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THE PREVALENCE OF BRUCELLOSIS IN CATTLE, SHEEP AND GOATS IN MAPUTO PROVINCE, MOÇAMBIQUE

By

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS .... **II**

TABLE OF CONTENTS .... **III**

LIST OF TABLES .... **V**

LIST OF FIGURES .... **VI**

LIST OF ABREVIATIONS .... **VII**

ABSTRACT .... **VIII**

CHAPTER 1 INTRODUCTION **1**

1.1 BACKGROUND 1
1.2 THE AIMS OF THE RESEARCH PROJECT 3

CHAPTER 2 LITERATURE REVIEW **4**

2.1 AETIOLOGY 4
2.2 EPIDEMIOLOGY 5
2.3 PATHOGENESIS 11
2.4 CLINICAL SIGNS 13
2.5 PATHOLOGY 14
2.6 DIAGNOSIS 16
2.7 CONTROL AND PREVENTION OF BRUCELLOSIS 24

CHAPTER 3 MATERIALS AND METHODS **25**

3.1 STUDY AREA 25
3.2 EXPERIMENTAL DESIGN 25
3.3 SAMPLE COLLECTION AND HANDLING 27
3.4 TOTAL NUMBER OF SAMPLES COLLECTED 29
3.5 SEROLOGY 29
3.6 BACTERIOLOGY 32
3.7 MOLECULAR DETECTION 34
3.8 STATISTICAL ANALYSIS 40

CHAPTER 4 RESULTS 41

4.1 SEROLOGICAL RESULTS.. 41
4.2 BACTERIOLOGICAL TEST RESULTS.. 46
4.3 NUCLEIC ACID DETECTION 47

CHAPTER 5 DISCUSSION.. 49

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS.............................. 54

REFERENCES.. 55

LIST OF TABLES

Page

Table 1 Number of cattle sampled in the study area 26

Table 2 Number of small ruminants sampled in the study area 27

Table 3 Results of DNA concentrations after extraction 37

Table 4 Primer sequences and melting temperatures 38

Table 5 Primer sets and amplicon sizes 38

Table 6 Apparent prevalence of brucellosis in the study area 42

Table 7 Apparent prevalence of bovine brucellosis by sex 43

Table 8 Apparent prevalence of bovine brucellosis in females by age 43

Table 9 Apparent prevalence in the cattle livestock production sector 44

Table 10 Agreement between three serological tests (RBT, iELISA and CFT) for bovine brucellosis as measured by percent agreement (above the diagonal) and Kappa coefficient (K) (below the diagonal)... 44

Table 11 Prevalence of brucellosis on dairy farms 45

Table 12 Agreement between four serological tests (MRT, RBT, iELISA and CFT) for bovine brucellosis as measured by percent agreement (above the diagonal) and Kappa coefficient (K) (below the diagonal)... 45

LIST OF FIGURES

Page

Figure 1 Hygromas of the carpal joints in a cow from Mahubo in the Boane District with brucellosis 15

Figure 2 PCR amplification products generated by *Brucella* genus-specific primers 47

Figure 3 PCR amplification products generated by *Brucella* species-specific primers 48

LIST OF ABREVIATIONS

AMOS – Abortus; Melitensis; Ovis; Suis

cELISA – Competitive enzyme-linked immunosorbent assay

CFT – Complement fixation test

CI – Confidence interval

DINAP – National Directorate of Livestock

ELISA – Enzyme-linked immunosorbent assay

HA – Haemagglutination

HRPO – Horseradish peroxidase

iELISA – Indirect enzyme-linked immunosorbent assay

INIVE – National Veterinary Research Institute

IU – International Units

LPS – Lipopolysaccharide

MRT – Milk ring test

OIE – Office International des Epizooties

OPS – O-polysaccharide

OVI – Onderstepoort Veterinary Institute

PCR – Polymerase chain reaction

RBT – Rose Bengal test

SAT – Serum agglutination test

SLPS – Smooth lipopolysaccharide

TIA – Trabalho de Inquérito Agrícola

WHO – World Health Organization

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The Prevalence of Brucellosis in Cattle, Sheep and Goats in Maputo Province, Moçambique

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ABSTRACT

Brucellosis is a constraint to livestock production in Moçambique. Reproductive failure due to brucellosis has been reported in the country and abortions occur in cattle, sheep and goats on many of the farms in Maputo Province of Moçambique. Brucellosis is an infectious disease affecting mainly sexually mature animals and is caused by organisms of the genus *Brucella*. *Brucella abortus* is the species that mainly affects cattle throughout the world, while *Brucella melitensis* affects mainly goats and sheep. Control of bovine brucellosis using *B. abortus* S19 vaccine was

undertaken before the civil war in Moçambique which started in 1978 and ended in 1992. During this period the vaccination status of animals was not known. The diagnosis of brucellosis is mainly based on serological surveys and conflicting results obtained previously emphasized the need for additional studies.

The aims of this research were to determine the prevalence of brucellosis in cattle, goats and sheep due to *B. abortus* and *B. melitensis* in five districts of Maputo Province based on serology, and to assess the relationship between abortions and reproductive failure occurring in the study area by isolation of *Brucella* organisms or nucleic acid detection.

A total of 971 adult beef and dairy cattle, 752 goats and 260 sheep from the study area were tested for *Brucella* antibodies using the Rose Bengal test (RBT), indirect ELISA and complement fixation test (CFT). Milk from 85 dairy cows and tissue samples from 32 cows and bulls from Gaza and Inhambane provinces slaughtered in Maputo municipal abattoir and Machava abattoir, and an aborted foetus were also examined.

All serological test results for small stock were negative. An overall apparent prevalence of 14.0% (n=971) was found in beef and dairy cattle in the study area based on a 95% confidence interval. The highest prevalence was observed in Manhiça District (27.4% (n=180)) with a significant difference compared to other districts ($p<0.05$). The lowest prevalence was observed in Magude District (7.1% (n=241)). The prevalence in the remaining districts were 14.5% (n=138) in Matutuine, 8.7% (n=173) in Moamba and 14.6% (n=239) in Boane. Apparent prevalences of 15.5% (n=161) in small, 14.0% (n=578) in medium and 12.9% (n=232) in large livestock production sectors where found in the study area with no significant differences ($p>0.05$) between them.

B. abortus was isolated from the spleen of an aborted foetus. A PCR amplification product of 600bp was generated from this isolate 584/05 and from the organs of five slaughtered cows using primers ISP1 and ISP2 for *Brucella* genus-specific sequences. The isolate 584/05 was confirmed as a *B. abortus* field strain when

fragments of 498bp and 178bp were generated using a PCR assay for differentiation of *B. abortus* field strains and vaccine strains 19 and RB51. A correlation was made between the 584/05 isolate and serum from the cow that aborted. The results confirmed that the isolate was not a vaccine strain. The results from this study will supplement existing epidemiological data and aid brucellosis control programmes in Moçambique.

CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

Livestock plays a crucial role in the livelihood of the majority of Africans including citizens from Moçambique. It is the main source of meat, milk, and draft power and cash income. It also plays an important role in many socio-cultural traditions. In Moçambique, as in the majority of sub-Saharan African countries, livestock are mainly kept by small farmers in arid and semi-arid regions. Livestock production sectors are defined in three categories as small, ranging from 0 to 30 animals, medium (31 to 100 animals) and large (more than 100 animals) based on total number of cattle, goats and sheep (Trabalho de Inquérito Agrícola -TIA).

Many well-known bacterial, viral and parasitic diseases (both directly and indirectly transmitted), occur commonly and are poorly controlled in both livestock and human populations (McDermott and Arimi 2002; Smits and Cutler 2004). These diseases have a great socio-economic impact such as low production due to abortion, reduced milk production, and loss of draft power, which have a negative effect on cash income. However, despite their economic and social importance, livestock management as well as programs to control infectious diseases in Moçambique, including brucellosis, have declined due to the civil war (between 1978 and 1992) and to decreasing government support particularly for operational costs of disease control.

Reproductive failures due to a variety of infectious agents occur worldwide, including Moçambique, where the most important cause has been recognised as brucellosis by the regulatory veterinary authorities and the National Veterinary Research Institute (INIVE reports 1997-2003). Brucellosis is an infectious disease affecting mainly sexually mature animals and is caused by organisms of

the genus *Brucella*. *Brucella abortus* is the species that mainly affects cattle throughout the world, while *Brucella melitensis* affects mainly goats and sheep. Abortion in cattle, goats and sheep due to brucellosis has been reported in Moçambique based on isolation of the organism and positive serological test results. Various authors have reported the occurrence of bovine brucellosis throughout the country. Abreu (1967) was the first to isolate *Brucella* in Moçambique. Isolation of organisms has also been reported by Mata (1989) and INIVE annual reports, while Neves (1968), Gradil (1968), Mario and Valadão (1972), Pereira and Schwalbach (1997), Manhiça (1998) and Maxlhuza (2002) reported positive serological reactors based on surveys carried out in the country with the emphasis on the southern part, where the majority of cattle are kept.

Efforts to control the disease in the country through organized vaccination programs were undertaken by the veterinary authorities before the civil war that started in 1978. After the end of the civil war in 1992, the vaccination status of animals was not clear as some commercial farmers used *B. abortus* S19 vaccine, while in the traditional sector (Family Sector), although vaccination programmes are undertaken by the veterinary services, there is little information is available on the status of *B. abortus* vaccination. This lack of information has created problems with the interpretation of serological test results when attempting to diagnose brucellosis.

The conflicting results previously obtained from different studies done in animals as well as reports of abortions in the country emphasized the need for additional studies.

1.2 THE AIMS OF THE RESEARCH PROJECT

The aims of the project were to:

- Provide estimates of the seroprevalence of brucellosis in cattle, goats and sheep in five districts of Maputo province.
- Culture and determine the biotypes of *B. abortus* and *B. melitensis* and its possible relation to abortion and reproductive failures.
- Identify and type *B. abortus* and *B. melitensis* in cattle and small ruminants by molecular methods.

CHAPTER 2
LITERATURE REVIEW

2.1 AETIOLOGY

Brucellae are Gram-negative coccobacilli or short rods with straight or slightly convex sides and rounded ends. They do not ferment carbohydrates in conventional media (Quinn *et al*. 1999). The genus *Brucella* contains six species: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae*. Seven biovars are recognized for *B. abortus*, three for *B. melitensis*, and five for *B. suis*. However, the degree of genetic relatedness, as shown by DNA hybridization studies, is consistent with the existence of a single species within the genus *Brucella* (Bricker 2002; OIE Manual 2004). *B. abortus, B. melitensis, B. suis* and *B. neotomae* generally occur in smooth form, while *B. ovis* and *B. canis* are invariable rough species (Nielsen 2002). A broad spectrum of smooth *Brucella* isolates has recently been described from a wide variety of cetacean and pinniped marine mammals. As their overall characteristics are not consistent with those of any of the six recognized *Brucella spp.* it has been suggested that they comprise more than one species. Two new species, *Brucella pinnipedialis for* pinniped isolates and *Brucella ceti* for cetacean isolates were identified (Ross *et al*. 1994; Godfroid *et al*. 2005).

