obtained. It is therefore suggested that similar studies be undertaken to cover larger areas of Nigeria. Such studies may identify more species and biovars and add to knowledge of the epidemiology of brucellosis in Nigeria.

In the present study more areas, including Bauchi, Adamawa, Kaduna, Kogi, Nassarawa and Sokoto States have been covered compared to previous reports (Eze, 1981; Bale and Kumi-Diaka, 1981).

The prevailing species and biotypes of *Brucella* commonly associated with infections among Nigerian livestock have not been extensively established. Previous

studies by Eze (1978) indicate that out of 20 strains of *B. abortus* isolated from cattle, 19 (95%) were of biovar 1 while only 1 (5%) was of biovar 2. Similarly, out of eight strains of *B. abortus* obtained from cattle by Bale and Kumi-Diaka (1981), 5 (62.5%) were of biovar 1, 2 (25%) were of biovar 3 and 1 (12.5%) was of biovar 4. It does appear from these previous reports and the present study that *B. abortus* biovar 1 may be the predominant strain associated with disease problems in livestock from States studied.

There are several bases for a diagnosis of brucellosis, but the only definitive one is isolation of the organism (Crawford *et al*., 1979). The identification and biotyping of *Brucella* strains from Nigerian livestock as indicated in this study are therefore very significant. Most previous reports on the investigation of brucellosis among animal population were based on serological diagnosis, and there have not been much effort to isolate brucellae from livestock in Nigeria (Eze, 1978; Bale and Kumi-Diaka, 1981).

The evolution of 18 of the isolates (Table 4.2) of the field strains of *B. abortus* biovar 1 to CO_2 independence as reported here appears to be unusual. It has been reported that spontaneous mutation rate of *B. abortus* for conventional characteristics occurs at the rate of 3×10^{-10} for CO_2 requirement (Merr and Wilson, 1950; Jones *et a*l., 1982) and 2.2×10^{-10} per cell division for basic fuschin resistance (Shibau *et al*., 1962). These mutation rates indicate that the phenotypic markers are quite stable (Jones *et al*., 1982). It is unlikely that spontaneous mutation occurred during the isolation and identification procedure, because the isolates were only subcultured twice.

Biovar classification of *B. abortus* is determined by quantitative phenotypic differences in growth requirements and sensitivity to dyes (Jones *et al*., 1982). Meyer (1964) suggested that biovar 1 and 2 are closely related, and proposed that biovar 1 may have evolved from biovar 2. Occasional isolation of multiple biovars from a herd

(Nicoletti, 1981) and tissues of a naturally infected cow (Jones *et al*., 1982) have been reported. This situation was not encountered in this study. The *B. abortus* strains isolated from both milk and vaginal swabs of 2 of the sheep were all of biovar 1.

The biotyping of *B. abortus* isolates is generally recommended as a valuable epizootiological tool (Alton *et a*l., 1988; Crawford et al., 1979). Using the routine identification procedures described previously (Alton *et al*., 1988), *B. abortus* strain 19 cannot be distinguished from other CO_2-independent field strains of *B. abortus* biovar 1. The first step in the differentiation of this strain isolates is by penicillin sensitivity assay (Corbel *et al.,* 1987). All the field isolates obtained in this study grew on *Brucella* basal media containing 5 i.u. of benzyl-penicillin per ml of media confirming that they were not S19 (Table 4.4). The result supported the hypothesis that biotyping can be useful in determining the source of *B. abortus* infection for cattle and in differentiating field from vaccine strains infections in vaccinated cattle (Crawford *et al*., 1979). Furthermore, this study emphasizes the usefulness of bacteriological examination in the diagnosis of *B. abortus* infection. Strain 19-infected cattle can be identified and retained for further production.

The maintenance host for *B. abortus* is cattle, but other animals can be infected (Zowgi and Ebadi, 1988; FAO, 2004). Horses are susceptible to *B. abortus*, and natural infection in horses has been associated with close contact with infected livestock. The prevalence of seropositive reaction for brucellosis in horses has been reported when samples were collected from areas with high infection rate in cattle (Crawford *et al*., 1979). The horses from which *Brucella* were isolated in this study were pastured on the same farm with infected cattle from which *B. abortus* biovar 1 was isolated. It is probable that the infected cattle were the source of infection for these horses.

The virulence of a *Brucella* strain is dependent on its ability to establish chronic

infection (Pizarro-Cerda *et al*., 1998). An important property of *Brucella* associated with virulence is the ability to survive and multiply intracellularly in host phagocytes (Thoen and Enright, 1986). The virulence of a *Brucella* strain in natural infection in cattle refers to its capacity to produce clinical manifestations such as abortion with retained placenta, etc. (Eze, 1981). Experimentally, it is rather expensive to use larger animals to estimate the virulence of brucellae. Most of the current understanding of the pathogenesis and immunity of animal brucellosis has been derived from studies in mice (Stevens *et al*., 1994). Studies in mice with strain 19 and RB51 have produced similar results to those seen in cattle (Tobias *et al*., 1992; Stevens *et al*., 1995). It is therefore a usual practice to use mice for virulence studies of *Brucella* as carried out in this study.

All the *Brucella abortus* used in this study could be cultured from the blood of infected mice at 4 days post-infection indicating that they could establish clinical infection as early as 4 days after infection.

Brucella abortus strain 19 accepted as a non-virulent attenuated strain was eliminated from the blood stream of inoculated mice within 2 weeks. The typical virulent strain of World Health Organization, *B. abortus* W.544 and the locally isolated strains SH_3, MK_5 and HS_2 produced in the inoculated mice a bacteraemia lasting for at least 6 weeks. It is thus probable that these local strains are virulent *Brucella* strains and even more virulent than strain W.544. *Brucella abortus* strain 19 was cleared from the spleen of mice by 12 weeks post-inoculation while strain W.544 and the local strains: SH_3, MK_5 and HS_2 were still present in the spleen as at 20 weeks post-inoculation which indicate that these local strains are virulent. The more rapid clearance of the S19 vaccine strain from the spleen of mice compared to the other strains indicates the attenuation of this strain. This is moreso given the fact that the dose of S19 was five fold those of w.544

and the field strains.

The development by BALB/c mice model of chronic infection with *B. abortus* without overt disease corresponds to chronic brucellosis in cattle. The relatively more rapid clearance of strain 19 compared with strain w.544 and the field strains of *B. abortus* also corresponds to the relative virulence of these strains in cattle and in this respect indicates that the BALB/c mouse might serve as a useful model for brucellosis of cattle and for evaluating vaccines intended for cattle (Montaraz and Winter, 1986).

The spleen/body weight index of mice infected with *B. abortus* S19 was generally similar to those of the non-infected control mice. The ratio for the mice infected with *B. abortus* W.544, SH_3, MK_5, SH_2 were on average generally higher than those of the control mice. This indicates that the spleens of the mice inoculated with virulent *Brucella* organisms had undergone some hypertrophy.

In this present study, the spleens of all infected mice exhibited severe lymphoid depletion at three to six weeks after infection. Lymphoid depletion in the spleens of the S19 infected mice had disappeared at 10 weeks post inoculation while lymphoid depletion in the spleens of strains W.544, SH_3, MK_5 and HS_2 disappeared only at 20 weeks. During the depletion, spleen cells are incapable of exhibiting cell-mediated immune response (Lauderdale *et al*., 1990). This indicates that mice infected with the virulent *Brucella* strains are not able to exhibit cell-mediated immune response at 20 weeks after infection.

The antibody response in strain W.544, SH_3, MK_5 and HS_2 infected mice was comparable to mice infected with S19. This suggests that the local field strains are immunogenic even though virulent.

The results obtained from this study are similar to that obtained by Eze (1983)

who carried out experimental virulence studies using guinea pigs, and observed that all the local strains of *Brucella* isolated from cattle were virulent.

The virulence of the local field strains of *Brucella* as indicated from this study makes them unsuitable as candidates for vaccine testing. However, they could be used as challenge strains in experimental vaccination of animals.

Brucellosis caused by species of bacterial organisms of the genus *Brucella*, is a zoonotic disease endemic in many areas of the world, characterized by chronic infections in animals leading to abortion and infertility, and a systemic febrile illness in humans (Paulsen *et al*., 2002).

In Nigeria, brucellosis is recognized to be endemic among livestock and human populations, and a major source of heavy economic losses to the livestock industry (Eze, 1981). A major issue regarding the situation of brucellosis in Nigeria today is the need to institute control and eradication programmes. Knowledge and understanding of the epidemiology of the disease among the livestock population is crucial to the necessary steps required for the formulation of strategies for the control and eradication of the disease in an animal population. Also, because of the complications involved in the diagnosis of the disease, including the difficulties in distinguishing between infected and vaccinated animals by conventional serological tests, bacteriological isolation and identification of the etiological agent are necessary steps in the design of epidemiological and eradication programmes (Plommet, 1986).

In this study, attempt has been made to isolate *Brucella* from clinical samples obtained from aborting animals. Although only twenty-five strains of *Brucella abortus* belonging to biovar 1 were isolated, lack of facilities prevented more extensive isolation work, or more isolation would probably have been made. The results obtained are in

agreement with previous works on the isolation of *Brucella* from livestock in Nigeria where the predominant species and biovar were *B. abortus* biovar 1(Eze, 1978; Bale and Kumi-Diaka, 1981).

Also the results obtained from the identification of stored isolates of *Brucella* indicate that all the strains were *Brucella abortus*. Out of a total of 32 isolates, 31 (96.9%) were of biovar 1 while only 1(3.1%) was biovar 2. These results together with other results obtained from the present study indicate that *B. abortus* biovar 1 may be the predominant strain of *Brucella* infecting the Nigerian livestock.

Reports from various areas of Nigeria reveal that animal brucellosis is widely distributed in the country. There is pattern of low, medium and high prevalence in specific areas of the country and prevalence also varies between herds in the same area (Nuru and Denis, 1975; Ocholi *et al*, 1996). Most of the reports are based on serological evidences and little effort has been made to isolate *Brucella* from the livestock (Bale and Kumi-Diaka; 1981; Halle and Ajogi, 1997). Relying on serological evidences alone can be potentially misleading. The confirmatory diagnosis of brucellosis is based on the isolation of the organism from infected animals. The isolation of *Brucella* strains from Nigerian livestock as undertaken in this project is therefore very significant.

Previous works on the isolation of brucellae from livestock in Nigeria are more than two decades old (Eze, 1978; Bale and Kumi-Diaka, 1981). Since then a lot of changes may have occurred in the epidemiology of the disease in the country. The present work provides current information and confirms the involvement of *Brucella* in some of the cases of abortion in animals frequently reported from the field.

