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CHARACTERISATION OF *BRUCELLA* SPECIES FROM ZIMBABWE

by

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Thesis Summary

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by

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Department: Veterinary Tropical Diseases

Degree: MSc (Veterinary Science)

Brucellosis is an important zoonotic disease of ruminants, suidae, canids, several wildlife species and humans caused by from the genus *Brucella* genus consisting of gram-negative bacteria that are facultative intracellular pathogens. Brucellosis is endemic in sub-Saharan African countries, which include Zimbabwe where *Brucella abortus* and *B. melitensis* have been reported. In order to control brucellosis, surveillance and identification of *Brucella* species is of paramount importance. The aim of the study was to carry out a survey of bovine brucellosis using serological tests, PCR assay and characterizing *Brucella* spp in Zimbabwe using molecular techniques. Serological tests and PCR based assay were used to detect brucellosis in cattle and wildlife in Chiredzi district of Zimbabwe. Blood and serum samples from cattle (n=700) from Chiredzi district (Malipati and Pesvi communal areas), as well as, African buffalo (n=10) and impalas (n=14) (from Gonarezhou National Park (GNP)) were tested for bovine brucellosis using rose bengal test (RBT), serum agglutination methods (SAT) and indirect enzyme linked immunoassays (iELISA). The seroprevalence of bovine brucellosis in cattle was found to be 8.3% using RBT and iELISA and 6.0% using RBT, SAT and iELISA. No antibodies for *Brucella* spp were detected in the African Buffaloes and impalas. *Brucella* strains were cultured from

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milk and blood sample from cows in Chiredzi district. DNA from the two *Brucella* strains was amplified using the ITS66 and ITS279 primers specific for *Brucella* 16-23S rDNA intergenic spacer (ITS) region. This *Brucella* specific PCR assay was also used to detect *Brucella* DNA in blood and buffy coat samples from cattle that were seropositive using RBT, SAT and iELISA. Despite the fact that the blood and buffy coat samples were from animals that were bacteriemia since Brucella was isolated from blood and milk and all samples had SAT titres above 148, no *Brucella DNA* were detected using the specific PCR assay. Furthermore *Brucella* strains (23) isolated at the Central Veterinary Laboratory (CVL) in Zimbabwe were identified using molecular techniques like MLVA, AMOS-PCR and Bruceladder PCR. Only a few isolates were classified up to species level using bacteriology method (biotyping). MLVA-16 was able to identify *B. abortus, B. melitensis, B. ovis* and *B. suis* strains amongst Zimbabwean isolates. The latter two species is the first report of these species in Zimbabwe. The MLVA assay furthermore indicated that some Zimbabwean isolates significantly differ from isolates of other origin in specific species clusters. These Zimbabwean isolates could not be speciated and future research using bacteriology and molecular characterization are necessary to characterize these isolates. A few strains identified using bacteriological methods and MLVA were also verified using AMOS-PCR and Bruceladder. For these results it is clear that MLVA, AMOS-PCR and Bruceladder can be used to identify *Brucella* species and biovars and can therefore contribute towards the control of brucellosis in African countries.

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CHAPTER 1

1. GENERAL INTRODUCTION

Brucellae are facultative, intracellular, non-motile, non spore-forming, Gram-negative coccobacilli bacteria that are pathogenic for humans and a variety of livestock animals and wildlife. Classification of *Brucella* species is mainly based on pathogenicity, host preferences, and conventional microbiological tests used for phenotyping (biotyping). General routine identification of *Brucella* species and biovars still relies on phenotyping which are time consuming. *Brucella* spp., the causative agents of brucellosis, is pathogenic to variety of domesticated and wild animals. There are ten species currently recognized, namely *B. abortus, B. melitensis, B. suis*, *B. ovis*, *B. canis*, *B. neotomae* , *B. pinnipedialis, B. ceti* , *B. microti* and B. *inopinata* (wound fluid from human). *Brucella. canis* and *B. ovis* are classified as rough species and the other species as smooth species because they possess the smooth lipopolysaccharide (S-LPS).

Identification of brucellosis is important to the accomplishment of surveillance and eradication efforts. Currently, serological screening of potential hosts, isolation and identification of the pathogen from potential hosts are used for the diagnosis of brucellosis. Isolation and identification of *Brucella* spp. is recognized as the 'gold standard'. However, it takes more than two weeks to culture the bacteria and complete 25 phenotypic traits used for species and biovar identification of cultured organisms. It is a lengthy process that requires experienced technicians with high risk of laboratory infections. The absence of bacterial isolation does not rule out brucellosis since some positive animals yield negative culture results. Therefore

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serological tests are used to define the brucellosis status of animals. For the control and diagnosis of brucellosis in small ruminants and cattle more than one serological test of which rose bengal test (RBT), buffered antigen plate agglutination test (BPAT), fluorescence immunosorbent assays (FPA) and enzyme linked immunoassays (ELISA) are suitable screening tests. Brucellosis serology in wildlife is performed using the same antigens as in domestic ruminant serology. Serological tests are genus and not species specific. Since serology is general used to diagnose brucellosis in animals, serum is the sample readily available. Serum samples restrict the analysis to PCR apart from serology. The method of extraction of bacterial DNA from blood may be crucial in the detection of the bacterium DNA during PCR. Additionally, the stage of infection of the animals since this may determine the amount and location of bacteria influence the detection of *Brucella* DNA during PCR.

Various molecular typing methods including PCR techniques differentiating *Brucella* at the species level and/or biovar level have been described. The development of discriminatory molecular tools for identification and typing of *Brucella* has been problematic, reflecting the lack of genetic polymorphism in *Brucella*. A high degree of homology initially implied by DNA-DNA hybridization has been confirmed by a variety of approaches, such as multilocus sequence analysis (MLSA) and 16S rRNA sequencing. These sequences were long considered highly conserved and have a high degree of genetic homology making identification and differentiation of *Brucella* species and biovars very challenging. Multiplex PCR assays have been developed that enable identification and differentiation of *Brucella* at the species / biovar level from culture or infected tissue. These include the AMOS-PCR consisting of specific primers that amplifies a locus containing a copy of the genetic element IS711 that

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distinguish *B. abortus* biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1 and vaccine strains due to the different copies of the element in each of these species / biovars. Another multiplex PCR assay known as Bruceladder targeting multiple genes of *Brucella* species, the vaccine strains S19 and RB51 of *B. abortus* as well as *B. melitensis Rev1* were based on species and strain specific genetic differences. Multiple-locus variable number of tandem repeats (VNTR) analysis (MLVA) has become a powerful tool for distinguishing among closely related strains of organism because VNTR loci typically mutate independently of one another. MLVA has proven to be highly appropriate for the typing of pathogenic bacteria species with a high genetic homogeneity, including the *Mycobacterium tuberculosis* complex, *Bacillus anthracis*, and *Yesinia pestis.*

Brucellosis is common in sub-Sahara Africa and is more significant in female domestic animals where it causes abortions. This disease is among the most important bacterial zoonoses especially in developing countries where the disease may have important veterinarian, economic and public health consequences. Few strains from southern African countries have contributed to the MLVA-16 *Brucella* database (http://mlva.u-psud.fr). The aims of the study were to carry out a survey of bovine brucellosis in cattle and wildlife from Chiredzi district in Zimbabwe using serology, bacteriology (to culture from infected animals) and *Brucella* specific PCR assay form blood DNA. The MLVA-16 scheme was applied to identify the species and / or biovar of *Brucella* strains isolated at the Central Veterinary Laboratory (CVL) in Zimbabwe and some of MLVA results were further verified using AMOS-PCR and Bruceladder.

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CHAPTER 2

2.0 LITERATURE REVIEW

2.1 *Taxonomy and etiology*

Brucellosis is caused by a group of bacteria belonging to the genus *Brucella,* which are gram negative coccobacili (Corbel and Brinley-Morgan, 1984; Walker, 1999). Ten species are currently recognized, namely *B. abortus* (8 biovars), *B. melitensis* (3 biovars), *B. suis* (5 biovars), *B. ovis., B. canis*, *B. neotomae* (classification of first six species reviewed in Osterman and Moriyon, 2006)*, B. pinnipedialis (Foster et al.,* 2007)*, B. ceti* (Foster *et al.,* 2007) and *B. microti* (Scholz et *al*, 2008b) and *B. inopinata* (wound fluid from human) (Scholz *et al.,* 2009). The traditional and current classification of *Brucella* species is largely based on its preferred host, pathogenicity and phenotypic laboratory tests (biotyping) (Corbel and Brinley-Morgan*,* 1984; Alton *et al.,* 1988; Verger *et al.* 1985).

Bovine brucellosis is caused by *B. abortus* (8 biovars) principally affects cattle and other bovidae e.g. African buffalo (*Syncerus caffer*). Brucellosis in cattle is usually caused by *B. abortus* biovar (bv.) 1 being the most frequently isolated biotype worldwide (Table 2.1.). *Brucella melitensis* (3 biovars*)* affects goats but can also infect sheep and cattle whereas *B. canis* affects dogs and *B. ovis* affects sheep (Keid *et al*., 2007; Whatmore, 2009) (Appendix Table 1). *Brucella suis* has been reported to be the causative agent of brucellosis in suis, hares, reindeer and other no primary hosts like dogs, horses, humans and cattle (Alton, 1990; Davis, 1990; Ewalt *et al.,* 1997). *Brucella suis* is currently divided into 5 biovars. Biovars 1–3 infect suidae (Table 2.1) of which bv. 1 and 3 may cause severe disease in humans and require high biosafety laboratory precautions (Walker, 1999). *Brucella suis* bv. 4

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infect reindeers and caribous *(Rangifer tarandus)* throughout the Arctic region and can be transmitted to cattle, canidae and occasionally to humans (Forbes, 1991; Godfroid, 2002), whereas *B. suis* bv. 5 have been reported from rodents (Alton *et al.* 1988; Fretin *et al*. 2008). *Brucella abortus* and *B. suis* have also been isolated world-wide from variety of wildlife species namely African buffalo (*Syncerus caffer*), eland *(Taurotragus oryx)*, wild boar (*Sus scrofa*) and water buck (*Kobus elipsiprymnus*) (Condry and Vickers, 1969; 1972; 1976; Godfroid, 2002), while *B. melitensis* is rarely reported in wildlife with a few cases reported in Europe in chamois and ibex in the Alps (Godfroid, 2002).

Table 2.1: Currently described *Brucella* species and host associations.

Species	**Biovars described**	**Major host(s)**
B. abortus	1–6, (7)*, 9	Cattle
B. melitensis	1–3	Sheep, goats
B. suis	1–5	Pigs, hares, reindeer, rodents
B. canis	–	Dogs
B. ovis	–	Sheep
B. neotomae	–	Rodents
B. pinnipedialis	–	Seals
B. ceti	–	Dolphins, porpoises
B. microti	–	Voles
B. inopinata	–	Not known

* See Whatmore, 2009.

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2.2 *Geographical distribution*

Brucellosis has a worldwide distribution. The species and biovars have different distributions. *Brucella abortus* bv. 1 and 2 have a worldwide distribution, while bv. 3 is commonly found in India, Egypt and Africa (Walker, 1999), whereas *B. abortus* bv. 5 are common in Germany and United Kingdom (Walker, 1999).

Brucella suis has a global distribution with a relatively low prevalence in domestic pigs, except in South-East India and South America (Godfroid, 2002; Godfroid *et al.,* 2005). *Brucella suis* bv. 2 appear to be restricted to Europe where it is frequently isolated from wild boars with no or mild clinical signs (Godfroid, 2002; Lagier *et al.,* 2005). This biovar can also infect pigs and hares but seems unable to infect healthy humans (Godfroid, 2002; Lagier *et al.,* 2005). *Brucella suis* bv. 4 infect reindeers, caribous and can be transmitted to cattle, canidae and occasionally to humans (Forbes, 1991; Godfroid, 2002; Godfroid *et al.,* 2005). *Brucella suis* bv. 5 have been isolated from rodents in Eastern Europe and may cause diseases in humans as well (Alton *et al.,* 1988; Godfroid *et al.,* 2005; Fretin *et al*., 2008).

Brucella melitensis has a global distribution, but does not occur in North America, Australia and New Zealand. Apart from affecting goats and sheep it also affects camels, alpacas and llamas (WHO, 1986; Walker, 1999; Godfroid, 2002). Both *B. ovis* and *B. canis* have a worldwide distribution. *Brucella neotomae* is found in desert wood rat limited to Salt Lake Desert of Utah, USA (Walker, 1999; Whatmore, 2009).

The first confirmed report of bovine brucellosis in cattle was in 1913 in Zimbabwe (Bevan, 1931). Since then *B. abortus* bv. 1 has been mainly isolated from cattle

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(Mohan *et al.,* 1996; Motope *et al.,* 2009) and wildlife (Condy and Vickers, 1969; 1976) and *B. abortus* bv. 2 to a lesser extent in cattle (Matope *et al.,* 2009). *Brucella* antibodies have been detected in various wildlife species including waterbuck, buffalo, eland, giraffe and impala in Zimbabwe (Condy and Vickers, 1969; 1972; Madsen and Anderson, 1995; Madsen *et al.,* 1996). *Brucella melitensis* was believed to be absent from Zimbabwe until 1987 when it was diagnosed (Madsen, 1989) and recently an isolate from a goat was identified as *B. melitensis* bv. 1 (Matope *et al.,* 2009). Corbel (1997) indicated *B. abortus* in bovine, *B. melitensis* in capine and *B. ovis* in ovine from official brucellosis reports in animals in 1994 in Zimbabwe. Furthermore there has been no official report of *B. suis* in Zimbabwe (Corbel, 1997).

2.3 *Transmission*

Brucellosis is a disease which infects multiple species and found in many continents even in marine ecosystem (Bengis *et al*., 2002). Several factors such as livestock production type, herd size, interaction with wildlife, ecological and social-economic factors play significant roles in the epidemiology of brucellosis (Muma *et al*., 2007; Matope *et al*., 2010 a; b). Dissemination of *Brucella* can be by direct or indirect contact with infectious animals. The major source of exposure to *B. abortus* infected cattle and *B. meiltensis* infected sheep and goats are through aborted fetuses, placenta and post-abortion uterine fluid (Walker, 1999). *Brucella* infection is principally transmitted through contact with foetal membranes, lochia, post parturient discharges and milk (Godfroid *et al*., 2002; Gorvel, 2008). Vaginal secretions and milk also represent potential routes of animal-to-animal transmission following close contact and venereal transmission can occur particularly in swine, ovine and canine brucellosis (Walker, 1999; Godfroid *et al*., 2002). Animals shed *Brucella* organisms in

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their blood at the early stages of the infection (Cordes and Carter, 1979; Ilhan *et al*., 2008). *Brucella canis* organisms appear also in the early stages of infection and may last for one year or more (Wanke, 2004). Furthermore dissemination of *B. canis* to other dogs can occur through urine (Walker, 1999; Keid *et al.,* 2007).

Brucellosis is directly and indirectly transmitted from animals to humans. Consumption of contaminated foods or occupational exposure remains the major source of infection to humans. The disease is primarily recognized as an occupational hazard for veterinarians, farmers, laboratory technicians, slaughterhouse workers, and others who work with animals (maximum danger during calving/lambing/kidding period) and their products. Infection occurs through the skin (intact or abraded), inhalation or conjunctiva (Walker, 1999). The main source of infection for the public is through ingestion of contaminated dairy product. The bacteria can also be transmitted in raw or undercooked meat from infected animals.

The species generally considered pathogenic for humans in order of virulence are *B. melitensis*, *B. suis* and *B. abortus* (Baldwin and Goenka, 2006). The naturally occurring pathogenic 'rough' strains are not considered highly pathogenic for humans although occasional human infections with *B. canis* are reported and there are suggestions that cases may be underestimated (Lucero *et al*., 2005a; 2005b). *Brucella canis* and *B. ovis* are classified as rough species and the other species as smooth species because they possess the S-LPS.

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2.4 *Clinical signs*

The disease can persist for periods ranging from days to years since it is an intracellular organism. The non-classical structure of *Brucella* LPS has direct effects on intracellular bacterial survival as it lacks many of the pathogen-associated molecular patterns (PAMPs) that are typical for other proteobacteria (Gorvel, 2008). A number of pattern recognition receptors on macrophages and dendtric cells, such as Toll-like receptors (TLRs), detect potential pathogens and mount an immune response specific to the detected patterns (Gorvel, 2008). *Brucella* LPS is poor inducer of TLRs mediating signaling (Barquero-Calvo *et al*., 2007: Gorvel, 2008). Organism can enter and replicate in variety of cells including epithelial cells, placental trophoblasts, dendritic cells and macrophages (Gorvel, 2008). Differences in disease manifestations may be accounted for either by differences in the species or by differences in the host that can involve various body organs and tissues (Young, 1995).

Human brucellosis can be an acute or chronic disease with variable clinical manifestations (Pappas *et al.,* 2005). Disease can occur at any age and affect any organ system (Young, 1995; Pappas *et al.,* 2005). Signs and symptoms of the disease include: continued, intermittent, or irregular fever of variable duration, headache, weakness, chills, depression, weight loss and generalized body aches. Some of the uncommon signs include joint manifestations, particularly spondylitis, neurological complications associated not only with personality changes (anxiety, amnesia, delusions, hallucinations, delirium phobias and irritability), but also pronounced and prolonged frontal or occipital headaches, anorexia, abdominal pain and arthralgia (Acha and Szyfres, 1987).

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Brucella abortus: *Brucella abortus* infection can lead to abortions (normally in second half of gestation), stillbirths, weak calves, reduced fertility, weight loss and reduced milk yield compounding the economic significance of this pathogen (Anonymous, 2007; Gorvel, 2008). Chronic infection can result in hygroma and are regarded as highly indicative of infection. The course of brucellosis in cattle is governed primarily by the age of the animal when exposed to infection and to a lesser extent by the severity of the challenge in terms of numbers of organisms and their virulence. Prepubescent calves lose the infection once removed from the source of contamination (Walker, 1999). After puberty the chances of cattle becoming permanently infected increase since the *B. abortus* organism enters the body via a mucous membrane and then multiply in the local lymph node (Walker, 1999). A bacteriemia ensues and *B. abortus* colonize predilection sites such as mammary gland, testes seminal vesicles, joints, bursae, liver and spleen and associated lymph nodes (Walker, 1999; Ilhan *et al.,* 2008). It is only where a placenta exists (i.e. second half of pregnancy) that the uterus is invaded and the classical signs of brucellosis are seen. The incubation period is about 6 weeks, thus animals abort from the fifth to seventh month onwards. During abortion, large numbers of brucellae are released which may in turn, cause infection of other animals in the herd (McGiven *et al.,* 2003). In less acute infection, cattle may give birth to full term but weak calf, or merely show a retained placenta. A necrotic placentitis which may be acute or widespread is characteristic. Necrotic placentitis occur due to cells of the villi and the walls or crypts becoming swollen and through considerable leucocyte infiltration leading to necrosis. Symptoms of brucellosis are mostly pathogenic, during the acute stage of the disease and the organism can be

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chronically located in the supramammary lymphatic nodes and mammary glands of 80% of infected animals and they continue to secrete the *Brucella* organism in their body fluids (Ilhan *et al.,* 2008). Arthritis and lameness contribute to emaciation as observed in chronic bovine brucellosis (Moreno and Moriyo, 2006).

Brucella suis: Bacteria of this species typically cause chronic inflammatory lesions in the reproductive organs of susceptible animals that may extend to joints and other organs. The most prominent clinical sign is abortion at any stage of gestation (Walker, 1999; Olsen, 2004; Godfroid *et al*. 2005). *Brucella suis* biovar 1 infections have been reported in cattle but have partial induced pathology, no induction of abortion despite the excretion of organisms in the milk (Godfroid, 2002). Evidence indicates transmission of *B. suis* biovar 1 to cattle by feral swine in USA (Van der Leek *et al*., 1993; Bricker and Halling, 1994).

Brucella ovis: It is transmitted venereally (Walker, 1999; Godfroid, 2002) causing ram epididymitis. Blindness and neurological signs, thick-walled carpal joints and enlargement of the testicle characterized by necrosis and fibrosis have been seldom reported (Ferroglio *et al*., 2000: Garin-Bastuji *et al*., 1990). Some rams can shed *B. ovis* for long periods without apparent clinical lesions (Anonymous, 2007).

Brucella canis: The disease causes epididymitis and orchitis in male dogs, endometritis, placentitis and abortions in females, and often presents as infertility in both sexes (Keid *et al.,* 2007). Abortion between 45 and 59 days of gestation is the primary clinical sign reported in canine brucellosis (Keid *et al*., 2007). Non-reproductive lesions are less common and include inflammation in eyes and axial

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and appendicular skeleton, lymphadenopathy and splenomegaly (Keid *et al*., 2007). In immature and non-pregnant bitch's lymph node enlargement may be the only clinical sign (Moore and Gupta, 1970; Keid *et al*., 2007).

Brucella melitensis: *Brucella melitensis* mainly cause abortions, stillbirths or the birth of weak offspring. Sheep and goats usually abort only once following repermeation of the uterus and shedding can occur with successive pregnancies. Milk production is significantly reduced in animals that abort or in animals after normal birth (Anonymous, 2007). It causes epididymitis and orchitis in rams and also causes abortion in cattle that were in contact with infected sheep (Verger *et al*., 1989). When *B. abortus* is eradicated in cattle population *B. melitensis* or *B. suis* may infect cattle and the disease is indistinguishable from *B. abortus* infection (Corbel, 1997). Both *B. melitensis* and *B. suis* can infect cattle and spread within herds but rarely cause abortion (Bricker and Halling, 1994).

2.5 *Diagnosis*

Symptoms of brucellosis are not pathognomomic, hence diagnosis relying mostly on laboratory test (O'Leary *et al*., 2006; Leyla *et al*., 2003). Diagnosis of brucellosis in cattle is complicated by variable incubation time and the absence of clinical sings other than abortion (McGiven *et al*., 2003). Therefore diagnosis of brucellosis is still based on serological tests. Smear or culture can be made to confirm brucellosis as the cause of abortion. Smears of placenta, foetal stomach, or vaginal discharge are stained by modified Ziehl- Nelson or Koster methods and examined for the presence of brucellae organisms. However *Brucella* may be confused with Q- Fever organisms (*Coxiella burnetti*) using this method (Walker, 1999). The gold standard in brucellosis

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diagnosis remains the isolation of brucellae. The classical bacteriology identification of *Brucella* strains is based on phenotypic characteristics, i.e. agglutination with monospecific sera, CO_2 requirement, H_2S production, urea hydrolysis, inhibition of growth by basic fuchsin and thionin, and phage typing (Alton *et al.,* 1988; Appendix Table 1). However, it takes more than two weeks to culture the bacteria and complete 25 phenotypic traits used for species and biovar identification of cultured organisms (Alton *et al.,* 1988; Bricker, 2004; Whatmore, 2009). Care should be taken since bacteriology methods depend on the bacterial viability hence pose a biosecurity risk to laboratory personnel (Wallach *et al*., 2004).