B. abortus B. melitensis and *B. suis* are morphologically and tinctorially indistinguishable. They are small, Gram-negative, non-sporulating, non-encapsulating cocci, coccobacilli or short rods, 0,6 to 1,5µm in length and 0,5 to 0,7µm in width. The organism is not acid-fast but does resist decolourization by weak acids and thus stains red with Stamp's modification of the Ziehl-Neelsen stain (Berman 1981; Godfroid *et al.* 2004a; b). Most wild strains of *B. abortus* are fastidious and slow-growing, and require carbon dioxide (5 to 10 percent)

supplementation for primary isolation at an optimal growth temperature of 36 to 38 °C, while growth of *B. melitensis* is not dependent on an atmosphere of 5 to 10 per cent of CO_2, although there might be some exceptions (Alton *et al*. 1988).

2.2 EPIDEMIOLOGY

Distribution

Brucellosis is a widespread disease and of major economic importance in most of the countries in the world, particularly among cattle. In small ruminants the disease is more restricted to the Mediterranean region including southern Europe, West and central Asia, South America and Africa (Nielsen and Duncan 1990; Corbel 1997; Godfroid *et al*. 2005), with considerable variation between herds, and between areas and countries.

In Africa, the whole south and east as well as west and northwest Africa were considered to have a moderate degree of infection with the incidence ranging from 11 to 20% of cattle herds. A high incidence (above 30% of herds) was described for a ring of countries situated in the wet and dry savanna areas and tropical rain forest zone of West, Central and East Africa (Thimm and Wundt 1976; Corbel 1997). The occurrence of brucellosis in sub-Saharan countries (either prevalence or incidence) is not well documented and reports submitted to the World Organisation for Animal Health (Office International des Épizooties) are largely confined to serological surveys, and mainly conducted for cattle and less for goats and sheep. McDermott and Arimi (2002) referred to a great variation in prevalence in sub-Saharan Africa (ranging from 4.8 to 41%) in pastoral systems. The latter are considered the livestock-dominated systems, which is also the case in Moçambique. In comparison with bovine brucellosis, brucellosis in sheep and goats caused mainly by *B. melitensis* has with only a few exceptions a low/sporadic degree of incidence throughout the African continent (Thimm and Wundt 1976; McDermott and Arimi 2002).

According to Thim and Wundt (1976), and Godfroid *et al.* (2004a), the disease has a relatively high prevalence in southern Africa, especially in intensively farmed areas. *Brucella* is the most important bacterium causing abortion in the sub-continent and has an important economic impact on the beef and dairy cattle industry and on public health. It is suspected that the disease was introduced into southern Africa from cattle imported from Europe, but there is also a possibility that it was introduced into the subcontinent much earlier, during the migration of the African tribes and their cattle herds from other African countries (Bishop *et al.* 1994).

Bovine brucellosis was also reported in the countries surrounding Moçambique. Hall (1913) cited by Godfroid *et al.* (2004a) confirmed the presence of brucellosis in South Africa when he isolated *B. abortus* from the stomach of an aborted bovine foetus. The presence of brucellosis in Zimbabwe was confirmed in 1914 (Bishop *et al.* 1994). The first documented report of the presence of *B. melitensis* in the southern part of Africa was in 1953 when the organism was isolated from karakul sheep in Namibia. In 1965 the organism was recovered from three outbreaks of abortion in sheep in Mpumalanga and Limpopo Provinces of South Africa. The histories of positive goats revealed that a number of goats had been purchased in Swaziland in which country the presence of disease has been confirmed (Godfroid *et al.* 2004b).

Based on this information, and the shared borders with Mpumalanga and Limpopo provinces, it was deemed justifiable to include the possible detection of *B. melitensis* in goats and sheep in the study. Also taken into consideration was the emigration of Moçambicans and their animals to South Africa and Swaziland during the civil war between 1978 and 1992.

The status of brucellosis in Mozambique

In Moçambique brucellosis is considered to be a constraint to livestock production. Bovine brucellosis was reported in Moçambique for the first time in 1952 by Abreu (1967) following isolation of the organism with concomitant signs of abortion on different farms throughout the country. To date few isolations have been made in the southern part of the country where biotype 1 was essentially isolated compared to other biotypes using classical typing methods (INIVE reports, 1997-2003). Mata (1989) reported the isolation and typing of biotype 6. Neves (1968) found positive reactors with the serum agglutination test (SAT) in southern Mozambique, while Gradil (1968) in a survey carried out between 1955 to 1957 using SAT, found 20-30% positive reactors. Between 1954 and 1984 the rate of positive reactors decreased from 30% to 5%. Pereira and Schwalbach (1997) reported a seroprevalence of 2.25% in dairy farms and 13.3% in beef cattle, using the complement fixation test (CFT).

From 1984 onwards, only a few studies were carried out in the country, due to the civil war. Manhiça (1998) reported the occurrence of positive reactors to CFT in the 10 provinces of the country ranging from 1.9% to 33.3% based on serum samples tested at the Serology Laboratory of the National Veterinary Research Institute (INIVE). No conclusions relating to the epidemiological situation were made due to lack of information on animal age, sex, vaccination status, livestock production systems and sampling methods used. Surveys carried out by the Veterinary Services in the country revealed the occurrence of brucellosis in the family sector as well as in the private sector, although this data was not quantified nor representative (DINAP reports, 1999-2004).

The prevalence of *B. melitensis* has not been thoroughly studied in Moçambique. A survey on brucellosis in small ruminants in southern Moçambique was carried out and serum samples tested using a slow agglutination test (Mário and Valadão 1972). Titres of up to 1280 International Units (IU) were found in Maputo

Province whereas titres of 50 IU were found in other studied regions (Mário and Valadão 1972). Results of *Brucella* positive cases in small ruminants were also reported during the last 10 years in Maputo Province where 9.90% positive reactors were detected from a population of 404 animals with a rapid agglutination test using *B. melitensis* antigen from a commercial source (Sommex (Pty) Ltd). The rapid agglutination test is not only specific for *B. melitensis.* The same samples reacted negatively when subjected to the Rose Bengal test using *B. abortus* antigen (Manhiça 2001; INIVE report 1997- 2003).

The prevalence of brucellosis in humans in Moçambique is not well known although humans, especially farm workers, often consume raw and fermented milk. Some workers have presented symptoms similar to those associated with brucellosis such as backache, rheumatic pains, tiredness, and insidious onset of fever. These symptoms are in most of the cases considered and treated as malaria. A study carried out in humans in Maputo Province showed an antibody prevalence of 10% (Dottorini *et al*. 1985) based on the Rose Bengal test (RBT), serum agglutination test (SAT) and complement fixation test (CFT), while subsequent studies in humans found titres that were too low to be considered positive (Nalá *et al*. 1999). Maxlhuza (2002) found a seroprevalence of 1% in a group of shepherds in Moamba District of Maputo Province.

The host-micro-organism relationship

Brucella species are intracellular micro-organisms, and each species has a preferred natural host that serves as a reservoir of infection. In domestic animals, cattle are mainly affected by *B. abortus*, sheep and goats are mainly affected by *B. melitensis* (Bishop *et al*. 1994). *Brucella* organisms have a predilection for ungulate placentas, foetal fluids and testes of bulls and rams. *B. abortus* is excreted in bovine milk and can remain viable in milk, water and damp soil for up to 4 months (Nielsen and Duncan 1990).

Transmission

Cattle, goats and sheep usually become infected after ingesting contaminated feed, water, or milk, sucking or licking an infected placenta, newborn or foetus, or the genitalia of an infected female soon after it has aborted or after birth, at which time very large numbers of *Brucella* are present, particularly in the placenta and lochia (Godfroid *et al.* 2004a;b). Animals may also become infected by inhaling organisms or through the conjunctiva. Calves and lambs can also become infected in the uterus (Nielsen and Duncan 1990).

Humans may become infected after ingesting unpasteurized milk and dairy products containing *Brucella*, direct contact with infected animals, animal carcasses and aborted materials, the organism itself in laboratories, as well as by accidental inoculation with *Brucella* vaccines (Nielsen and Duncan 1990).

Risk factors for transmission

The epidemiological variables which are considered to affect the initiation, spread, maintenance and/or control of brucellosis can be categorized into those related to the animal population, to management, or to biology of disease (Nicoletti 1980; Radostits *et al.* 1994a). The factors influencing the transmission of *Brucella* spp in a geographical region can be classified into two categories: those associated with transmission of disease between herds (purchase of infected animals, proximity of infected herds to clean herds, sharing pastures, dip tanks, watering points, and strays of infected animals into clean herds), and those influencing the maintenance and spread of infection within herds (unvaccinated animals in infected herds, herd size, population density, method of housing and use of maternity pens) (Nicoletti 1980; Radostits *et al.* 1994a).

Host factors

Susceptibility of livestock to *Brucella* infection is influenced by the age (young animals are less susceptible to *Brucella* than older animals), sex and reproductive status of the individual animal (sexually mature, pregnant animals are more susceptible to infection with the organism than sexually immature animals) (Nicoletti 1980). Placental trophoblasts produce erythritol in increasing amounts during the later stages of pregnancy which coincides with the period when pregnant cattle are more susceptible to infection with *B. abortus*. The preferential utilization of erythritol rather than glucose is characteristic of pathogenic *Brucella* strains. Erythritol promotes the growth of some strains of *Brucella* and the metabolic pathway for degradation of erythritol has been described previously. However, as *Brucella* has also been found in the reproductive tract of animals with no detectable levels of erythritol, the role of this sugar in the virulence of the organisms has been put into question (Sangari *et al.* 2000). *B. abortus* strain 19 is a spontaneous attenuated mutant widely used to vaccinate cattle. S19 is the only *B. abortus* strain that is inhibited by erythritol (Sangari *et al.* 2000).

Management of risk factors

The spread of disease from one herd to another and from one area to another is most commonly due to the movement of an infected animal from an infected herd or area into a non-infected herd or area (Radostits *et al.* 1994a).