The result obtained in this present study conforms to what is known about the epidemiology of bovine brucellosis caused by *B. abortus* reported from other countries

of the world. Although reported incidence and prevalence of the disease vary widely from country to country, brucellosis caused mainly by *B. abortus* biovar 1 is still the most widespread form recognized worldwide (Corbel, 1997). Specific biovars tend to predominate in certain geographical areas. For example, in the USA, 85% of bovine infections involve *B. abortus* biovar 1 (Bricker *et al*, 2003).

In this study, successful isolation of *Brucella* was possible from cattle, sheep and horses. In a similar study, isolation of *Brucella* was possible only from cattle (Eze, 1981). The isolation of *Brucella* from goats (Falade, 1981) and pigs (Bale and Nuru, 1985) have been reported but this was not achieved in this present work. More work on the isolation of *Brucella* from these species is required. Such works should also include dogs and camels. This is particularly important as there is a need to establish the role of these animal species in the epidemiology of brucellosis in Nigeria. The extension of brucellosis among sheep and horses may not only be a source of infection for other animals but also for human beings. This is particularly so since traditional animal husbandry practices encourage herding of animals, which may promote the spread of the disease not only among the animal species but also to man. The recovery of *Brucella* from horses is also very significant. This is particularly important in view of its public health significance especially as horses have recently gained popularity with the increasing interest in polo games and horse racing (Oladosu *et al*., 1986.)

The isolation of *Brucella* predominantly (48%) from milk samples is of particular zoonotic and public health importance. In most parts of the Northern States of Nigeria, local customs encourage the consumption of raw bovine milk. Under such conditions public health hazards due to brucellosis definitely exist. In the Western States of Nigeria large-scale community outbreaks of brucellosis among human beings as a result of

consumption of animal products have been reported (Alausa, 1983). Serological survey for *Brucella* antibodies in pregnant women and high-risk groups has been undertaken (Baba *et al.,* 2001). The extent of *Brucella* infections among human populations in Nigeria is not known. Large scale country-wide studies to determine the prevalence of *Brucella* infections among human population by cultural isolation and serological surveys are required. These should include veterinarians, herdsmen, livestock attendants, abattoir workers and laboratory workers.

The results of this study and the confirmation of existing records indicate that brucellosis in Nigerian livestock has existed for a long time, and is still spreading despite efforts by the government to improve the livestock industry (Eze, 1985; Bale and Kumi-Diaka, 1981). One major factor contributing to the spread of the disease is the free movement of animals practiced by the nomadic Fulani herdsmen who are accustomed to the traditional extensive system of management, and own about 95% of all food animal populations in Nigeria (Rikin, 1988b). Another factor is the herding of animals like cattle, sheep and goats together, and sometimes with horses. These factors need to be taken into consideration in the planning and execution of control programmes.

The *Brucella* strains isolated showed virulence for mice, persisting in the blood stream after infection. They showed even greater virulence than the World Health Organization (WHO) standard virulent strain *B abortus* W.544. Though this result may not be directly extrapolated to cattle, which is the traditional host of this pathogen, the isolates obtained from sheep and cattle were isolated from hosts that had history of abortion and this confirms their virulence. Similarly, one of the isolates from the horses was obtained from a host that had severe bursitis and hygroma. The virulence of the field isolates of *Brucella* obtained in this study indicates that they cannot be used for testing as

vaccine candidates. The search for local isolates as vaccine candidate is suggested.

Out of a total of 25 strains of *Brucella* isolated from livestock in this study, 17 (68%) were from cattle. Also, in locations where *Brucella* was isolated from other animals, sheep and horses, epizootiological history indicates that such animals were in contact with infected cattle. It would appear from these studies and other reports (Eze, 1978; Bale and Kumi-Diaka, 1981) that bovine brucellosis caused by *B. abortus* is the major problem among livestock in Nigeria. Also, it appears that cattle are the main reservoir of *Brucella* infection for other animals in the country. It is therefore suggested that control efforts should concentrate mainly on this species as the target.

Apart from causing heavy losses to the livestock industry, brucellosis poses serious human health hazards. The role played by brucellosis in limiting livestock production, and its economic impact on the livestock industry in Nigeria is widely recognized. An annual loss due to this disease is estimated to be about 224 million Dollars (Esuruoso, 1979). There is a need to institute a control programme for this disease in Nigeria if the unrealized economic potential is to be derived from the livestock industry.

The benefit-cost analysis for the control of brucellosis in Nigeria has been reported (Rikin, 1988a). Suggestions on the practical strategies and measures to be followed in the control of the disease have been made (Esuruoso, 1979; Eze, 1981; Rikin, 1988b; Ajogi, 1998). Additional suggestions should include the control of movement of animals by appropriate legislation. There should be improvement in the management of animals by discouraging herding of different animal species together to reduce the risk of transmission of infection among the animal populations. These measures must go along with mass vaccination of cattle of all ages using the *B. abortus*

strain RB51. This can be continued until prevalence of the disease has fallen about 2%, an economically feasible level at which infected animals must be slaughtered. Such control measures combined with hygienic measures and education of government, herdsmen and communities on brucellosis, if coordinated with full cooperation of livestock owners, and local, state and Federal Government could lead to a control programme that is national in scope (Rikin, 1988b).

5.2 SUMMARY AND CONCLUSIONS

In this project, the objectives listed have been largely achieved. Twenty-five *Brucella* strains were isolated from clinical specimens obtained from various sources including aborting animals in various States of Nigeria. The isolates were biotyped, and they all belonged to *B. abortus* biovar 1. All the 32 stored cultures of *Brucella* isolated from cattle in previous studies were re-biotyped. They all belong to one species, *B. abortus*. Thirty-one of the isolates were biovar 1 while one was biovar 2. Experimental work indicates that the field isolates were virulent for mice and cannot be used as candidates for vaccine testing. Mice infected with these isolates produced humoral immune response measured by conventional serological tests for brucellosis (RBPT and SAT). Antibody reaction to the local *Brucella* strains was comparable to reaction produced by imported W.H.O. reference strain w.544 and Strain 19 vaccine.

Further work is required to:

i. Undertake molecular characterization of *Brucella* strains obtained from livestock in Nigeria

ii. Produce an effective *Brucell*a vaccine using local isolate of *Brucella*.

iii. Investigate the role of camels, dogs, pigs and wild life in the epidemiology of brucellosis in Nigeria.

iv. Undertake studies on the prevalence of *Brucella* infections among human population in Nigeria.

v. Evaluate the immune response of mice, guinea pigs and Nigerian cattle to *Brucella abortus* SRB51 and compare it with that of S19 before it can be commercially accepted for use in Nigeria.

vi. Establish Central Brucellosis Laboratory that is adequately equipped with facilities and well-trained staff to undertake *Brucella* reference works.

vii. Institute an extensive functional surveillance programme monitoring, and the campaign for control and eradication of brucellosis in Nigeria.

REFERENCES

1. Adams, G., Ficht, T. and Allen, C. (1998). Derivation and evaluation of the rough *rfb*K brucellosis vaccine in cattle. III Foro Nacional de Brucellosis. Mexico, pp141-158.

2. Adams, I. W. and McKay, I. (1966) *Brucella* in Government-owned livestock in Eastern Nigeria. *Nature* (London), 212: 217-218.

3. Adamu, N. B., Ajogi, I., Bale, J. O. and Mosimabale, F. O. (1997). Camel (*Camelus dromedarie*s) brucellosis in three Northern States of Nigeria. *Bulletin of Animal Health and Production in Africa,* 45: 193-197.

4. Adesiyun, A. A., Abdullahi, S. U. and Adeyanju, J. B. (1986). Presence of *Brucella abortus* and *Brucella canis* antibodies in dogs in Nigeria. *Journal of Small Animal Practice,* 27: 31-37.

5. Agunloye, C. A., Esuruoso, G. O., Ajogi, I., Ogudipe G.A. T., Agbede, S. A., Oyekole, O. D. and Ezeugwu, R. U. (1988) Bovine brucellosis surveillance in 2 N'dama breeding herds in Nigeria. *Bulletin of Animal Health and Production in Africa*, 36:63-68.

6. Ajogi, I., Adesiyun, A. A. and Esuruoso, G. O. (1996). Settling the nomads in Wase and Wawa-Zange grazing reserves in the savannah zone of Nigeria. II. The prevalence of bovine brucellosis. *Nigerian Veterinary Journal*, 1: 80-84.

7. Ajogi, I. (1997). Sero-prevalence of brucellosis in slaughtered cattle in four Northern States of Nigeria. *Tropical Veterinarian*, 15: 21-24.

8. Ajogi, I. (1998). Settling the nomads in Wase and Wawa-Zange grazing reserves in the savannah zone of Nigeria. IV. Strategies for the control of bovine brucellosis. *Nigerian Veterinary Journal*, 19: 40-48.

9. Ajogi, I., Akinwumi, J. A., Esuoroso, G. O. and Lamorde, A. G. (1998). Settling the nomads in Wase and Wawa-Zange grazing reserves in the savannah zone of Nigeria. III. Estimated financial losses due to bovine brucellosis. *Nigerian Veterinary Journal*, 19: 86-94.

10. Ajogi, I. and Akinwumi, J. A. (2001). Cash-flow model of the cost of brucellosis in traditionally managed cattle herds in Nigeria. *Bulletin of Animal Health and Production in Africa*, 49: 169-173.

11. Alausa, K. O. (1979). The investigation and control of a large scale community outbreak of brucellosis in Nigeria. *Public Health*, 185-189

12. Alausa, K. O. (1980). Incidence and seasonal prevalence among occupationally-exposed population to brucellosis. *Tropical Geographical Medicine*, 32: 12.

13. Alausa, K. O. and Osoba, A. O. (1975). *Brucella* seroreactivity in Western

Nigeria (An epidemiological study). *Transactions of the Royal Society for Tropical Medicine and Hygiene*, 69: 259-260.

14. Alausa, K. O. and Awoseyi, A. O. (1976). Brucellosis: Situation in Western Nigeria. *Tropical Geographical Medicine*, 28: 54-59.

15. Alausa, K.O. (1983). Brucellosis in Nigeria: Epidemiology and practical problems of control. In: Human ecology and Infectious diseases. Academic Press, London. Pp315-332

16. Allardet-Servent, A., Bourg, G., Ramuz, M., Pages, M., Bellis, M and Roizes, G. (1988). DNA polymorphism in strains of the genus *Brucella*. *Journal of Bacteriol*ogy, 170: 603-607.

17. Alton, G. G. (1985). The epidemiology of Brucella melitensis in sheep and goats. In : *Brucella melitensis* by Verger, J. M. and Plommet, M. (eds.). CEC Seminar, Martinus Nijoff, Dordrecht-Boston-Lancaster, pp187-196.