Tissues for culture include foetal stomach contents or lung, placenta, vaginal discharge, milk and semen (Walker, 1999). At post mortem the best samples are mammary tissue and supramammary or iliac lymph nodes (Walker, 1999). However the absence of bacterial isolation does not rule out brucellosis since some serological positive animals yield negative culture results (Leal-Klevezas *et al.,* 1995; Godfroid, 2002).

2.5.1 *Serology*

Identification of *Brucella* is important for surveillance and eradication efforts. Due to the time consuming isolation, identification and bioyping of *Brucella* species ('gold standard') that takes more than two weeks days, serological test are commonly used (Jacobson, 1998; Gall and Nielsen, 2004). Serological techniques are therefore the basis of diagnosis and mass testing programs. The OIE (2008) indicates a combination of serological tests suitable for screening livestock for brucellosis. These serological tests include serum agglutination methods (SAT), rapid slide

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agglutination test (RSAT) (with or without 2-mercaptoethanol), tube agglutination test (TAT) (with or without 2-mercaptoethanol), CFT, BPAT, FPA and ELISA. The control and diagnosis of brucellosis in small ruminants and cattle requires the use of more than one serological test of which RBT, BPAT, FPA and ELISA are suitable screening test (OIE, 2008). The serological tests are genus and not species specific.

Serological tests are based on the detection of the LPS that is a dominant antigen in all smooth *Brucella* strains. The structure of the LPS in Brucella is conserved and the LPS of smooth *B.abortus, B melitensis and B. suis* strains all share common epitopes (Abdoel *et al*., 2008). The presence of S-LPS in the vaccine strains, *B. abortus* S19 and *B. melitensis* Rev1 may interfere with the discrimination between infected and vaccinated individuals and impair the test and slaughter strategy. *Brucella ovis and B. canis* are rough strains (Abdoel *et al*., 2008) and may require the use of a different antigen (Blasco, 1990; Carmichael and Shin, 1996; Abdoel *et al.,* 2008).

2.5.1.1 Rose Bengal Test (RBT)

RBT is an agglutination test where drops of stained antigen are mixed with serum and any agglutination signifies a positive reaction. RBT is often used as a first screening assay, because it is easy, quick and not expensive to perform (Madsen, 1989; Abdoel *et al*., 2008). Cross reaction with Gram negative bacteria (*Yesinia enterocolitica* 0:9, *Escherichia coli* 0:157, *Salmonella* spp occurs (WHO, 1986; Alton *et al*., 1988; Nielsen, 2002; Nielsen *et al.,* 2004). RBT antigen has been found to deteriorate when repeatedly cycled between refrigerator and room temperature during use (Gall and Nielsen, 2004). Gall and Nielsen (2004) reviewed the sensitivity

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and specificity of 12146 bovine samples tested using RBT that was 81% and 86%, respectively. Higher sensitivity and specificity for the RBT test has been reported in other studies (Nielsen *et al*., 1995, 1996; McGiven *et al*., 2003). However the specificity of RBT is low due to false positives (Madsen, 1986).

2.5.1.2 Complement fixation tests (CFT)

It is a precipitation test that consists of a complex series of proteins which, if triggered by an antigen-antibody complex react in a sequential manner to cause cell lysis. The CFT is highly sensitive and specific and it is accepted as confirmatory test for bovine brucellosis (Abdoel *et al*., 2008; OIE, 2008), but is labour intensive, difficult to standardize and produces anti-complimentary results which requires repeating the test that increases the cost (Samertino *et al*., 1999).

2.5.1.3 Enzyme-linked immunosorbent assay (ELISA)

Primary binding tests used in the diagnosis of brucellosis that are able to recognize all isotype antibodies of *Brucella* (Tizard, 1996). Examples of these assays are the indirect ELISA (iELISA) and competitive ELISA (c-ELISA). ELISA directly measures the interaction of antibody and antigen (Gall and Nielsen, 2004) and was developed to be more sensitive and specific than conventional tests. ELISAs have a distinct advantage over conventional serological tests since they do not depend on agglutinate or precipitation of antibodies (Gall *et al.,* 1998). Secondly, ELISAs can be customized to be more specific by using highly purified reagents such as antigens and monoclonal antibodies. The iELISA was introduced in 1976 for detection of antibody to *B. abortus* (Carlsson *et al.,* 1976). Samartino *et al.* (1999) indicated that the iELISA is a useful screening test in areas where vaccination with S19 is not

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mandatory and brucellosis prevalence is low. The iELISA is most specific and detects antibody, including IgG1 that is produced against other bacteria such as *Yesinia enterocolitica 0:9.* The iELISA cannot discriminate between brucellosis and other antibodies (Nielsen *et al*., 1996; Samartino *et al*., 1999; Godfroid *et al.,* 2002). The c-ELISA was reported to eliminate cross-reaction, although competitive antibodies of *Y. enterocolitica* O: 9 have been observed (Nelson, 1990). The use of the both ELISAs could help in improving the quality of results and reduce overall cost of diagnosis (Samartino *et al*., 1999). c-ELISA can distinguish vaccine and field strains. This test would be very useful and cost-effective in the diagnosis of bovine brucellosis in countries where vaccination is still mandatory (Samartino *et al*., 1999). The c-ELISA is best when testing poor quality serum samples (Stack *et al*., 1999; McGiven *et al.,* 2008). A new assay called the AlphaLISA was developed. The existing c-ELISA was converted to an AlphaLISA homogenous proximity based assay, which requires no separation steps (McGiven *et al*., 2008). AlphaElisa perform the same as established ELISAs, the elimination of coating and separation steps in the AlphaLISA reduces the opportunity for error and improves precision (McGiven *et al*., 2008).

2.5.1.4 Fluorescence Polarisation assays (FPA)

FPA is a primary binding assay that requires less incubation time and no serum dilution (McGiven *et al*., 2008). FPA has equal or greater diagnostic accuracy than the other primary binding assays (iELISA and c-ELISA) and is less costly (easier to perform and adaptable to automation). FPA requires two measurement of sample (pre- and post- antigen addition) (McGiven *et al*., 2008).

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2.5.2 PCR

Although identification of bacteria at the species level is usually sufficient for short-term health care response, the tracing of bacterial pathogens or the identification of emerging clones escaping prophylactic or therapeutic strategies require precise strain identification (fingerprinting) tools (Grissa *et al*., 2007). Bacterial culture and isolation remains the irrefutable gold standard for the diagnosis of *Brucella* spp. Currently the diagnosis of brucellosis is based primarily on serological tests (Godfroid *et al.,* 2002; Whatmore, 2009). Both bacteriology and serology methods have their limitations. There has been increased investigation in the use of sensitive, specific, rapid and relatively inexpensive method like PCR for the detection of *Brucella* antigen (O'Leary *et al*., 2006). PCR has several advantages over the current bacteriology methods for identification of *Brucella* spp. With PCR, as few as 10^4 bacteria can be added directly to the reaction mixture. Furthermore it is less expensive than conventional methods and is very amenable to automation and robotics that also reduce costs and contamination (Bricker and Halling, 1994). A major advantage of PCR is the speed of the assay since results can be obtained in less than a single day with minimal sample preparation (Bricker and Halling, 1994).

Various samples such as blood (Leal-Klevezas, 1995; Ilhan *et al.,* 2008), milk (Leal-Klevezas, 1995; Romero *et al.,* 1995b), lymphoid tissue (Gupta, 2006), stomach contents and aborted foetuses (Centinkaya *et al.,* 1999; Cortez *et al.,* 2001) can be used in PCR assays detecting *Brucella* antigens. The selection of regions or gene targets also influences the sensitivity of the PCR assay. Some of the PCR targets used for identifying *Brucella* spp. include:

1. IS711 (Ouahrani-Bettache *et al*., 1996)

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2.16S rRNA (Herman and De Ridder 1992; Romero *et al*., 1995a)

3. 31kDa outer membrane protein (*omp*) / BCSP-31 (Baily *et al*., 1995a; Gallien *et al*., 1998; Guarino *et al*., 2000; Sreevatsan *et al*., 2000; Cortez *et al*., 2001; O' Leary *et al.* 2006)

4. 43kDa *omp* (Fekete *et al*, 1990)

5. *Omp*2 gene (Leal-Klevezas *et al*., 1995a; 2000)

6. *AlkB* gene primers used in real time PCR (Probert *et al*., 2003)

Multilocus PCR based methods include:

1. SNP based typing to identify *Brucella* isolates up to species level (Gopaul *et al*., 2008).

2. AMOS-PCR based on IS711 element (Bricker and Halling, 1994; 1995)

2. Bruceladder (Garcia-Yoldi *et al*., 2006)

3. Modified Bruceladder (Huber *et al.,* 2009)

Simple genus-specific PCR assays have been investigated for screening livestock for brucellosis. The 43 kDa *omp* is a patented PCR based test that was used on aborted foetuses and maternal tissue with 98% sensitivity and 96% specificity (Fekete *et al*., 1992). However, the exact assay was never published due to the patent (Fekete *et al*., 1992; Bricker, 2004). Various other gene targets like the 16S rDNA (Herman and De Ridder, 1992; Cetinkaya *et al*., 1999; O'Leary *et al*., 2006), rRNA operon (Romero *et al*., 1995a; b), 16S-23S rRNA intergenic spacer region (Rijpens *et al*., 1996; Fox *et al* 1998), BCSP31 / 31 kDa omp (Baily *et al*., 1992; 1995a; Gallien *et al*., 1998; Guarino *et al*., 2000; Sreevatsan *et al*., 2000; Cortez *et al*., 2001; O' Leary *et al.* 2006) and omp2a and omp2b genes (Leal-Klevezas *et al*.,

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1995) on aborted foetuses, maternal tissue, cultures, milk and stomach contents (Centinkaya *et al*., 1999; Cortez *et al.,* 2001; O' Leary *et al.,* 2006) have been used. The detection levels of the mentioned PCR assays were similar to culture. The 16S-23S rRNA intergenic spacer region had no false positive results with *Ochrobactrum. anthropi* and could detect 2 CFU (colony forming units)/ml with aborted bovine foetal tissues and stomach contents (Cortez *et al*. 2001). The BCSP31 gene was applied to buffalo blood and found to be significantly less sensitive than either the ELISA or CFT assays (Guarino *et al*., 2000). The PCR assay that detect a homologous region in *omp*2a and 2b genes provide twice the number of target per bacterium and was applied to blood and found to be more sensitive than serology and culture (Leal-Klevezas *et al*.1995). PCR assays like 16S-23S rRNA intergenic spacer region, 31 kDa omp / BCSP31 gene and *omp*2a and *omp*2b genes have similar detection levels to culture results from infected tissue, milk and aborted foetus and are specific to *Brucella* spp.

Blood samples collected for serology are most often the only available sample for further analysis. Since *Brucella* spp. are facultative intracellular pathogens the inoculum found in the host with brucellosis is normally very low. Whole blood samples are mainly used in diagnostics of human brucellosis (Queipo-Ortuno *et al.,* 2008; Navarro *et al.,* 1999). Whole blood samples provide the maximum number of target bacteria, but PCR inhibitors like anticoagulants, haemoglobin, host DNA or other substances are also present in whole blood (Navarro *et al.,* 1999; 2002; Ilhan *et al.,* 2008). O'Leary *et al.* (2006) found that whole blood is not a good template for the detection of *B. abortus* n DNA in cattle. Greater sensitivity than whole blood was reported in serum samples (Zerva *et al.,* 2001) despite the fact that DNA in serum is

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presumable lower (Queipo-Ortuno *et al.,* 2008). O'Leary *et al.,* (2006) suggested using the buffy coat (white cell pellet) may be a better template since bacteria are taken up by macrophages and non-professional phagocytes. Blood PCR assays are therefore less sensitive than other samples since the stage of infection may influence the number and location of *Brucella* organisms in white blood cells and lymphoid tissue glands (bacteriemia may not be constant) (O'Leary *et al.,* 2006) or the extraction is not optimal (Queipo-Ortuno *et al.,* (2008). The latter might be crucial since bacterial DNA in host blood is present at low numbers and thus the DNA extraction must be maximal to increase the likelihood of detection (O'Leary *et al.,* 2006; Queipo-Ortuno *et al.,* 2008). Queipo-Ortuno *et al.* (2008) reported a variation in detection of *Brucella* DNA from spiked serum samples using PCR amongst seven commercial DNA extraction kits. Therefore optimization of DNA extraction could be an important factor in detection of *Brucella* DNA from blood. Other factors affecting the performance of both PCR and culture techniques include sample collection, storage and handling.

Multiplex PCR assays like AMOS PCR distinguish IS711 genetic elements of *B. abortus* (bv. 1, 2, and 4), *B. melitensis* (bv. 1, 2, and 3), *B. ovis*, and *B. suis* (bv. 1) (AMOS abbreviation based on the basis of the initials of the species that it identifies) (Halling *et al.,*1993; Bricker and Halling, 1994; 1995;) and Bruceladder (Garcia-Yoldi *et al*., 2006) enable differentiation of *Brucella* strains at the species level and could be used to differentiate vaccine strains from wild type field strains. The Bruceladder, a single tube PCR assay that can differentiate all six classical *Brucella* spp. from marine mammals isolates and the vaccine strains *B. abortus* RB51 and S19 as well as *B. melitensis* Rev 1, was recently described (García-Yoldi *et al*., 2007).

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Historically, the most widely used assay is AMOS-PCR (Whatmore, 2009). One of the major disadvantages of this PCR assay is that it fails to detect all *Brucella* species or all biovars of some species (Whatmore, 2009). In addition to the multiplex PCRs discussed above some single target PCRs have proven particularly useful. Of note here is the so-called *bp26* PCR which utilises a copy of IS*711* located downstream of the *bp26* gene in marine mammal isolates to distinguish marine mammal *Brucella* from those associated with terrestrial mammals (Cloeckaert *et al*., 2000). This is in fact the marker for marine mammal isolates utilised in 'Bruceladder' but has proven useful as a stand-alone assay to distinguish marine mammal *Brucella* in a number of studies and notably as an unexpected cause of human infection (Sohn *et al*., 2003; McDonald *et al*., 2006). A 19-primer multiplex PCR (modified Bruceladder) was developed which can distinguish all the *Brucella* species (Huber *et al*., 2009). The multiplex assay can identify *B. neotome, B pinnipedialis, B. ceti, B. microti* as well as differentiate *B. abortus* biovars 1, 2, 4 from biovars 3,5,6,9 and also *B. suis* biovar 1, biovars 3, 4 and biovars 2 and 5 (Huber *et al*., 2009).

The development of discriminatory molecular tools for identification and typing of *Brucella* has been problematic, reflecting the lack of genetic polymorphism in *Brucella*. A high degree of homology initially implied by DNA-DNA hybridization has been confirmed by a variety of approaches, such as multilocus enzyme electrophoresis (MLEE) and 16S rRNA sequencing (Gandara *et al.,* 2001; Al Dahouk *et al.,* 2007). Other molecular sub-typing systems include pulfied–field gel electrophoresis (PFGE) and insertional element characterization-systems also have difficulties in distinguishing between *B canis* and *B. suis* (Huynh *et al.,* 2007). These sequences were long considered highly conserved and have a high degree of

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genetic homology making identification and differentiation of *Brucella* spp. and biovars very challenging. Multiplex PCR assays have been developed that enable identification and differentiation of *Brucella* at the species / biovar level from culture or infected tissue.

The genome sequences of *B. melitensis* 16M and *B. suis* (Delvecchio *et al*., 2002; Paulsen *et al*., 2002) facilitated the search for DNA sequence variability that enables differentiation of *Brucella* strains by PCR. Hypervariability among short tandem repeat sequences has been exploited for strain-typing of several bacterial pathogens (Bricker *et al*., 2003). MLVA is a typing technique based on the polymorphism of certain tandemly repeated DNA sequences. VNTRs are found in all organisms and have diverse practical applications including strain identification in bacterial epidemiology. Tandem repeat sequences (VNTR) are useful markers, since multiple alleles can be present at a single locus of various strains and size differences are easily resolved by electrophoresis. Tandem repeats are often classified as microsatellites (repeat units up to 8 bp) and minisatellites (more than 8bp repeat units) and mutate at a higher rate than the rest of the genome (Vergnaud and Denoeud, 2000; Le Fleche *et al*., 2006). MLVA has become a powerful tool for distinguishing among closely related strains of organism because VNTR loci typically mutate independently of one another. In a typical MLVA assay, a few to more than twenty VNTRs, distributed over the entire bacterial genome, are analyzed, and a code corresponding to the number of repeats at each locus is determined. This code is easily stored into databases and can be used for strain clustering and epidemiological studies (Grissa *et al.,* 2007). MLVA has proven to be highly appropriate for the typing of pathogenic bacterial species with a high genetic

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homogeneity, including the *Mycobacterium tuberculosis* complex (Le Fleche *et al*., 2002), *Bacillus anthracis* (Keim *et al*., 2000; Le Fleche *et al*., 2001), and *Yesinia pestis* (Pourcel *et al*., 2004). . MLVA is best applied within a highly homogeneous group of strains, typically with genomes showing an average similarity well above 98%. Some MLVA assays have been developed in species with an internal genome homogeneity in the 95% to 98% range (as illustrated for instance in *Legionella pneumophila*; Pourcel *et al*., 2007).

Several loci containing tandem repeats were found in the *B. abortus* genome sequence (Halling *et al*. 2003) and these DNA sequences were compared with homologous sequences of published genome sequences of *B. suis* (Paulsen *et al*., 2002) and *B. melitensis* (Delvecchio *et al*., 2002). This led to the discovery of several VNTR loci. A subset of 16 VNTR loci was selected and applied to the typing of *Brucella* isolates (Le Fleche *et al.,* 2006; Al Dahouk *et al.,* 2007). With the analysis of the MLVA-16 assay the generated clusters correspond to the classical biotyping scheme of *Brucella* species. The 16 markers consist of 8 minisatellites markers that enable species identification and another group of 8 microsatellite markers with higher discriminatory power (Le Fleche *et al*., 2006; Al Dahouk *et al.,* 2007). The MLVA assay is rapid, highly discriminatory, and reproducible within animal *Brucella* isolates. MLVA can significantly contribute to epidemiological trace-back analysis of *Brucella* infections and may advance surveillance and control of human brucellosis (Marianelli *et al.,* 2007; Smits *et al.,* 2009; Whatmore, 2009). MLVA has a number of other technical advantages, most notably its relative simplicity, fast turnaround time, amenability to high-throughput approaches, applicability to nonviable and/or crude preparations, and the ability to easily compare digital results between laboratories

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(Le Fleche *et al.,* 2006). MLVA is replacing or at least complementing traditional genotyping methods.

2.6 *Control and prevention*

In order to control brucellosis, comprehensive surveillance, pre- and post import testing is of paramount importance (Corbel, 1997). Control of brucellosis is usually based on vaccination, serology testing and culling (Marin *et al*., 1999). Testing of livestock is cumbersome when dealing with farms located in remote areas or with animals from nomadic populations and migratory farmers. *Brucella abortus* is adapted to cattle as its primary host, and control strategies have focused on elimination of the disease from cattle populations. The identification of genus and species of field isolates will benefit brucellosis eradication programs (Bricker and Halling, 1994) since correct vaccination and control management will be possible. Many countries have implemented eradication programs resulting in the reduction or elimination of the disease, but the disease remains enzootic in many regions of the world. In those countries where the disease has been eradicated or strictly controlled, continued surveillance is essential to preventing re-emergence of the disease. Microbial genome typing or DNA fingerprinting is important for the delineation of outbreaks of infectious diseases and for the universal tracing of virulent or multi-resistant pathogens (van Belkum, 2003). It is equally important to determine by epidemiological trace-back analysis where the infection originated, how it was spread, and what measures are needed to prevent additional spread of the disease from this primary source. It is generally recognized that the prevention of human brucellosis is best achieved by control or eradication of the disease in animals but this strategy is not relevant for protection against a bioterrorist attack on

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military or civilian populations. Renewed interest in *Brucella* as a potential biological warfare agent has also drawn attention to the need for an effective vaccine for human use. A human vaccine could possibly be an effective countermeasure for prevention of naturally occurring or deliberately induced human infections (Corbel, 1997).

Currently, three vaccine strains (*Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1) are recommended by the World Organization for Animal Health (Office International des Epizooties—OIE) for use in the control of brucellosis in livestock (Garin-Bastuji and Blasco, 2008; Nielsen and Ewalt, 2008). Knowledge of the spread and prevalence of the infection is essential when planning control measures. It is generally acknowledged that all of the available brucellosis vaccines are only effective in specific hosts, and cross-protection is not readily achieved (Corbel, 1997). At present, no effective vaccine is available for protection of swine from brucellosis (Cutler and Commander 2005). Vaccination of sheep is by smooth *B.melitensis* Rev1 vaccine but does not provide 100% protection and it interferes with common serological test use in sheep (RBT and CFT) (Marin *et al*., 1999). *Brucella melitensis* Rev1 is one of the most commonly used attenuated live vaccines against caprine brucellosis and induces high level of protection in goat. Rev1 vaccine has suffered from a lack of coordinated standardization in production methods leading to considerable variability in efficiency of different preparations (Garcia-Yoldi,2007; Blasco, 1997) and carries resistance to streptomycin, an antibiotic that is therapeutically useful in man (Cloeckaert *et al*., 2002). Despite the availability of two smooth live vaccine strains, *B. abortus* S19 for cattle and *B. melitensis* Rev1 for small ruminants and a further rough attenuated strain, *B. abortus* RB51 for cattle, the

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search for improved vaccines has continued. The vaccine strains can cause abortion when administered to pregnant animals and is still fully virulent (Blasco, 1997; Ashford *et al*., 2004). There is a substantial economic burden of brucellosis reflected by the costs of attaining and maintaining disease free status, or the cost of disease in terms of loss of productivity and control costs (Whatmore *et al*., 2007).

2.7 References

Abdoel T., Dias I.T., Cardoso R., and Smits H.L. (2008). Simple and rapid field tests for brucellosis in livestock, *Veterinary Microbiology,* **130**: 312-219.

Acha P.N. and Szyfres B. (1987). Zoonoses and Communicable Diseases Common to Man and Animals 2nd edition. Pan American Health Organization, USA, 24-44.

Al Dahouk S., Le Flèche P., Nöckler K., Jacques I., Grayon M., Scholz H.C, Tomaso H., Vergnaud G. and Neubauer H. (2007). Evaluation of *Brucella* MLVA typing for human brucellosis. *Journal of Microbiological Methods*, **69**: 137-145.