Pathogen risk factors and immune mechanisms

Brucella is a facultative intracellular organism which is capable of multiplication and survives within host phagocytes. The organisms are phagocytosed by polymorphonuclear leucocytes in which some survive and multiply. These are then transported to lymphoid tissue and foetal placenta. The inability of the

leukocytes to effectively kill virulent *Brucella* at the primary site of infection is a key factor in the dissemination to regional lymph nodes, mononuclear phagocytic system, and organs such as the uterus and udder. The ability to survive within macrophages and leukocytes enables the organism to be protected from humoral and cellular bactericidal mechanisms during the periods of haematogenous spread (Nielsen and Duncan 1990).

Naturally infected animals and those vaccinated as adults with strain 19 remain positive to the serum and other agglutination tests for long periods. The antibody response to *Brucella* consists of an early IgA and IgM isotype response, the timing of which depends on the route of exposure, the dose of bacteria and the health status of the animal. The IgM response is followed shortly by production of IgG1 antibody and later by IgG2 (Nielsen 2002). The total concentration of IgG2 increases with age. Most cross-reacting antibody, resulting from exposure to micro-organisms other than *Brucella* spp., consist of IgM, making serological tests which measure IgM not specific as false positive results occur, leading to low assay specificity. In the case of *Brucella* infection, the concentration of anti-*Brucella* total IgG2 increases with the level of antigen exposure, therefore the monitoring of IgG1 and IgG2 *Brucella* antibody levels is relevant for detection of *Brucella*-infected cattle (Saegerman et al. 2004).

2.3 PATHOGENESIS

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

nodes may be followed by bacteraemia which may persist for several months, resolve itself, or be recurrent for at least two years in 5 to 10 per cent of animals. Recurrence occurs particularly during pregnancy (Radostits *et al.* 1994a; Godfroid *et al.* 2004a). During the bacteraemic phase, organisms are carried intracellularly in neutrophils and macrophages, or free in the plasma and localize in various organs, especially the pregnant uterus, udder and supramammary lymph nodes and the spleen, and in males in the testes, and male accessory sex glands (Godfroid *et al.* 2004a). Occasionally bacterial localization occurs in synovial structures, causing a purulent tendovaginitis, arthritis or bursitis.

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

2.4 CLINICAL SIGNS

For brucellosis, the incubation period usually implies the period from exposure to infection to the time at which clinical or serological evidence indicates that infection has occurred. The length of the incubation period of bovine brucellosis varies considerably and to some extent is also affected by the size of the infective dose, the age, and sex, stage of gestation and immunity of the infected animal (Bishop *et al.* 1994). In cows that eventually abort, the usual length of the incubation period varies according to the time at which infection occurred. The first sign of disease in a susceptible herd of cattle is abortion at late stages of gestation (Godfroid *et al.* 2004a). In bulls an acute to chronic, uni- or bilateral orchitis and epididymitis, with frequent sterility may occur (Nielsen and Duncan 1990). The abortion rate in infected herds also depends on many factors and varies according to the susceptibility of the pregnant animals, management practices, the period for which the herd has been infected and various environmental factors (Nicoletti 1980). About 20% of animals do not abort, while 80% abort only once as a result of *Brucella* infection. The placenta is not consistently retained after abortion, but if it occurs metritis is common. Early abortion may result in a considerable reduction in milk yield (Godfroid *et al.* 2004a).

The presence of the disease in a susceptible herd of goats or flock of sheep is usually an abortion storm, during which a high proportion of pregnant animals abort, generally late in gestation. In some cases, subsequent retention of the placenta and foetal membranes occur. The milk yield following abortion is poor, and quality of the milk may be reduced (Godfroid *et al.* 2004b). In males, localization of *B. melitensis* in the testis, epididymis and accessory sex organs is common and may result in acute orchitis and epididymitis and subsequent reduction in fertility (Herr 1994).

In humans acute brucellosis is an illness characterised by acute or insidious onset of fever, night sweats, undulant fever, fatigue, anorexia and weight loss, headache and joint pain. The disease may last for several days, months, or years if not adequately treated (Nielsen and Duncan 1990).

2.5 PATHOLOGY

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

There is considerable variation in the severity of the uterine lesions at abortion. As the disease progresses lesions advance from acute to chronic endometritis. Microscopically, the endometrium is infiltrated by lymphocytes and plasma cells and some neutrophils. Microgranulomas may be scattered in the endometrium. The chorion is not uniformly affected and large parts may appear quite normal. The lesions in and at the periphery of the cotyledons, as well as those in the intercotyledonary area vary in extent, appearing to be most severe adjacent to cotyledons. The affected cotyledons, or parts of them, are covered by sticky, odourless, brownish exudates, and are yellowish-grey as a result of necrosis. Parts of the intercotyledonary placenta are thickened, oedematous, yellow-grey and may contain exudates on the surface. The udder in infected ruminants does not show any gross lesions, although supramammary lymph nodes may be somewhat enlarged (Godfroid *et al.* 2004a; b). Some aborted foetuses have varying degrees of subcutaneous oedema and blood-tinged fluid in the thoracic and abdominal cavities, while the abomasal content is sometimes turbid, bright yellow and flaky (Nielsen and Duncan 1990). Microscopically most aborted foetuses reveal a multifocal bronchopneumonia, small foci of necrosis or

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

In bulls, acute to chronic, uni- or bilateral orchitis, epididymitis, and seminal vesiculitis occasionally occur. Orchitis is characterized by multifocal or diffuse necrosis of the testicular parenchyma, and a focal necrotizing epididymitis may occur. The scrotal circumference in these animals may be normal or severely increased (Bishop *et al.* 1994). Strain 19 vaccine may also cause orchitis.

Uni- or bilateral hygromas, especially of the carpal joints and other bursae, may be evident (Figure 1) in some animals in chronically infected herds, or may occasionally follow inoculation of heifers with strain 19 vaccine. A progressive, erosive, non-suppurative arthritis of the stifle joints has been reported in young cattle from brucellosis-free herds that were vaccinated with strain 19 vaccine (Godfroid *et al.* 2004a).

Figure 1: Hygromas of the carpal joints in a cow from Mahubo in the Boane district with brucellosis. It was not possible to obtain samples for bacterial culture from this animal.

2.6 DIAGNOSIS

All abortions in cattle should be treated as suspicious for brucellosis and should be investigated, as the clinical signs are not pathognomonic. Diagnostic tests for brucellosis are sub-divided into three groups – tests for the detection of *Brucella*, tests to detect immunoglobulins and those dependent on allergic reactions to *Brucella* (Alton *et al.* 1988; Saegerman *et al*. 1999; OIE Manual 2004). There is no single available test that completely covers all requirements for diagnosing brucellosis. The ideal diagnostic test should detect infection early during the long and variable incubation period, not be influenced by the presence of non-specific antibodies, detect carriers, and differentiate between responses to vaccination and those to field infection (Alton *et al.* 1988; Nielsen 2002).

Tests for the detection of Brucella

Because of variable incubation periods and the often subclinical nature of the disease in most animals, a definitive diagnosis should be based on isolation and identification of *Brucella* (Alton *et al.* 1988; OIE Manual 2004). The isolation and characterization of the disease-causing agent by bacteriological methods is still considered the "gold standard" for the diagnosis of brucellosis (Bricker 2002). However, the method also has drawbacks. Due to the biological properties of *Brucella,* the cultural methods are time-consuming and when the level of infection is low they may fail (Gallien *et al.* 1998; Fekete *et al.* 1990). Thus, highly sensitive diagnostic tests for detection of *Brucella* based on the polymerase chain reaction (PCR) have been developed and reported and were reviewed by Bricker (2002).

Specimens

The specimens of choice include foetal membranes, lung, stomach content, liver and spleen of aborted foetuses and full-term calves and from live cows uterine discharge, milk or colostrum. Other specimens which may be submitted include supramammary lymph nodes, retropharyngeal, mandibular, iliac, or prescapular and parotid lymph nodes, udder tissue, fluid aspirated from hygromas, male sex glands, testes and semen or seminal plasma and external inguinal lymph nodes of infected bulls (Alton *et al.* 1988; OIE Manual 2004).

Bacteriological detection

Direct microscopic examination

Using Stamp's modification of Ziehl-Neelsen stain, *Brucella* stains red against a blue background in tissue sections and smears. However, other organisms such as *Coxiella burnetii*, *Chlamydophila abortus* and *Nocardia* spp. are also acid-fast.

Isolation

Brucella grows on serum dextrose agar, but growth from primary isolation is seldom clearly visible before 48 hours of incubation at which stage colonies are usually 0.5 to 1.0 mm in diameter. The use of selective media such as Farrel's medium may substantially enhance the chances of isolation by inhibiting the growth of contaminants, although the growth of *Brucella* may be markedly slower. For this reason the cultures should be incubated for five days or longer before being discarded as negative. Most wild strains are fastidious and slow-growing, and require supplementary carbon dioxide (5 to 10 per cent) for primary isolation at an optimal growth temperature of 36 to 38℃ (Alton *et al.* 1988; Quinn *et al.* 1999).

Identification and typing

Colony morphology

After 3-5 days incubation on selective serum agar, pinpoint, smooth, glistening, bluish, translucent colonies appear. As they age the colonies become opaque and about 2-3 mm in diameter. Smooth colonies in a clear growth medium such as serum-dextrose agar, are convex, entire-edged, have a smooth shiny surface and are pale yellowish-brown when viewed under transmitted light. Smooth forms are often markedly pathogenic whereas the rough variants are usually less pathogenic (Quinn *et al.* 1999).

Microscopic appearance

MZN-stained smear from suspected colonies show small red-staining (MZN-positive) coccobacilli. *Brucella* is Gram-negative.

Biochemical tests

For routine identification, a combination of growth characteristics, colonial and cellular morphology, staining properties, agglutinating antiserum and biochemical reactions allow an accurate identification (Quinn *et al.* 1999). *Brucella* is non-motile, catalase-positive, and oxidase-positive, give a rapid urease activity (except some *B. melitensis* strains), reduce nitrate and are indole-negative.

Molecular detection

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a highly sensitive, very specific, rapid, and easily adapted to high volume demand diagnostic tool to detect slow growing

bacteria including *Brucella* (Bricker 2002). These methods can detect a few bacteria in a sample and are as sensitive as classical culture-based techniques. Furthermore, it is possible to detect dead bacteria reducing the necessity of careful sample conservation before analysis (Ouahrani-Bettache *et al* 1996). As long as careful attention is given to avoid contamination in the laboratory, the method is very reliable and usually highly reproducible at any properly equipped laboratory (Bricker 2002).