18. Alton, G. G. (1990). *Brucella melitensis*. In: Animal Brucellosis by K. H. Nielsen and J. R. Duncan (Eds.). CRC Press, Boca Raton. Pp383-409.

19. Alton, G. G., Corner, L. A. and Plackett, P. (1983). Vaccination of cattle against brucellosis using either a reduced dose of strain 19 or one or two doses of 45/20 vaccine. *Australian Veterinary Journal,* 60: 175 – 177.

20. Alton, G. G., Jones, L. M., Angus, R. D., Verger, J. M. (1988). Techniques for the brucellosis Laboratory, Institut National de la Recherché Agronomique, Paris, France. Pp63-129.

21. Anon (1927) Annual Report of the Veterinary Department of Nigeria for the year 1927.

22. Anon (1947) Annual Report of the Veterinary Department of Nigeria for the year 1947

23. Anon (1995) Animal Health Yearbook. FAO Animal Production and Health series. No. 34, pp143.

24. Anon (1997). The development of new/improved brucellosis vaccines. In: Proceedings of the Report of a WHO Meeting, Geneva, Switzerland, 11-12 December, WHO/EMC/ZDI/98.14. World Health Organization, Geneva, Switzerland.

25. Anon (2000). Suspected brucellosis case prompts investigation of possible bioterrorism-related activity. *Morbidity and Mortality Weekly Report,* 49: 509-512.

26. Anon (2001). Brucellosis in Sheep and Goats (*Brucella melitensis*). In: Report of the European Commission Scientific Committee on Animal Health and Animal

Welfare. SANCO.C.2/AH/R23/2001, Pp1-88.

27. Ariza, J., Guido, F., Pallares, R., Rufi, G. and Fernandez-Viladrich, P. (1992). Treatment of human brucellosis with doxycycline plus rifampicin or doxycycline plus streptomycin: A randomized, double-blind study. *Annals of Internal Medicine,* 117: 25-30.

28. Baba, M. M., Sarkinared, S. E. and Brisbe, F. (2001). Serological evidence of brucellosis among predisposed patients with pyrexia of unknown origin in North-Eastern Nigeria. *Central European Journal of Public Health,* 9: 158-161.

29. Baily, G. G., Krahn, J. B., Drasar, B. S. and Stocker, N. G. (1992). Detection of *Brucella melitensis* and *B. abortus* by DNA amplification. *Journal of Tropical Medicine and Hygiene,* 95: 217-275.

30. Bale, J. O. and Kumi-Diaka, J. (1981) Serological and bacteriological study of bovine brucellae from livestock investigation and breeding centers in Nigeria. *British Veterinary Journal,* 137, 256-261.

31. Bale, J.O., Nuru, S. and Addo, P.B. (1982). Serological study of sheep and goats brucellosis in Northern Nigeria. *Bulletin of Animal Health and Production in Africa*, 30, 73-79.

32. Bale, J. O. and Kwanashie, G. G. (1984). Sero-prevalence of brucellosis among horses in Northern Nigeria. *Journal of Animal Production Research,* 4: 161-164

33. Bale, J.O. and Nuru, S. (1985). Porcine brucellosis: Bacteriological and serological investigation of naturally infected pigs from six piggeries in Northern Nigeria. *Journal of Animal Production Research*, 5:193-199.

34. Bale, J. O., Nuru, S., Addo, P. B. and Adeyinka, A. (2003). Bacteriological investigation of sheep and goats milk for brucellosis in government farms in Northern Nigeria. *Journal of Animal Production*, 30: 107-116.

35. Bang, B. and Stribolt, S. (1897). The etiology of epizootic abortion. *Journal of Comparative Pathology and Therapy*, 10: 125.

36. Beh, K. J. (1974). Quantitative distribution of *Brucella* antibody among immunoglobulin classes in vaccinated and infected cattle. *Research in Veterinary Science*, 17:1-4.

37. Bercovich, Z. (1998). Maintenance of *Brucella abortus*-free herds: A review with emphasis on the epidemiology and the problems in diagnosing brucellosis in areas of low prevalence. *Veterinary Quarterly,* 20: 81-88.

38. Bercovich, Z. (1999). The use of skin-delayed type hypersensitivity as an adjunct test to diagnose brucellosis in cattle. PhD. Thesis, University of Utrecht, The Netherlands.

39. Bercovich, Z. and Taaijke, R. (1990). Enzyme immunoassay using mouse monoclonal anti-bovine antibodies in cow milk. *Journal of Veterinary Medicine*, 37: 753-759.

40. Blasco, J. M. (1990). *Brucella ovis*. In: Animal Brucellosis by Nielsen, K. H. and Duncan, J. R. (Eds), CRC Press, Boca Raton. Pp353-378.

41. Blasco, J. M., Gamazo, C., Winter, A. J., Jimenez de Bagues, M. P., Man, C., Moriyon, I., Alonso-Umeneta, B. and Diaz, R. (1993). Evaluation of whole cell and sub-cellular vaccines against *Brucella ovis* in rams. *Veterinary Immunology and Immunopathology*, 37: 257-270.

42. Bowden, R. A., Verger, J. M., Grayon, M. and Cloeckaert, A. (1997). Rapid identification of rough *Brucella* isolates by a latex co-agglutination assay with 25kDa outer membrane protein and rough lipopolysaccharide-specific monoclonal antibodies. *Clinical Diagnostic and Laboratory Immunol*ogy, 4: 611-614.

43. Bricker, B. J. (1999). Differentiation of hard-to-type bacterial strains by RNA mismatch cleavage. *Biotechnques*, 27:321-324.

44. Bricker, B. J. and Halling, S. M. (1994). Differentiation of *Brucella abortus* biovar 1, 2 and 4, *Brucella melitensis*, *Brucella ovis* and *Brucella suis* biovar 1 by PCR. *Journal of Clinical Microbiology*, 32: 2660-2666.

45. Bricker, B. J. and Halling, S. M. (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *Journal of Clinical Microbiology*, 33: 1640-1642.

46. Bricker, B. J., Ewalt, D. R., Macmillan, A. P., Foster, G. and Brew, S. (2000). Molecular characterization of *Brucella* strains isolated from marine mammals. *Journal of Clinical Microbiology*, 38: 1258-1262.

47. Bricker, B. J., Ewalt, D. R. and Halling, S. M. (2003). *Brucella* "HOOF-Prints" strain typing by multilocus analysis of variable number tandem repeats (VNTRs). *BMC Microbiology*, 3: 15-29.

48. Brinley-Morgan, W. J., and Corbel, M. J. (1990). *Brucella* infections in man and animals: contagious equine metritis, In: Topley and Wilson's principles of bacteriology, virology and immunology by M. T. Parker and L. H. Collier (eds.), 8th ed., Edward Arnold, London, pp. 547–570.

49. Brisbie, F., Nawathe, D. R. and Bot, C. J. (1993) Serological prevalence of brucellosis in sheep, goats and human beings in Maiduguri metropolis. *Tropical Veterinarian*, 11: 27-33.

50. Brown, G. M., Ranger, C. R. and Kelly, D. J. (1971). Selective media for the isolation of *Brucella ovis*. *Cornell Veterinarian*., 61: 265-280.

51. Bruce, D. (1887). Notes on the discovery of a micro-organism in Malta fever.

Practitioner (London), 39: 161.

52. Buck, J. M. (1930). Studies of vaccination during calfhood to prevent bovine infectious abortion. *Journal of Agricultural Research*, 41: 667.

53. Buddles, M. R. and Boyes, R. W. (1953). A *Brucella* mutant causing genital infections in sheep in New Zealand. *Australian Veterinary Journal*, 29: 143-153.

54. Canning, P. C., Roth, J. A., Tabatabai, L. B. and Deyoe, B. L. (1985). Isolation of components of *Brucella abortus* responsible for inhibition of function in bovine neutrophils. *Journal of Infectious Diseases,* 152:913–921.

55. Canning, P. C., Roth, J. A. and Deyoe, B. L. (1986). Release of 5-guanosine monophosphate and adenine by *Brucella abortus* and their role in the intracellular survival of the bacteria. *Journal of Infectious Diseases,* 154:464–470.

56. Carmichael, L. E. (1990). *Brucella canis*. In: Nielsen, K. H. and Duncan, J. R. (Eds.), Animal Brucellosis. CRC Press, Boca Raton. Pp336-350.

57. Carmichael, L. E. and Kenny, M. R. (1968). Canine abortion caused by *Brucella canis*. *Journal of American Veterinary Medical Association,* 152: 605-616.

58. Catlin, J. E. and Sheehan, E. J. (1986). Transmission of bovine brucellosis from dam to calf. *Journal of American Veterinary Medical Association,* 188: 867-869.

59. Chan, R. and Hardiman, R. P. (1993). Endocarditis caused by *Brucella melitensis*. *Medical Journal of Australia,* 58: 631-632.

60. Cheville, N. F., Stevens, M. G., Jensen, A. E., Tatum, F. M. and Halling, S. M. (1993). Immune responses and protection against infection and abortion in cattle experimentally vaccinated with mutant strains of Brucella abortus. *American Journal of Veterinary Research,* 54: 1591-1597.

61. Cheville, N. F., Olsen, S. C., Jensen, A. E., Stevens, M. G., Palmer, M. V. and Florance, A. M. (1996). Effects of age at vaccination on efficacy of *Brucella abortus* RB51to protect cattle against brucellosis. *American Journal of Veterinary Research,* 57: 1153-1156.

62. Chima, J.C. (1988) Mycoplasmas as etiological agents of caprine vulvovaginitis in Nigeria. PhD Thesis, University of Ibadan.

63. Chukwu, C. C. (1985a). Brucellosis in Africa. Part 1. The prevalence. *Bulletin of Animal Health and Production in Africa,* 33: 193-198.

64. Chukwu, C. C. (1985b). Brucellosis in Africa. Part 2. The importance. *Bulletin of Animal Health and Production in Africa,* 35: 92-98.

65. Chukwu, C. C. (1985c). Serological investigation on cattle vaccinated with a killed *Brucella abortus* strain 45/20 adjuvant vaccine. *International Journal of*

Zoonoses, 12: 14-21.

66. Chukwu, C. C. (1987a). Comparison of cell-mediated immune responses to *Brucella abortus* strain 19 vaccine and *Brucella abortus* 45/20 killed vaccine in cattle. *Microbioscience Letters*, 34: 93.

67. Chukwu, C. C. (1987b). Studies on sero-prevalence of bovine brucellosis in Enugu and Nsukka. *Zariya Veterinarian*., 2: 383-387.

68. Cloeckaert, A., Verger, J. M. Grayon, M. and Grapinnet, O. (1996). Polymorphism at the *dnaK* locus of *Brucella* species and identification of a *Brucella melitensis* species-specific marker. *Journal of Medical Microbiology,* 45:200-205.