Alton G. G. (1990). *Brucella suis*. In: Animal brucellosis (K. Nielsen and J. R. Duncan, eds.). CRC Press, 411-422.

Alton G., Jones L.M., Angus R.D. and Verger J.M. (1988). Techniques for the brucellosis laboratory, Institut National de la Recherche Agronomique, Paris, France, 81-134.

© University of Pretoria

Ashford D.A., Di Pietra J., Lingappa J., Woods C., Noll H., Neville B., Weyant R., Bragg S.L., Spiegel R.A., Tappero J. and Perkins B. A. (2004). Adverse events in humans associated with accidental exposure to the livestock brucellosis vaccine RB51. *Vaccine,* **22**: 3435-3539.

Baily G. G., Krahn J. B., Drasar B. S. and Stoker, N. G. (1992). Detection of Brucella melitensis and *Brucella abortus* by DNA amplification. *Journal of Tropical Medicine and Hygine,* **95**: 271–275.

Baldwin C.L. and Goenka R. (2006). Host immune responses to the intracellular bacteria *Brucella*: does the bacterium instruct the host to facilitate chronic infection, *Critical- Review Immunology,* **26**: 407–442.

Barquero-Calvo E., Chaves-Olarte E., Weiss D.S., Guzman-Verri C., Chaco´n-Dı´az C., Rucavado A., Moriyo´n I. and Moreno E. (2007). *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection, PLoS ONE, **2**: e631.

Bengis R.G., Kock R.A. and Fischer J. (2002). Infectious animal diseases: the Wildlife/livestock interface. *Office of International Epizootics,* **21**: 53–65.

Blasco J. M. (1997). A review of the use of *Brucella melitensis* Rev. 1 vaccine in adult sheep and goats. *Preventive Veterinary Medicine*, **31**: 275-283.

© University of Pretoria

Blasco J.M. (1990). *Brucella ovis*. In: Animal Brucellosis (K. Nielsen and J.R. Duncan, eds.). CRC Press, 351–378.

Bricker B.J. (2004). Molecular Diagnostics of Animal Brucellosis: A Review of PCR-Based Assays and Approaches. In: *Brucella* Molecular and Cellular Biology (I.Lopez-Goni and I. Moriyon eds.). Horizon Bioscience, England, 25-51.

Bricker B.J, Ewalt D.R. and Halling S.M. (2003). *Brucella* (HOOF-prints: strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BioMed Central Microbiology*, **3**:15.

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

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

Carlsson H. E., Hurvell B. and Lindberg A.A. (1976). Enzyme linked immunosorbent assay (ELISA) for titration of antibodies against *Brucella abortus* and *Yersinia enterocolitica*. *Acta Patholology and Microbiology*. Scandanivia, **84**:168–176.

Carmichael L.E. and Shin S.J. (1996). Canine brucellosis: a diagnostician's dilemma. *Seminar. Veterinary Medicne Surgery. (Small Animal),* **11**:161–165.

© University of Pretoria

Cetinkaya B., Ongor H., Muz A., Ertas H. B., Kalender H. and Erdogan H. M. (1999). Detection of *Brucella* species DNA in stomach content of aborted sheep foetuses by PCR. *Veterinary Records*, **144**: 239-240.

Cloeckaert A. and Vizcaino N. (2004). DNA polymorphism and taxanomy of *Brucella* species. In: *Brucella* molecular and cellular biology (I. Lopez- Gani and Moriyon I. eds.). Horizon Bioscience, England.

Cloeckaert A., Vizcaino N., Paquet J-Y., Bowden R. A. and Elzer P. H. (2002). Major outer membrane proteins of *Brucella* spp.: past, present and future. *Veterinary Microbiology*, **90**: 229-247.

Cloeckaert A., Verger J.M., Grayon M. and Grépinet O. (2000) An IS*711* element downstream of the *bp26* gene is a specific marker of *Brucella* spp. isolated from marine mammals, *Clinical Diagnostics and Laboratory Immunology,* **7**:835–839.

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

Corbel M. J. (1997). Brucellosis: an overview. *Emerging Infectious Disseases,* **3**: 213-221.

© University of Pretoria

Corbel M.J. and Brinely-Morgan W.J. (1984). Genus *Brucella* Meyer and Shaw 1920, p 377-388. In: Bergey's manual of systematic bacteriology*, vol. 1* (.W. R. Krieg and J. G. Holt eds.). The Williams and Wilkins Co., Baltimore, USA.

Cordes D.O. and Carter M.E. (1979). Persistency of Brucella abortus infection in six herds of cattle under brucellosis eradication. *New Zealand Veterinary Journal*, **27**: 255–259.

Cortez A., Scarcelli E., Soares R.M., Heinemann M.B., Sakamoto S.M., Genovez M.E., Ferreira F. and Richtzenhain L.J. (2001). Detection of *Brucella* DNA from aborted bovine foetuses by polymerase chain reaction. *Australian Veterinary Journal,* **79**: 500-501.

Cutler S J. and Commander N.J. (2005). Brucellosis new aspects of an old disease. *Journal of Applied Microbiology,* **98**:1270-1281.

Cutler S. and Whatmore A. (2003). Progress in understanding brucellosis. *Veterinary Records*, **153**: 641–642.

Davis D.S. (1990). Brucellosis in wildlife.In Animal brucellosis (K. Nielsen and R.J. Duncan, eds). CRC Press, Florida, 321-334.

DelVecchio V.G., Kapatral V., Redkar R.J., Patra G., Mujer C., Los T., Ivanova N., I. Anderson I., Bhattacharyya A., Lykidis A., Reznik G., Jablonski L., Larsen N., D'Souza M., Bernal A., M. Mazur M., Goltsman E., Selkov E., Elzer P.H., Hagius S.,

© University of Pretoria

O'Callaghan D., Letesson J.J., Haselkorn R., Kyrpides N. and R. Overbeek (2002). The genome sequence of the facultative intracellular pathogen *Brucella melitensis*, *Proceedings of the National Academy of Sciences, USA*, **99**: 443–448.

DelVecchio V.G., Kapatral V., Elzer P., Patra G. And Mujer C.V. (2001). The genome of *Brucella melitensis. Veterinary Microbiology*, **90**: 587-592.

Ewalt D.R., Payeur J.P., Rhyan J.c. and Geer P.L. (1997). *Brucella suis* biovars 1 in naturally infected cattle: a bacteriology, serological and histological study, *Journal of Veterinary Diagnostics and Investigations*, **9**:417-420.

Fekete A., Bantle J.A. and Halling S. M. (1992). Detection of *Brucella* by polymerase chain reaction in bovine fetal and maternal tissue. *Journal of Veterinary Diagnostics and Investigations,* **4**: 79-83.

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

Ferroglio E., Rossi L. and Gennero S. (2000). Lung-tissue extracts as an alternative to serum for surveillance fro brucellosis in chamois, *Preventive Veterinary Medicine*, **43**:117-122.

Forbes L. B. (1991). Isolates of *Brucella suis* biovar 4 from animals and humans in Canada, 1982– 1990. *Canadian Veterinary Journal,* **32**: 686–688.

© University of Pretoria

Foster G., Osterman B.S., Godfroid J., Jacques I. and Cloeckaert A. (2007). *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts, *International Journal Systemic Evolution and Microbiology,* **57**: 2688–2693.

Fox K. F., Fox A., Nagpal M., Steinberg P. and Heroux K. (1998). Identification of *Brucella* by ribosomal spacer region PCR and differentiation of *Brucella canis* from other *Brucella* spp. pathogenic for humans by carbohydrate profiles.*Journal of Clinical Microbiology*, **36**: 3217-3222.

Fretin D., Whatmore A.M., Al Dahouk S., Neubauer H., Garin-Bastuji B., Albert D., Van Hessche M., Ménart M., Godfroid J., Walravens K. and Wattiau P. (2008). *Brucella suis* identification and biovar typing by real-time PCR, *Veterinary Microbiology,* **131**: 376–385.

Gall D., Colling A., Marino O., Moreno E., Nielsen K., Perez B. and Samartino L. (1998). Enzyme immunoassays for serological diagnosis of bovine brucellosis: a trial in Latin America. *Clinical Diagnosis and Laboratory Immunology*, **5**: 654-661.

Gallien P., Dorn C., Alban G., Staak C. and Protz D. (1998). Detection of Brucella species in organs of naturally infected cattle by polymerase chain reaction. *Veterinary Record*, **142**: 512–514.

Gall D. and Nielsen K. (2004). Serological diagnosis of bovine brucellosis: A review of test performance and cost comparison. *Reveiw.-Office of International Epizootics,* **23**: 989-1002.

© University of Pretoria

Gandara B., Merino A.L., Rogel A. and Martinez-Romero E. (2001). Limited Genetic Diversity of *Brucella* spp. *Journal of Clinical Micobiology,* **39**: 235-240.

García-Yoldi D., Marín C.M., de Miguel M.J., Muñoz P.M., Vizmanos J.L. and López-Goñi I. (2006). Multiplex PCR Assay for the identification and differentiation of all *Brucella* species and the Vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clinical Chemistry,* **52**: 779-781

García-Yoldi D., Le Fleche P., Marín C.M., De Miguel M.J., Muñoz P.M., Vergnaud G. and I. López-Goñi I. (2007). Assessment of genetic stability of *Brucella melitensis* Rev 1 vaccine strain by multiple-locus variable-number tandem repeat analysis, *Vaccine* **25**: 2858–2862.

García-Yoldi D., Le Fleche P., De Miguel M.J., Muñoz P.M., Blasco J.M., Cvetnic Z., Marín C.M., Vergnaud G.and López-Goñi I. (2007). Comparison of multiple-locus variable-number tandem-repeat analysis with other PCR-based methods for typing *Brucella suis* isolates, *Journal of Clinical Microbiology,* **45**: 4070–4072.

Garin-Bastuji B. and Blasco J.M. (2008). Chapter 2.7.2. Caprine and ovine brucellosis (excluding *Brucella ovis*). In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (6th ed.), OIE, Paris, France: 974–982.

Garin-Bastuji B., Ondar J., Richard Y. and Gastillin J. (1990). Isolation of Brucella melitensis biovars 3 from a chamois (*Rupicapra rupicapra*) in the southern French Alps, *Journal of Wildlife Diseases*, **26**:116-118.

© University of Pretoria

Gee E.J., De Barun K. B., Levett P.N., Whitney M.A., Novak T.R. and Popovic T. (2004). Use of 16S rRNA Gene Sequencing for Rapid Confirmatory Identification of *Brucella* Isolates, *Journal of Clinical microbiology*, **42 (8)**: 3649–3654.

Godfroid J. (2002). Brucellosis in wildlife. *Revue Scientifique Et Technique De Office International Des Epizooties*, **21**: 277-286.

Godfroid J., Saegerman C., Wellemans V., Walravens K., Letesson J.J., Tibor A., McMillan A., Spencer S., Sanna M., Baller D., Pouillot R. and Garin-Bastuji B. (2002). How to substantiate eradication of bovine brucellosis when aspecific serological reactions occur in the course of brucellosis testing. *Veterinary Microbiology,* **90**: 461-447.

Godfroid J., Cloeckaert A., Liautard J-P., Kohler S., Fretin D., Walravens K., Garin-Bastujie B. and Letesson J.J. (2005). From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Veterinary Research,* **36**: 313-326.

Gopaul K.K., Koylass M.S., Smith C.J. and Whatmore A.M. (2008). Rapid identification of *Brucella* isolates to the species level by real time PCR based single nucleotide polymorphism (SNP) analysis, *BioMed Central Microbiology,* **8**: 86.

Gorvel J.P. (2008). *Brucella*: a Mr "Hide" converted into Dr Jekyll, *Microbes and Infections,* **10**: 1010–1013.

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Grissa I.P., Bouchon P., Pourcel C. and Vergnuad G. (2007).On line resources for bacterial micro-evolution studies using MLVA or CRISPR typing. *Biochimie*, **90**: 660-668.

Groussaud P., Shankster S.J., Koylass M.S.and Whatmore A.M. (2007). Molecular typing divides marine mammal strains of *Brucella* into at least three groups with distinct host preferences, *Journal of Medical Microbiology.* **56**: 1512–1518.

Guarino F., Serpe L., Fusco G., Scaramuzzo A. and Gallo P. (2000). Detection of *Brucella* species in buffalo whole blood by gene-specific PCR. *Veterinary Records*, **147**: 634-636.

Gupta V.K., Verma D.K., Singh K., Kumari R., Singh S.V. and Viham V.S. (2006). Single-step PCR for detection of Brucella melitensis from tissue and blood of goats. *Small Ruminants Research*, **66**: 169–174.

Halling S. M., Tatum F. M. and Bricker B. (1993). Sequence and characterization of an insertion sequence IS711, from *Brucella ovis*. *Gene*, **133**: 123-127.

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

Her M., Kang S., Cho D., Cho Y., Hwang I., Heo Y., Jung S. and Yoo H. (2009). Application and evaluation of the MLVA typing assay for the *Brucella abortus* strains isolated in Korea, *BioMed Central Microbiology,* **9**:230.

© University of Pretoria

Hinić V., Brodard I., Thomann A., Cvetnić Z., Makaya P.V., Frey J.and Abril C. (2008). Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems, *Journal of Microbiological Methods,* **75**: 375–378.

Hirsh C. D and Zee C.Y. (1999).Veterinary Microbiology, Blackwell Science, incorporation: **196**-203.

Huber B., Scholz C.H., Lucero N. and Busee H. (2009). Development of a PCR assay for typing and subtyping of Brucella species. *International Journal of Medical microbiology*, **288(8**): 563-573.

Huynh Y.L., Van Ert N.M., Hafield T., Prbert S.W., Bellaire H.B., Dobson M., Burgess J.R., Weyant S.R., Popovic T., ZaneckiS., Wagner M.D. and Keim, P. (2007). Multiple locus Variable Number Tandem Repeat (VNTR) Analysis (MLVA) of *Brucella* spp.Identifies Species-Specific Markers and Insights into Phylogenetic Relationships In: National Institute of Allergy and infectious Diseases, NIH Volume 1 Frontiers in Research (V.S. Georgiev, K.A. Western and J.J. McGowan, eds). Humana Press USA, Chapter 5, 47-54.

Ilhan Z., Aksakal A., Ekin I.H., Gulhan T., Solmaz H. and Erdenlig S. (2008). Comparison of culture and PCR for the detection of *Brucella melitensis* in blood and lymphoid tissues of serologically positive and negative slaughtered sheep. *Applied Microbiology,* **46**: 301-306.

© University of Pretoria

Jacobson R. H. (1998). Validation of serological assays for the diagnosis of infectious diseases. In: Veterinary laboratories for infectious diseases (J.E. Pearson, ed.). *Revue Scientifique Et Technique D'Office International Des Epizooties,* **17**: 469-486.

Keid L.B., Soares R.M., Vasconcellos S.A., Chiebao D.P., Salgado V.R., Megid J. and Richtzenhain L.J. (2007). A polymerase chain reaction for detection of *Brucella canis* in vaginal swabs of naturally infected bitches. *Theriogenology,* **68**: 1260–1270.

Keim P., Price L.B., Kleyvtska A.M., Smith K.L., Schupp J.M., Okinaka R., Jackson P.J. and Hugh-Jones M.E. (2000). Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *Journal of Bacteriology,* **182**: 2928-2936.

Koylass S.M., King C.A., Edwards-Smallbone J., Gopaul K. K., Perret L.L. and Whatmore M.A. (2010). Comparative performance of SNP typing and Bruce-Ladder in the discrimination of *Brucella suis* and *Brucella canis*, *Veterinary Microbiology,* **142**:450-454.

Lagier A., Brown S., Soualah A., Julier I., Tourrand B., Albert D., Reynes J. and Garin-Bastuji B. (2005). Brucellose aigue¨ a` B.suis biovar 2 chez un chasseur de sanglier. *Medecine et Maladies Infectieuses,* **35**: 185.

Le Fleche P., Jacques I., Grayon M., Dahouk S. A., Bouchoni P., Denoeud F., Nockler K., Neubauer H., Guilloteau L. A., and Vergnaud G. (2006). Evaluation and

© University of Pretoria

selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BioMed Central Microbiology,* **6**:9.

Le Flèche P., Fabre M., Denoeud F., Koeck J.L., and Vergnaud G. (2002). High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. *BioMed Central Microbiology,* **2**:37.

Le Flèche P., Hauck Y., Onteniente L., Prieur A., Denoeud F., Ramisse V., Sylvestre P., Benson G., Ramisse F. and Vergnaud G. (2001). A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BioMed Central Microbiology,* **1**:2.

Leal-Klevezas D. S., Martinez-Vazques I. O., Lopez-Merino A. and Martinez-Soriano J. P. (1995). Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *Journal of Medical Microbiology,* **33**: 3087-3090.

Leyla G., Kadri G. and Umran O. (2003). Comparison of polymerase chain reaction and bacteriological culture for the diagnosis of sheep brucellosis using aborted fetus samples, *Veterinary Microbiology*, **93**: 53–61.

Lucero N.E., Escobar G.I., Ayala S.M. and Jacob N. (2005a).Diagnosis of human brucellosis caused by *Brucella canis*, *Journal of Medical Microbiology,* **54**: 457–461.

Lucero N.E., Jacob N.O., Ayala S.M., Escobar G.I., Tuccillo P. and Jacques I. (2005b). Unusual clinical presentation of brucellosis caused by *Brucella canis*, *Journal of Medical Microbiology,* **54**: 505–508.

© University of Pretoria

Lucero N.E., Greco G. and Carrete P.A. (1999).*Brucella melitensis* biovar 3 in Argentina? *Review- Argentina Microbiology,* **31 (suppl)**: 58–59.

MacFarlane D., Salibury R.M., Osborne H.G. and Jebson J.L (1952).Investigation into sheep abortion in New Zealand during the 1950 lambing season. *Austration Veterinary Journal,* **28**:221-226.

Matope G., Bhebhe E., Muma J.B., Lund A., and Skjerve E. (2010a).Risk factors for Brucella spp. infection in smallholder household herds, *Epidemiology and Infectious Diseases*, 1-8.

Matope, G., Bhebhe, E., Muma, J.B., Lund, A. and Skjerve, E. (2010b). Herd-level factors for Brucella seropositivity in cattle reared in smallholder dairy farms of Zimbabwe. *Preventive Veterinary Medicine,* **94**:213–221.

Matope G., Bhebhe E., Muma J.B., Skjerve E. and Djønne B. (2009). Characterization of some *Brucella* species from Zimbabwe by biochemical profiling and AMOS-PCR. *BMC Research Notes*, **2:**261.

Marı´n C. M., Moreno E., Moriyo´n I., Dı´az R. and Blasco J. M. (1999). Performance of competitive and indirect enzyme-linked immunosorbent assays, gel immunoprecipitation with native hapten polysaccharide, and standard serological tests in diagnosis of sheep brucellosis. *Clinical Diagnostics and Laboratory Immunology,* **6**:269–272.

© University of Pretoria

Maquart M., LeFlech P., Foster G., Tryland M., Ramisse F., Djonne B., Al Dahouk S., Jacques I., Neubauer H., Walravens K., Godfroid J., Cloeckaert A. and Vergnaud G. (2009).MLVA-16 typing of 295 marine mammal Brucella isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis*, *BioMed Central Microbiology,* **9**:145.

Mayer-Scholl A., Draeger A., Göllner C., Scholz C,H., Nöckler K. (2010). Advancement of a multiplex PCR for the differentiation of all currently described Brucella species, *Journal of Microbiological Methods*, **80**: 112–114

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

McDonald W.L., Jamaludin R., Mackereth G., Hansen M., Humphrey S., Short P., Taylor T., Swingler J., Dawson C.E., Whatmore A.M., Stubberfield E., Perrett L.L. and Simmons G. (2006). Characterization of a *Brucella* sp. strain as a marine-mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand, *Journal of Clinical Microbiology,* **44**: 4363–4370.

McGiven J. A., Sawyer J., Perrett L.L., Brew S. D., Commander N. J., Fisher A., McLarnon S., Harper K. and Stack J. A. (2008). A new homogeneous assay for high throughput serological diagnosis of brucellosis in ruminants, *Journal of Immunological Methods,* **337 (1)**: 7-15.

McGiven J.A., Tucker J.D., Perrett L.L., Stack J.A. Brew S.D. and MacMillan A.P. (2003). Validation of FPA and cELISA for the detection of antibodies to *Brucella*

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abortus in cattle sera and comparison to SAT, CFT and iELISA. *Journal of Immunological. Methods*, **278**: 171-178.

Moore J.A. and Gupta B.N. (1970). Epizootiology, diagnosis, and control of *Brucella canis. Journal of American Veterinary Medicine Association,* **156**:1737–40.

Muma J.B., Munyeme M., Samui K.L., Skejerve E. and Oloya B.C. (2007). Risk factors for brucellosis in indigenous cattle reared in livestock–wildlife interface areas of Zambia. *Preventive Veterinary Medicine*, **80**: 306–317.

Navarro E., Escribano J., Ferna¤ndez J.A. and Soler J. (2002). Comparison of three different PCR methods for detection of Brucella spp. in human blood samples, *FEMS Immunology and Medical Microbiology,* **34**: 147-151.

Nielsen K. and Ewalt D.R. (2008). Chapter 2.4.3. Bovine Brucellosis, *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (6th edition), OIE, Paris, France, pp.624–660.

Nielsen K., Smith P., Widdison J., Gall D., Kelly L., Kelly W. and Nicoletti P. (2004). Serological relationship between cattle exposed to *Brucella abortus*, *Yersinia enterocolitica* O: 9 and *Escherichia coli* O157: H7. *Veterinary Microbiology*, **100**: 25-30.

Nielsen K. (2002). Diagnosis of brucellosis by serology. *Veterinary Microbiology,* **90**: 447–459.

© University of Pretoria

Nielsen K., Gall D., Jolley M., Leishman G., Balsevicius S.,Smith P., Nicoletti P. and Thomas F. (1996). A homogeneous fluorescence polarisation assay for detection of antibody to Brucella abortus, *Journal of Immunological Methods,* **195**: 161.

Nielsen K., Kelly L., Gall D., Nicoletti P. and Kelly W. (1995). Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. *Veterinary Immunology and Immunopathology*, **46 (3-4):** 285-291.

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2008). Chapter 2.4.3.Bovine Brucellosis. OIE, Paris, 409–438.

Olsen S. (2004). Porcine brucellosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, Paris, France.

O'Leary S., Sheahan M. and Sweeney T. (2006). *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows, *Research in Veterinary Science,* **81**: 170–176.

Osterman B. and Moriyón I. (2006). International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of *Brucella*. Report of the meeting, 17 September 2003, Pamplona, Spain, *International Journal of System Evoution and Microbioogy,* **56**: 1173–1175.