PCR is the amplification of a DNA sequence to high copy number. This amplification involves two oligonucleotide primers that hybridize to opposite strands of the target sequence and repeated heating cycles to denature the DNA and subsequent annealing of the primers to their complementary sequences. This is followed by extension of annealing primers with DNA polymerase. A heat-stable polymerase (Taq polymerase) derived from a bacterium adapted to hot springs, *Thermus aquaticus,* has been used to extend the primers. The successive cycling doubles the amount of DNA synthesized to amplification greater than 10^5, which permits the detection of a small number of bacteria including *Brucella*. A prerequisite for PCR is that the nucleotide sequence for the DNA of interest is known (Fekete *et al*. 1990).

Numerous PCR based assays for *Brucella* have been developed and published. The assays were designed to exploit a single unique genetic locus that was highly conserved in *Brucella* targeting *BCSP31* or the 16S rRNA genes (Herman and De Ridder 1992; Bricker 2002). The advantage of these assays is that they tend to have a simple design and are very robust. They are useful for screening or for identification when species or biovar designations are not critical (Bricker 2002). In order to differentiate *Brucella* species and/or biovars several PCR assays were developed. These assays are directed toward genetic loci that are variable among species and/or biovars. Such targets are uncommon in *Brucella* since the genus is remarkably homogeneous and has been proposed to be a single species, while some large deletions and rearrangements have been

reported within a species or biovar, most genetic differences consist of single nucleotide polymorphisms (Bricker 2002). Differential PCR-based assays are particularly useful for epidemiological trace back, or for species-specific eradication programs.

The different PCR assays that have been developed can be categorised as genus-specific and species-specific. Strategically, for the species-specific tests three approaches were reported (1) assays designed with highly specific primers and stringent assay conditions; (2) assays designed with semi-specific primers and mildly permissive assay conditions; and (3) assays based on amplification with random primers under very permissive conditions (Bricker 2002).

Tests for the detection of specific immunoglobulins

Serological tests

Serological tests are widely used for detection of infected animals. However, no serological test is appropriate in each and every epidemiological situation. Consideration should therefore be given to all factors that impact on the relevance of the test method and test results for a specific diagnostic interpretation or application (Dohoo *et al.* 1986). Most of the serological tests, particularly those using whole-cell suspensions as antigen, such as the serum agglutination test (SAT), Rose Bengal test (RBT), complement fixation test (CFT), most enzyme-linked immunosorbent assays (ELISA) and milk ring test (MRT) have been developed against the O-polysaccharide (OPS) moiety of the smooth lipopolysaccharide (SLPS) (Nielsen 2002).

ELISAs (iELISA and cELISA) are methods that involve the immobilisation of one of the active components on a solid phase. Since the conventional serological tests and iELISA cannot distinguish vaccinal antibody produced by strain 19 vaccine, a competitive enzyme immunoassay (cELISA) was developed. The

main rationale for this assay was that vaccines induced antibody of lower affinity due to the shorter exposure to antigen due to immune elimination compared to field infection in which antigen persisted resulting in increased antibody affinity. Thus a competing antibody could be selected to inhibit binding of vaccinal but not field strain induced antibody. Because of their inherent supply and uniformity advantages, monoclonal antibodies were selected as competitive antibodies that should be specific for a common epitope of the OPS molecule, allowing its use for *B. abortus*, *B. melitensis* and *B. suis* (Nielsen 2002)

Serological tests may show cross-reactions with other Gram-negative organisms such as *Salmonella* group N, *Eschericia coli* O:157, *E. coli* O:116, and *Pseudomonas maltophilia*. However, the most notable cross-reaction is between smooth lipopolysacharide (SLPS) found in *Brucella* and *Yersinia enterocolitica* O:9 making diagnosis difficult due to the sharing of antigenic determinants in the O-polysaccharide (OPS) molecule, which is the basis for most serological tests (Corbel *et al.* 1983; Muñoz *et al.* 2005; Nielsen *et al.* 2006).

Rose Bengal test (RBT)

RBT is a spot agglutination test used to screen herds. The test is highly sensitive for individual diagnosis, especially in cattle vaccinated with strain 19 (Alton *et al.* 1988; Nielsen 2002; OIE Manual 2004) and can be performed in the field. The antigen stained with Rose Bengal stain, is buffered at a pH of 3.65 (Alton *et al.* 1988). At this level of activity non-specific agglutinins are destroyed and IgG, the most abundant antibody in the serum of infected animals, agglutinates strongly (Alton *et al.* 1988; OIE Manual 2004). This test is prescribed by the OIE for international trade in cattle.

Complement fixation test (CFT)

The complement fixation test is very specific and sensitive and is regarded throughout the world as being the confirmatory test of choice for serological detection of infected animals. According to Radostits *et al.* (1994a; b), the CFT rarely exhibits non-specific reaction and is useful in differentiating the titres of calfhood vaccination from those due to infection. The reactions to the CFT recede sooner than those to the serum agglutination test after calfhood vaccination with the strain 19 vaccine. The test has been modified, standardized and adapted to microtitre systems (Alton *et al.* 1988). Results are expressed in International Units (IU) and a cut-off point of 20 IU has been defined, which is rigorously applied where strain 19 vaccine has not been used for several years (Alton *et al.* 1988). The CFT has been a valuable asset in control and eradication programs as a confirmatory test and it is recommended by the OIE Manual as a test prescribed for international trade.

Indirect enzyme-linked immunosorbent assay (iELISA)

Indirect ELISAs are tests in which antigen is bound to a solid phase, usually a polystyrene microtitre plate so that the antibody, if present in a sample, binds to the immobilised 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 (OIE Manual 2004). The iELISA is a highly sensitive test but it is sometimes not capable of differentiating between antibody resulting from S19 vaccination or other false-positive reactions induced by pathogenic *Brucella* strains. Therefore it should be considered more as a screening test than a confirmatory test in the testing of vaccinated herds affected by false-positive results (Nielsen 2002; OIE Manual 2004). This test is prescribed by the OIE for international trade in cattle.

Milk ring test (MRT)

The MRT is used to detect antibodies in milk. The development of a positive reaction is dependent on two reactions: (i) aggregation of fat globules in the milk and (ii) stained *Brucella* cells (antigen) which are added to the milk, and are agglutinated by the *Brucella* antibody-fat globule complexes which rise to form a coloured cream layer at the top. This is a screening test used on bulk milk to detect infected animals on a herd basis or to monitor clean herds. Herds of which the MRT is positive should be examined by serological tests to identify the infected animals (Alton *et al.* 1988). While it is a relatively insensitive test subject to wrong interpretation caused by various milk conditions such as mastitis, colostrum and milk at the end of the lactating cycle, it is recommended by the OIE as a screening test for bovine brucellosis.

Test to demonstrate an allergic reaction to Brucella

The brucellin skin test is an alternative immunological test which can be used to screen unvaccinated herds, provided that a purified, free, smooth LPS antigen is used. This test has a very high specificity, such that serologically negative animals that are positive reactors to the brucellin test should be regarded as infected animals (Bercovich and Muskens 1999; Saegerman *et al.* 1999; Godfroid *et al.* 2004). Results of this test may also aid the interpretation of serological reactions thought to be due to infection with cross-reacting bacteria, especially in brucellosis-free areas (Godfroid *et al.* 2002). Although the brucellin intradermal test is one of the most specific tests for brucellosis (in nonvaccinated animals), diagnosis should not be made solely on the basis of positive intradermal reactions given by animals in the herds, but should be supported by a reliable serological test. The allergic test should be used as a flock or herd test rather than as a test for individual animals (Alton *et al.* 1988).

2.7 CONTROL AND PREVENTION OF BRUCELLOSIS

Brucellosis is an infectious disease which has been controlled and eradicated in some countries in the world (Godfroid *et al.* 2004a). In sub-Saharan Africa, animal health services delivered by the public sector have greatly decreased over the last 20 years due to various factors such as decreasing government budgets, particularly for operational costs of disease control. Thus, programs that require coordinated surveillance, information exchange and application of control measures are not implemented in many sub-Saharan countries (McDermott and Arimi 2000; Smits and Cutler 2004). The main objectives for control and prevention of brucellosis are centred on the economic impacts of disease and its public health consequences. Control activities mainly reported by countries include: surveillance, movement control within and outside herds, vaccination, test and slaughter programmes, treatment of meat and milk products or a mixture of the above (Nicoletti 1980; McDermott and Arimi 2000; Godfroid *et al.* 2004a).

In Moçambique control of brucellosis was well organized until 1980 and the S19 vaccine was used in cattle. A strain 19 vaccine produced by the Onderstepoort Veterinary Institute in South Africa containing 4 - 12 x 10^{10} viable *Brucella* cells per dose was used. The vaccine was administered subcutaneously to heifer calves at four to eight months of age. However, recently some private farmers have used S19 vaccination in adult cows and RB51 vaccine on a very small and controlled scale. Surveillance and movement control were also implemented but on a very low scale.

CHAPTER 3
MATERIALS AND METHODS

3.1 STUDY AREA

The study was carried out in five districts of Maputo Province namely Magude, Matutuine, Moamba, Boane and Manhiça Districts. The districts cover northern, central and southern Maputo province. The selection of districts was done in order to involve distinct ecological conditions such as lowlands (areas within river valleys) and highlands (areas outside river valleys).

3.2 EXPERIMENTAL DESIGN

Animals

Adult beef and dairy cows and bulls, sheep and goats (where cattle and small ruminants are kept together) from the livestock production sector from Magude, Moamba, Matutuine, Boane and Manhiça districts of Maputo Province, as well as cattle slaughtered in Maputo municipal abattoir and Machava abattoir on the outskirts of Maputo were randomly sampled. Details of the study population for the 5 districts screened for brucellosis are summarized in Table 1.

Table 1: Number of the cattle sampled in the study area

Districts/Area	No. herds	No. of animals sampled		
		Total	Cows and heifers	Bulls
Overall	**79**	**971**	**920**	**51**
Manhiça	**12**	**180**	**166**	**14**
Palmeira Sede	1	7	5	2
3 de Fevereiro Mongue	3	29	23	6
Xinavane	2	96	90	6
Ilha Josina Machel	6	48	48	0
Moamba	**17**	**173**	**168**	**5**
Muxia/Goane	9	88	83	5
Sabie/Ncomanine	6	60	60	0
Pessene	2	25	25	0
Magude	**23**	**241**	**223**	**18**
Matlotlo	3	30	23	7
Facazissa	5	62	59	3
Pomono	8	96	90	6
Duco/Moine	7	53	51	2
Boane	**16**	**239**	**229**	**10**
Mahanhane	5	26	25	1
Mahubo	9	92	87	5
Umbeluzi	1	93	91	2
Massaca	1	28	26	2
Matutuine	**11**	**138**	**134**	**4**
Mudada	6	78	77	1
Hindane	2	22	22	0
Tinonganine	3	38	35	3

The selection of cattle herds was based on sharing of facilities such as the use of the same dip tanks, sharing of kraals (pens), use of communal pastures, sex, age, vaccination status and occurrence of abortion based on state veterinary reports and discussions with farmers and animal owners. The cattle population from the study area were not vaccinated from 2002 to 2005. For goats and sheep, selection of herds was based on the above-mentioned criteria and on the fact that they were reared together with cattle.