69. Clavareau, C., Wellemans, V., Walravens, K., Tryland, M., Verger, J. M. Grayon, M., Cloeckaert, A., Letesson, J. J. and Godfroid, J. (1998). Phenotypic and molecular characterization of a *Brucella* strain isolated from a mink whale (*Baleonoptera acutorostrata*). *Microbiology*, 144, 3267-3273.

70. Cloeckaert, A., Grayon, M. and Grepinet, O. (2000). An IS711 element downstream of the bp26 gene is a specific marker of species isolated from marine mammals. *Clinical Diagnostic and Laboratory Medicine,* 7: 835-839.

71. Cloeckaert, A., Verger, J. M., Grayon, M., Paquet, J. Y., Garin-Bastuji, B., Foster, G. and Godfroid, J. (2001). Classification of *Brucella* spp. Isolated from marine mammals by DNA polymorphism at the *omp*2 locus. *Microbial Infections,* 3: 729-738.

72. Cloeckaert, A., Verger, J.M., Grayon, M., Grepinet, O. (1995). Restriction site polymorphism of the genes encoding the major 25 and 36 kDa outer-membrane proteins of *Brucella*. *Microbiology*, 141: 2111–2121.

73. Colby, L. A. (1997). The humoral response of Elk (*Cervus elaphus nelsoni*) and mice to vaccination with *Brucella abortus* strain RB51. Msc. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA.

74. Collard, P. (1962). Antibodies against brucellae in healthy persons in various parts of Nigeria. *West African Medical Journal,* 11: 172-174.

75. Corbel, M. J. (1997). Brucellosis: An overview. *Emerging Infectious Diseases,* 3, 213-221.

76. Corbel, M. J., Bracewell, C. D., Thomas, E. L. and Gill, K. P. W. (1979). Techniques in the identification and classification of *Brucella*. In: Identification methods for Microbiologists. 2nd Edition. Academic Press, London.

77. Corbel, M. J. and Brinley-Morgan, W. J. (1984) Genus *Brucella*. Meyer and Shaw 1920. In: Bergey's Manual of systematic Bacteriology by Kreig, N. R. Holt, J. G. (Eds.). Williams and Wilkins, Baltimore. Pp.377-388

78. Corbel, M. J., Stuart, F. A., and Brewer, R. A. (1984). Observations on serological cross-reactions between *Brucella* species and organisms of the other genera. *Development in Biological Standardization,* 56: 314-348.

79. Corbel, M. J., Gill, K. P. W. and Thomas, E. L. (1987). Methods for the identification of *Brucella*. Ministry of Agriculture, Foods and Fisheries, London.

80. Crawford, R. P., Williams, J. D., Huber, J. D. and Childers, A. B. (1979). Biotypes of *Brucella abortus* and their value in epidemiological studies of infected cattle herds. *Journal of American Veterinary Medical Association,* 1274-1277.

81. Crawford, R. P., Huber, J. D. and Adams, B. S. (1990). Epidemiology and surveillance. In: Animal Brucellosis by Nielsen, K. H. and Duncan, J. R. (Eds.). CRC Press, Boca Raton. Pp 131-151.

82. Da Costa, M., Guillou, J. P., Garin-Bastuji, B., Thiebaud, M. and Dubray, G. (1996). Specificity of six gene sequences for the detection of the genus *Brucella* by DNA amplification. *Journal of Applied Bacteriology,* 81:267-275.

83. Davies, G. and Casey, A. (1973). The survival of *Brucella abortus* in milk and milk products. *British Veterinary Journal,* 129: 345-353.

84. Dees. C., Fountain, M. W., Talylor, J. R. and Schultz, R. D. (1985). Enhancing intraphagocytic killing of *Brucella abortus* in bovine mononuclear cells by liposome-containing gentamicin. *Veterinary Immunology and Immunopathology,* 8: 171-182.

85. DelVecchio, V. G., Kapatral, V., Redkar, R. J., Patra, G., Mujer, C., Los, T., Ivanova, N., Anderson, I., Bhattacharyya, A., Lykidis, A., Reznik, G., Jablonski, L., Larsen, N., D'Souza, M., Bernal, A., Mazur, M., Goltsman, E., Selkov, E., Elzer, P., Hagius, S., O'Callaghan, D., Letesson, J. J., Haselkorn, R., Kyrpides, N. and Overbeek, R. (2002). The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. *Proceedings of National Academy of Sciences* (USA), 99: 443-448.

86. Detilleux, P. G., Deyoe, B. L. and Cheville, N. F. (1990). Penetration and Intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. *Infection and Immunity,* 58:2320–2328.

87. Eaglesome, M. D. and Garcia, M. M. (1992). Microbial agents associated with bovine genital tract infections and semen. Part I. *Brucella abortus, Leptospira, Campylobacter fetus and Trichomonas foetus*. *Veterinary Bulletin,* 62: 744-751.

88. Elberg, S. S. and Faunce, W. K. (1957). Immunization against *Brucella* infection. IV. Immunity conferred on goats by a non-dependent mutant from a streptomycin dependant mutant strain of *Brucella melitensis*. *Journal of Bacteriology,* 3: 211.

89. Elegbe, I.A., Juga, A and Adebayo, J.O. (1987). Species and serotypes of *Campylobacter* from domestic animals in Nigeria. *Journal of Diarrhea Diseases*

Research, 5:97-101.

90. Elzer, P. H, Jacobson, R. H. Nielsen, K. H. Douglas, J. T.and Winter, A. J. (1994). BALB/c mice infected with *Brucella abortus* express protracted polyclonal responses of both IgG2a and IgG3 isotypes. *Immunological Letters,*. 42:145–150.

91. Enright, F.M. (1990). The pathogenesis and pathobiology of *Brucella* infection in domestic animals. In: Nielsen, K., Duncan, J.R. (eds.) Animal Brucellosis, Boca Raton, Florida, CRC Press Inc. pp301-320.

92. Erasmus, J. A. and Bergh, E. C. (1985). Ovine brucellosis: repeated vaccinations with Rev. 1vaccine and the prevalence of the disease in the Winburg district. *Journal of South African Veterinary Association,* 56: 205-208.

93. Esuruoso, G. O. (1974) Bovine brucellosis in two Southern States of Nigeria: II. Incidence and implications of infection in range cattle. *Bulletin of Epizootic Diseases in Africa,* 22: 34-35.

94. Esuruoso, G. O. (1979). Current situation in Nigeria and preliminary evaluation of the probable costs and benefits of a proposed brucellosis control programme for the country. *Veterinary Epidemiology and Economics,* Pp. 644-649.

95. Esuruoso, G. O. and Hill, D. H. (1971). Sero-epidemiological survey of bovine brucellosis in dairy herds in the Western States of Nigeria. *Nigerian Agricultural Journal*, 8: 147-154.

96. Esuruoso, G. O. and Van Blake, H. E. (1972). Bovine brucellosis in two Southern States of Nigeria: I. An investigation of selected herds. *Bulletin of Epizootic Diseases in Africa*, 20: 269-274.

97. Evans, A. (1918). Further studies on *Bacterium abortus* and related bacteria II: A comparison of *Bacterium abortus* with *Bacterium brochosepticus* and with organism which causes Malta fever. *Journal of Infectious Diseases,* 22: 580-593.

98. Ewalt, D. R., Payeur, J. B., Martin, B. M., Cummins, D. R. and Miller, W. G. (1994) Characteristics of a *Brucella* species from a bottle nose dolphin (*Tursiops truncatus*). *Journal Veterinary Diagnostic Investigation,* 6: 448-452.

99. Eze, E. N., (1978). Isolation of brucellae from the Nigerian livestock and the typing of such isolates. *Bulletin of Animal and Production in Africa,* 26: 29-36

100. Eze, E. N. (1981). Studies of the Immune response guinea pigs and cattle to *Brucella* antigens. Ph.D. Thesis, University of Ibadan.

101. Eze, E. N., Esuruoso, G. O. and Sansi, K. A. O. (1983). The virulence of some locally isolated *Brucella abortus* strains in guinea-pigs. *Nigerian Veterinary Journal*, 12: 44-46.

102. Eze, E. N. (1985). Problems of brucellosis control in Nigeria. *Nigerian Livestock Farmer*, 2:19-20

103. Ezeh, A.O. (1988). Isolation of Leptospiras from Nigerian cattle. PhD Thesis, Ahmadu Bello University, Zaria.

104. Falade, S. (1974). *Brucella* agglutination antibodies in the sera of persons dwelling in Ibadan and surrounding districts. *Journal of Nigerian Veterinary Medical Association,* 3: 21-23.

105. Falade, S. (1977). Absence of *Brucella* antibodies in dogs. *Bulletin of Animal Health and Production in Africa,* 25: 97.

106. Falade, S. (1981). Brucellae isolated from goats. *Zentralblatt VeterinaerMedizine, B20*: 205-209.

107. Falade, S., Ojo, M. O. and Sellers, K. C. (1975) A serological survey of caprine brucellosis in Nigeria. *Bulletin of Animal Health and Production in Africa,* 22: 335-339.

108. Falade, S. and Shonekan, A. O. (1981) A serological survey of *Brucella abortus* infection in Nigerian sheep. *Nigerian Veterinary Journal,* 2: 50-52.

109. FAO (2004). Bovine brucellosis. In: Animal Health and Disease Cards. Pp1-18.

110. Farber, J. M. (1996). An introduction to the hows and whys of molecular typing. *Journal Food Protection*, 59: 1091-1101.

111. Farrell, I. D. (1974). The development of a new selective medium for the isolation of *Brucella abortus* from contaminated sources. *Research in Veterinary Science,* 16: 280-286.

112. Farrell, I. D. and Robinson, L. (1972). A comparison of various selective media, including a new selective medium for the isolation of brucellae from milk. *Journal of Applied Bacteriology,* 35: 625-630.

113. Fekete, A., Bantle, J. A., Halling, S. M. and Sanborn, M. R. (1990). Preliminary development of a diagnostic test for *Brucella* using polymerase chain reaction. *Journal of Applied Bacteriology*, 69: 216-227.

114. Fekete, A., Bantle, J. A., Halling, S. M. and Stich, R. W. (1992). Amplification fragment length polymorphism in Brucella stains by use of polymerase chain reaction with arbitrary primers. *Journal of Applied Bacteriology,* 174: 7778-7783.

115. Fernandes, D. M., R. Benson, and C. L. Baldwin. 1995. Lack of a role for natural killer cells in early control of *Brucella abortus* 2308 infections in mice. *Infection and Immunity,* 4029–4033.