© University of Pretoria

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

Pappas A., Papadimitriou P., Akritidis N., Christou L. and Tsianos E.V. (2006). The new global map of human brucellosis. *Lancet Infectious Diseases,* **6**: 91–99.

Pappas G., Akritidis A., Bosilkovski M. and Tsianos E. (2005). Brucellosis. *New England Journal of Medicine*, **352**: 2325-2336.

Paulsen I.T., Shadri R., Nelson K.E., Eisen J.A., Heidelberg J.F., Read T.D., Dodson R.J., Umayam L., Brinkac L. M, Beanan M. J., Daugherty S.C. and Deboy R.T. (2002). The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proceedings of the National Academy of Sciences,*
USA, **99**: 13148-13153.

Pourcel C., Andre-Mazeaud F., Neubauer H., Ramisse F. and Vergnaud G. (2004). Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BioMed Central Microbiology,* **4:**22.

Probert W.S., Schrader K.N., Khuong N.Y., Bystrom S.L. and Graves M.H. (2004). Real-time multiplex PCR assay for detection of Brucella spp., *B. abortus*, and *B. melitensis. Journal of Clinical Micobiology,* **42**: 1290–1293.

© University of Pretoria

Rijpens N.P., Jannes G., Van Asbroeck M., Rossau R. and Herman L. M. (1996). Direct detection of *Brucella* spp in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. *Applied Environmental Microbiology,* **62**: 1683-1688.

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

Romero C. Pardo M., Grillo M.J., Diaz R., Biasco J.M., and Lopez-Goni I. (1995b). Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *Journal of Clinical Micobiology,* **33**: 3198-3200.

Samartino L., Gall D., Gregoret R. and Nielsen K. (1999). Validation of enzyme-linked immunosorbent assays for the diagnosis of bovine brucellosis. *Veterinary Microbiology*, **70 (3-4)**:193-200.

Stack J.A., Perrett L.L., Brew S.D. and MacMillan A.P. (1999). Competitive ELISA for bovine brucellosis suitable for testing poor quality samples. *Veterinary Records,* **145**: 735.

Scholz H.C., Hubalek Z., Nesvadbova J., Tomaso H., Vergnaud G., Flèche P., Whatmore M.A., Al Dahouk S., Lodri C. K. M. and Pfeffer, M. (2008). Isolation of *Brucella microti* from Soil, *Emerging Infectious Diseases*, **14 (8)**: 1317.

Sohn A.H., Probert W.S., Glaser C.A, Gupta N., Bollen A.W., Wong J.D., Grace E.M. and McDonald W.C. (2003). Human neurobrucellosis with intracerebral granuloma

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caused by a marine mammal *Brucella* spp, *Emerging Infectious Diseases,* **9**: 485–488.

Sreevatsan S., Bookout J.B., Ringpis F., Perumaalla V.S., Ficht T.A., Adams G.L., Hagius S.D., Elzer P.H., Bricker B.J., Kumar G.K., Rajasekhar M., Isloor S. and Barathur R.R.(2000). A multiplex approach to molecular detection of Brucella abortus and/or Mycobacterium bovis infection in cattle. *Journal of Clinical Microbiology,* **38**: 2602–2610.

Ten Queipo-Ortuño M. I., Tena F., Colmenero J. D. and Morata P. (2008). Comparison of seven commercial DNA extraction kits for the recovery of Brucella DNA from spiked human serum samples using real-time PCR. European Journal of Clinical Microbiology and Infectious Diseases, **27(2)**:109-114.

Wanke M.M. (2004). Canine brucellosis, *Animal Reproduction Science,* **82–83:**195–207.

Walker, R.L. (1999). Brucella. In: *Veterinary Microbiology* (D.C. Hirsh and Y.C Zee, eds.). Blackwell Science Inc., Massachusetts, USA, pp196-203.

Wallach J.C., Giambartolomei G.H., Baldi P.C. and Fossati C.A. (2004). Human infection with M-strain of *Brucella canis*. *Emerging Infectious Disease*, **10:** 146–148.

Weynants V., Tibor A., Denoel P.A., Saegerman C., Godfroid J., Thiange P. and Letesson J.J., (1996). Infection of cattle with Yersinia enterocolitica O: 9 a cause of

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the false positive serological reactions in bovine brucellosis diagnostic tests. *Veterinary Microbiology*, **48**: 101–112.

Whatmore A. M. (2009). Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens ·FAO/WHO Collaborating Centre for Brucellosis, OIE Brucellosis Reference Centre, Veterinary Laboratories Agency, Addlestone, Surrey KT15 3NB, United Kingdom.

Whatmore A.M., Perrett L.L. and Macmillan A.P. (2007). Characterisation of the genetic diversity of *Brucella* by multilocus sequencing, *BioMed Central Microbiology,* **7**: 34.

Van Belkum A. (2007). Tracing isolates of bacterial species by multilocus variable number of tandem repeat analysis (MLVA), *Federation of European Microbiological Societies, Immunological Medical Microbiology*, **49**:22-27.

van der Leek M. L., Becker H. N., Humphrey P., Adams C. L., Belden R. C., Frankenberger W. B. and Nicoletti P. L. (1993). Prevalence of Brucella sp. antibodies in feral swine in Florida, *Journal of Wildlife Diseases*, **29**:410-415.

Verger J. M., Garin-Bastuji B., Grayon M., and A. M. Mahe´. (1989). La brucellose bovine a` *Brucella melitensis* en France. *Annales de Recherches Veterinaires*, **20**:93–102.

Verger J. M. (1985). *B. melitensis* infection of cattle, p. 197–203. In: *Brucella melitensis* (J. M. Verger and M. Plommet, eds.). Martinus Nijhoff, Dordrecht, The Netherlands.

© University of Pretoria

Verger J.M., Grimont F., Grimont P.A.D and Grayson M. (1985). *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *International Journal of Systemic Bacteriology*, **35**:292-295.

Young E.J. (1995). An overview of Human Brucellosis. *Clinical Infectious Diseases,* **21:** 283-289.

Zerva L., Bourantas K., Mitka S., Kansouzidou A. and Legakis N.J. (2001). Serum Is the Preferred Clinical Specimen for Diagnosis of Human Brucellosis by PCR. Journal of Clinical Microbiology, **39(4)**: 1661–1664.

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CHAPTER 3

DETECTION OF BRUCELLOSIS IN CHIREDZI DISTRICT IN ZIMBABWE.

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ABSTRACT

Brucellosis is an endemic disease in Zimbabwe caused by the genus *Brucella.* *Brucella abortus* biovar (bv.) 1 has been reported in cattle and wildlife in this country. A survey of brucellosis using classical serological and bacteriology methods was conducted in wildlife in Gonarezhou National Park (GNP) and cattle in Chiredzi district bordering GNP. Additionally the 16-23S intergenic spacer (ITS) region specific to *Brucella* was used as a PCR assay on DNA extracted from blood and cultures to determine whether PCR can be used to confirm the serological and bacteriology test results. The rose bengal test (RBT) and indirect enzyme linked immunoassays (iELISA) serological tests of bovine serum show that 8.3% of the bovine tested were seropositive for *Brucella* spp. antibodies in the Chiredzi district. No *Brucella* antibodies were found in African buffalo and impala in GNP that borders the Chiredzi district using RBT and iELISA. *Brucella abortus* was isolated from blood and milk collected from seropositive cows. The *Brucella* specific 16-23S ITS PCR assay detected *Brucella* DNA in the two cultures, but not from DNA extracted from serum and Buffy coat. This indicates that the PCR assay is not sensitive enough to

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identify brucellosis from blood samples in cattle since various samples were seropositive for RBT, serum agglutination tests (SAT) and iELISA. Brucellosis was not detected in sampled wildlife in GNP whereas 8.3% prevalence of bovine brucellosis was detected in communal cattle in the Chiredzi district. Public awareness to the communities in the Chiredzi district should be increased to reduce the risk of human exposure to *Brucella* infection.

INTRODUCTION

Brucellosis is a bacterial contagious disease of great economic importance affecting animal health and production, in addition to its zoonotic importance affecting public health (WHO, 1998; Godfroid *et al*., 2005; Pappas *et al*., 2005). Brucellosis is transmitted among livestock and wild life primarily through contact with aborted fetal membranes and postparturient discharges, milk and during mating (Godfroid, 2002). This disease cause late-term abortions, birth of weak calves with poor survival rate, testicular infections, arthritis and lameness are some of the clinical symptoms observed in wildlife and livestock (Davis, 1990; Bischop *et al.,* 1994). Based on pathogenicity and host preference, six classical species are recognized namely *Brucella abortus, B. melitensis, B. suis, B. canis* and *B. neotomae* (Alton *et al.,* 1988), of which *B. abortus* and *B. melitensis* is the most prominent on the African continent.

Brucellosis is endemic in sub-Saharan African countries which include Zimbabwe where it was first diagnosed in cattle which aborted in 1913 (Bevan, 1931). Samples submitted to the Central Veterinary Laboratory (CVL) in Zimbabwe had a higher *Brucella* infection in commercial areas than communal areas (Swanepoel *et al.,*

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1976; Bryant and Norval, 1985; Madsen 1989; Matope *et al*., 2010a). Madsen (1989) and Mohan *et al.* (1996) identified cultures isolated *B. abortus* from aborted foetuses as *B. abortus* bv. 1. The same biovar was also isolated from a waterbuck in Wankie National Park in 1968 (Condy and Vickers, 1969) and from eland hygroma fluid (Condy and Vickers, 1972). Furthermore, sero-surveys in wildlife in Zimbabwe detected *Brucella* antibodies in various wildlife species including African buffalo, zebra, eland, kudu, giraffe, and impala (Condy and Vickers, 1972; Madsen and Anderson, 1995).

The intensity of contact with infected animals and/or herds and with contaminated environmental sources, as well as, interfase interaction between domestic animals and wildlife has been implicated to play a role in the spread of *Brucella* infections (Madsen, 1989; Madsen and Anderson, 1995). As already mentioned brucellosis occurs in wildlife in Zimbabwe, but most researchers consider wildlife only as a reservoir host, that could circulate *Brucella* infection within the population which then is a positive source of reinfection for domestic animals (Davis, 1990; Madsen and Anderson, 1995; Godfroid, 2002).

Identification of *Brucella* species is important in surveillance and eradication efforts. Currently, serological screening of potential hosts, as well as, isolation and identification of the pathogen (gold standard) from potential hosts are used for the diagnosis of brucellosis (Alton *et al*., 1988; OIE, 2008). Bacteriology method (gold standard) is time consuming (Alton *et al.,* 1988) and subjective (Whatmore, 2009). High throughput serological testing is therefore a key tool in the surveillance and control of brucellosis. Classical tests recommended by OIE (2008) like rose bengal test (RBT), serum agglutination test (SAT) and complementation fixation test (CFT)

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employ a whole cell antigen as the key diagnostic reagents (McGiven *et al*., 2008). These classical tests as well as the indirect enzyme linked immunoassays (iELISA), competitive ELISA (cELISA) and fluorescence immunosorbent assays (FPA) are recommended by (OIE, 2008) to establish the brucellosis status of cattle.

PCR has also been investigated as a quick and accurate diagnostic method since it has shown to be valuable methods for detection of DNA from different bacteria. Simple genus-specific PCR assays have been investigated for screening livestock for brucellosis on a wide range of *Brucella* infected samples such as blood (Leal-Klevezas, 1995; Ilhan *et al*., 2008), milk (Leal- Klevezas, 1995; Romero *et al*., 1995), lymphoid tissue (Gupta, 2006), stomach contents and aborted foetuses (Centinkaya *et al.,* 1999; Cortez *et al*., 2001). The most commonly used genus-specific PCR assays for *Brucella* detection includes 16S-23S rRNA intergenic spacer (ITS) region (Keid *et al.,* 2007), 31 kDa omp / BCSP31 gene (Baily *et al*., 1992; Cortez *et al*., 2001; O'Leary *et al.,* 2006), as well as omp2a and omp2b genes (Leal-Klevezas *et al*., 1995; Centinkaya *et al*., 1999; Cortez *et al.,* 2001; O'Leary *et al.,* 2006). Most PCR assays are not sensitive and specific enough to identify brucellosis in blood samples. Unfortunately blood samples are most often the only available sample. The aims of the study were to carry out a survey of bovine brucellosis in cattle and wildlife from Chiredzi district and GNP using classical and bacteriology methods. *Brucella* specific PCR assay using blood and culture DNA was investigated to determine whether PCR can be used to complement serological and bacteriology tests.

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MATERIALS AND METHODS

Study site

The Chiredzi district is in the southeast lowveld and shares boundary with GNP in Zimbabwe and unfenced part of northern part of Kruger National Park (KNP) (separated by Limpopo River). The Chiredzi district was selected since there was reports of clinical cases of abortion in the area and animals with abortion history were included in the sampling (personnel communication Chiredzi Veterinary Services, 2009; Table 3.1). There is furthermore no history of vaccination in the Chiredzi district (Veterinary services Harare, 2007). Bovine were sampled in the Malipati and Pesvi communal areas in the Chiredzi district in Zimbabwe (Fig. 1). Impala and buffalo were sampled in the GNP. According to an aerial survey done in 2009 the impala and buffalo population in GNP were 6005 and 2274, respectively (Dunham *et al.,* 2010).

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Figure 1: The location of the two communal areas (Malipati and Pesvi, ●) that were surveyed in the Chiredzi District that borders the Gonarezhou National Park.

Samples

Serum and whole blood from bovine (n=700) as well as milk (n=10) from lactating cows were collected in the Chiredzi district (Malipati and Pesvi communal areas) in Zimbabwe during 2009. Serum and whole blood were also collected from African buffalo (*Syncerus caffer*) (n=12) and impalas *(Aepyceros melampus*) (n=14) in the GNP in Zimbabwe during 2009 (Fig. 1). The sample size of wildlife in this study was small due to cost associated with sampling from wildlife.

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Serology

Serum from bovine, African buffalo and impala were tested for antibodies of *Brucella* using RBT, SAT and iELISA. RBT was done at the CVL in Zimbabwe and Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI), South Africa as described by (Alton *et al*. 1988). Briefly, the test was performed in plates where equal volumes (25 µl) of the serum and stained rose bengal antigen (pH 3.65) were mixed with a shaker for five minutes. Any agglutination was regarded as positive and all doubtful reactions were recorded as negative or zero scores. SAT was done as described by the OIE (2008) at the ARC-OVI. The bovine brucellosis iELISA kit (Institut Pourquier) was used. In this study, samples positive for both the RBT and iELISA were considered positive.

Cultures

Milk

Milk samples (n=10) were collected from animals which had a history of abortion and tested positive for *Brucella* antibodies using RBT in the Chiredzi district to establish cultures from milk (Table 3.1). Milk samples were centrifuged at 6000–7000 g for 15 minutes. The skim milk was discarded and the cream and sediment were mixed and spread on selective media. Samples were inoculated onto *Brucella* selective medium, blood agar (BA) (Quinn *et al*., 1994) and MacConkey agar. The inoculated *Brucella* selective media and BA plates were placed in a jar with gas (6% O_2, 10% CO_2 and 84% N_2), while MacConkey plates were placed in air. All plates were incubated at 37°C and examined daily for the first three days and again on day 4 and 5 as well as on day 8 to 10. Plates that did not show any growth after 10 days were discarded as negative. Suspected *Brucella* colonies were transfer to blood agar on

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which *Brucella* appear small (1 mm diameter), round, grey and non-haemolytic. Suspected colonies were Gram and modified Ziehl Neelsen (Stamp's) stained and the reaction to oxidase and catalase were observed (Quinn *et. al*., 1994).

Blood

The culturing was also done at CVL in Harare, Zimbabwe. Blood sample (n=20) (5 ml blood from animals which had tested positive for brucellosis using the RBT) was added to biphasic medium (trypticase soy solid and liquid phase; Ruiz *et al.,* 1961) and incubated at 37℃ with 5% CO_2 atmosphere. The solid phase was prepared with 12 ml of trypticase soy agar and the liquid phase consisted of 30 ml trypticase soy broth. Inoculated solid and liquid phase bottles were checked every 24 hours to evaluate haemolysis and turbidity. Once the bacterial growth within medium was detected by turbidity and haemolysis, the colony was subcultured and Gram stains were performed to confirm the presence of Gram-negative rods in the broth and on agar slants. Colonies were Gram and modified Ziehl Neelsen (Stamp's) stained and the reaction to oxidase and catalase were observed (Quinn *et. al*., 1994).

PCR

DNA was extracted from blood (n=20), Buffy coat (n=20) and cultures (n=2) of animals that tested positive with the RBT, SAT, and iELISA using Qiagen DNA mini kit (Qiagen) according to the manufacturer's instructions. PCR amplification reaction mixture was prepared in a total volume of 25 µl containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 2.0 mM $MgCl_2$, 200 µM of each dNTP's, 0.4 µM of each primer, 2.5 µl DNA template and 1.5 U GoTaq® Hot Start Polymerase (Promega). The reaction was carried out in a DNA thermal cycler (Applied Biosystems 2720). DNA of *B.*

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melitensis 16M reference strain was used as the positive control. After an initial denaturation at 95℃ for 5 minutes, 35 cycles consisting of 30 seconds of denaturation at 95℃, 30 seconds of annealing at 56℃, and 30 seconds of elongation at 72℃, followed by a final elongation at 72℃ for 5 minutes. The samples were analyzed by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 mg/ml), and the DNA bands were visualized under UV light (UVP transilluminator model TM-20).

RESULTS

Serology

The prevalence based on RBT and iELISA in Malipati and Pesvi communal areas in the Chiredzi district was 8.3% in the interfase region where domestic animals can come in contact with wildlife from GNP and KNP. The prevalence of the individual communal areas was 9% and 6.7% in Malipati and Pesvi, respectively. Table 3.2 indicates the seropositive results of the bovine using RBT, SAT, iELISA. None of the African buffaloes and impala was positive for any of the serological tests.

Table 3.1: Information of RBT seropositive bovine samples in the Pesvi and malipati communal regions in Zimbabwe

Number	Other strain number[b]	Location	Age in months	Sex[c]	RBT[d]	SAT[e]	iELISA[f]
1	8	Pesvi	14	F	P	148	P
2	9	Pesvi	54	M	P	108	P
3	17	Pesvi	84	F	P	N	P
4	41	Pesvi	132	F	P	N	N
5	42	Pesvi	60	F	P	N	P
6	48	Pesvi	36	F	P	N	N

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Number[a]	Other strain number[b]	Location	Age in months	Sex[c]	RBT[d]	SAT[e]	iELISA[f]
7*	139	Pesvi	72	F	P	42	N
8*	150	Pesvi	36	F	P	108	P
9	152	Pesvi	24	M	P	108	N
10*#!	43	Malipati	72	F	P	430	P
11*	357	Pesvi	72	F	P	108	N
12	384	Pesvi	24	M	P	42	N
13*	437	Pesvi	84	F	P	N	N
14	456	Pesvi	156	F	P	42	P
15	458	Pesvi	120	F	P	42	P
16*	462	Pesvi	72	F	P	42	P
17*$!	323	Pesvi	84	F	P	2376	P
18	474	Pesvi	36	F	P	N	P
19	487	Pesvi	144	F	P	42	P
20!	494	Pesvi	168	F	P	430	P
21*!	500	Malipati	72	F	P	512	P
22	503	Malipati	72	F	P	42	P
23!	505	Malipati	48	F	P	1720	P
24!	508	Malipati	72	F	P	2376	N
25	509	Malipati	36	F	P	108	P
26	519	Malipati	84	F	P	42	P
27!	529	Malipati	72	F	P	596	P
28!	554	Malipati	36	F	P	430	P
29	557	Malipati	108	F	P	N	N
30	562	Malipati	84	F	P	42	N
31	564	Malipati	60	F	P	42	P
32!	577	Malipati	48	F	P	2048	N
33	588	Malipati	36	F	P	42	P
34!	593	Malipati	144	F	P	148	P
35	597	Malipati	84	F	P	128	P
36	607	Malipati	144	F	P	108	P
37	611	Malipati	60	M	P	108	N
38	614	Malipati	28	F	P	42	P
39!	615	Malipati	48	F	P	2704	P
40!	617	Malipati	48	F	P	430	P
41	621	Malipati	36	F	P	128	P
42	717	Pesvi	156	F	P	N	P
43	721	Malipati	24	F	P	N	N
44	722	Malipati	144	F	P	N	P
45	726	Malipati	60	F	P	42	P
46!	728	Malipati	48	F	P	148	P
47	732	Malipati	86	F	P	128	P
48	738	Malipati	60	F	P	N	P
49	745	Malipati	30	F	P	128	P
50	757	Malipati	36	M	P	N	P

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Number[a]	Other strain number[b]	Location	Age in months	Sex[c]	RBT[d]	SAT[e]	iELISA[f]
51	758	Malipati	36	M	P	N	P
52	760	Malipati	48	F	P	N	P
53	762	Malipati	60	F	P	N	P
54!	769	Malipati	60	F	P	148	P
55	779	Malipati	36	F	P	42	N
56	781	Malipati	48	F	P	42	P
57!	787	Malipati	48	F	P	168	P
58!	800	Malipati	60	F	P	168	P
59	802	Malipati	36	F	P	N	P
60	805	Malipati	24	F	P	N	N
61!	806	Malipati	36	F	P	168	P
62	813	Malipati	24	F	P	42	P
63	818	Malipati	24	M	P	N	P
64	819	Malipati	24	F	P	N	P
65!	820	Malipati	48	M	P	168	P
66	853	Malipati	60	M	P	108	P
67	854	Malipati	48	F	P	42	P
68	857	Malipati	60	F	P	N	P
69	859	Malipati	24	M	P	N	P
70	860	Malipati	24	F	P	108	N
71*!	861	Malipati	48	F	P	1720	P
72!	863	Malipati	48	F	P	2048	P
73	864	Malipati	24	F	P	N	P
74	867	Malipati	36	F	P	N	P
75	911	Pesvi	84	F	P	N	P
76	913	Pesvi	84	F	P	N	N

[a] The (*) indicate the animals with previous abortion history and *Brucella* cultures were isolates from milk (#) and blood ($). The samples with SAT titers of ≥ 148 were tested for *Brucella* DNA using the PCR assay (!).
[b] Strain number used by collector.
[c] F = female and M = Male.
[d] Only positive (P) RBT cattle listed since seronegative cattle were not analyzed further.
[e] N indicated negative SAT results and titers are indicated for positive samples.
f N indicate negative iELISA results and P indicate positive iELISA result

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Table 3.2: Seroprevalence of cattle in the Malipati and Pesvi communal areas in the Chiredzi district, Zimbabwe using different serological test.