Table 2: Number of small ruminants sampled in the study area

Districts	No of goats sampled	No of sheep sampled
Manhiça	62	14
Moamba	214	109
Magude	166	52
Boane	65	13
Matutuine	245	72
Total	**752**	**260**

Samples were collected from a total population of 43 172 bovines distributed as follows: Magude (20 652), Moamba (11 081), Manhiça (1 686), Boane (3 095) and Matutuine (6658). A total of 41 292 goats were distributed as follows: Magude (8 753), Moamba (13 557), Manhiça (3 722), Boane (613) and Matutuine (14 647) and a total of 4 177 sheep distributed as follows: Magude (1 098), Moamba (1 603), Manhiça (650), Boane (168) and Matutuine (1 175).

3.3 SAMPLE COLLECTION AND HANDLING

All samples were collected using adequate equipment and handled according to OIE requirements. The samples were carefully collected and packed, avoiding any possibility of leakage or cross-contamination. Individually identified containers were placed in large and strong outer containers and packed with enough absorbent material to protect from damage. Immediately after collection, samples were transported to the laboratory and stored as recommended in the OIE Manual (2000). For transport the specimens, blood, milk, or organs were packed in a cooler bag with ice packs and kept cool during transport from the place of collection to the laboratory.

Blood samples

Blood samples from cattle (n=971) were collected from the caudal tail vein and from sheep (n=752) and goats (=260) were collected directly from the jugular vein, using individual needles and sterile plain vacuum tubes (Vacutainer). The tubes containing blood were left to stand until a complete clot was formed, and serum was decanted into a vial for storage at -20ºC until processed serologically.

Milk samples

Since infection of the udder may be confined to one quarter, milk samples (n=85) were obtained from each healthy quarter separately (Alton *et al.* 1988). Samples were collected in individual sterile flasks from each healthy quarter of 121 dairy cows and kept refrigerated at 4 °C until processed serologically and bacteriologically.

Hygroma sample

A sample from a hygroma on the leg of a bull was collected by aspiration using a sterile syringe and submitted for bacterial isolation and identification.

Tissue samples

Tissue samples (n=108) from retropharyngeal, external and internal inguinal, medial iliac and mammary lymph nodes, udder, uterine caruncles, testes, epididymis of 32 cattle from the southern provinces of the country (Gaza and Inhambane Provinces), slaughtered in the Maputo municipal abattoir and Machava abattoir, and samples from an aborted foetus (stomach content, spleen, liver, lung and heart) from a farm in Matutuine district were collected and processed for bacterial isolation according to Alton *et al.* (1988), and nucleic acid

detection as recommended by Ouahrani-Bettache *et al.* 1996; Bricker and Halling 1994; 1995; Ewalt and Bricker 2000.

3.4 TOTAL NUMBER OF SAMPLES COLLECTED

Cattle

A total of 971 adult beef and dairy cows, heifers and bulls were sampled in Maputo province and distributed as follows: 241 in Magude, 138 in Matutuine, 239 in Boane, 173 in Moamba and 180 in Manhiça districts.

Blood and tissue samples from 32 animals (9 females and 23 male) from Gaza Province (Massangena, Mabalane, Massingir, Chokwé, Magude and Chicualacuala Districts) and Inhambane Province (Zavala District) were collected in abattoirs. One aborted foetus was collected from a farm in the Matutuine District. Milk from 85 dairy cows and hygroma fluid from a cow was collected during the study.

Goats and sheep

Blood from a total of 752 goats were collected as follows: Magude (166), Matutuine (245), Moamba (214), Boane (65) and Manhiça (62). A total of 260 sheep from Magude (52), Matutuine (72), Moamba (109), Boane (13) and Manhiça (14) were bled.

3.5 SEROLOGY

B. abortus antigen and 2nd International Standard Anti-*Brucella abortus* serum produced at Onderstepoort Veterinary Institute (OVI) and supplied by Sommex (Pty) Ltd (South Africa) were used for the RBT, CFT and MRT and the tests done

at the Serology Section of INIVE. A negative control serum from a bovine that repeatedly tested negative was included and was obtained from INIVE.

Rose Bengal test (RBT)

The RBT was performed in WHO haemagglutination (HA) plates according to Alton *et al.* (1988) and the OIE Manual (2004). Equal volumes of 25 µl of serum and antigen were added to the wells. The plates were agitated by vigorously tapping the side for 20 counts in order to mix antigen and serum thoroughly, and results read after a 4 min rotation. Any degree of agglutination was recorded as positive, from a clear rimming to full agglutination with clear supernatant fluid.

Complement fixation test (CFT)

CF reagent preparation

The preparation of diluent, sheep erythrocytes, complement and haemolysin and standardization of erythrocytes and titrations were processed as described by Alton *et al.* (1975). Repeated dispensing of fixed volumes of 0.25 ml of diluent, haemolysin, antigen, complement and sensitized erythrocyte suspensions were done with a 1 ml glass tuberculin syringe fitted with a short length of fine polythene tubing. Dispensing and dilution of serum was done by a hand operated pipetting unit delivering 0.25 ml.

Reaction procedure

The CFT was carried out in three tubes, two for serial dilutions and one for anti-complementary (serum) control according to Alton *et al.* (1975) modified by Rodriguez (1983). Briefly, 1 ml of each serum sample was inactivated by heating in an incubator for 30 min at 56 °C. In the case of goat and sheep sera, inactivation was done at 62 °C. Addition of 0.25 ml of diluent was done for each

of the test tubes. An amount of 0.1 ml of inactivated sera was added to 0.4 ml diluent in a separate tube to obtain a dilution of 1:5. After mixing the contents of the first tube, 0.25 ml was transferred to tube number 2, mixed and 0.25 ml discarded. It resulted in the first 2 tubes containing doubling dilutions of serum of 1:5 and 1:10, and was the only dilutions used for the test. Standard positive sera were included in each set of tests (serum control tube) with a 1:5 dilution of serum where 0.25 ml diluent was added in lieu of antigen. A total of 0.25 ml of diluted antigen was added to all tubes except the anti-complementary controls, and 0.25 ml of complement was added to all tubes. Three complement control tubes were included in the test. Test tubes were incubated for 30 min at 37 °C after which sensitized erythrocyte suspensions (0.25 ml) were added to all tubes, including all controls and incubated for 30 min at 37 °C after mixing thoroughly. The test was read by eye. Complete lack of haemolysis was recorded as 4+ and complete haemolysis was recorded as 0. The CFT is used as a confirmatory serological test in Moçambique and titres of 1:5 were recorded as positive.

Indirect enzyme-linked immunoabsorbent assay (iELISA)

An indirect ELISA (iELISA) kit (HerdChek®, IDEXX), supplied by DEHTEQ (Pty) Ltd (South Africa) was used. Indirect ELISA (iELISA) was done according to the manufacturer's recommendations. Briefly, serum samples were diluted one-hundred fold (1:100) with sample diluent in tubes in order to standardize incubation times, and transferred to the antigen coated plates. Positive and negative controls (100 µl) were dispensed respectively into wells A1 and A2 and wells A3 and A4. Diluted serum (100 µl) was dispensed into the remaining wells and plates incubated for 90 min at 20-25 °C. Liquid contents of all wells were discarded in a waste reservoir and plates washed four times with approximately 300 µl wash solution, discarding the liquid content after each wash. Plates were firmly tapped onto absorbent material after final discarding to remove residual wash fluid. A total of 100 µl of anti-bovine IgG horseradish peroxidase (HRPO) conjugate was dispensed into each well and plates incubated for 30 min at

20–25 °C after which the washing process was repeated, followed by addition of 100 µl TMB substrate solution into each well. Plates were incubated for 15 min at 20–25 °C when 100 µl of stop solution was dispensed into each well to stop the reaction. The absorbance at a wavelength of 650 nm was measured using a spectrophotometer. Serum samples with a sample positive (S/P) ratio of 1.0 and greater were considered positive.

Milk ring test (MRT)

Milk samples and antigen were kept at room temperature for at least 30 min before testing. The test was performed according to Alton *et al.* (1988). Milk samples were thoroughly mixed to disperse the cream and 1 ml dispensed into each tube test. One drop (0.03 ml) of ring test antigen was dispensed into each tube and swirled gently to ensure that the antigen and milk were thoroughly mixed. Test tubes were incubated in a water bath for 1 hour at 37 °C. The test was considered positive when the intensity of blue colour in the cream layer was equal to or deeper than the skim portion.

3.6 BACTERIOLOGY

Standard bacteriological procedures were used and performed at the Bacteriology Section of INIVE.

Microscopical examination of collected specimens

Direct smears from tissues collected from an aborted foetus and organs from abattoirs were made according to the OIE Manual (2004) and stained with Gram’s stain. Whether the results from microscopic examination were positive or negative, samples were cultured for confirmation.

Isolation and typing of collected specimens

Milk samples

Milk samples were centrifuged at 6000 g for 15 min. The cream and deposit obtained after the skim milk was discarded were mixed and spread with a swab-stick on a solid tryptose medium (Difco Laboratories) as recommended by Alton *et al.* 1988.

Foetal stomach contents

Foetal stomach contents was collected from the stomach of an aborted foetus with a sterile Pasteur pipette and three drops placed and spread on the surface of tryptose agar (Difco Laboratories) and processed according to Alton *et al* (1988).

Hygroma fluid

Hygroma fluid was collected with a syringe and spread directly on the surface of tryptose agar with a swab-stick as described by Alton *et al.* (1988).

Tissue samples

Organs collected in abattoirs included mammary, inguinal, retropharyngeal, mandibular and medial iliac lymph nodes, udder, uterine caruncles, testes, and lung, liver and spleen from an aborted foetus. They were immersed in alcohol and flamed before being cut in small pieces and spread on the surface of tryptose agar (Alton *et al.* 1988).