116. Festerbank, R. (1987). Some aspects of experimental bovine brucellosis. *Annales*

Recherche Veterinaire, 18: 421-428.

117. Ficht, T. A., Bearden, S. W., Sowa, B. A. and Marquis, H. (1990). Genetic variation at the *omp*2 porin locus of the brucellae: species-specific markers. *Molecular Microbiology,* 4: 1135-1142.

118. Ficht, T. A., Husseinen, H. S., Derr, J. and Bearden, S. W. (1996). Species-specific sequence at the omp2 locus of *Brucella* type strains. *International Journal of Systematic Bacteriology,* 46: 329-331.

119. Finlay, B. B. and Falkow, S. (1989). Common themes in microbial pathogenicity. *Microbiological Review*, 53: 210-230.

120. Foster, G., Jahans, K. L., Reid, R. J. and Ross, H. M. (1996). Isolation of *Brucella* species from cetaceans, seals and an otter. *Veterinary Record*, 138: 583-586.

121. Garcia-Carrillo, C. (1980). Comparison of *Brucella melitensis* Rev. 1 and *B. abortus* strain 19 as a vaccine against brucellosis in cattle. *Zentralblatt Veterinaemedicine*, 27: 131-138.

122. Gay, K. and Damons, S. R. (1955). Use of the embryonating egg for the isolation of *Brucella*: A yolk sac technique. Proceedings of the 3rd International American. Congress on Brucellosis, Washington, DC, Pp157-164.

123. Grillo, M. J., Barberan, M. and Blasco, M. (1997). Transmission of *Brucella melitensis* from sheep to lambs. *Veterinary Record,* 140: 602-605.

124. Grimmont, F., Verger, J. M., Cornelis, P., Limet, J., Lefevre, M., Grayon, M., Regnault, B., Van Broeck, J. and Grimmont, P. A. D. (1992). Molecular typing of *Brucella* with cloned DNA probes. *Research in Microbiology*, 143: 55-65.

125. Hadjichristodolou, C., Voulgaris, P., Toulieres, L., Babalis, T., Manetas, S., and Goutziana, G. (1994). Tolerance of the human brucellosis vaccine and the intradermal reaction test for brucellosis. *European Journal of Clinical Microbiology and Infectious Diseases,* 13: 129-134.

126. Halle, P. D. and Ajogi, I. (1997) Brucellosis in Nigeria: A review of recent developments. *Israel Journal of Veterinary Medicine,* 52: 125-131.

127. Halling, S. M., Detilleux, P. G., Tatum, F. M., Judge, B. A. and Mayfield. J. E. (1991). Deletion of the BCSP31 gene of *Brucella abortus* by replacement. *Infection and Immunity,* 59:3863–3868.

128. Halling, S. M. and Bricker, B. J. (1994). Characterization and occurrence of two repeated palindromic DNA elements of *Brucella* spp.: Bru-RS1and Bru-RS2. *Molecular Microbiology,* 14: 681-689.

129. Harmon, B. G., Adams. L. G., Templeton, J. W. and Smith, R. (1989).

Macrophage functions in mammary gland of *Brucella abortus*-infected cows and cows that resisted infection after inoculation of *Brucella abortus*. *American Journal of Veterinary Research,* 50: 459-465.

130. Herman, L. and De Ridder, H. (1992). Identification of *Brucella* spp. by using the polymerase chain reaction. *Applied Enviromental Microbiology,* 58: 2099-2101.

131. Herr, S., Ribeiro, L. M. M. and Chaparro, F. (1990). Persistence infection with *Brucella abortus* biotype 1 in a cow. *Journal of South African Veterinary Association,* 61: 77.

132. Ho, M. and C. Cheers. (1982). Resistance and susceptibility of mice to bacterial infection. IV. Genetic and cellular basis of resistance to chronic infection with *Brucella abortus*. *Journal of Infectious Diseases,* 146:381–387.

133. Hoffmann, E. M. and J. J. Houle. (1995). Contradictory roles for antibody and complement in the interaction of *Brucella abortus* with its host. *Critical Review in Microbiology,* 21:153–163.

134. Hong, P. C., Tsolis, R. M. and Ficht, T. A. (2000). Identification of genes required for chronic persistence of *Brucella abortus* in mice. *Infection and Immunity,* 68:4102–4107.

135. Hornitzky, M. and Searson, J. (1986). The relationship between the isolation of *Brucella abortus* and serological status of infected, non-vaccinated cattle. *Australian Veterinary Journal,* 63: 172-174.

136. Horrocks, W. H. (1905) A preliminary notes on goats as a means of propagation of Mediterranean fever. Report of the Commission for the Investigation of Mediterranean fever. Part III. Pp84.

137. Hoyer, B. H. and McCullough, N. B. (1968a). Homologies of deoxyribonucleic acids from *Brucella ovis*, canine abortion organisms, and other *Brucella* species. *Journal of Bacteriology,* 96: 1783-1790.

138. Hoyer, B. H. and McCullough, N. B. (1968b). Polynucleotide homologies of deoxyribonucleic acids. *Journal of Bacteriology,* 95: 444-448.

139. Jahans, K. L., Foster, G. and Broughton, E. S. (1997). The characteristics of *Brucella* strains isolated from marine mammals. *Veterinary Microbiology,* 57: 373-382.

140. Jensen, A.E., Cheville, N. F., Ewalt, D. R., Thoen, C. O. and Payeur, J. B. (1995). Application of pulsed-field gel electrophoresis for differentiation of vaccine strain RB51 from field isolates of *Brucella abortus* from cattle, bison, and elk. *American Journal of Veterinary Research,* 56: 308-312.

141. Jensen, A. E., Cheville, N. F., Thoen, C. O., Macmillan, A. P.and Miller, W. G. (1999). Genomic fingerprinting and development of a dendrogram for *Brucella* species isolated from seals, porpoises, and dolphins. *Journal of Veterinary*

Diagnostic Investigation, 11: 152-157.

142. Jepson, P. D., Brew, S., Macmillan, A. P., Baker, J. R., Barnett, J., Kirkwood, J. K., Kuiken, T., and Robinson, I. R. (1997). Antibodies to *Brucella* in marine mammals around the coast of England and Wales. *Veterinary Record,* 141: 513-515.

143. Jiang, X., B. Leonard, R. Benson, and C. L. Baldwin. 1993. Macrophage control of *Brucella abortus*: role of reactive oxygen intermediates and nitric oxide. *Cellular Immunology,* 51:309–319.

144. Jones, L. M., Montgomery, V. and Wilson, J. B. (1965). Characteristics of carbon dioxide independent cultures of *Brucella abortus* isolated from cattle vaccinated with strain 19. *Journal of Infectious Diseases,* 115: 312-320.

145. Jones, R. L., Deyeo, B. L., Meyer, M. E., Buening, G. M. and Fales, W. H. (1982). Isolation of two *Brucella* biotypes from tissues of a naturally infected cow. *Journal of Clinical Microbiology,* 16: 641-643.

146. Jones, S. M., and A. J. Winter. (1992). Survival of virulent and attenuated strains of *Brucella abortus* in normal and gamma interferon-activated murine peritoneal macrophages. *Infection and Immunity,*. 60:3011–3014.

147. Kerkofs, P., Botton, Y., Thiange, P., Dekeyser, P. and Limet, J. N. (1990). Diagnosis of bovine brucellosis by enzyme immunoassay of milk. *Veterinary Microbiology,* 24: 73-80.

148. Kiss, Z. (1971). Value of antiglobulin test in brucellosis eradication in a one-year field study. *Veterinary Bulletin,* Abstract .5609.

149. Ko, J. and Splitter, G. A. (2003). Molecular host-pathogen interaction in brucellosis: Current understanding and future approaches to vaccine development for mice and humans. *Clinical Microbiological Review,* 16: 65-78.

150. Kolar, J. (1989). Brucellosis in Eastern European countries. In: Brucellosis: clinical and laboratory aspects by E. J. Young and M. J. Corbel. CRC Press, Boca Raton. Pp163-172.

151. Kramer, J. W., Nduaka, O. and Uzoukwu, M. (1967) Serological survey of diseases of cattle, sheep and goats in Eastern Provinces of Nigeria. *Bulletin of Epizootic Diseases in Africa,* 15: 25-29.

152. Kuzdas, C. D. and Morse, E. V. (1953). A selective medium for the isolation of brucellae from contaminated materials. *Journal of Bacteriology,* 66:502-504.

153. Lantier, F. and Fensterbank, R. (1985). Kinetics of Rev. 1 infection in sheep. In: *Bruclla melitensis* by M. Plommet and J. M. Verger (Eds.). Martinus Niijhoff, Dordrecht, pp247 -251.

154. Lauderdale, T. L., Jones, S. M. and Winter, A. J. (1990). Response of murine T cells to antigens of *Brucella abortus* at sequential periods after infection. *Immunology and Infectious Diseases,*1: 59-66.

155. Levieux, D. (1974). Bovine immunoglobulins and brucellosis. II. IgG1, IgG2 and IgM in agglutination, Coombs, Complement fixation and rose Bengal plate tests. *Annales Recherche Veterinaire,* 5: 343-353.

156. Lopez-Goni, I., Guzman-Verri, C., Manterola, L., Moriyon, I. and Moreno, E. (2002). Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. *Vetetrinary Microbiology,* 90: 329-339.

157. Lord, V. R., Schurig, G. G., Cherwonogrodzky, J. W. Marcano, M. J. and Melendez, G. E. (1998). Field study of vaccination of cattle with *Brucella abortus* strains RB51 and 19 under high and low disease prevalence. *American Journal of Vetrinary Research,* 59: 1016-1020.

158. Lu, S. L. and Zhang, J. L. (1989). Brucellosis in China. In: Brucellosis: Clinical and laboratory aspects by E. J. Young and M. J. Corbel. CRC Press, Boca Raton, pp173-180.

159. Lubani, M. M., Dudkin, K. K. and Sharda, D. C. (1989). A multi-center therapeutic study of 1100 children with brucellosis. *Pediatric Infectious Diseases,* 8: 75-78.

160. Luzzi, G. A., Brindle, R., Socket, P. N., Solera, J., Klenerman, P. and Warren, D. A. (1993). Brucellosis: imported and laboratory-acquired case, and an overview of treatment trials. *Transactios of Royal Society for Tropical Medicine and Hygiene,* 87: 138-141.

161. Macmillan, A. P. (1990). Conventional serological tests. In: Animal Brucellosis by K. Nielsen and J. R. Duncan (Eds.). CRC Press, Boca Raton, Florida. Pp153-190.

162. Macmillan, A. P., Baskerville, A., Hambleton, P. and Corbel, M. J (1985). Experimental *Brucella abortus* infection in the horse: Observations during the 3 months following inoculation. *Research in Veterinary Science*, 33:351-359.