Test	Number tested	Positive	Seroprevalence (%)
RBT	700	76	10.9
SAT	700	51	7.3
iELISA	700	59	8.4
RBT and iELISA	700	58	8.3
RBT, iELISA and SAT	700	42	6.0

Culture

One *Brucella* isolate was cultured from a cow's milk and another from blood collected from a cow that was seropositive with RBT, SAT and iELISA (Table 3.1). The isolates had the microscopic and bacteriological characteristics typical of the *Brucella* genus namely Gram-negative coccobacilli; non-motile, positive for modified Ziehl-Neelsen staining; with oxidase and catalase production (Alton *et al.,* 1988; Quinn *et al*., 1994).

PCR

Blood samples (n=20) from 58 seropositive animals using RBT, SAT (high titers of ≥ 148), iELISA were screened for *Brucella* DNA using the 16S-23S rRNA ITS region (Keid *et al.,* 2007). The results were negative since no amplification was obtained from blood (results not shown). The same results were obtained with PCR using buffy coat DNA (n=20) (results not shown). DNA extracted from isolates from blood, milk and *B. melitensis* reference strain 16M produced a 214 bp product that is

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specific to *Brucella* using the ITS66 and ITS279 primers for the 16-23S rDNA ITS region.

DISCUSSION

Brucellosis is present in cattle in the Chiredzi districts (Malipati and Pesvi communal areas) in Zimbabwe. Serological test (RBT and iELISA) indicated that 7.6% of the tested cattle were *Brucella* seropositive in the Chiredzi district in Zimbabwe. Furthermore, *Brucella* cultures were isolated from milk and blood from seropositive cows. These cultures were identified as *Brucella* (Chapter 4). Mainly *B. abortus* bv 1 has been isolated from aborted fetuses and milk from cattle (Mohan *et al.,* 1996; Matope *et al.,* 2010b) and to a lesser extent *B. abortus* bv 2 in commercial and communal farms in Zimbabwe (Matope *et al.,* 2010b).

About 60-80% of cattle in Zimbabwe are found in communal areas (Anonymous, 2001). Previously bovine brucellosis was absent from communal lands in some provinces in Zimbabwe (Bryant and Norval, 1985), but more recently the individual and herd seroprevalence of brucellosis in communal areas in Zimbabwe was 5.5–22.9 % (Matope *et al.,* 2010b). The *Brucella* prevalence in this study in the Chiredzi district was therefore in the reported ranges of previous studies of brucellosis in Zimbabwe. This is an increase tendency from studies by Madsen (1989) an Mohan *et al.* (1996) that reported 1-16% *Brucella* seroprevalence in communal areas and 10% - 53% sero prevalence in commercial farms. This increase in seroprevalence in communal areas could be attributed to various factors such as geographical area, contact with wildlife, herd size, bread type and management factors (Muma *et al*., 2006; Matope *et al.,* 2010b). Brucellosis control strategy was based on calf hood

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vaccination with the S19 vaccine that was compulsory for commercial herds and optional in the communal areas in Zimbabwe since the 1980s (Madsen, 1989). The land reform programme in 2000 caused an increase in cattle between commercial and communal farming sectors. *Brucella* seroprevalence studies indicated that the disease is more prominent in the commercial than communal farming sector (Madsen, 1989; Mohan *et al.,* 1996) and animal movement could have resulted in the increase of brucellosis in communal areas. Furthermore economic depression has coincided with the land reform program since 2000 and resulted in reduces veterinary services like vaccination programes. Matope *et al.* (2010b) identified risk factors during 2004-2005 in communal housheld and diary herds. The study indicated that density, herd size, age of cow and geographical area are associated with *Brucella* prevalence (Matope *et al.,* 2010b). The seroprevalence were lower in the Pesvi region (6%) than the Malipati region (8.3%). The reason for the higher prevalence might be due to small sample size in Pesvi and different management strategies in the individual communal regions. Since *Brucella* was isolated in the Chiredzi district from milk and blood (indicating that some animals are bacteriemic) it is crucial that public awareness should be strengthened to reduce the risk of human exposure to *Brucella* infection.

The diagnosis of brucellosis in small ruminants and cattle still requires the use of more than one serological test like CFT, RBT and SAT (most commonly used tests for routine diagnosis. The SAT was not used in this study to determine *Brucella* seroprevalence as it recognized as inferior to other tests (Corbel and MacMillan, 1996; McGiven *et al* 2003). RBT and SAT is done as combined test at ARC-OVI, South Africa. High SAT titre was used as indication of vaccination and/or

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bacteraemia, since SAT is more sensitive to immunoglobulin M (IgM) isotype (WHO, 1986) that persists in animals vaccinated with S19 vaccine (WHO 1986). RBT is a rapid screening test, but is known to produce false positives (Gall and Nielsen, 2004). Therefore a secondary screening test is recommended with a higher sensitivity and specificity. The primary binding assay, iELISA, was developed as a more sensitive and specific alternative to conventional tests (Gall and Nielsen, 2004). The iELISA eliminates false positives (Gall and Nielsen, 2004) that are also observed in this study (Table 3.1 and 3.2). Both RBT and iELISA cannot distinguish between natural infection and vaccination with S19, but since there was no history of vaccination in the Chiredzi district, the two tests (RBT and iELISA) were used in this study.

No *Brucella* antibodies were found in impala and buffalo in the GNP in this study. The same African buffalo and impala serum samples were also found to be seronegative by other researcher's using RBT and cELISA (Personal communication (Degarine-Wichatitsky, 2010). Madsen and Anderson (1995) found *Brucella* antibodies in sera of Zimbabwean wildlife in national parks, hunting areas and game ranches collected in 1990-1991. African buffalo had the highest seroprevalence (6.5%; 29 of 444) followed by eland (1.4%; 8 of 555) and impala had the lowest (0.05%; 1 of 2068). The wildlife sample size in this study (10 impala and 10 African buffalo) was small due to high cost of sampling wildlife and therefore no conclusion can be made as far as brucellosis is concerned in wildlife in this study. Future investigation in seroprevalence of *Brucella* antibodies in wildlife should take in consideration that the prevalence is low in impala.

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Cultures were obtained from blood and milk from two cows in the Pesvi and Malipati communal areas. It is known that infected animals yield negative culture results. Reasons for this may be the condition of the tissues submitted, improper storage of tissues, not selecting an appropriate variety of tissues, not selecting a sufficient amount of tissues, selecting samples from uninfected tissues (Gall and Nielsen, 2004), selective media used, growth conditions or inhibition of growth by contaminants (Gall and Nielsen, 2004). Since bacteriology is time consuming and inconsistent, serology is mainly used to diagnose brucellosis in animals. Blood samples are therefore most often the only available sample. PCR assay has been reported not to be sensitive enough as a diagnostic tool. This was confirmed in this study since no amplification from seropositive blood DNA was obtained using the *Brucella* specific 16-23S ITS PCR assay. Blood samples for PCR investigation were selected from samples with high SAT titers since high titers indicate bacteriemia and/or vaccination. As SAT is most sensitive to the detection of IgM that are present in early infection and usually remain detectable for several weeks to months, and in some cases IgM antibodies may even persist for 1 year after the infection (Buchanan *et al*., 1974). We thought that successful PCR from blood DNA can be obtained from bacteriemic animals (acute brucellosis). Cultures were isolated from milk and blood from seropositive cows, indicating that the animals must be bacteriemic. However the PCR assay did not detect *Brucella* DNA in the blood of these bacteriemic animals. O'Leary *et al.* (2006) reported that whole blood is not suitable for detection of *Brucella* DNA but suggested using the buffy coat since bacteria are taken up by macrophages and non professional phagocytes. The PCR assay was not sensitive enough to detect *Brucella* DNA from Buffy coat DNA. This could be due to various parameters that influence PCR such as the stage of infection that influence the

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amount and location of bacteria, possible inhibitory effect of host species genomic DNA, extraction method (Leal-Klevezas *et al*., 2000; Al Nakkas *et al*., 2002;O'Leary *et al.,* 2006). The PCR assay could only detect *Brucella* DNA from culture isolated from seropositive animals. The *Brucella* specific PCR assay can not be used as confirmation of serological test. PCR assays using other samples like fetus swabs, vaginal swabs and milk have been shown to exceed serological and bacteriological methods in experimentally and naturally infected animals (Leal-Klevezas *et al*., 1995; Hamdy and Amin, 2002). The 16-23S rRNA ITS PCR assay is a good confirmation assay for *Brucella* cultures but not for blood. However further studies on the influence of DNA extraction methods from blood and Buffy coat on the sensitivity of PCR assay is necessary.

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REFERENCES

Al Nakkas A.F., Wright S.G., Mustafa A.S. and Wilson S. (2002). Single-tube, nested PCR for the diagnosis of human brucellosis in Kuwait. *Annals of Tropical Medicine and Parasitiology,* **96**: 397-403.

Alton G., Jones L.M., Angus R.D. and Verger, J.M. (1988). Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France, 81-134.

© University of Pretoria

Anonymous. (2001). Annual Census Report for 2001. Department of Veterinary Services, Harare, Zimbabwe.

Baily G. G., Krahn J. B., Drasar B. S. and Stoker, N. G. (1992). Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *Journal of Tropical Medicine and Hygiene,* **95**: 271–275.

Bevan L.E.W. (1931). Annual Report of the Veterinary Bacteriologist. Department of Veterinary Services, Southern Rhodesia.

Bishop G.C., Bosman P.P. and Herr S. (1994). Bovine brucellosis. In: Infectious diseases of livestock with special reference to Southern Africa, Volume 2 (J.A.W. Coetzer, G.R. Thomson and R. C. Tustin, eds). Oxford University Press, Cape Town, 1053-1066.

Bryant B.A. and Norval R.A.I. (1985). Diseases affecting domestic animals in communal lands in Manicaland. *Zimbabwe Veterinary Journal,* **16**: 9–17.

Buchanan T.M., Sulzer C.R., Frix M.K. and Feldman R. A. (1974). Brucellosis in the United States, 1960–1972: an abattoir-associated disease. Part II. Diagnostic aspects. *Medicine*, **53**: 415–426.

Cetinkaya B., Ongor H., Muz A., Ertas H.B., Kalender H. and Erdogan, H. M. (1999). Detection of *Brucella* species DNA in stomach content of aborted sheep foetuses by PCR, *Veterinary Records,* **144**: 239-240.

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Condy J.B. and Vickers D.B. (1969). The isolation of *Brucella abortus* from a waterbuck. *The Veterinary Record*, **85**: 200.

Condy J.B. and Vickers D.B. (1972). Brucellosis in Rhodesian wildlife. *Journal of the South African Veterinary Association*, **43**: 175-179.

Corbel M.J. and MacMillan A.P. (1996). Bovine brucellosis. In: Office International Des Epizooties Manual of Standards for Diagnostic Tests and Vaccines (R. Reichard, Ed.). Office International Des Epizooties, Paris, France, 248.

Cortez A., Scarcelli E., Soares R.M., Heinemann M.B., Sakamoto S.M., Genovez M.E., Ferreira F. and Richtzenhain L.J. (2001). Detection of *Brucella* DNA from aborted bovine foetuses by polymerase chain reaction. *Australian Veterinary Journal,* **79**: 500-501.

Davis D.S. (1990). Brucellosis in wildlife. In: Animal brucellosis (K. Nielsen and R.J. Duncan, eds). CRC Press, Florida, 321-334.

Dunham K.M., van der Westhuizen E., van der Westhuizen H.F. and Gandiwa E. (2010). Aerial survey of elephant and other large herbivores in Gonarezhou National Park (Zimbabwe), Zinave National Park (Mozambique) and surrounds: 2009. Frankfurt Zoological Society.

© University of Pretoria

Gall D. and Nielsen K. (2004). Serological diagnosis of bovine brucellosis: A review of test performance and cost comparison. *Revue Scientifique et Technique D'Office International des Epizooties,* **23***: 989-1002.*

Godfroid J. (2002). Brucellosis in wildlife. *Revue Scientifique et Technique D'Office International des Epizooties,* **21**: 277-286.

Godfroid J., Cloeckaert A., Liautard J-P., Kohler S., Fretin D., Walravens K., Garin-Bastujie B. and Letesson J.J. (2005). From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Veterinary Research,* **36**: 313-326.

Gupta V.K., Verma D.K., Singh K., Kumari R., Singh S.V. and Viham V.S. (2006). Single-step PCR for detection of Brucella melitensis from tissue and blood of goats. *Small Ruminants Research*, **66**: 169–174.

Hamdy M.E.R. and Amin A.S. (2002). Detection of Brucella species in the milk of infected cattle, sheep, goats and camels by PCR. *Veterinary Journal*, **163**: 299–305.

Ilhan Z., Aksakal A., Ekin I.H., Gulhan T., Solmaz H. and Erdenlig S. (2008). Comparison of culture and PCR for the detection of *Brucella melitensis* in blood and lymphoid tissues of serologically positive and negative slaughtered sheep. *Letter of Applied Microbiology,* **46**: 301-306.

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Keid L.B., Soares R.M., Vasconcellos S.A., Chiebao D.P., Salgado V.R., Megid J. and Richtzenhain L.J. (2007). A polymerase chain reaction for detection of *Brucella canis* in vaginal swabs of naturally infected bitches. *Theriogenology,* **68**: 1260–1270.

Leal-Klevezas D.S., Martinez-Vazquez I.O., Garcia-Cantu J., Lopez-Merino A. and Martinez-Soriano, J.P. (2000). Use of polymerase chain reaction to detect *Brucella abortus* biovar 1 in infected goats. *Veterinary Microbiology,* **75**: 91-97.

Leal-Klevezas D.S., Martinez-Vazques I.O., Lopez-Merino A. and Martinez-Soriano J.P. (1995). Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *Journal of Clinical Microbiology,* **33**: 3087-3090.

Madsen M. (1989). The current state of brucellosis in Zimbabwe. *Zimbabwe Veterinary Journal*, **20**: 133-149.

Madsen M. and Anderson E.C. (1995). Serological survey of Zimbabwe Wildlife for brucellosis. *Journal of Zoo and Wildlife Medicine*, **26**: 240-245.

Martin S.W. (1977). The evaluation of tests. *Canadian Journal of Comparative Medicine*, **41**: 19-25.

Matope G., Bhebhe E., Muma J.B., Lund A. and Skjerve, E. (2010a). Herd-level factors for Brucella seropositivity in cattle reared in smallholder dairy farms of Zimbabwe. *Preventive Veterinary Medicine,* **94**: 213–221.

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Matope G., Bhebhe E., Muma J.B., Lund A. and Skjerve, E. (2010b). Risk factors for *Brucella* spp. infection in smallholder household herds. *Epidemiology and Infection,* **139**: 157-164.

McGiven J. A., Sawyer J., Perrett L.L., Brew S.D., Commander N.J, Fisher A., McLarnon S., Harper K. and Stack J. A. (2008). A new homogeneous assay for high throughput serological diagnosis of brucellosis in ruminants, *Journal of Immunological Methods,* **337**: 7-15.

McGiven J.A., Tucker J.D., Perrett L.L., Stack J.A. Brew S.D. and MacMillan A.P. (2003). Validation of FPA and cELISA for the detection of antibodies to *Brucella abortus* in cattle sera and comparison to SAT, CFT and iELISA. *Journal of Immunological Methods*, **278**: 171-178.

Mohan K., Makaya P.V., Muvavarirwa P., Matope G., Mahembe E. and Pawandiwa, A. (1996). Brucellosis surveillance and control in Zimbabwe: bacteriological and serological investigation in dairy herds. *Onderstepoort Journal of Veterinary Research*, **63**: 47-51.

Muma J.B., Samui K.L., Siamudaala V.M., Oloya J., Matope G., Omer M.K., Munyeme M., Mubita C. and Skjerve E. (2006). Prevalence of antibodies to *Brucella* spp. and individual risk factors of infection in traditional cattle, goats and sheep reared in livestock-wildlife interface areas of Zambia. *Tropical Animal Health and Production*, **38**: 195-206.

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O'Leary S., Sheahan M. and Sweeney T. (2006). *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows, *Research Veterinary Science,* **81**: 170–176.

OIE. (2008) Bovine Brucellosis. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. OIE, Paris, 409–438.

Pappas G; Akritidis A., Bosilkovski M., Tsianos E. (2005). Brucellosis. *New England Journal of Medicine*, **352**: 2325-2336.

Quinn P.J., Carter M.E., Markey B. and Carter G.R. (1999). In: Clinical Veterinary Microbiology. Mosby International Limited, Edinburgh, 261-267,648.

Romero C., Pardo M., Grillo M.J., Diaz R., Biasco J.M. and Lopez-Goni I. (1995). Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *Journal of Clinical Microbiology,* **33**: 3198-3200.

Ruiz C. M. (1961). Laboratory Diagnosis of brucellosis in man. *Bulletin if World Health Organization,* **24**: 73-84.

Swanepoel R., Blackburn N.K. and Lander K.P. (1976). The occurrence, diagnosis and control in cattle in Rhodesia. *Zimbabwe Veterinary Journal,* **7**: 24-31.

© University of Pretoria

Whatmore A. M. (2009). Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infection, Genetics and Evolution* **9**: 1168-1184.

WHO. (1986). Joint FAO/WHO Expert Committee on Brucellosis Sixth Report, Technical Report Series 740, Geneva, Switzerland.

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CHAPTER 4

CHARACTERIZATION OF *BRUCELLA* SPECIES FROM ZIMBABWE USING PCR ASSAYS.

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ABSTRACT

Brucella species are responsible for brucellosis worldwide, making it one of the world's most widespread zoonotic diseases with enormous economic losses. Identification of *Brucella* species is mainly based on difference in pathogenecity, host preferences, and microbiological phenotypic tests. Molecular PCR assays have been developed that can characterize *Brucella* species and biovars. The multi-locus variable number tandem repeats analysis (MLVA) consist of DNA fingerprinting using variable number of tandem repeats (VNTRs) that allows for delineation of outbreaks of infectious diseases and epidemiological trace back of outbreaks. Twenty three *Brucella* strains isolated at Central Veterinary Laboratory in Zimbabwe from various hosts were characterized using MLVA-16. MLVA-16 was able to identify *B. ovis, B. suis, B. melitensis* and *B. abortus* strains amongst the Zimbawean isolates. The MLVA assay also indicated that some Zimbabwean isolates significantly differ from isolates of other origin. These Zimbabwean isolates could not be speciated and future research using bacteriology and PCR assays for accurate identification is necessary. A few strains identified using bacteriology and MLVA were also verified

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using AMOS-PCR and Bruceladder. MLVA, AMOS-PCR and Bruceladder can be used to identify *Brucella* species and biovars. This could be used as an important resource since some of the laboratories in Africa lack adequate resources and expertise to do biotyping of *Brucella* species and biovars.

INTRODUCTION

Brucellosis is a worldwide zoonotic disease of mainly ruminant animals including wildlife which can act as reservoirs (Godfroid, 2002) that also affects humans. Brucellosis is caused by species in the genus *Brucella.* This genus consists of six classic species, *Brucella abortus*, *B. melitensis*, *B. suis, B. ovis, B. canis, B. neotome* (Osterman and Moriyon, 2006). The recently described species in marine animals are *B. ceti* and *B. pinnipedialis* (Foster *et al.,* 2007) as well as *B. microti* at first isolated from the common vole (*Microtus arvalis)* (Scholz *et al.,* 2008) and later from red foxes (Scholz *et al.,* 2009) that are host specific like the classical terrestrial strains (Whatmore, 2009). *Brucella inopinata* is the most recently described species from a human breast implant (De *et al*., 2008; Scholz *et al*., 2009).

General classification of genus *Brucella* into species and biovars is still based on phenotypic characteristics (biotypes) that is difficult to interprete since there is no standardization of the typing reagents (Le Fleche *et al*., 2006; Whatmore, 2009). The resolution of biotyping is moderate and also requires the manipulation of the living agent that poses a biosafety risk when handling the samples (Le Fleche *et al*., 2006; Al Dahouk *et al.,* 2009; 2010).

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Different molecular techniques have been employed with variable success due to fact that terrestrial *Brucella* spp. are homogenous and harbor >80% interspecies homology by DNA-DNA hybridization studies (Kattar *et al*., 2008; Scholz *et al*., 2008: Verger *et al*., 1985). Low resolution species-specific multiplex PCR assays have also been developed to distinguish species and biovars of *Brucella.* Multiplex PCR includes the AMOS-PCR that differentiates *B. abortus* biovar 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1 based on the genetic element IS711 (Bricker and Halling, 1994) and later advanced the AMOS PCR to differentiate also the *B. abortus* vaccine strains S19 and RB51 (Bricker and Halling, 1995);1995). Another multiplex PCR developed by Garcia-Yoldi *et al. (*2006) used species- and strain-specific genetic differences to distinguish among *Brucella* species. This multiplex PCR assay identifies all biotypes of *B. abortus, B. melitensis, B. ovis, B. canis, B. neotomae, B. suis* and some vaccine strains (Garcia-Yoldi *et al.,* 2006). However Bruceladder does not differentiate between *B. ceti* and *B. pinnipedialis* as well as *B. microti* and *B. suis* and was not tested to identify *B. inopinata* (Huber *et al.,* 2009; Mayer-Scholl *et al.,* 2010). A high resolution molecular technique namely multilocus variable number of tandem repeats (VNTR) assay (MLVA) has been found to have enough discrimination power in the *Brucella* genus and can identify species and biovars (Le Fleche *et al.,* 2006). The MLVA consist of 16 VNTR markers, consisting of 8 moderately variable minisatellites (panel 1) and 8 highly polymorphic microsatellites (panel 2A and 2B) (Le Fleche *et al.,* 2006; Al Dahouk *et al.,* 2007) that distinguish *Brucella* based on genus and biovars as well as distinguish widespread temporal and geographical origins of very close origins of *Brucella* spp. (Kattar *et al*., 2008; Smits *et al*., 2009).

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In Zimbabwe, only *B. abortus* and *B. melitensis* have been reported to cause brucellosis in animals (Madsen, 1989; Matope *et al.,* 2009). Matope *et al.* (2009) reported that *B. abortus* biovar (bv.) 1 is the most prominent cause of bovine brucellosis and to a lesser extent *B. abortus* bv. 2 using biotyping and AMOS-PCR to identify *Brucella* isolates from commercial and smallholder (communal) cattle farms in Zimbabwe. These authors also reported a single *B. melitensis* bv. 1 isolated from a goat in Zimbabwe. In wildlife, *B. abortus* bv. 1 was isolated from waterbuck and eland in Zimbabwe (Condy and Vickers, 1969; 1976). Moreover brucellosis in various wildlife including African buffalo, eland, zebra, giraffe and impala has only been demonstrated in Zimbabwe by serology (Madsen and Anderson, 1972; Madsen and Anderson, 1995). *Brucella* isolates from 1990 to 2009 have been isolates from various host animals throughout the country at the Central Veterinary Laboratory (CVL) in Zimbabwe. Some of the isolates using biotyping have been identified to the species level. The aim of this study was to characterize these *Brucella* isolates using MLVA and to verify MLVA results using AMOS-PCR and Bruceladder. This work explores the suitability of these PCR assays for both typing and species identification.