Identification and typing of isolates

The plates were incubated at 37 ℃ in air with 5 to 10% CO_2, and examined for the presence of *Brucella* colonies after 2, 4 and 7 days. For identification, colonial morphology, staining properties and biochemical tests were used, followed by biotyping of isolates (CO_2 requirement, urease activity growth in the presence of sensitivity for thionine and basic fuchsin), as recommended by Quinn *et al.* (1999).

Biological test

A guinea-pig were inoculated subcutaneously with 1 ml of homogenized suspension of stomach content and spleen from an aborted foetus and euthanized after 6 weeks. Serum was collected and submitted for serology and spleen submitted for bacteriological examination as described by Quinn *et al.* (1999).

3.7 MOLECULAR DETECTION

The PCR assay (Brucella AMOS) used in this study is based on the observation that the repetitive genetic element IS*711* also known as IS*6501* (Ouahrani-Bettache *et al.* 1996) is unique to *Brucella* species, and for most species at least one copy of the element occurs at a unique species- or biovar-specific chromosomal locus.

The assay is a rapid screening method for differentiation of *B. abortus* field and vaccine strains 19 and RB51 (Ewalt and Bricker 2000). It is based on the insertion of the genetic element IS*711* at a unique chromosomal locus in *B. abortus* and the double insertion of IS*711* at a specific locus in *B. abortus* RB51. One PCR primer is anchored within the IS*711* sequence, while the differentiating primers are localized in the unique chromosomal DNAs adjacent to

the insertion. The primers used amplify a 498 bp product present in *B. abortus* and the vaccine strains S19 and RB51. The identification of S19 is based on the absence of the amplification of a short sequence (178 bp) of the *eri* gene (essential for erythritol catabolism) present in all *Brucella* strains except *B. abortus* S19. Thus, the identification of S19 is based on the absence of amplification of this target (Ewalt and Bricker 2000).

Samples

Samples that were analysed for nucleic acid detection include organs of 5 selected slaughtered animals (uterus and mammary, inguinal, retropharyngeal lymph nodes) and lung, liver and spleen from an aborted foetus.

DNA extraction

DNA extraction from organs of slaughtered animals

The DNeasy Blood & Tissue Kit (QIAGEN) supplied by Inqaba (South Africa) was used for extraction. The extraction was done according to the manufacturer's recommendations. Briefly, after cutting 25 mg of tissue into small pieces it was placed in a 1.5 ml centrifuge tube and 180 µl Buffer ATL added. Twenty µl of proteinase K was added to the mixture and vortexed. The mixture was incubated overnight at 56 °C in a water bath for complete lysis of the tissue. The mixture (lysate) was vortexed for 15 sec and 200 µl Buffer AL was added and vortexed. Then 200µl ethanol (96%) was added and mixed thoroughly by vortexing. The mixture (including precipitate) was then transferred into a DNeasy Mini spin column placed in a 2 ml collection tube followed by centrifugation at 8000 rpm for 1 min. The DNeasy Mini spin column was placed in a new 2 ml collection tube where 500 µl Buffer AW1 was added, centrifuged for 1 min at 8000 rpm after which the DNeasy Mini spin was placed in a new 2 ml collection tube. Buffer AW2 (500 µl) was added and centrifuged for 3 min at 14000 rpm to dry the

DNeasy membrane. DNeasy Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl Buffer AE was directly added onto the DNeasy membrane by pipeting. It was then incubated at room temperature for 1 min, centrifuged for 1 min at 8000 rpm to elute and kept at minus 20°C for further use.

DNA extraction from an isolated bacterium

DNA from an isolate from an aborted foetus was extracted according to the protocol described by Ouahrani-Bettache *et al.* (1996). Briefly, the isolate was resuspended in 200 µl PBS, centrifuged at 14 000 rpm for 10 min and the supernatant discarded. The pellet was resuspended in 100 µl PBS and boiled for 10 min, immediately placed on ice for 5 min and centrifuged after cooling for 1 min at 14 000 rpm. The supernatant was collected and kept at -20 °C for further use.

Calculation of extracted DNA concentration

The concentration of DNA was determined by absorbance measurement at 260 nm, and purity by the ratio of absorbence at A_{260}, A_{280}, and A_{320} using a spectrophotometer (BIORAD/SmartSpec^{TM} 3000). Ratio values and concentrations are shown in Table 3.

Table 3: Results of DNA concentrations after extraction

DNA Sample	A_{260}	A_{280}	Ratio	A_{320}	DNA concentration (µg/ml)
1	0.221	0.133	1.6641	0.011	221.3788
2	0.451	0.247	1.8266	0.019	451.6940
3	0.270	0.161	1.6790	0.019	269.9399
4	0.130	0.085	1.5349	0.20	130.0710
5	0.223	0.125	1.7794	0.010	223.0589
584/05	0.168	0.089	1.8881	0.020	167.8109

Ratio value considered between 1.70 minimum values to 2.0 maximum values.

DNA samples identified as: 1. Mammary lymph node; 2. Inguinal lymph node; 3. Mammary lymph node; 4. Uterus; and 5. Retropharyngeal lymph node; 584/05. Isolate from an aborted foetus

Polymerase chain reaction (PCR)

Standard positive control

Standard positive controls for *B. abortus*, *B. melitensis*, *B. abortus* S19 and RB51 obtained from the Onderstepoort Veterinary Institute (OVI) were used and, were kindly donated by the Biotechnology Laboratory from the Department of Veterinary Tropical Diseases, University of Pretoria.

Primers

Genus-specific primers (ISP1; ISP2) for identification of *Brucella* sequences and species-specific primers (IS711; AB; BM, ERI1; ERI2 and RB51) for species-specific sequences (Table 4) were used according to Ouahrani-Bettache *et al.* (1996) and Bricker and Halling (1994; 1995).

Table 4: Primer sequences and melting temperatures recommended

Name of sequence	Nucleotide sequence 5' → 3'	Tm	
		Min	Max
ISP1	GGT TGT TAA AGG AGA ACA GC	58.35	58.35
ISP2	GAC GAT AGC GTT TCA ACT TG	58.35	58.35
IS711	TGC CGA TCA CTT AAG GGC CTT CAT	64.57	64.57
AB	GAC GAA CGG AAT TTT TCC AAT CCC	62.86	62.86
BM	AAA TCG CGT CCT TGC TGG TCT GA	64.55	64.55
ERI1	GCG CCG CGA AGA ACT TAT CAA	62.57	62.57
ERI2	CGC CAT GTT AGC GGC GGT GA	66.55	66.55
RB51	CCC CGG AAG ATA TGC TTC GAT CC	66.33	66.33

Table 5: Primer sets and amplicon sizes

Type	Primer set	No of base pairs
Brucella group specific	ISP1 + ISP2	600 bp
B. abortus	IS*711* + AB	498 bp
B. melitensis	IS*711* + BM	731 bp
B. abortus vaccine strain S19	ERI1 + ERI2	178 bp
B. abortus vaccine strain RB51	IS*711* + RB51	364 bp

PCR reaction

A PCR kit (Fermentas Life Sciences) supplied by Inqaba Biotec (South Africa) containing Taq DNA Polymerase (recombinant), 10X Taq Buffer with $(NH_4)_2SO_4$, $MgCl_2$, dNTP Mix (consisted of aqueous solutions [pH 7.0] of dATP, dCTP, dGTP and dTTP each at a final concentration of 2mM), and water nuclease free, was used. A master Mix of a total volume of 25 µl was used, consisting of 10X PCR reaction buffer (50mM Tris, 2.5 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, [pH 8.3]); 0.5 µl dNTPs; 0.5 µl of primers each; 17.75 µl water; 0.25 µl Taq DNA polymerase and 0.5 µl template DNA was prepared in a single tube and aliquoted into individual tubes for several parallel reactions. PCR amplification was performed in a BIORAD thermocycler.

PCR profile

Brucella genus-specific sequences

The PCR was performed according to Ouahrani-Bettache *et al.* (1996), and adjusted by including an initial denaturation step, addition of 5 cycles and a final extension step.

The PCR profile was set as follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 95 °C for 35 sec, annealing at 56 °C for 45 sec and extension at 72 °C for 45 sec. Finally an extension step of 7 min at 72 °C was performed.

Brucella species-specific sequences

PCR was performed according to Ewalt and Bricker (2000) and modified at OVI. The assay was adjusted by increasing the annealing temperature to 60 °C and including a final extension step of 7 min.

The PCR profile was set as follows: initial denaturation at 94°C for 2 min and a final extension at 72 °C for 7 min at the end. A total of 35 cycles divided in the three following phases; denaturation at 95 °C for 20 sec, annealing at 60 °C for 20 sec, extension at 72 °C for 30 sec according to Ewalt and Bricker (2000).

Analysis

An agarose gel (1.5%) was prepared using 1x TBE buffer (10xTBE buffer: 1 M Tris, 1 M Boric Acid, 50 mM EDTA, [pH 8.3]). The gel was stained with 3 µl ethidium bromide (10 mg/ml). An amount of 10 µl of PCR product was added to 3 µl of 6X Orange Loading Dye Solution**,** mixed and loaded. A 100bp DNA molecular weight marker was used as a size standard. Gel electrophoresis was

performed at 100 V for 30 to 45 min and amplicons analysed under UV light and photographed using a *BIORAD* Smart Spec$^{TM\,3}$ 3000 with computer software.

3.8 STATISTICAL ANALYSIS

The sample size was obtained from a computer programme WinEpiscope, using a level of confidence of 95% with an expected error of 2%. An estimated prevalence of 10%, based on systematic sampling for estimation of disease prevalence and detection of *Brucella* was used.

Data were analysed using SPSS software version 13.0 for the prevalence of brucellosis in the study area and test results compared using the Kappa (K) coefficient test with the statistical program STATA version 9.0. The significance level considered was 5% for the Kappa test.

CHAPTER 4
RESULTS

4.1 SEROLOGICAL RESULTS

Overall prevalence in districts

Cattle

An overall apparent prevalence of 14.0% (n=971) was found in beef and dairy cattle sera processed serologically using RBT, iELISA and CFT in the studied districts. The RBT and iELISA were used as screening tests and the CFT as the confirmatory test. CFT titres of 1:5 and above were recorded as positive. The results are shown in table 6.