163. Madkour, M. M. 2001. Madkour's Brucellosis. Springer Verlag, New York. pp11

164. Marin, C. B., Alabart, J. L. and Blasco, J. M. (1996a). Effect of antibiotics contained in two *Brucella* selective media on growth of *Brucella abortus, B. melitensis and B.ovis*. *Journal of Clinical Microbiology,* 34: 426-428.

165. Marin, C. M., Jimenez de Bagues, M. P., Barberan, M. and Blasco, J. M. (1996b). Comparison of two selective media for the isolation of *Brucella melitensis* from naturally infected sheep and goats. *Veterinary Record*, 138: 409-411.

166. Marston, J. A. (1861). Report on fever (Malta). Army Med. Rep., 3: 486-521.

167. McCullough, N. B. and Dick, L. A. (1942). Physiological studies of *Brucella* I: Quantitative accessory growth factor requirements of certain strains of *Brucella. Journal of Infectious Diseases,* 71: 193-197.

168. McDermott, J. J. and Arimi, S. M. (2002). Brucellosis in sub-Saharan Africa: Control and impact. *Veterinary Microbiology,* 90: 111-134.

169. McEwen, A. D. (1940). Experiments on contagious abortion: Immunization studies with vaccines of graded virulence. *Veterinary Record,* 52: 815.

170. Mercier, E., Jumas, B. E., Allardet-Servent, A., O'Callaghan, D. and Ramuz, M. (1996). Polymorphism in *Brucella* strains detected by studying distribution of two short repetitive DNA elements. *Journal Clinical Microbiology,* 34: 1299-1302.

171. Merr, A. G. and Wilson, J. B. (1950). Genetic aspects of the added carbon dioxide requirement of *Brucella abortus*. *Proceedings of Society for Experimental Biology and Medicine,* 75: 438-440.

172. Mettam, R. W. M. (1947) Annual Report of the Veterinary Department of Nigeria for the year 1947.

173. Meyer, M. E. (1964). The epizootiology of brucellosis and its relationship to the identification of *Brucella* organisms. *American Journal Veterinary Research,* 25:553-557.

174. Meyer, K. F. and Shaw, F. B. (1920) Characteristics of *Brucella abortus* and *Brucella melitensis*: A comparison of the morphologic, cultural and biological characteristics of *Brucella abortus* and *Brucella melitensis*. *Journal of Infectious Diseases*, 27: 173-184.

175. Michaux-Charachon, S., Jumas-Bilak, E., Allardet-Servent, A., Bourg, G., Boschiroli, M. L., Ramuz, M. and O'Callaghan, D. (2002). The *Brucella* genome at the beginning of the post-genomic era. *Veterinary Microbiology*, 90: 581-585.

176. Milward, F. W., Nicoletti, P. and Hoffmann, E. (1984). Effectiveness of various therapeutic regimens for brucellosis. *American Journal of Veterinary Research*, 45: 1825-1828.

177. Montaraz, J. A., and A. J. Winter. 1986. Comparison of living and nonliving vaccines for *Brucella abortus* in BALB/c mice. *Infection and Immunity,* 53:245–251.

178. Montejo, J. M., Alberola, I. and Glez, Z. P. (1993). Open, randomized therapeutic trial of six antimicrobial regimens in the treatment of human brucellosis. *Clinical Infectious Diseases*, 6: 671-676.

179. Moreno, E. (1998). Genome evolution within the alpha-*Proteobacteria*: why do some bacteria not possess plasmids and others exhibit more than one different chromosome? *FEMS Microbiological Review*, 22:255-275.

180. Moreno, E. and Moriyon, I. (2001). *Brucella melitensis*: A nasty bug with hidden credentials for virulence. *Proceedings of National Academy of Science,* (USA), 99: 1-3.

181. Moreno, E., Cloeckaert, A. and Moriyon, I. (2002). *Brucella* evolution and taxonomy. *Veterinary Microbiology*, 90: 209-227.

182. Moreno, E., Stackebrandt, E., Dorsch, M., Wolters, J., Busch, M. and Mayer, H. (1990). *Brucella abortus* 16S rRNA and lipid A reveal a phylogenetic relationship with members of the alpha-2 subdivision of the class *Proteobacteria*. *Journal of Bacteriology*, 172: 3569-3576.

183. Morris, R. S. (1999). The application of economics to animal health programmes: a practical guide. *Revue Scientific Technical Office International des Epizooties*, 18: 305-314.

184. Murray, C. J. l. and Lopez, A. D. (1996). The global burden of disease: A comprehensive assessment of mortality and disability from disease, injuries and risk factors in 1990 and projected to 2020. Harvard University Press, Boston.

185. Mustafa, A. A. and Abusowa, M. (1993). Field-oriented trial of the Chinese *Brucella suis* strain 2 vaccine in sheep and goats in Libya. *Annales Recherche. Veterinaire*, 24: 422-429.

186. Nicoletti, P. (1980). Epidemiology of bovine brucellosis. *Advance in Veterinary Science and Comparative Medicine*, 24: 69-98.

187. Nicoletti, P. (1981). Prevalence and persistence of *Brucella abortus* strain 19 infections and prevalence of other biotypes in vaccinated dairy herds. *American Journal Veterinary Research*, 178: 143-145.

188. Nicoletti, P. (1989). Relationship between animal and human disease. In: Brucellosis: Clinical and laboratory aspects by E. J. Young, and M. J. Corbel (Eds.). CRC Press, Boca Raton, USA, pp. 41-51.

189. Nicoletti, P. (1990). Vaccination. In: Animal brucellosis by K. Nielsen and J. R. Duncan (Eds.), CRC Press, Boca Raton. Pp284-299.

190. Nicoletti, P. (1993). Eradication of brucellosis in animals. *Saudi Medical. Journal*, 14: 288-292.

191. Nicoletti, P., Lenk, R. P., Popescu, M. C. and Swenson, C. E. (1989). Efficacy of various treatment regimens, using liposomal streptomycin in cows with brucellosis. *American Journal Veterinary Research*, 50: 1004-1007.

192. Nicoletti, P., Milward, F. W., Hoffmann, E. and Altvater, L. (1985). Efficacy of long-acting oxytetracycline alone or combined with streptomycin in the treatment of bovine brucellosis. *Journal of American Veterinary Medical Association*, 187: 493-495.

193. Nielsen, K. H., Wright, P. F. and Cherwonogrodzky, J. H. (1988). A review of enzyme immunoassay for detection of antibody to *Brucella abortus* in cattle. *Veterinary Immunology and Immunopathology,* 18: 331-347.

194. Nielsen, O., Stewart, R. E., Nielsen, K., Measures, L. and Duignan, P. (2001). Serologic survey of *Brucella* spp. antibodies in some marine mammals of North America. *Journal of Wildlife Diseases*, 37: 89-100.

195. Nuru, S. (1982). Problems and prospects of the Nigeria beef industry. In: Proceedings of the National Conference on beef production held at NAPRI, Shika.

196. Nuru, S. and Dennis, S.M. (1975). Serological survey of brucellosis in slaughtered cattle in North Central State of Nigeria. *Journal of Nigerian Veterinary Medical Association*, 4: 3-8.

197. Nuru, S. and Schnurrenberger, P. (1975). The present status of bovine brucellosis in Nigeria: its prevalence, significance and areas of research needs. *Bulletin of Office of International Epizootics*, 83: 1113-1123.

198. Ocholi, R. A., Ezeokoli, C. D., Akerejola, O. O. and Saror, D. I. (1996) Use of the enzyme-linked immunosorbent assay for screening cattle for *Brucella* antibodies in Nigeria. *Veterinary Quarterly*, 18: 22-24.

199. Ocholi, R. A., Kalejaye, J. O. and Okewole, P. A. (1993) Brucellosis in Nigeria: A review. *Tropical Veterinarian*, 11: 15-26.

200. Okewole, P. A., Eze, E. N., Okoh, A. E. J., Oyetunde, I. L. and Odeyemi, P. S. (1988) Small ruminant brucellosis in some parts of Northern Nigeria. *Bulletin of Animal Health Production Africa*, 36: 251-254.

201. Okoh, A. E. J. (1978) A survey of brucellosis in camels in Kano, Nigeria. *Tropical Animal Health and Production*, 11: 213-214.

202. Okoh, A. E. J. (1980) Abortion in sheep near Kano, Nigeria. *Tropical Animal Health and Production*, 12: 11-14.

203. Okoh, A. E. J., Alexei, I. and Agbonlahor, D. E. (1979) Brucellosis in dogs in Kano State, Nigeria. *Tropical Animal Health and Production,* 10: 219-220.

204. Oladosu, L. A., Falade, S. and Akpokodje, U. (1986). Equine brucellosis in Nigeria. *Zariya Veterinarian*, 1: 129-133.

205. Oni, O.O., Adesiyun, A.A., Adekeye, J.O. and Saidu, S.N.A. (1989). Prevalence and some characteristics of *Listeria monocytogenes* isolated from cattle and milk in Kaduna state, Nigeria. *Isreal Journal of Veterinary Medicine,* 45:12-17.

206. Osori, D.I.K. (1976) Seasonal variation in reproductive activity of indigenous cattle in Northern Nigeria. PhD Thesis, Ahmadu Bello University, Zaria.

207. Ouahrani-Bettache, S., Soubrier, M. P. and Liautard, J. P. (1996). IS6501-anchored PCR for the detection and identification of *Brucella* species and strains. *Journal of Applied Bacteriology,* 81:154-160.

208. Oyedipe, E.O., Bavanendran, V. and Eduvie, L.O. (1981) Factors affecting the reproductive performance of Fulani Cattle. NAPRI Seminar.

209. Palmer, M. V., Olsen, S. C. and Cheville, N. F. (1997). Safety and immunogenicity of *Brucella abortus* strain RB51 vaccine in pregnant cattle. *American Journal of Veterinary Research,* 58: 472-477.

210. Patterson, J. M. and Deyeo, B. L. (1976). Effect of physical properties of milk fat globules on *Brucella* ring test sensitivity. *Journal of Dairy Science,* 60: 851-856.

211. Paulsen, I. T., Sheshadri, R., Nelson, K. E., Eisen, J. A., Heidelberg, J. F., Read, T. D., Dobson, R. J., Umayam, M., Brikac, L. M., Beanan, M. J., Daugherty, S. C., Deboy, R. T., Durkin, A. S., Kolonay, J. F., Madupu, R., Nelson, W. C., Ayodeji, B., Kraul, M., Shetty, J., Malek, J., Van Aken, S. C., Reidmuller, S., Tetelin, H., Gill, S. R., White, O., Salzberg, S. L., Hoover, D., Lindler, L. E., Halling, S. M., Boyle, S. M. and Fraser, C. M. 2002). The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogen and symbionts. *Proceedings of National Academy of Sciences (USA),* 99: 13148-13153.