MATERIALS AND METHODS

Bacterial strains and bacteriology

Twenty three *Brucella* cultures from domestic and wildlife animals in Zimbabwe were investigated (Table 4.1, Appendix Table 2). The isolates where isolated at CVL in Zimbabwe. The isolates were characterized as *Brucella* by bacteriological methods (urease, catalase, oxidase, H_2S, indole and sensitivity to dyes (thionin and basic fuchsin)) as indicated by Alton *et al.* (1988). Only 9 of the 23 cultures were classified

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to species level according to bacteriology methods (Alton *et al*., 1988), mainly due to financial constrains in the country. Five reference strains DNA obtained from Brucellosis OIE, FAO and EU Reference Laboratory in France were included as controls for PCR assays (Table 4.2). Information of *Brucella* isolates from previously published studies (Le Fleche *et al*., 2006; Al Dahouk *et al.,* 2007) that were used in MLVA analysis in this study are indicated in Appendix Table 2.

MLVA

DNA was extracted from each isolate grown on Brucella selective media and blood agar using Qiagen DNA mini kit (Qiagen). DNA concentrations were determined using spectrophotometer. MLVA-16 was performed as previously described (Le Fleche *et al*., 2006; Al Dahouk *et al.,* 2007). The 16 primer set were divided in 3 groups namely panel 1 (bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45 and bruce55) indicate primers), panel 2A (bruce18, bruce19, bruce21) and panel 2B (bruce04, bruce07, bruce09, bruce16 and bruce30). PCR was performed in 15 µl reactions containing 3-15 ng of DNA template, 1X PCR buffer (Promega), 200µM of each deoxynucleotide triphosphate, 0.5 µM of each flanking primer (Le Flèche *et al.,* 2006; Al Dahouk *et al.,* 2007) and 1U Go*Taq* Hotstart polymerase (Promega). The PCR conditions included an initial denaturation step of 96℃ for 5 minutes, followed by 30 cycles of 96℃ for 30 seconds, 60℃ for 30 seconds, extension at 72℃ for 1 minute, followed by a final extension step of 72℃ for 5 minutes. The PCR reaction products (5µl) were separated on 2-3% agarose in 1x TAE buffer using electrophoresis until the bromophenol blue has run for 20 cm on the agarose gel. The 16M *B. melitensis* reference strain was included as a control since each VNTR loci size is known. *Brucella* reference strains that have already

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been characterized using the MLVA-16 markers panel 1, panel 2A (bruce18; bruce19; bruce21) and panel 2B (bruce04; bruce07; bruce09; bruce16; bruce30) were included to ensure accurate evaluation of field strain analysis. For Panel 1 VNTRs, 2% agarose gel was used with Fermentas 1000 bp PCR Molecular Ruler and Bioline Hyperladder II. For panel 2 VNTRs, 3% standard agarose gel was used Biolabs low molecular weight DNA ladder 766-25 bp. The ethidium bromide stained gels were visualized by UV light Genotype was scored by visual analysis of the gel images and Bioumeric software version 5.1 (Applied Maths).

Data analysis

Band size estimates were converted to a number of units (repeat units) following the published allele numbering system (http://mlva.u-psud.fr) (Appendix Table 3). MLVA data was analysed using the character data set within Bionumeric software (version 5.1) (Applied Maths). Clustering analysis was performed used the categorical coefficient and UPMGA (unweighted pair group method using arithmetic averages). A different weight was given to the markers depending on the panel they belong to: Panel 1 markers got an individual weight of 2 (total weight for panel 1: 16), panel 2A markers got a weight of 1 (total weight for panel 2A: 3), and markers of panel 2B got a weight of 0.2 (total weight for panel 2B: 1) (Al Dahouk *et al.,* 2007). The MLVA results were compared with MLVA-16 published data of *Brucella* reference and other strains by Le Fleche *et al.* (2006), Al Dahouk *et al.* (2007) and Maquart *et al.* (2009) (Appendix Table 3). The published repeats of copy number of reference and other strains were obtained from Maquart *et al.* (2009).

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Table 4.1: Information of *Brucella* isolates from Zimbabwe used in the study.

Strains	Species	Origin in Zimbabwe	Animal / culture source
ZW377	*Brucella canis*	Harare	Dog
ZW011	*Brucella suis*	Shamva	Pig
ZW323	*Brucella abortus*	Chiredzi	Cow
ZW283	*Brucella abortus*	Gwanda	Cow
ZW248	*Brucella*	Mazowe	Cow
ZW053	*Brucella*	Matabeland	Cow
ZW043	*Brucella*	Chiredzi	Cow
ZW040	*Brucella*	Bindura	Testis
ZW045	*Brucella*	Bindura	Bull testicle
ZW046	*Brucella*	Norton	Bovine
ZW047	*Brucella*	Unknown	Unknown
ZW048	*Brucella*	Unknown	Unknown
ZW050	*Brucella*	Handerson research	Unknown
ZW052	*Brucella*	Handerson research	Milk sample
ZW100	*Brucella canis*	Harare (Highlands)	Dog
ZW003	*Brucella*	Unknown	Eland
ZW0105	*Brucella melentisis*	Chiredzi district	Goat
ZW600	*Brucella melentisis*	Chiredzi district	Cow
ZW201	*Brucella suis*	Norton	Pig
ZW002	*B ovis*	Gwanda	Sheep
ZW001	*Brucella melentisis*	Unknown	Unknown
ZW004	*Brucella melentisis*	Unknown	Unknown
ZW005	*Brucella ovis*	Insiza	Sheep

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Table 4.2: List of *Brucella* reference isolates used in the study

Brucella species	Biovar	Reference number (REF)
Brucella melitensis	1	16M
Brucella abortus	1	544
Brucella abortus	2	86/8/59
Brucella abortus	4	292
Brucella suis	1	1330
Brucella ovis	-	63/290
Brucella canis	-	RM6/66

AMOS-PCR

The AMOS-PCR was done as previously described by Bricker and Halling (1994; 1995). The PCR reaction mixture consist of 1.5mM $MgCl_2$, 1X PCR buffer (Promega), 250 µM deoxynucleotide triphosphates (dNTPS), 5 primer cocktail consisting of *B. abortus, B. melitensis, B. ovis* and *B. suis* specific primers each (0.2 µM) and 1 µM IS711-specific primer, 1U Go*Taq* Hotstart polymerase (Promega) and 2.5µ DNA per 25 µl reaction. The PCR conditions consisted of an intial denaturing at 95˚C for 15 minutes followed by 35 cycles of 95˚C for 1 minute, 55.5˚C for 2 minutes and 72˚C for 2 minutes. PCR products were separated by gel electrophoresis on a 1.5% agarose gel that was stained with ethidium bromide and photographed under UV light.

Bruceladder

The Bruceladder PCR assay was done as previously described by Garcia-Yoldi *et al.* (2006). The 8 primer set PCR conditions included an initial denaturation step at 95°C

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for 15 minutes followed by 95℃ for 35 seconds, 64℃ for 90 seconds, 72℃ for 3 minute for 25 cycles, with a final extension 72℃ for 6 minutes. PCR products were separated by gel electrophoresis on a 1.5% agarose gel that was stained with ethidium bromide and photographed under UV light.

RESULTS

Bacteriology

All the *Brucella* spp. isolates from Zimbabwe were non-motile, Gram-negative coccibacilli, positive for modified Ziehl-Neelsen staining, negative indole production, oxidase and catalase production negative. Only a few of the isolates were further characterized using growth characteristics and biochemical profiles (Table 4.1) (phages lyses were not determined). One of these isolates, ZW600, was classified as *B. melitensis* using growth characteristics and biochemical profiles.

MLVA

All strains from Zimbabwe could be types at all loci (Fig. 4.1 show example of Bruce19 for Zimbabwean isolates), with a two exceptions namely ZW100 that did not amplify Bruce12 of panel 1 and ZW103 did not amplify Bruce21 (panel 2A), as well as non of panel 2B loci (Bruce04, 07, 09, 16 and 30) despite various optimization attempts (Appendix Table 3). The 23 Zimbabwean *Brucella* isolates from cattle, milk, goats, pigs, dogs and Eland consisted of 21 genotypes (20 new genotypes) and clustered into 7 groups (Fig 4.2) when analyzed with 228 *Brucella* strains (including reference strains) (Appendix Table 2). The groups consisted of *B. abortus* bv 1, 2, 4 (predominantly although some bv 3 (BCCN 93-26 and REF Tulya) and bv. 6 (bruc0308 and 0309) isolates also group into this cluster), *B. abortus* bv 3, 5, 6, 7

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and 9, *B. melitensis* West Mediterranean, *B. melitensis* East Mediterranean, *B. melitensis* Americas that includes ZW105, Zimbabwean cluster composed of *B. abortus* (ZW040, ZW048, ZW043, ZW045, and ZW046) that groups with *B. abortus* clusters, another Zimbabwean cluster (ZW283, ZW323, ZW052, ZW053 and ZW248) that forms a bigger clusters with *B. abortus* and *B. melitensis,* two Zimbabwean isolates ZW050 and ZW0600 that forms a bigger cluster with *B. suis* bv 1, 3, 4 and *B. canis*, *B. canis* group made up of ZW100, ZW103 and ZW377 and *B. suis* group made up of ZW001, ZW201, ZW004 and ZW011 that cluster in *B. suis* bv. 1, 3, 4 and *B. canis* cluster, *B. ovis* group was made up of ZW005 and ZW002 isolate clustered with *B. suis* bv 2.

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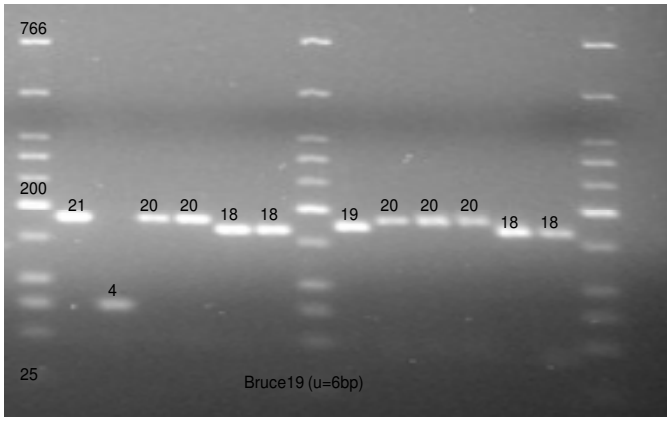
Figure 4.1: VNTR locus Bruce19 PCR products from various Zimbabwean isolates including *B. melitensis* 16M (REF 16M) and *B. abortus* bv. 1 (REF 544) reference strains. Lane 1, 8 and 15 Biolabs low molecular weight DNA ladder 25-766bp); lane 2-7: REF 544, ZW002, ZW283, ZW323, ZW011, 16M; lane 9-14: ZW377, ZW043, ZW042, ZW248, ZW201 and REF16M.



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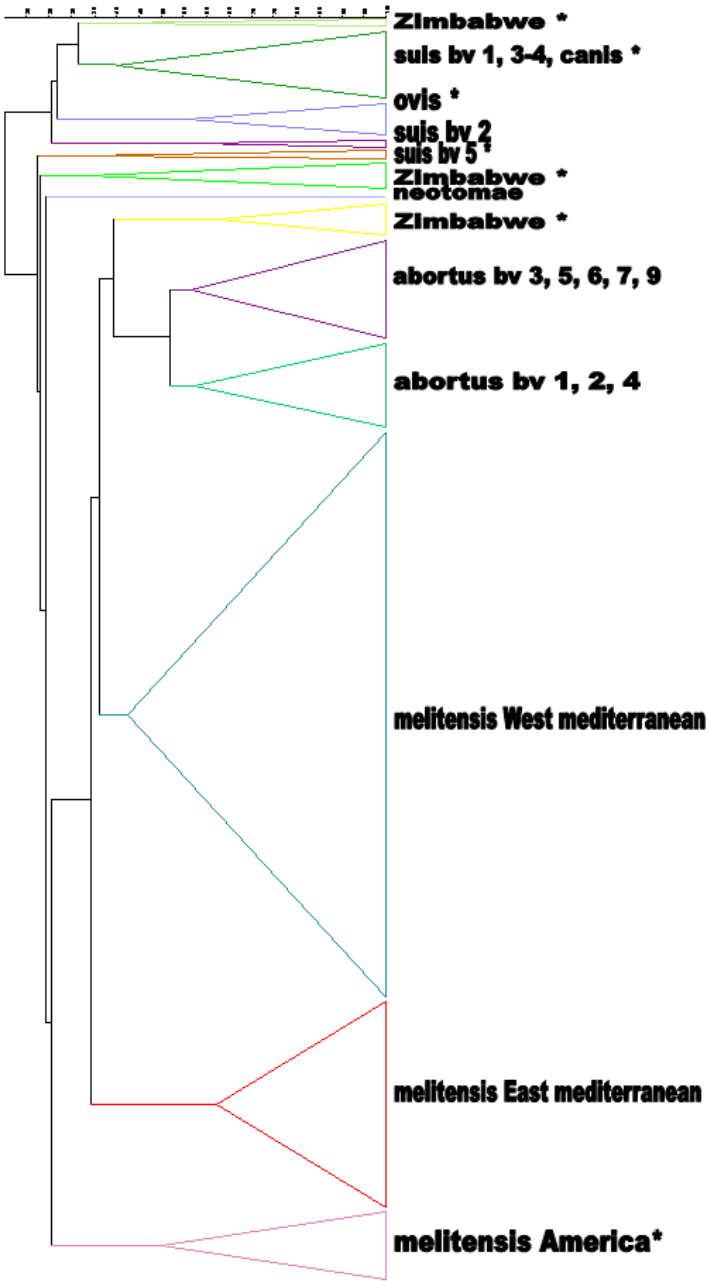

Figure 4.2: MLVA-16 cluster analysis of 23 Zimbabwean *Brucella* strains clustering amongst 228 *Brucella* isolates in 7 groups (* indicate groups where Zimbabwean isolates group). Clustering was done using UPGMA algorithm. The dendrogram is based on 225 genotypes from 228 strains (Appendix Table 2 and Appendix Fig. 1).

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AMOS-PCR and Bruccellader

A few strains that were already identified to species level using bacteriology tests were selected to verify MLVA results. The strains inlcudes *B. canis* (ZW100 and 377), *B. suis* (ZW001, 011 and 201), *B. ovis* (ZW002 and 005), *B. abortus* (ZW283 and 323) and reference strains. The *B. canis* isolates (ZW100 and 377) produced no band, *B. ovis* (ZW002 and 005) produced 916 bp PCR fragment, while ZW001, ZW201, ZW011 produced a 285 bp fragment significant to *B. suis* and *B. abortus* isolates (ZW283 and ZW323) produced a 498 bp fragment

Isolates (ZW100 and ZW377) produced band sizes 1682, 1071**,** 587, 450 and 152 bp that identify isolates as *B. canis* with Bruceladder multiplex PCR assay. The *B. abortus* isolates (ZW283 and 323) are characterized by band sizes 1652, 794, 587, 450 and 152 bp and isolates ZW001, ZW201 and ZW011 produced 1652, 1071, 587, 450, 292 and 152 bp fragment sizes typical to *B. suis* using Bruceladder. Isolates ZW002 and ZW005 produced 1071, 794, 587, 450 and 152 bp fragments that is typical to *B. ovis*. An extra band of size larger than 2000 bp was obtained with *B. canis* and *B abortus* isolates. The marker size used was Fermentas 1 kb plus DNA ladder.

DISCUSSION

The purpose of the study was to explore the suitability of MLVA as well as multiplex PCR assays (AMOS-PCR and Bruceladder) for identification of *Brucella* species. *Brucella* strains (23) isolated from cattle, pigs, dogs, goats, eland, milk and tissue at the CVL in Zimbabwe were identified using PCR assays. *Brucella abortus and B. melitensis* are the most prominent species in Africa that were previously reported in

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Zimbabwe from livestock and wildlife (Condy and Vickers, 1969; 1976; Madsen, 1989; Matope *et al.,* 2009). *Brucella abortus* was identified in this study from cattle, milk and testes from various regions in Zimbabwe (Table 4.1). The MLVA grouped *B. abortus* strains into *B. abortus* bv. 1, 2, 4 (predominantly although some bv. 3 (BCCN 93-26 and REF Tulya) and bv. 6 (bruc 0308 and 0309) isolates also group into this cluster) cluster and *B. abortus* bv 3, 5, 6, 7, 9. Isolates ZW040, ZW048, ZW043, ZW045, and ZW046 formed a larger cluster with *B. abortus* strains and Zimbabwean isolates ZW283, ZW323, ZW052, ZW053 and ZW248 formed a separate subcluster and grouped closely to *B. abortus* and *B. melitensis* clusters (Appendix Fig. 2). Isolates ZW283 and ZW323 were identified using bacteriology, AMOS-PCR and Bruceladder suggesting that this group (ZW283, ZW323, ZW052, ZW053 and ZW248) consist of *B. abortus* strains. The MLVA assay confirms that some African strains significantly differ from isolates of other origin and that *B. abortus* bv. 3 is a heterogeneous group (Verger *et al*., 1985; Le Fleche *et al*., 2006). Furthermore it has been shown that bruce43 is a highly variable marker and is sometimes found to have a different copy number on the same farm suggesting that it can serve as a discriminating marker rather than a species identification marker for *B. abortus* strains (Kattar *et al*., 2008).

Brucella melitensis was isolated from goat in Chiredzi, Zimbabwe. This isolate (ZW105) was original speciated as *B. melitensis* (ZW105) (Table 4.1) and clustered with *B. melitensis* bv. 1 reference strain 16M in the *B. melitensis* America group using MLVA data (Appendix Fig 2). *Brucella melitensis* Le Fleche *et al*. (2006) initially observed that *B. melitensis* group is very heterogenous. The same observation was made by Al Dahouk *et al.* (2007) that observed three subgroups

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(American, West- and East Mediterranean), which did not associate with classical biotyping results. Al Dahouk *et al*. (2007) also observed Fuchsin sensitive *B. melitensis* strains were found in close clusters that suggested an association between VNTRs and phenotypic characteristics. The other isolates (ZW600), which was initial classified as *B. melitensis,* together with ZW050 (from unknown host) grouped together and formed a bigger cluster with *B. suis* bv. 1, 3, 4 and *B. canis*. Le Fleche *et al*. (2006) reported *B. melitensis* strain (BCCN 84-3) from dog in Costa Rica to group with *B. suis* and *B. canis.* This interesting case is similar to the two Zimbabwean isolates, ZW050 (unknown host) and 600 (cattle) that clustered with *B. suis* bv. 1, 3, 4 and *B. canis.* These two isolates can not be speciated based on the MLVA data. Further research using biotyping, sequencing, and PCR assays like AMOS-PCR and Bruceladder is necessary for accurate speciation.

MLVA results for *B. suis* was experimentally shown to be accurate. An experimental infection of pigs with *B. suis* showed that the strains that were re-isolated from four of six infected animals showed some minor changes, an increase or decrease in one tandem repeat copy number (Whatmore *et al*., 2006; Her *et al*., 2009). Zimbabwean isolates ZW001, ZW004, ZW011, ZW100 ZW103, ZW201 and ZW377 were included in the *B. suis* bv 1, 3, 4 and *B. canis* cluster. ZW001 (unknown), ZW004 (unknown), ZW011 (pig) and ZW201 (pig) formed a subcluster in the *B. suis* bv. 1, 3, 4 and *B. canis* cluster with *B. suis* bv. 1 reference strain (REF 1330). Since most of these isolates were isolated from pigs in Zimbabwe and the AMOS-PCR (identify *B. suis* bv. 1) and Bruceladder also identify ZW011 and ZW201 as *B. suis* these isolates is most probably *B. suis* bv. 1. This will be the first report of *B. suis* in Zimbabwe. The status of *B. suis* as a single species has been questioned in light of a broader host

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specificity and as no species specific markers for *B. suis* have been identified contrary to other classical species (Moreno *et al*., 2002). Independent studies revealed that *B. suis* bv. 5 is more related to *Brucella* species infecting marine mammals than to other four other *B. suis* biovar (Marianelli *et al*., 2006; Whatmore *et al*., 2006, 2007). Further investigation is necessary to determine the species of isolate ZW047 (milk) that group with *B. suis* bv. 5 reference strain (Appendix Fig. 2). However this phylogeny is weakly supported (<35%; Appendix Fig. 2). ZW100 was the same genotype as the *B. canis* reference strain (REF RM6/66), isolated from a dog and identified by AMOS-PCR and Bruceladder as *B. canis*. This will also be the first report of *B. canis* in Zimbabwe. The other two isolates ZW 103 (eland) and ZW377 (dog) grouped with *B. canis* and *B. suis* bv 3, 4 subcluster. However isolate ZW103 did not amplify Bruce21 (panel 2A) and panel 2B loci (Bruce04, 07, 09, 16 and 30) using MLVA-16. The MLVA-16 data of this isolate as well as ZW377 cannot be used to accurately determine the species of these isolates. Further research using biotyping, sequencing, and PCR assays like AMOS-PCR and Bruceladder is necessary for accurate speciation of ZW103 as well as ZW377. *Brucella canis* group is close to *B suis* group as expected (Whatmore, 2009). Independent studies have demonstrated that, with exception of *B. suis* bv. 5, all *B suis* and *B. canis* strains form a consistent group of organisms within the *Brucella* cluster (Fretin *et al*., 2008). There are case of *B. suis* and *B. canis* closely related (Gandara *et al*., 2001, Whatmore *et al*., 2005: Koylass *et al.,* 2010) as observed in this study. For example, *B. canis* has long been considered very closely related to *B. suis* on the basis of a number of approaches including chromosomal maps (Michaux-Charachon *et al.,* 1997), *omp* profiling (Cloeckaert *et al.,* 1995), MLEE (Gandara *et al*., 2001), AFLP (Whatmore *et al*., 2005) and insertion sequence typing (Ouahrani *et al*., 1993) that

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raised questions regarding its status as a distinct species (Whatmore *et al*., 2007). The AFLP and MLEE studies indicated that *B. suis* bv. 5 is distinct from other *B. suis* isolates (Gandara *et al*.,2001;Whatmore *et al*., 2005) and it is not clear whether there is justification for including *B. suis* bv. 5 in a taxonomic group with *B. suis*. Single nucleotide polymorphisms (SNP) has been found to be suitable for characterization of *B. suis* at biovar level (Fretin *et al*., 2008) and could be used in future to characterize Zimbabwean isolates in the *B. suis* bv 1. 3, 4 cluster as well as the *B. suis* bv. 5 cluster.