Table 6: Apparent prevalence of brucellosis in the study areas (CFT as confirmatory test), using a 95% confidence interval (CI)

District	Locality	n	Prevalence (%)
Manhiça	Manhiça-sede	7	0.0
	3 de Fevereiro	29	17.2
	Xinavane	96	32.3
	Ilha Josina Machel	48	27.1
Subtotal		**180**	**27.2[a]**
Moamba	Muxia/Goane	88	13.6
	Sábie/Ncomanine	60	3.3
	Pessene	25	4.0
Subtotal		**173**	**8.7[bc]**
Matutuíne	Tanque Mudada	78	24.6
	Tinonganine	38	7.9
	Tanque C. Hindane	22	4.5
Subtotal		**138**	**14.5[b]**
Magude	Matlotlo	30	3.3
	Facazissa	62	6.5
	Tanque C. Pomono	96	1.0
	Duco Moiane	53	20.8
Subtotal		**241**	**7.1[c]**
Boane	Tanque Mahanhane	26	19.2
	Mahubo	37	0.0
	Tanque C. Mahubo	55	29.1
	Umbelúzi	93	15.1
	Massaca	28	0.0
Subtotal		**239**	**14.6[b]**
Total		**971**	**14.0**

[a,b,c] Different letters on the same column means significant differences between districts ($p<0.05$)

The prevalence differed between districts. A significant difference ($p<0.05$) was observed in Manhiça district (27.2% (n=180)) compared to the other districts. No significant differences were observed between Moamba (8.7% (n=173)), Matutuine (14.5% (n=138)) and Boane (14.6% (n=239)) districts ($p>0.05$). The lowest prevalence was observed in Magude (7.1% (n=241)) district which represented a significant difference ($p<0.05$) when compared to Manhiça,

Matutuine and Boane districts. There was no significant difference (p>0.05) between Moamba (8.7% (n=173)) and Magude (7.1% (n=241)) districts.

Apparent prevalence between sex and age

An apparent prevalence of 13.49% (n=905) was found in females as compared to males where a prevalence of 0.42% (n=66) was found for cattle tested with the CFT as confirmatory test (table 7).

Table 7: Apparent prevalence of bovine brucellosis by sex (95% CI)

		n	Positive	Prevalence (%)
Sex	Male	66	4	0.42
	Female	905	131	13.49
	Total	**971**	**135**	**13.91**

The apparent prevalence found in cows was 12.77% (n=783) compared to heifers where a prevalence of 0.72% (n=122) was found for cattle tested with the CFT as confirmatory test (table 8).

Table 8: Apparent prevalence of bovine brucellosis in females by age (95% CI)

		n	Positive	Prevalence (%)
Sex	Male	122	7	0.72
	Female	783	124	12.77
	Total	**905**	**131**	**13.49**

Sheep and goats

No positive reactors were found in goat (n=749) and sheep (n=260) sera tested serologically (RBT, ELISA and CFT) using *B. abortus* antigen.

Prevalence in the cattle livestock production sectors

The apparent prevalence found were as follows: 15.5% (n=161) in small, 14.0% (n=578) medium and 12.9% (n=232) large cattle livestock production sectors respectively. From the results obtained, there was no significant difference ($p>0.05$) among the cattle livestock production sectors in the study area, as shown in Table 9.

Table 9: Apparent prevalence in the cattle livestock production sector based on CFT (confirmatory test) using a 95% CI

Farm size	CFT	
	n	**Positive (%)**
Small	161	15.5
Medium	578	14.0
Large	232	12.9
Total	**971**	**14.0**

Comparative results of RBT, ELISA and CFT

The statistical analysis of the serological results showed complete agreement of 100% between iELISA and CFT. The agreement between RBT/CFT and RBT/iELISA was 99.64% as shown in Table 10.

Table 10: Agreement among three serological tests (RBT, iELISA and CFT) for bovine brucellosis as measured by percent agreement (above the diagonal) and Kappa coefficient (K) (below the diagonal)

	RBT	**iELISA**	**CFT**
RBT	**-**	**99.64**	**99.64**
iELISA	**0.9858**	**-**	**100**
CFT	**0.9858**	**1**	**-**

The prevalence of brucellosis on dairy farms in Boane District

A prevalence of 15.1% (n=93) was found on the Texeira dairy farm based on CFT as the confirmatory test, while in Casa do Gaiato no positive reactors were found (0% (n=28)) as shown in Table 11. There was a significant difference between the two dairy farms ($p<0.05$).

Table 11: Prevalence of brucellosis on dairy farms

District	Dairy farm	CFT	
		n	Positive (%)
Boane	Texeira	93	15.1
	Casa do Gaiato	28	0.0
	Total	**121**	**12.5**

Comparative results of MRT with serum based tests (RBT, IELISA and CFT)

The results for milk samples tested by MRT showed an agreement of 100%, when compared to results from the same cows tested by iELISA and CFT. A significant difference was observed between MRT and RBT ($p<0.05$), with a Kappa coefficient of 70.5% for MRT and RBT, as shown in Table 12.

Table 12: Agreement among the four serological tests (MRT, RBT, iELISA and CFT) for bovine brucellosis as measured by percent agreement (above the diagonal) and Kappa coefficient (K) (below the diagonal)

	MRT	RBT	iELISA	CFT
MRT	**-**	**70.25%**	**100%**	**100%**
RBT	**0.3134**	**-**	**-**	**-**
iELISA	**1**	**-**	**-**	**-**
CFT	**1**	**-**	**-**	**-**

4.2 BACTERIOLOGICAL TEST RESULTS

Isolation and typing of Brucella

Successful isolation of *Brucella abortus* was only obtained from the liver and spleen of an aborted foetus. Smooth translucent pinpoint colonies were observed. Results obtained with biochemical tests together with colony morphology and staining properties were as follows: Gram-negative coccobacilli; non-motile, catalase and oxidase-positive, urease-positive (after 2 hours) and KNO_3 positive. Growth in the presence of basic fuchsin (20µg/ml) was observed while no growth was observed in the presence of thionin (20µg/ml). The isolate was designated isolate 584/05 and freez-dried for further use. The cow from which the *B. abortus* isolate 584/05 was obtained, tested positive with the CFT at a screening dilution of 1:10.

Biological test

Brucella was isolated from the spleen of a guinea-pig slaughtered 6 weeks after inoculation with a suspension from the aborted foetus. Serum of the guinea-pig reacted positively to CFT with a titre of 1:10.

Isolation from tissue samples from slaughtered cattle

No isolation was done from organs (lymph nodes, udder, uterine caruncles, testes, epididymis) from 32 animals slaughtered at the Maputo municipal abattoir and Machava abbatoir. A representative group of specimens (n=11) were sent to the OVI for an interlaboratory comparison, but the results came back as negative. The sera of the 32 animals were tested and 5 animals had reactions with the RBT but were negative with the CFT. The ELISA was not available at the time of testing these samples. No isolation was made from milk samples, or from the hygroma fluid collected from a bull.

4.3 NUCLEIC ACID DETECTION

Detection of genus-specific sequences

A PCR amplification product of 600 bp was generated from the 584/05 isolate and from the organs of the 5 selected animals collected in the abattoirs for detection of *Brucella* using primers ISP1 and ISP2 (genus-specific sequences) (Figure 2).

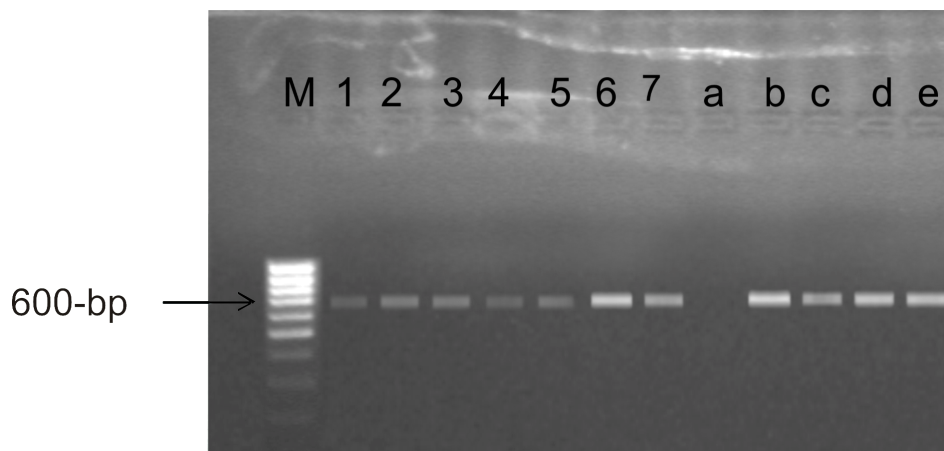

Figure 2: PCR amplification products generated by *Brucella* genus-specific primers

PCR was performed with 7 DNA samples: lanes 1, 2, 3, 4, 5 represent extracted DNA from organs collected in the abattoirs; lane 6, DNA of 584/05 isolate; lane 7, DNA from vaccine S19; lane a, negative control of water containing no template; lane b, *B. abortus*, lane c, *B. abortus* strain 19; *lane* d*, B. abortus* strain RB51, lane e, *B. melitensis.* First lane (M) contains a 100-bp ladder as a size standard.

Detection of species-specific sequences

A 498 bp fragment typical for *B. abortus* was amplified from isolate 584/05 and the positive controls *B. abortus, B. abortus* S19 and RB51 when subjected to PCR using primers for *B. abortus* (IS711/AB) as shown in Fig. 3 (A). Fig. 3 (B) shows a 178 bp fragment amplified from isolate 584/05 and controls *B. abortus, B. abortus* vaccine RB51, except S19 when subjected to PCR using primers for *B. abortus* S19 (ERI1/ERI2).

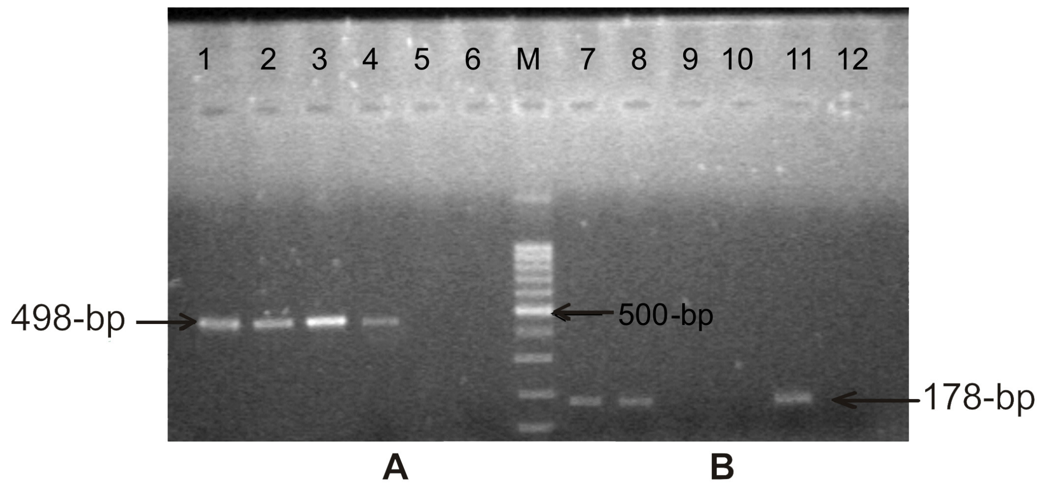

Figure 3: PCR amplification products generated by *Brucella* species-specific primers

The (M) lane contains a 100-bp ladder as a size standard.