212. Phillipon, A., Renoux, G. and Plommet, M. (1970). Experimental bovine brucellosis. III. Vaginal excretion of *Brucella abortus* before and after calving. *Annales Recherch Veterinaire*, 1: 215-224.

213. Phillips, M., B. L. Deyoe, and Canning, P. C. (1989). Protection of mice against *Brucella abortus* infection by inoculation with monoclonal antibodies recognizing *Brucella* O-antigen. *American Journal of Veterinary Research,* 50:2158–2161.

214. Pizarro-Cerda, J., Meresse, S., Parton, R. G., van der Goot, G., Sola-Landa, A., Lopez- Goni, I., E. Moreno, E. and Gorvel, J. P. (1998). *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infection and Immunity,* 66:5711-5724.

215. Plommet, P. (1986). Development of brucellosis control programmes: Principles and strategies for control. Workshop on Brucellosis Control in Countries of the Mediterranean Area and the Arab Penninsula, Amman, Jordan.

216. Plommet, M. (1987). Brucellosis and immunity: humoral and cellular components in mice. *Annales Institut Pasteur Microbiology*, 138: 105-110.

217. Plommet, M. (1991a). Minimal requirements for growth of *Brucella suis* and other species. *Zentrablatt fur Bakteriologie*, 275: 436-450.

218. Plommet, M. (1991b). New animal vaccines. In: Brucella and brucellosis in man and animals. Proceedings of a symposium held in Izmir, Turkey, 24-26

September. Pp77-85.

219. Plommet, M., Festerbank, R., Vassal, L., Auclair, J. and Mocquot, G. (1988). Survival of *Brucella abortus* in ripened soft cheese made from naturally infected cow's milk. *Le Lait*, 8: 115-120.

220. Poester, F. P., Goncalves, V. S. P. and Lage, A. P. (2002). Brucellosis in Brazil. *Veterinary Microbiology*, 90: 55-62.

221. Porte, F., Liautard, J. P. and Kohler, S. (1999). Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. *Infection and Immunity*, 67:4041–4047.

222. Poste, G., Kirsh, R. and Koestler, T. (1984). The challenge of liposome targeting *in vivo*. In: Liposome Technology by G. Gregoriadis (Ed.). Vol. 3, Boca Raton, Florida. Pp2-28.

223. Radostits, O. M., Blood, D, D. C. & Gay, C. (1997). Diseases caused by *Brucella* species. In: Veterinary Medicine. 8th edition, W.B. Saunders Company Ltd, London, Pp79.

224. Radwan, A. I., Bekairi, S. I and Mukayel, A. A. (1992). Treatment of *Brucella melitensis* infection in sheep and goats with oxytetracycline combined with streptomycin. *Revue Science Technical*, 11: 845-857.

225. Radwan, A. I., Bekairi, S. I., al-Bokmy, A. M., Prasad, P. V., Mohammed, O. M. and Hussain, S. T. (1993). Successful therapeutic regimens for treating *Brucella melitensis* and *Brucella abortus* infections in cows. *Revue Science Technical*, 12: 909-922.

226. Ragan, V. E. (2002). The Animal and Plant Health Inspection Service (APHIS) brucellosis eradication program in the United States. *Veterinary Microbiology*, 90: 11-18.

227. Ray, W. C., Brown, R. R., Stringfellow, D. A., Schnurrenberger, P. R. Scalan, C. M. and Swan, A. I. (1988). Bovine brucellosis: An investigation into progeny of culture positive cows. *Journal of American Veterinary Medical Association,* 192:182-186

228. Renoux, G. and Mahaffey, L. W. (1955). Brucellosis in sheep and goats II. Establishment of the LD_{50} of a strain *B. melitensis* for sheep and goats. Archives of. Institut. Pasteur, Tunis, 32: 65-79.

229. Rikin, E. U. (1988a). Benefit-cost analysis for a brucellosis control program in Nigeria. *Acta Veterinaria Scandinavia* (supplement), 84: 371-373.

230. Rikin, E.U. (1988b). Brucellosis of cattle in Nigeria: proposal for a control programme under intensive and extensive husbandry systems. *Acta Veterinaria Scandinavia* (supplement), 84: 95-97.

231. Romero, C., Gamazo, C., Pardo, M. and Lopez-Goni, I. (1995). Specific detection of *Brucella* DNA by PCR. *Journal of Clinical Microbiology*, 33: 615-617.

232. Roop, R. M., Prestom-Moore, D., Bagchi, T. and Schurig, G. G. (1987). Rapid identification of smooth *Brucella* species with a monoclonal antibody. *Journal of Clinical Microbiology,* 25:2090-2093.

233. Ross, H. M., Foster, G., Reid, R. J., Jahans, K. L. and Macmillan, A. P. (1994) *Brucella* species infection in sea mammals. *Veterinary Record*, 138: 647-648.

234. Ross, H. M., Jahans, J. L., Macmillan, A. P., Reid, R. J., Thompson, P. M. and Foster, G. (1996). *Brucella* species infection in North Sea seal and cetacean populations. *Veterinary Record,* 138: 647-648.

235. Rossi, C. and Cantini, G. (1969). Mercaptoethanol in the diagnosis of bovine brucellosis. *Veterinary Bulletin,* 39: Abtrar.4874.

236. Roth, F., Zinsstag, J., Orkhon, D., Chimed-Ochir, G., Hutton, G., Cosivi, O., Carrin, G. and Otte, J. (2003). Human health benefits from livestock vaccination for brucellosis: case study. *Bulletin of World Health Organization,* 81: 867-876.

237. Roux, J. and Serre, A. (1971). Prophylaxie individuelle et vaccination contre les brucellosis humaines par une fraction antigenique de Brucella melitensis (complexe proteins-glycoproteines- glycosaminopeptides). *Revue. Epidemiologie. Medicine des. Society. Sante Public.*, 19: 503-516.

238. Ryan, W. J. (1967). A selective medium for the isolation of *Brucella abortus* from milk. *Monthly Bulletin of Ministry of Health,* 26: 33-39.

239. Sanchez, D. O., Zandomeni, R. O., Cravero, S., Verdun, R. E., Pierrou, E., Faccio, P., Diaz, G., Lanzavecchia, S., Aguero, F., Frasch, A. C. C., Andersson, S. G. E., Rossetti, O. L., Grau, O. and Ugalde, R. A. (2001). Gene discovery through sequencing of *Brucella abortus*. *Infection and Immunity,* 69: 865-868.

240. Santos, J. M., Verstreate, D. R., Perera, V. Y. and Winter, A. J. (1984). Outer membrane proteins from rough strains of four Brucella species. *Infection and Immunity*, 46: 188-194.

241. Schnurrenberger, P., Sharman, R. and Wise, G. (1987). Attacking Animal diseases: Concepts and strategies for control and eradication. Iowa State University Press, Ames, Iowa.

242. Schurig, G. G., Hammerberg, C. and Finkler, B. R. (1984). Monoclonal antibodies to *Brucella* surface antigens associated with smooth lipopolysaccharide complex. *American Journal of Veterinary Research,* 45: 967-971.

243. Schurig, G. G., Roop, Jr., R. M., Bagchi, T., Boyle, S., Buhrman, D. and Sriranganathan, N. (1991). Biological properties of RB51; a stable rough strain of

Brucella abortus. *Veterinary Microbiology,*. 28:171–188.

244. Schurig, G. G., Sriranganathan, N. and Corbel, M. J. (2002). Brucellosis vaccines: past, present and future. *Veterinary Microbiology*, 90: 479-496.

245. Seifert, H. S. H. (1996). Diseases caused by aerobic rods. I. Brucellosis. In: Tropical Animal Health. Kluwer Academic Publishers, Dordrecht. Pp356-367.

246. Shibau, S., Isayama, Y. and Shimizu, T. (1962). A possibility of variation in *Brucella abortus* from type II to type I. *National Institute of Animal Health Quarterly,* (Tokyo), 2: 10-14.

247. Smith, R. (1990). T-lymphocyte-mediated mechanisms of acquired protective immunity against brucellosis in cattle. In: Advances in Brucellosis Research by J. G. Adams (Ed.). Texas A & M University Press, Texas. Pp169-190.

248. Stevens, M. G., Olsen, S. C., Palmer, M. V. and Cheville, N. F. (1997) *Brucella abortus* strain RB51: Anew brucellosis vaccine for cattle. *The Compendium on Continuing Education*, 19: 766-774.

249. Stevens, M. G., Olsen, S. C., Pugh, Jr., G. W. and Brees, D. (1995). Comparison of immune responses and resistance to brucellosis in mice vaccinated with *Brucella abortus* 19 or RB51. *Infection and Immunity,* 63:264–270.

250. Stevens, M. G., S. C. Olsen, G. W. Pugh, Jr., and M. V. Palmer. (1994). Immune and pathologic responses in mice infected with *Brucella abortus* 19, RB51, or 2308. *Infection and Immunity,* 62:3206–3212.

251. Stoener, H. H. and Lackman, D. B. (1957). A new species of *Brucella* isolated from the desert wood rat, *Neotoma lepida*. *American Journal Veterinary Research,* 18: 947-951.

252. Suarez, F., Soberon, A., Diaz-Aparicio, E. and Adams, G. (1998). Evaluacion de la vacuna RB51 y la vacuna experimental *rfb*K para su uso en caprinos. III. Foro Nacional de brucellosis, pp191-204.

253. Tanaka, S., Suto, T., Isayama, Y., Azuma, R. and Hatakeyama, H. (1977). Chemo-taxonomical studies on fatty acids of "*Brucella*" species. Ann. Sclavo, 19: 67-82.

254. Taylor, A. W. and McDiarmid, A. (1949). The stability of the avirulent characters of *Brucella abortus* strain 19 and strain 45/20 in lactating pregnant cows. *Veterinary Record,* 61: 317-318.

255. Tcherneva, E., Rijpens, N., Jersek, B. and Herman, L. M. (2000). Differentiation of *Brucella* species by randomly amplified polymorphic DNA analysis. *Journal of Applied Microbiology,* 88:69-80.

256. Tcherneva, E., Ripjens, N., Naydensky, C. and Herman, L. (1996). Repetitive

element sequence based polymerase chain reaction for typing of *Brucella* strains. *Veterinary Microbiology,* 51: 169-178.

257. Thayer, J. D. and Martin, J. E. (1964). A selective medium for the cultivation of *Nisseria gonorrhea and N. meningitides*. *Public Health Report*, 79: 49.