In this study, MLVA-16 was able to group two *B. ovis* strains (ZW002 and ZW005) into *B. ovis* cluster. Isolate ZW002 grouped on its own and ZW005 grouped together with *B. ovis* reference strain (REF 63/290) and *B. ovis* BCCN 97-41 isolated from sheep in Argentina (Le Fleche *et al*., 2006) (Appendix Fig. 2). *Brucella ovis* has been indicated to be present in Zimbabwe from OIE report indicated by Corbel (1997).

The high variability of VNTR loci has already been exploited for *Brucella* strain typing (Bricker, 2004; Le Fleche *et al*., 2006; Whatmore *et al*., 2006). Panel 1 of MLVA-16 was shown to be useful for species identification; Le Fleche *et al*. (2006) described panel 1as comprising 8 user-friendly minisatellites markers with a good species identification capability. Pane 2 was described by Le Fleche *et al*. (2006) as a complementary group with microsatellite markers with higher discriminatory power. Panel 2A and 2B increased the discriminatory power. Panel 2B contains highly variable markers; this is why the panel is often given a lower weight in clustering analysis (Maquart *et al*., 2009). Al Dahouk *et al*. (2007) assign different weights to

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different panels (Panel 1, 2A and 2B), taking into account the mutation rate and the associated levels of homoplasy. The MLVA loci stability was confirmed via in-vitro and in-vivo passages and their possible use as epidemiological markers for trace-back origin (Her *et al*., 2009). This molecular epidemiology of MLVA contributes to the analysis and understanding of infections caused by pathogenic bacteria (van Belkum, 2003; Al Dahouk *et al*., 2007).

AMOS-PCR results were consistent with those obtained by Bricker and Halling (1994). The assay amplifies DNA from *B. abortus* bv. 1, 2, and 3 (498bp); *B. melitensis* bv. 1, 2, 3 (731bp); *B. ovis* (976bp) and *B. suis* bv. 1 (285bp) (Bricker and Halling, 1994). Matope *et al.* (2009) used AMOS-PCR and biotyping to characterize 14 *Brucella* isolates from cattle and goat in Zimbabwe and identified *B. abortus* bv. 1 and 2 and *B. melitensis* bv. 1. Our situation was different since all our isolates where not speciated up to biovar level using biochemical profiling as described by Matope *et al*. (2009). The Bruceladder assay was also done to resolve species *B. canis and B. neotomae* that AMOS-PCR cannot identify. The Bruceladder identified *B. canis* (ZW100) as well as verify AMOS-PCR identification of *B. suis, B. abortus* and *B. ovis* as reported by Garcia-Yoldi *et al*. (2006). Isolates initial classified as *B. canis* by bacteriological typing were found to be *B.canis* and no species was classified as *B. neotomae* since this species has only been reported in desert rat in USA. The use of the different PCR assays demonstrates the importance and ability to use DNA for further analysis of *Brucella* spp.

The MLVA-16 assay provides a clustering of strains that is in accordance with currently recognized *Brucella* species and biovars isolated from terrestrial mammals

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(Le Fleche *et al.,* 2006; Maquart *et al*., 2009). AMOS-PCR and Bruceladder can also be use to identify specific species and / or biovar. This study has also shown that MLVA can be used to complement identification of *Brucella* species up to biovars and or species level. The MLVA results were verified by AMOS-PCR and Bruceladder. Since most of the laboratories in Africa lack resources and expertise as (evidenced in study) to do biotyping of *Brucella* up to species level, PCR assays like MLVA, AMOS-PCR and Bruceladder can contribute to the identification and MLVA can furthermore be used as an epidemiological tool and trace back of outbreaks. These PCR assays can therefore add to the control and eradication of brucellosis, since *B. abortus, B. melitensis, B. ovis*, *B. suis* and *B. canis* could be identified. The two latter species were reported for the first time in Zimbabwe. More research needs to be done on various isolates of which the speciation could not be resolved using MLVA data and that were not included in the verification using AMOS-PCR and Bruceladder. Additionally more strains and epidemiological data from Zimbabwe are needed in order to accurately draw conclusions on the clustering and circulation of strains.

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REFERENCES

Al Dahouk S., Le Flèche P., Nöckler K., Jacques I., Grayon M., Scholz H.C, Tomaso H., Vergnaud G. and Neubauer H. (2007). Evaluation of *Brucella* MLVA typing for human brucellosis. *Journal of Microbiology Methods*, **69:** 137-145.

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Baily G.G., Krahn J.B., Drasar B.S. and Stoker N.G. (1992). Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *J Trop Med Hyg*, 95, 271-275.
Blasco J. M. (1997) A review of the use of *Brucella melitensis* Rev. 1 vaccine in adult sheep and goats. *Preventive Veterinary Medicine*, **31**: 275-283

Bricker B.J. (2004). Molecular Diagnostics of Animal Brucellosis: A Review of PCR-Based Assays and Approaches. In: *Brucella*: Molecular and Cellular Biology. I. Lopez-Goni and I. Moriyon (Eds). Horizon Bioscience, England, 25-51.

Bricker B.J, Ewalt D.R. and Halling S.M. (2003). *Brucella* (HOOF-prints: strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). *BioMed Central Microbiology*, **3**: 15.

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

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

Cetinkaya B., Ongor H., Muz A., Ertas H. B., Kalender H. and Erdogan, H. M. (1999). Detection of *Brucella* species DNA in stomach content of aborted sheep foetuses by PCR. *Veterinary Records*, **144**: 239-240.

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

Condy J.B. and Vickers D.B. (1972). Brucellosis in Rhodesian Wildlife. *Journal of the South African Veterinary Association*, **43**: 175-179.

Condy J.B. and Vickers D.B. (1969). The isolation of *Brucella abortus* from a waterbuck. *The Veterinary Record*, **85**: 200.

© University of Pretoria

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

Corbel M.J. and Brinely-Morgan W.J. (1984). Genus *Brucella* Meyer and Shaw 1920, p 377-388. In: Bergey's manual of systematic bacteriology, vol. 1. (W. R. Krieg and J. G. Holt eds.). The Williams and Wilkins Co., Baltimore, USA.

Cortez A., Scarcelli E., Soares R.M., Heinemann M.B., Sakamoto S.M., Genovez M.E., Ferreira F. and Richtzenhain L.J. (2001). Detection of *Brucella* DNA from aborted bovine foetuses by polymerase chain reaction. *Australian Veterinary Journal,* **79**: 500-501.

Dawson C.E., Stubberfield E.J., Perrett L.L., King A. C., Whatmore A.M., Bashiruddin J.B., Stack J.A. and MacMillan A.P. (2008). Phenotypic and molecular characterisation of *Brucella* isolates from marine mammals. *BioMed Central Microbiology,* **8**: 224.

DelVecchio V.G., Kapatral V., Elzer P., Patra G. and Mujer C.V. (2001). The genome of *Brucella melitensis. Veterinary Microbiology*, **90**: 587-592.

Fekete A., Bantle J.A. and Halling S. M. (1992). Detection of *Brucella* by polymerase chain reaction in bovine foetal and maternal tissue. *Journal of Veterinary Diagnostics and Investigation,* **4**: 79-83.

Fretin D., Whatmore A.M., Al Dahouk S., Neubauer H., Garin-Bastuji B., Albert D., Van Hessche M., Ménart M., Godfroid J., Walravens K. and P. Wattiau. (2008). *Brucella suis* identification and biovar typing by real-time PCR. *Veterinary Microbiology,* **131**: 376–385.

Foster G., Osterman B.S., Godfroid J., Jacques I. and A. Cloeckaert. (2007). *Brucella* ceti sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. *International Journal of Systemic Evolution and Microbiology,* **57**: 2688–2693.

© University of Pretoria

Gall D. and Nielsen K. (2004). Serological diagnosis of bovine brucellosis: A review of test performance and cost comparison. *Revue Scientifique Et Technique De Office International Des Epizooties,* **23**: 989-1002.

Gandara B., Merino A.L., Rogel A. and Martinez-Romero E. (2001). Limited Genetic Diversity of *Brucella* spp. *Journal of Clinical Microbiology,* **39**: 235-240.

García-Yoldi D., Marín C.M., de Miguel M.J., Muñoz P.M., Vizmanos J.L. and López-Goñi, I. (2006). Multiplex PCR Assay for the identification and differentiation of all *Brucella* species and the Vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. *Clinical Chemistry,* **52**: 779-781.

Godfroid, J. (2002). Brucellosis in wildlife. *Revue Scientifique Et Technique De Office International Des Epizooties*, **21**: 277-286.

Godfroid J., Saegerman C.,Wellemans V.,Walravens K., Letesson J-J., Tibor A., McMillan A., Spencer S., Sanna M., Baller D., Pouillot R. and Garin-Bastuji B. (2002). How to substantiate eradication of bovine brucellosis when aspecific serological reactions occur in the course of brucellosis testing. *Veterinary Microbiology,* **90**: 461-447.

Godfroid J., Cloeckaert A., Liautard J-P., Kohler S., Fretin D., Walravens K., Garin-Bastujie B. and Letesson J.J. (2005). From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. *Veterinary Research,* **36**: 313-326.

Guarino F., Serpe L., Fusco G., Scaramuzzo A. and Gallo, P. (2000). Detection of *Brucella* species in buffalo whole blood by gene-specific PCR. *Veterinary Records*, **147**: 634-636.

Halling S.M., Tatum F.M. and Bricker B. (1993). Sequence and characterization of an insertion sequence IS711, from *Brucella ovis*. *Gene*, **133**: 123-127.

© University of Pretoria

Her M., Kang S., Cho D., Cho., Y., Hwang I., Heo Y., Jung S. and Yoo H. (2009). Application and evaluation of the MLVA typing assay for the *Brucella abortus* strains isolated in Korea. *BioMed Central Microbiology,* **9**: 230.

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

Keid L.B., Soares R.M., Vasconcellos S.A., Chiebao D.P., Salgado V.R., Megid J. and Richtzenhain L.J. (2007). A polymerase chain reaction for detection of *Brucella canis* in vaginal swabs of naturally infected bitches. *Theriogenology,* **68**: 1260–1270.

Koylass S.M., King C.A., Edwards-Smallbone J., Gopaul K.K., Perret L.L. and Whatmore M.A. (2010). Comparative performance of SNP typing and Bruce-Ladder in the discrimination of *Brucella suis* and *Brucella canis*. *Veterinary Microbiology,* **142**:450-454.

Le Flèche P., Fabre M., Denoeud F., Koeck J.L., and Vergnaud G. (2002). High resolution, on-line identification of strains from the *Mycobacterium tuberculosis* complex based on tandem repeat typing. *BioMed Central Microbiology,* **2**: 37.

Le Flèche P., Hauck Y., Onteniente L., Prieur A., Denoeud F., Ramisse V., Sylvestre P., Benson G., Ramisse F. and Vergnaud G. (2001). A tandem repeats database for bacterial genomes: application to the genotyping of *Yersinia pestis* and *Bacillus anthracis*. *BioMed Central Microbiology,* **1**:2.

Le Fleche P., Jacques I., Grayon M., Dahouk S. A., Bouchoni P., Denoeud F., Nockler K., Neubauer H., Guilloteau L. A., and Vergnaud G. (2006). Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. *BioMed Central Microbiology,* **6**:9.

Leal-Klevezas D. S., Martinez-Vazques I. O., Lopez-Merino A. and Martinez-Soriano J. P. (1995). Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. *Journal of Clinical Microbiology,* **33**: 3087-3090.

© University of Pretoria

Madsen M. and Anderson E .C. (1995). Serological survey of Zimbabwe wildlife for brucellosis. *Journal of Zoo and Wildlife Medicine*, **26:** 240-245.

Matope G., Bhebhe E., Muma J.B. Skjerve E and Djonne B. (2009). Characterization of some *Brucella* species from Zimbabwe by biochemical profiling and AMOS-PCR. *BMC Research Notes,* **2**: 261-267.

Maquart M., LeFlech P., Foster G., Tryland M., Ramisse F., Djonne B., Al Dahouk S., Jacques I., Neubauer H., Walravens K., Godfroid J., Cloeckaert A. and Vergnaud G. (2009). MLVA-16 typing of 295 marine mammal Brucella isolates from different animal and geographic origins identifies 7 major groups within *Brucella ceti* and *Brucella pinnipedialis. BioMed central Microbiology,* **9**:145.

Marianelli C., Ciuchini F., Tarantino M., Pasquali P. and Adone R. (2006). Molecular characterization of the rpoB gene in *Brucella* species: new potential molecular markers for genotyping. *Microbes Infections*. 8: 860–865.

Mayer-Scholl A., Draeger A., Göllner C., Scholz C,H., Nöckler K. (2010). Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species, *Journal of Microbiological Methods*, **80**: 112–114.

Michaux-Charachon S., Bourg G., Jumas-Bilak E., Guigue-Talet P., Allardet-Servent A., O'Callaghan D.and Ramuz M. (1997). Genome structure and phylogeny in the genus *Brucella*, *Journal of Bacteriology.* **179**: 3244–3249.

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

OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2008). Chapter 2.3.1. Bovine Brucellosis. OIE, Paris, 409–438.

Osterman B. and Moriyón I. (2006). International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of *Brucella*. Report of the meeting, 17

© University of Pretoria

September 2003, Pamplona, Spain. *International Journal of System Evoution and Microbioogy,* **56**: 1173–1175.

Paulsen I.T., Shadri R., Nelson K.E., Eisen J.A., Heidelberg J.F., Read T.D., Dodson R.J., Umayam L., Brinkac L. M., Beanan M. J., Daugherty S.C. and Deboy R.T. (2002). The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proceedings of the National Academy of Sciences, USA*, **99**: 13148-13153.

Pourcel C., Andre-Mazeaud F., Neubauer H., Ramisse F. and Vergnaud G. (2004). Tandem repeats analysis for the high resolution phylogenetic analysis of *Yersinia pestis*. *BioMed Central Microbiology,* **4**:22.

Rijpens N.P., Jannes G., Van Asbroeck M., Rossau R. and Herman L. M. (1996). Direct detection of *Brucella* spp in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. *Applied Environmental Microbiology,* **62**: 1683-1688.

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

Romero C. Pardo M., Grillo M.J., Diaz R., Biasco J.M., and Lopez-Goni I. (1995b). Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. *Journal Clinical Microbiology,* **33**: 3198-3200.

Smits H.L., Espinosa B., Castillo R., Hall E., Guillen A., Zevaleta M., Gilman R.H., Melendez P., Guerra C., Draeger A., Broglia A. and Nöckler K. (2009). MLVA genotyping of human *Brucella* isolates from Peru. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **103**: 399–402.

Tscherneva E., Rijpens N., Jersek B., Herman L.M.F. (2000). Differentiation of Brucella species by random amplified polymorphic DNA analysis. *Journal of Applied Microbiology*, **88**: 69–80.

© University of Pretoria

Whatmore A.M. (2009). Current understanding of the genetic diversity of *Brucella*, an expanding genus of zoonotic pathogens. *Infection Genetics and Evolution* **9**: 1168-1184.

Whatmore M.A., Al Dahouk S., Lodri C. K. M. and Pfeffer M. (2008). Isolation of *Brucella microti* from Soil. *Emerging Infectious Diseases*, **14**: 1317.

Whatmore A.M., Perrett L.L. and Macmillan A.P. (2007). Characterisation of the genetic
diversity of *Brucella* by multilocus sequencing. *BioMed Central Microbiology,* **7**:34.

Whatmore A.M., Shankster S.J., Perrett L.L., Murphy T.J., Brew S.D., Thirlwall R.E., Cutler S.J. and Macmillan A.P. (2006). Identification and characterization of variable number tandem-repeat markers for typing of *Brucella* spp. *Journal of Clinical Microbiology,* **44:**1982–1993.

Whatmore A.M., Murphy T.J., Shankster S., Young E., Cutler S.J. and Macmillan A.P. (2005). Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. *Journal of Clinical Microbiology.* **43**: 761–769.

Verger J. M. (1985). *B. melitensis* infection of cattle. In: *Brucella melitensis* (J. M. Verger and M. Plommet eds.). Martinus Nijhoff, Dordrecht, the Netherlands.

Verger, J.M., Grimont, F., Grimont, P.A.D and Grayson, M. (1985) *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *International Journal of Systemic Bacteriology*, **35**:292-295.

Yagupsky P. (1999). Detection of Brucellae in blood cultures. *Journal of Clinical Microbiology,* **37**: 3437-3442.

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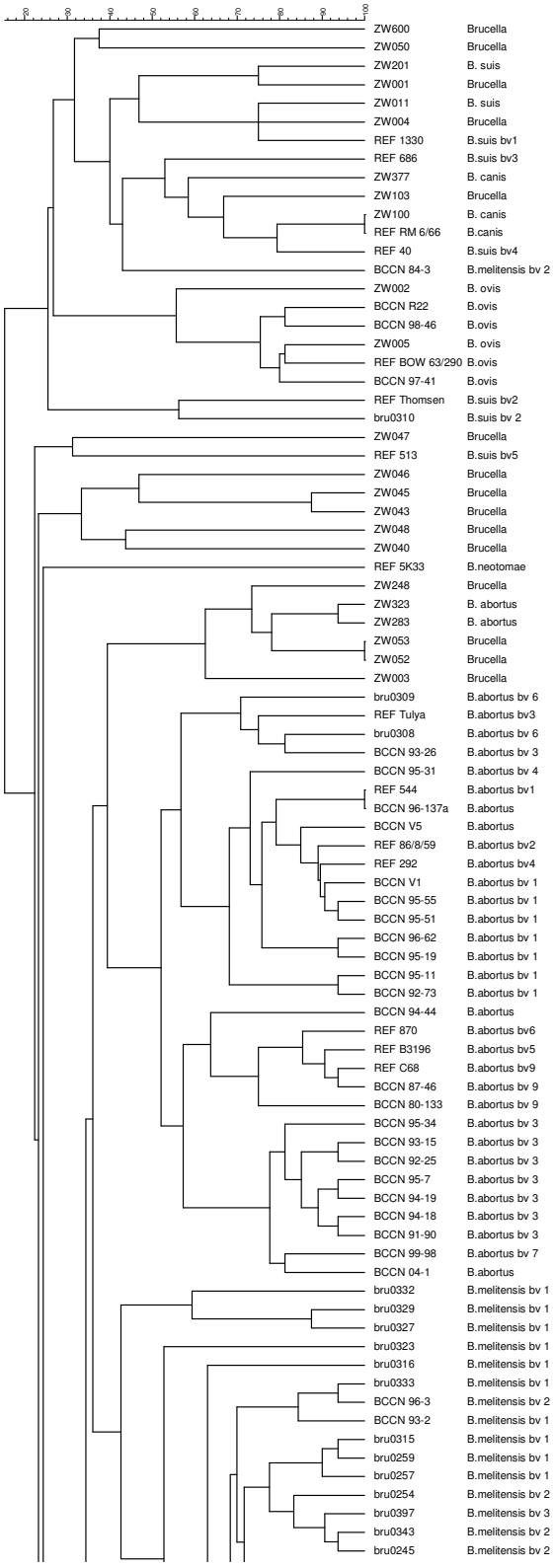
Appendix Figure 1: Cluster analysis using UPGMA algorithm of 228 *Brucella* strains. This dendrogram is based on 225 genotypes obtained from 228 strains (Appendix Table 2 and 3)



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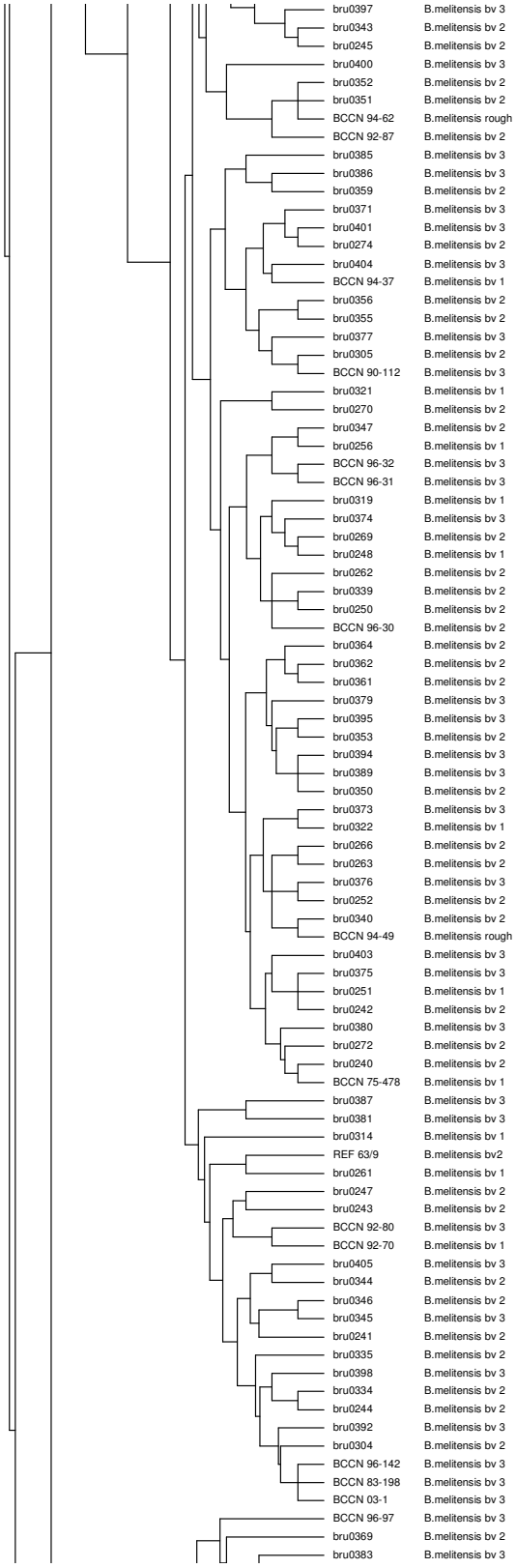

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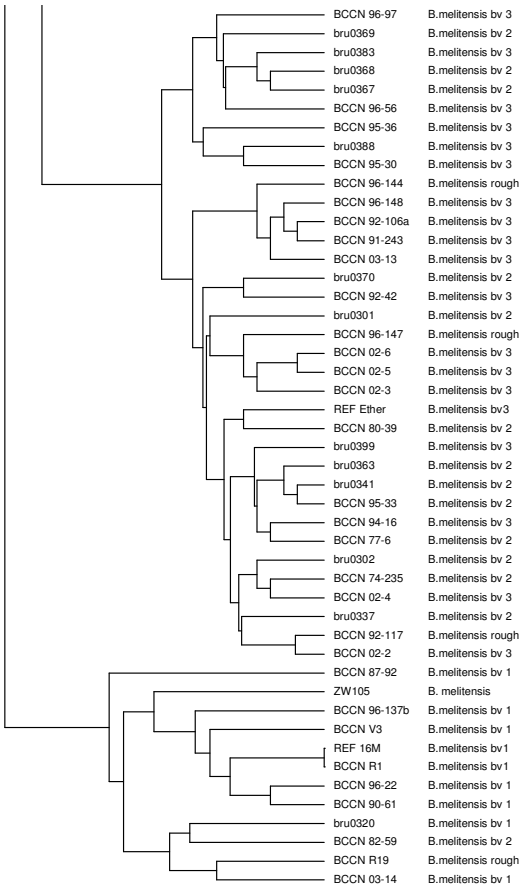

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Appendix Table 1: Classical biotyping of *Brucella* species as indicated by Whatmore (2009).