Fig. 3(A): Lane 1, DNA from isolate 584/05; controls are indicated as lane 2, *B. abortus*; lane 3, *B. abortus* strain 19; lane 4, *B. abortus* strain RB51; lane 5, *B. melitensis,* lane 6, negative control of water containing no template.

Fig. 3(B): Lane 7, DNA from isolate 584/05; controls are indicated as lane 8, *B. abortus*; lane 9, *B. abortus* strain 19; lane 10, *B. melitensis*; lane 11, *B. abortus* strain RB51; lane 12, negative control of water containing no template.

CHAPTER 5
DISCUSSION

The results from this study indicate that bovine brucellosis is prevalent in the five districts under investigation, with differences between the districts. An overall apparent prevalence of 14.2% was found in the study area based on serological tests. Although the results obtained were different from those previously reported by Màrio and Valdão (1972), it is in concordance with results reported in other studies (Pereira and Schwalbach 1997; Manhiça 1998; INIVE reports 1997-2003. Based on these results it is reasonable to say that the current results are an indication of the absence of brucellosis in sheep and goats in the study area. According to Thimm and Wundt (1976) the incidence of brucellosis in cattle herds was considered to be moderate (ranging from 11 to 20%) for the southern region of Africa. McDermott and Arimi (2002) referred to a prevalence greater than 5% (ranging from 4.8 to 41%) in extensive pastoral systems that are the most dominant livestock systems in Africa, including Moçambique.

All serum samples from this study were subjected to RBT and iELISA as screening tests and CFT as the confirmatory test, as recommended by the OIE Manual (2004) and reported by Nielsen (2002). All samples reacting positively to the screening tests were submitted for CF testing where titres of 1:5 and above were recorded. Sensitivity and specificity of these tests have been reported in previous studies (Dohoo *et al.* 1986; Nielsen 2002; Gall and Nielsen 2004).

It was not possible to express the CFT results in units due to the fact that it was performed in tubes and not in microtitre plates. It utilized only two dilutions, namely 1:5 and 1:10. That rules out the possibility to convert the volumes into units, as sera would have to be titrated out to be able to convert the sample titres into international units. This approach was developed and documented by

Rodrigues in 1983 as a result of constraints experienced during the Moçambican civil war that included *inter alia* the unavailability of a micro-shaker, plate centrifuge and spectrophotometer. The adapted method is the standard method used at INIVE and is still in use today. However, it is conceded that the very good concordances of the tests is surprising and may bring the validity of the adapted CFT into question as the ELISA was performed according to the manufacturer's instructions with its built-in controls.

Although it is not possible to distinguish between vaccine-induced antibody and antibody from natural infection, the RBT, ELISA and CFT have been valuable assets in control/eradication programs (Nielsen 2002). A high degree of agreement between the tests used is normally expected when dealing with known herd vaccination status with regards to strain 19 vaccines as reported by Dohoo *et al.* (1986) and Nielsen (2000), supporting results from this study where reactor animals in reactor herds were found in different districts. Animal vaccination with S 19 was undertaken in the study area until 2003. The positive results with the CFT can be considered to be from infected animals taking into consideration the age and sex of animals where a prevalence of 12.77% was found in adult cows as compared to young heifers vaccinated at the age of 4 to 8 months and a prevalence of 0.72% was found. The positive reactors (0.42%) found in bulls which are not vaccinated in the country also provides an idea of the real status of the disease in the study area.

The highest prevalence was observed in Manhiça district (27.4%) with a significant difference compared to other districts ($p<0.05$), while the lowest prevalence was observed in Magude district (7%) followed by Moamba district (8.7%). These results are in agreement with those previously reported by Manhiça (1998), Maxlhuza (2002) and reports (INIVE 1997-2003). Abortions have been reported to occur in all districts where the study was carried out, thus the positive reactors found in the study area can be considered to be from infected animals, although Godfroid *et al.* (2002) referred to the presence of anti-

brucellae antibodies as not necessarily meaning that the animal have current or active infection at the time of sampling but that it may be a result of past infection resulting in a “self-limiting” disease. The possible explanation for the relatively high and variable prevalence within the study area might be those related to transmission of disease between herds due to the proximity between the herds in the communal grazing areas and watering points, in shared kraals and dip tanks, as well as purchasing of infected animals. Other risk factors might be those influencing the maintenance and spread of infection within herds due to the presence of unvaccinated animals in infected herds, herd size, which often occur in the country and are reported by Thimm and Wundt (1976); Nicoletti (1980); McDermott and Arimi (2002); Unger *et al.* 2003; Smits and Cutler (2004).

Possible explanations for the results obtained from the cattle production sector might be that the relatively high prevalence may be due to the risk factors mentioned above, related to the transmission of disease between herds and maintenance and spread of infection within herds. However, it is important to mention that on some farms where cattle are kept with little contact with other animals, the prevalence was low or there were no reactors at all.

The results obtained from goats and sheep provide strong support for the absence of brucellosis in small ruminants in the study area. It is unlikely that the results were influenced by the fact that the tests used (RBT, iELISA and CFT) were not sensitive enough to detect animals with low infection doses as reported by Blasco *et al.* (1994); Bercovich *et al.* (1998). Although goat and sheep herds share communal grazing and watering points with cattle herds, it is likely that abortions reported among small ruminants in the study area are due to agents other than *Brucella*.

Results from dairy farms in the Boane district namely "Texeira" and "Casa do Gaiato" agree with those reported by Pereira and Schwalbach (1997). The prevalence found on "Texeira" dairy farm can be explained by the management practices used, while in "Casa do Gaiato", animals are kept in a closed environment and under control, avoiding contact with infected herds. On "Texeira" dairy farm the management practices are relatively poor, allowing animals to be in close contact with other animals from the area that may be infected (Nicoletti 1980; McDermott and Arimi 2002). Abortions were reported to occur at "Teixeira" while in "Casa do Gaiato" there was no report of abortion. The inability to isolate the *Brucella* from milk may have been due to factors such as the fastidious nature of the organism, the low number of viable organisms in the samples collected, or collection of inappropriate samples, or that the media and culture techniques used were not sensitive or accurate enough for the detection of the agent, all aspects that have to be considered when interpreting the results as reported by Alton *et al.* (1988). The close agreement between MRT/CFT and MRT/iELISA (100%) as compared to MRT/RBT (70.25%) has also been reported (Unger 2003).

The isolation of a *B. abortus* field strain from an aborted foetus from a farm in Matutuine district and its detection by means of PCR which differentiates *B. abortus* field strains and vaccine strains 19 and RB51, was in accordance with Ewalt and Bricker (2000) and Sangari *et al.* (2000) who referred to a fragment of 498 bp DNA to be typical for *B. abortus* field strains when species-specific primers IS*711*/AB are used. When using primers for vaccine strain S19 (ERI1/ERI2) a fragment of 178 bp DNA is amplified. According to Ewalt and Bricker (2000) and Sangari *et al.* (2000) identification of vaccine strain 19 is based on a PCR primer pair which amplifies a short sequence (178 bp) of the *eri* gene essential for erythritol catabolism present in all *Brucella* except *B. abortus* S19. Therefore the identification of S19 is based on the absence of amplification of this target due to the inability of S19 to catabolize erythritol.

A correlation was made between the isolate 584/05 and serum from the cow that aborted. The results confirmed that the abortion was due to infection by a field strain of *B. abortus*, although it was not possible to identify the biovar involved. This result can also support the findings based on serology, where a 14.0% overall prevalence obtained are likely to be from infected animals rather than reactions to vaccination with S19.

The inability to isolate *Brucella* from samples collected in abattoirs was disappointing and unexpected. It may have been due to the factors mentioned previously. The same organs when subjected to PCR assay for detection of *Brucella* using *Brucella* genus-specific primers (ISP1/ISP2) amplified a fragment of 600bp as described by Ouahrani-Bettache *et al.* (1996). From the results obtained it is reasonable to conclude that brucellosis is prevalent in the areas where the animals originated from (Gaza province) although isolation was not successful.

The PCR assay was shown to be a valuable tool for the detection of *Brucella* organisms from organs as reported by Ouahrani-Bettache *et al.* (1996) as well as for its specificity to differentiate *Brucella abortus* field strains from vaccine strain S19 as reported by Ewalt and Bricker (2000). Several advantages have been reported for the PCR assay over the current conventional methods used to identify *Brucella* species. The major advantage being the time taken compared to conventional methods which require several days to isolate and identify the organism (Fekete *et al* 1992; Ouahrani-Bettache *et al.* 1996; Bricker and Halling 1994; Ewalt and Bricker 2000); Güler *et al.* (2003). The other advantage is the need for minimal sample preparation because isolation of living organisms is not necessary. The assay is also not affected by contamination with other microbes that might be present in tissue samples used for isolation.

CHAPTER 6
CONCLUSIONS AND RECOMMENDATIONS

The isolation of a *Brucella abortus* field strain by conventional methods and its detection by PCR from a case of abortion, and the positive serological result from the cow that aborted, confirmed that the isolate was a *Brucella* field strain and not a vaccine strain. The PCR assay was shown to be a valuable tool for the detection of *Brucella* organisms as well as to differentiate *Brucella abortus* field strains from vaccine strains. The PCR assay can be used to supplement other diagnostic tests and epidemiological data (such as herd history and serological test results) aiding brucellosis control programs in Moçambique before the conventional identification methods are completed. However, further research is recommended for confirmation of serological and bacteriological results obtained and for standardization of PCR assays in the country.

Strain 19 vaccine is still considered the most acceptable and the most widely used vaccine against bovine brucellosis. Although inducing post-vaccinal antibodies that are detected in serological tests, its use should be recommended for control of brucellosis in the country. Strategies based on prevention of spread of the disease between animals, monitoring of negative herds for brucellosis and avoiding contact with infected herds, control of the movement of infected or suspected animals, vaccination of heifer calves, good records of vaccination status, and marking of infected and vaccinated animals are suggested for the success of a control program.

Due to the public health risks associated with consumption of milk, studies of brucellosis in humans involving dairy farm workers are suggested in order to support the findings from previous studies in Moçambique.

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