258. Thoen, C. O. and Enright, F. (1986). *Brucella*. In: Pathogenesis of bacterial infections in animals by C. L. Gyles and C. O. Thoen (Eds.). Iowa State University Press, Ames. Pp160-180.

259. Thrusfield, M. (1995). Veterinary epidemiology. Blackwell Scientific, UK.

260. Tobias, L., Schurig, G. G. and Cordes, D. O. (1992). Comparative behaviour of *Brucella abortus* strain 19and RB51in the pregnant mouse. *Research in Vetrinary Science,* 53: 179-183.

261. Traum, J. (1914). Annual Report of the Bur. Animal Industry, US Department of Agriculture, Pp30.

262. Tryland, M., Kleivane, L., Alfredsson, A., Kjeld, M., Arnason, A., Stuen, S. and Godfroid, J. (1999). Evidence of *Brucella* infection in marine mammals in the North Atlantic Ocean. *Veterinary Record,* 144: 588-592.

263. Tsolis, R. M. (2002). Comparative genome analysis of alpha-proteobacteria: Relationships between plant and animal pathogens and host specificity. *Proceedings of National Academy of Science* (USA), 99: 12503-12505.

264. Velasco, J., Bengoechea, J. A., Brandenburg, K., Lidner, B., Seydel, U., Gonzalez, D., Zahringer, U., Moreno, E. and Moriyon, I. (2000). *Ochrobactrum* species differ in outer membrane permeability and cationic peptide resistance. *Infection and Immunity,* 68: 3210-3218.

265. Velasco, J., Romero, C., Lopez-Goni, I., Leiva, J., Diaz, R. and Moriyon, I. (1998). Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov. a new species with a closer relationship to *Brucella* spp. *International Journal Systematic Bacteriogy,* 48: 759-768.

266. Verger, J. M., Grimont, F., Grimont, F. A. and Grayon, M. (1985). *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *InternalJournal of Systematic Bacteriology,* 35: 292-295.

267. Verger, J. M., Grimont, F., Grimont, F. A. and Grayon, M. (1987).Taxonomy of the genus *Brucella*. *Annales Institut Pasteur Microbiologie,* 138: 235-238.

268. Verger, J. M., Grayon, M., Zundle, E., Lechopier, P. and Olivier-Bernardin, V. (1995). Comparison of the efficacy of *Brucella suis* strain 2 and *Brucella melitensis* Rev. 1 live vaccines against a *Brucella melitensis* experimental infection in pregnant ewes. *Vaccine*, 13: 191-196.

269. Verger, J. M., Grayon, M., Cloeckaert, A., Lefevre, A. Ageron, M. and Grimont, F. (2000). Classification of *Brucella* strains isolated from marine mammals using DNA-DNA hybridization and ribotyping. *Research in Microbiology,* 151: 797-799.

270. Warner, D. (2001). Brucellosis in animals and humans. In: Zoonoses: animal diseases that affect man by D. E. Goodman (Ed.). Christian Veterinary Mission, Seattle. Pp23-32.

271. Wergifosse, P., Lintermans, P., Limet, J. N. and Cloeckaert, A. (1995). Cloning and nucleotide sequence of the gene coding for the major 25-kilodalton outer membrane protein of *Brucella abortus*. *Journal of Bacteriology,* 177: 1911-1914.

272. WHO (1986). Joint FAO/WHO Expert Committee on Brucellosis (Sixth Report). WHO Technical Report Series No. 740, Geneva.

273. WHO/MZCP (1998). Human and animal brucellosis. Report of a WHO/MZCP workshop, Damascus, Syria, 4-5 May.

274. Winter, A. J. (1990). Mechanism of protective immunity against *Brucella abortus* in the mouse model system of infections. In: Advances in Brucellosis Research by L. G. Adams (Ed.). Texas A & M University Press, Texas. Pp137-163.

275. Winter, A. J., Schurig, G. G., Boyle, S. M., Sriranganathan, N., Bevins, J. A. , Enright, F. M. , Elzer, P. H. and J. D. Kopec. 1996. Protection of BALB/c mice against homologous and heterologous species of *Brucella* by rough strain vaccines derived from *Brucella melitensis* and *Brucella suis* biovar 4. *American Journal of Veterinary Research,* 57: 677–683.

276. Xie, X. (1986). Orally administrable brucellosis vaccine: *Brucella suis* strain 2 vaccine. *Vaccine*, 4: 87-91.

277. Yabu, K., Youngner, K. S., Feingold, D. S., Keleti, G. and Gorelik, E. (1991). Augumentation of natural killer cells activity in mice by Bru-Pel. *Journal of Immunotherapy*, 10: 307-312.

278. Young, E. J. (1989). Treatment of brucellosis in humans. In: Brucellosis: Clinical and laboratory aspects by E. J. Young and M. J. Corbel (Eds.). CRC Press, Boca Raton, USA, pp. 127-141.

279. Young, M. (1995). An overview of human brucellosis. *Clinical Infectious Diseases*, 21: 283-290.

280. Young, J. and Corbel, M. J. (1989). Brucellosis: Clinical and laboratory aspects. E. J. Young and M. J. Corbel (Eds.). CRC Press, Boca Raton. pp. 41-51.

281. Zammit, T. (1905). A preliminary note on the examination of blood of goats suffering from Mediterranean fever. Report of Commission of Investigation of Mediterranean fever, Part III, Pp83.

282. Zhan, Y., and C. Cheers. 1995. Endogenous interleukin-12 is involved in resistance to *Brucella abortus* infection. *Infection and Immunity*, 63:1387–1390.

283. Zhan, Y., and C. Cheers. 1998. Control of IL-12 and IFN-γ production in response to live or dead bacteria by TNF and other factors. *Journal of Immunology*, 161:1447–1453.

284. Zobell, C. E. and Meyer, K. (1932). Metabolism studies on *Brucella* group. VIII: Nutrient requirements in synthetic mediums. *Journal of Infectious Diseases*, 51: 344-360.

285. Zowghi, E. and Ebadi, A. E. (1988). Abortion due to *Brucella abortus* in sheep in Iran. Revue Scientifique Technical Office International des Epizooties, 79-382.

APPENDIX

Nutrient Agar (NA)

The required amount of Nutrient agar powder was suspended in distilled or deionized water and allowed to dissolve completely. The solution was autoclaved at 1kg/cm^2 equivalent to 121°C for 15 minutes.

Serum Dextrose Agar (SDA)

To the nutrient agar, while it was still molten at 60^{o}C was added: 1% sterile glucose and 5% bovine or equine Serum (*Brucella* agglutinins negative, inactivated at 56°C for 30 minutes).

Serum Dextrose Antibiotic Agar (SDAA):

This was a modification of Farrell's medium (Farrell and Robinson, 1972; Farrell, 1974). To melted SDA at 56 - 60°C, the following antibiotics at indicated quantities per ml of media were added:

Bacitracin	-	25 International Units (I.U.)
Polymyxin B Sulphate	-	5 i.u.
Cycloheximide	-	100 Φg
Nalidixic acid	-	5 Φg
Nystatin	-	100 i.u
Amphotericin B	-	4 Φg
Vancomycin	-	20 Φg

A commercial preparation of ready-mixed supplement based on Farrell's formulation obtained from Oxoid (Oxoid Limited, Bakingstoke, U.K.) was also used. This was re-constituted on 50% methanol and added to the basal medium at the required concentration.

Tryptone Soya Agar (TSA)

It was a modification of the Brodie and Sinton's medium (Corbel et al., 1987). *Brucella* Tryptone Soya broth powder (Difo Laboratories) suspended in the distilled water and was allowed to stand for 10 minutes to dissolve the powder. To this, 1.5% Agar powder was added. The mixture was autoclaved at 103.5 kPa (121^{o}C) for 15 minutes. After cooling the broth to 60^{o} C, 5% sterile inactivated bovine or equine serum was added.

Tryptone Soya Antibiotic Agar (TSAA):

To TSA at 56 – 60° C antibiotics were added as described for Farrell's modified SDA medium.

Serum Dextrose Agar VCN (VCN):

This medium was as modification of the variation of the Thayer Martin medium (Brown *et al*., 1971). It was prepared by addition of antibiotics to SDA basal medium at the following concentrations per ml. of medium:

Vancomycin	-	3 Φg
Colistin	-	7.5 Φg
Nystatin	-	12.5 I.U

Culture Media

While the serum dextrose agar, serum dextrose agar with antibiotics, tryptose soya agar with antibiotics and modified Thayer-Martin medium were still molten after the addition of necessary ingredients, 20 - 25ml were poured aseptically into sterile plastic Petri or glass dishes on a level bench in a sterile cabinet. When the agar had set, the plates were transferred to a 37°C incubator with the plates turned upside down, to dry and incubated for 24 hours as a check for sterility.

Lead Acetate Paper Strips

This was prepared according to Corbel *et al*. (1987). Sheets of filter paper were soaked in 10% w/v solution of lead acetate and hung up to dry at 37^{o} C in air. When dry, the sheets were cut into strips of 12 cm x 1 cm. The strips were sterilized in specimen bottles at 103.5 kPa (121^{o}C) for 15 minutes.

Dye Solutions

A 0.1% stock solution of each dye was made in distilled water and sterilized by holding in boiling water for 1 hour. Stock solutions were kept in screw-capped bottles until required. From the stock solution, graded dilutions of the dyes at 1:25,000 and 1:50,000 were made.

Dye Plates

From the stock solution, graded dilutions of the dyes at 1:25,000 and 1:50,000 were made and were added to the SDA at 60^{o}C, to give a final concentration of 4-Φg and 20Φg per ml of media respectively. After thorough mixing, they were poured into Petri dishes and allowed to set. The plates were placed in incubator at 37^{o}C overnight to dry.

Nutrient Agar Slopes

Required nutrient Agar Solution was autoclaved at 103.5 kPa (121°C) for 15 minutes while still molten at 60°C, 5% horse serum was added and 3 mls each was dispensed into sterile bijou bottles. The medium was allowed to solidify with the tubes in a slop position.

U**rea Agar Slopes**

This was prepared by the use of Christensen's medium as described by Alton *et al*. (1988). The required amount of the medium was dissolved in distilled water. About 5

ml of the solution was distributed into test tubes and sterilized at 120°C for 20 minutes. After the tubes had cooled to 50°C, urea solution was added to given a final concentration of 2% of Urea. The medium was allowed to solidify with the test tubes in a sloped position.

SDA Containing Penicillin

To SDA while still molten at 60°C, 1ml of Benzyl-penicillin solution containing 500 i.u/ml of the antibiotic solution was added to give a final concentration of 5 i.u/ml of media. After thorough mixing, they were poured into Petri dishes and allowed to set. The plates were placed in incubator at 37°C overnight to dry..