Species	Biotype	Urease	CO_2 requirement	H_2S production	Growth on media containing		Agglutination with monospecific antisera			Lysis by phage at RTD					
					Thionin[a]	Fuchsin[a]	A	M	R	Tb	Wb	Bk_2	Fi	R/C	Tb × 10^4
Classical species															
B. abortus	**1**	**(+)[b]**	**(+)**	**+**	**–**	**+**	**+**	**–**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	2	**+**	**(+)**	**+**	**–**	**–**	**+**	**–**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	3[c]	**+**	**(+)**	**+**	**+**	**+[d]**	**+**	**–**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	4	**+**	**(+)**	**+**	**–**	**+**	**–**	**+**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	5	**+**	**–**	**–**	**+**	**+**	**–**	**+**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	6[c]	**+**	**–**	**(+)**	**+**	**+**	**+**	**-**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	7	**+**	**–**	**(+)**	**+**	**+**	**+**	**+**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
	9	**+**	**–**	**+**	**+**	**+**	**–**	**+**	**–**	**L**	**L**	**L**	**L**	**NL**	**L**
B. suis	**1**	**+**	**–**	**+**	**+**	**–[e]**	**+**	**–**	**–**	**NL**	**L**	**L**	**PL**	**NL**	**L**
	2	**+**	**–**	**–**	**+**	**–**	**+**	**–**	**–**	**NL**	**L**	**L**	**PL**	**NL**	**L**
	3	**+**	**–**	**–**	**+**	**+**	**+**	**–**	**–**	**NL**	**L**	**L**	**PL**	**NL**	**L**
	4	**+**	**–**	**–**	**+**	**(–)**	**+**	**+**	**–**	**NL**	**L**	**L**	**PL/L**	**NL**	**L**
	5	**+**	**–**	**–**	**+**	**–**	**–**	**+**	**–**	**NL**	**L**	**L**	**PL**	**NL**	**L**

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Species	Biotype	Urease	CO_2 requirement	H_2S production	Growth on media containing		Agglutination with monospecific antisera			Lysis by phage at RTD					
					Thionin[a]	Fuchsin[a]	A	M	R	Tb	Wb	Bk_2	Fi	R/C	Tb × 10^4
B. melitensis	**1**	**+**	**–**	**–**	**+**	**+**	**–**	**+**	**–**	**NL**	**NL**	**L**	**NL**	**NL**	**NL**
	2	**+**	**–**	**–**	**+**	**+**	**+**	**–**	**–**	**NL**	**NL**	**L**	**NL**	**NL**	**NL**
	3	**+**	**–**	**–**	**+**	**+**	**+**	**+**	**–**	**NL**	**NL**	**L**	**NL**	**NL**	**NL**
B. ovis		**–**	**+**	**–**	**+**	**(+)**	**–**	**–**	**+**	**NL**	**NL**	**NL**	**NL**	**L**	**NL**
B. canis		**+**	**–**	**–**	**+**	**–**	**–**	**–**	**+**	**NL**	**NL**	**NL**	**NL**	**L**	**NL**
B. neotomae		**+**	**–**	**+**	**–**	**–**	**+**	**–**	**–**	**NL/PL**	**L**	**L**	**L**	**NL**	**L**
Newly described species															
B. ceti		**+**	**(–)**	**–**	**(+)**	**(+)**	**+**	**(–)**	**–**	**NL$^{\alpha}$**	**L$^{\beta}$**	**L$^{\beta}$**	**NL/PL**	**NL**	
B. pinnipedialis		**+**	**(+)**	**–**	**+**	**+**	**(+)**	**(–)**	**–**	**NL$^{\alpha}$**	**L$^{\beta}$**	**L$^{\beta}$**	**NL/PL**	**NL**	
B. microti		**+**	**–**	**–**	**+**	**+**	**–**	**+**		**NL**	**L**		**NL**		**L**
B. inopinata		**+**	**–**	**+**	**+**	**+**	**–**	**+[f]**		**NL**			**NL**		**PL**

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Phage lysis: RTD=routine test dilution, Tb =Tbilisi, Wb=Weybridge, Bk_2=Berkeley, Fi=Firenze, R/C=rough strains, $Tb\times10^4$=$RTD\times10^4$, L=confluent lysis, PL=partial lysis, NL=no lysis, α=most strains no lysis, β=most strains lysis, (+)=most strains positive, (–)=most strains negative.

[a] Concentration=1/50,000 w/v.

[b] Strain is negative but most field strains are positive.

[c] For more certain differentiation of biotype 3 and 6, thionin at 1/25 000 (w/v) is used in addition. Biovar 3=+, biovar 6=–.

[d] Some strains of this biotype are inhibited by fuchsin.

[e] Some isolates may be resistant to fuchsin.

[f] Weak agglutination.

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Appendix Table 2: Information of *Brucella* isolates included in data analysis.

Strain[A]	Other number[a]	*Brucella* species	Host	Country	Reference
ZW001	BM	*Brucella*	unknown	Zimbabwe (ZW)	This study
ZW201	201	*B. suis*	Norton	Zimbabwe (ZW)	This study
ZW248	248	*Brucella*	Cattle	Mazowe, ZW	This study
ZW052	52	*Brucella*	Milk	Handerson, ZW	This study
ZW053	53	*Brucella*	Cattle	Matabeland, ZW	This study
ZW323	323	*B. abortus*	Cattle	Harare, ZW	This study
ZW283	283	*B. abortus*	Cattle	Gwanda, ZW	This study
ZW002	Bo	*B. ovis*	Sheep	Zimbabwe (ZW)	This study
ZW600	600	*B. melitensis*[b]	Cattle	Chiredzi, ZW	This study
ZW040	40	*B. abortus*	Testis	Bindura, ZW	This study
ZW048	48	*Brucella*	unknown	Zimbabwe (ZW)	This study
ZW105	105	*B. melitensis*	Goat	Chiredzi, ZW	This study
ZW043	43	*Brucella*	Cattle	Chiredzi, ZW	This study
ZW045	45	*Brucella*	Bull testicle	Bindura, ZW	This study
ZW046	46	*Brucella*	Cattle	Norton, ZW	This study
ZW047	47	*Brucella*	Milk	Zimbabwe (ZW)	This study
ZW050	50	*Brucella*	unknown	Handerson, ZW	This study
ZW004	BM1	*B. melitensis*[b]	unknown	Zimbabwe (ZW)	This study
ZW011	11	*B. suis*	Pig	Shamwa, ZW	This study
ZW377	377	*B. canis*	Dog	Harare, ZW	This study
ZW005	B1	*B. ovis*	Sheep	Zimbabwe (ZW)	This study
ZW100	100	*B. canis*	Dog	Harare / Highlands, ZW	This study
ZW103	103	*Brucella*	Eland	Zimbabwe (ZW)	This study
REF 16M	ATTC 23456; BCCN R1	*B.melitensis* bv.*1*	goat	United States	Le Flèche et al., (2006)
REF 544	ATCC 23448; BCCN R4	*B.abortus* bv.*1*	cattle	England	Le Flèche et al., (2006)
REF 86/8/59	ATCC 23449; BCCN R5	*B.abortus* bv.*2*	cattle	England	Le Flèche et al., (2006)
REF Tulya	ATCC 23450; BCCN R6	*B.abortus bv3*	human	Uganda	Le Flèche et al., (2006)

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Strain[A]	Other number[a]	*Brucella* species	Host	Country	Reference
REF 292	ATCC 23451; BCCN R7	*B.abortus* bv. *4*	cattle	England	Le Flèche et al., (2006)
REF B3196	ATCC 23452; BCCN R8	*B.abortus* bv. *5*	cattle	England	Le Flèche et al., (2006)
REF C68	ATCC 23455; BCCN R11	*B.abortus* bv. *9*	cattle	England	Le Flèche et al., (2006)
REF Ether	ATCC 23458; BCCN R3	*B.melitensis* bv. *3*	goat	Italia	Le Flèche et al., (2006)
REF 870	ATCC 23453; BCCN R9	*B.abortus* bv. *6*	cattle	Africa	Le Flèche et al., (2006)
REF 63/9	ATTC 23457; BCCN R2	*B.melitensis* bv. *2*	goat	Turkey	Le Flèche et al., (2006)
REF BOW 63/290	ATCC 25840; BCCN R17	*B.ovis*	sheep	Australia	Le Flèche et al., (2006)
REF 1330	ATCC 23444; BCCN R12	*B.suis* bv. *1*	swine	United States	Le Flèche et al., (2006)
REF Thomsen	ATCC23445; BCCN R13	*B.suis* bv. *2*	swine	Denmark	Le Flèche et al., (2006)
REF 686	ATCC 23446; BCCN R14	*B.suis* bv. *3*	swine	United States	Le Flèche et al., (2006)
REF 40	ATCC 23447; BCCN R15	*B.suis* bv. *4*	reindeer	Former USSR	Le Flèche et al., (2006)
REF 513	BCCN R21	*B.suis* bv. *5*	wild rodent	Former USSR	Le Flèche et al., (2006)
REF RM 6/66	ATCC 23365; BCCN R18	*B.canis*	dog	United States	Le Flèche et al., (2006)
REF 5K33	ATCC 23459; BCCN R16	*B.neotomae*	desert rat	United States	Le Flèche et al., (2006)
BCCN 92-73		*B.abortus* bv. *1*	cattle	France (87)	Le Flèche et al., (2006)
BCCN 94-44		*B.abortus*	human	Algeria	
BCCN 95-11		*B.abortus* bv. *1*	cattle	France (63)	Le Flèche et al., (2006)
BCCN 95-19		*B.abortus*	chamois	France (73)	Le Flèche et al., (2006)
BCCN 95-51		*B.abortus*	cattle	Argentina	Le Flèche et al., (2006)
BCCN 95-55		*B.abortus*	cattle	Costa Rica	Le Flèche et al., (2006)
BCCN 96-62		*B.abortus*	cattle	Italia (Sicilia)	Le Flèche et al., (2006)
BCCN 96-137a		*B.abortus*	human	Argentina	
BCCN V1	B19, BCCN V1 (vac)	*B.abortus*	cattle	United States	Le Flèche et al., (2006)
BCCN R1	ATTC 23456	*B.melitensis*	goat	United States	
BCCN 91-90		*B.abortus* bv. *3*	cattle	Greece	Le Flèche et al., (2006)
BCCN 92-25		*B.abortus*	cattle	France (85)	Le Flèche et al., (2006)
BCCN 93-15		*B.abortus* bv. *3*	cattle	Spain (Pamplona)	Le Flèche et al., (2006)
BCCN 93-26		*B.abortus* bv. *3*	dromedary	Sudan	Le Flèche et al., (2006)
BCCN 94-19		*B.abortus* bv. *3*	cattle	France (87)	Le Flèche et al., (2006)
BCCN 95-7		*B.abortus* bv. *3*	cattle	France (87)	Le Flèche et al., (2006)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
BCCN 95-34		*B.abortus* bv. *3*	cattle	Italia (Sicilia)	Le Flèche et al., (2006)
BCCN 95-31		*B.abortus*	cattle	Italia (Sicilia)	Le Flèche et al., (2006)
BCCN 80-133		*B.abortus*	dog	Belgium	Le Flèche et al., (2006)
BCCN 87-46		*B.abortus*	cattle	France (65)	Le Flèche et al., (2006)
BCCN 99-98		*B.abortus*	cattle	Mongolia	Le Flèche et al., (2006)
BCCN V5	RB51, BCCN V5 (vac)	*B.abortus*	cattle	United States	Le Flèche et al., (2006)
BCCN R22	Reo 198, BCCN R22	*B.ovis*	sheep	United States	Le Flèche et al., (2006)
BCCN 97-41		*B.ovis*	sheep	Argentina	Le Flèche et al., (2006)
BCCN 98-46		*B.ovis*	sheep	Argentina	Le Flèche et al., (2006)
BfR 97	Rind Nr.8	*B.abortus*	cattle	Zimbabwe	Le Flèche et al., (2006)
BfR 103	9673	*B.abortus*	cattle	Germany (Frankfurt/O.)	Le Flèche et al., (2006)
BfR 104	9676	*B.abortus*	cattle	Germany (Frankfurt/O.)	Le Flèche et al., (2006)
BfR 105	9676	*B.abortus*	cattle	Germany (Frankfurt/O.)	Le Flèche et al., (2006)
BfR 106	2215	*B.abortus* bv. *3*	cattle	Germany (Frankfurt/O.)	Le Flèche et al., (2006)
BfR 107	2,240,148	*B.abortus* bv. *3*	unknown	Germany (Frankfurt/O.)	Le Flèche et al., (2006)
BfR 108	ZW 371	*B.abortus*	cattle	Germany (Frankfurt/O.)	Le Flèche et al., (2006)
BfR 110	28842	*B.abortus*	cattle	Germany (Uckermark)	Le Flèche et al., (2006)
BfR 111	28842	*B.abortus*	cattle	Germany (Uckermark)	Al Dahouk et al., (2007)
BfR 112	28842	*B.abortus*	cattle	Germany (Uckermark)	Al Dahouk et al., (2007)
BfR 113	24879	*B.abortus*	cattle	Germany (Uckermark)	Le Flèche et al., (2006)
BfR 114	24879	*B.abortus*	cattle	Germany (Uckermark)	Al Dahouk et al., (2007)
BfR 115	24879	*B.abortus*	cattle	Germany (Uckermark)	Al Dahouk et al., (2007)
BfR 116	24880	*B.abortus*	cattle	Germany (Uckermark)	Al Dahouk et al., (2007)
BfR 117	24880	*B.abortus*	cattle	Germany (Uckermark)	Le Flèche et al., (2006)
BfR 118	24880	*B.abortus*	cattle	Germany (Uckermark)	Al Dahouk et al., (2007)
BfR 120	Tgb.Nr.292/85	*B.abortus*	human	Germany (Lützenkirchen)	Al Dahouk et al., (2007)
BfR 121	Nr.6796	*B.abortus*	cattle	Unknown	Le Flèche et al., (2006)
BfR 122	Kuh 796	*B.abortus*	cattle	Unknown	Al Dahouk et al., (2007)
BfR 123	Di 5232	*B.abortus* bv. *6*	unknown	Unknown	Le Flèche et al., (2006)
BfR 124	Rind PN-89-225GTZ	*B.abortus*	cattle	Syria	Le Flèche et al., (2006)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
BCCN 99-98		*B.abortus*	cattle	Mongolia	Le Flèche et al., (2006)
BCCN 04-1		*B.abortus*	unknown	Turkey	Le Flèche *et al.* (2006)
BCCN V3	bru0128	*B.melitensis* bv. *1*	human	Mexico	Al Dahouk *et al.* (2007)
BCCN 87-92	bru0129	*B.melitensis* bv. *1*	human	United States	Al Dahouk *et al.* (2007)
BCCN 96-22	bru0131	*B.melitensis* bv. *1*	sheep	Israel	Le Flèche *et al.* (2006)
BCCN 03-14	bru0139	*B.melitensis* bv. *1*	human	France (91) presumably Portugal	Al Dahouk *et al.* (2007)
BCCN 75-478	bru0140	*B.melitensis* bv. *1*	sheep	Israel	Le Flèche *et al.* (2006)
BCCN 90-61	bru0141	*B.melitensis* bv. *1*	unknown	South Africa	Le Flèche *et al.* (2006)
BCCN 92-70	bru0142	*B.melitensis* bv. *1*	human	France (75) presumably Iran	Al Dahouk *et al.* (2007)
BCCN 93-2	bru0143	*B.melitensis* bv. *1*	human	France (86)	Al Dahouk *et al.* (2007)
BCCN 94-37	bru0145	*B.melitensis* bv. *1*	human	France (75) presumably Algeria	Al Dahouk *et al.* (2007)
BCCN 96-137b	bru0146	*B.melitensis* bv. *1*	human	Argentina	Al Dahouk *et al.* (2007)
BCCN 74-235	bru0147	*B.melitensis* bv. *2*	human	France (69)	Al Dahouk *et al.* (2007)
BCCN 77-6	bru0148	*B.melitensis* bv. *2*	goat	France (06)	Le Flèche *et al.* (2006)
BCCN 80-39	bru0149	*B.melitensis* bv. *2*	sheep	France (04)	Le Flèche *et al.* (2006)
BCCN 82-59	bru0151	*B.melitensis* bv. *2*	human	Spain (Madrid)	Al Dahouk *et al.* (2007)
BCCN 84-3	bru0152	*B.melitensis* bv. *2*	dog	Costa Rica	Le Flèche *et al.* (2006)
BCCN 92-87	bru0153	*B.melitensis* bv. *2*	sheep	Spain (Aragon)	Le Flèche *et al.* (2006)
BCCN 95-33	bru0154	*B.melitensis* bv. *2*	sheep	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN 96-3	bru0155	*B.melitensis* bv. *2*	human	France (65) presumably Middle-East	Al Dahouk *et al.* (2007)
BCCN 96-30	bru0157	*B.melitensis* bv. *2*	human	Israel	Al Dahouk *et al.* (2007)
BCCN 83-198	bru0159	*B.melitensis* bv. *3*	human	Spain	Al Dahouk *et al.* (2007)
BCCN 91-243	bru0160	*B.melitensis* bv. *3*	sheep	Tunisia	Le Flèche *et al.* (2006)
BCCN 92-42	bru0161	*B.melitensis* bv. *3*	human	Tunisia	Al Dahouk *et al.* (2007)
BCCN 95-30	bru0163	*B.melitensis* bv. *3*	sheep	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN 95-36	bru0164	*B.melitensis* bv. *3*	goat	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN 96-31	bru0165	*B.melitensis* bv. *3*	sheep	Israel	Le Flèche *et al.* (2006)
BCCN 96-32	bru0166	*B.melitensis* bv. *3*	sheep	Israel	Le Flèche *et al.* (2006)
BCCN 96-56	bru0167	*B.melitensis* bv. *3*	sheep	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN 96-97	bru0169	*B.melitensis* bv. *3*	cattle	Italia (Sicilia)	Le Flèche *et al.* (2006)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
BCCN 96-142	bru0170	*B.melitensis* bv. *3*	human	France (69) presumably Portugal	Al Dahouk *et al.* (2007)
BCCN 02-2	bru0171	*B.melitensis* bv. *3*	human	Tunisia	Al Dahouk *et al.* (2007)
BCCN 02-3	bru0172	*B.melitensis* bv. *3*	human	Tunisia	Al Dahouk *et al.* (2007)
BCCN 02-4	bru0173	*B.melitensis* bv. *3*	human	Tunisia	Al Dahouk *et al.* (2007)
BCCN 02-5	bru0175	*B.melitensis* bv. *3*	human	Tunisia	Al Dahouk *et al.* (2007)
BCCN 02-6	bru0176	*B.melitensis* bv. *3*	human	Tunisia	Al Dahouk *et al.* (2007)
BCCN 03-1	bru0177	*B.melitensis* bv. *3*	human	France (75)	Al Dahouk *et al.* (2007)
BCCN 03-13	bru0178	*B.melitensis* bv. *3*	human	France (06)	Al Dahouk *et al.* (2007)
BCCN 90-112	bru0179	*B.melitensis* bv. *3*	cattle	Greece	Le Flèche *et al.* (2006)
BCCN 92-80	bru0181	*B.melitensis* bv. *3*	sheep	Spain (Basque Country)	Le Flèche *et al.* (2006)
BCCN 92-106a	bru0182	*B.melitensis* bv. *3*	unknown	Algeria	Le Flèche *et al.* (2006)
BCCN 94-16	bru0183	*B.melitensis* bv. *3*	cattle	France (12)	Al Dahouk *et al.* (2007)
BCCN 96-148	bru0184	*B.melitensis* bv. *3*	human	Algeria	Al Dahouk *et al.* (2007)
BCCN R19	bru0185	*B.melitensis rough*	goat	Malta	Le Flèche *et al.* (2006)
BCCN 92-117	bru0187	*B.melitensis rough*	sheep	France (12)	Al Dahouk *et al.* (2007)
BCCN 94-62	bru0188	*B.melitensis rough*	human	France (47)	Al Dahouk *et al.* (2007)
BCCN 96-144	bru0189	*B.melitensis rough*	human	Algeria	Al Dahouk *et al.* (2007)
BCCN 96-147	bru0190	*B.melitensis rough*	human	Algeria	Al Dahouk *et al.* (2007)
BCCN 94-49	bru0191	*B.melitensis rough*	sheep	India	Le Flèche *et al.* (2006)
BCCN 92-73	bru0193	*B.abortus* bv. *1*	cattle	France (87)	Le Flèche *et al.* (2006)
BCCN 95-11	bru0195	*B.abortus* bv. *1*	cattle	France (63)	Le Flèche *et al.* (2006)
BCCN 95-19	bru0196	*B.abortus* bv. *1*	chamois	France (73)	Le Flèche *et al.* (2006)
BCCN 95-51	bru0197	*B.abortus* bv. *1*	cattle	Argentina	Le Flèche *et al.* (2006)
BCCN 95-55	bru0199	*B.abortus* bv. *1*	cattle	Costa Rica	Le Flèche *et al.* (2006)
BCCN 96-62	bru0200	*B.abortus* bv. *1*	cattle	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN V1	bru0202	*B.abortus* bv. *1*	cattle	United States	Le Flèche *et al.* (2006)
BCCN 91-90	bru0205	*B.abortus* bv. *3*	cattle	Greece	Le Flèche *et al.* (2006)
BCCN 92-25	bru0206	*B.abortus* bv. *3*	cattle	France (85)	Le Flèche *et al.* (2006)
BCCN 93-15	bru0207	*B.abortus* bv. *3*	cattle	Spain (Pamplona)	Le Flèche *et al.* (2006)
BCCN 93-26	bru0208	*B.abortus* bv. *3*	dromedary	Sudan	Le Flèche *et al.* (2006)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
BCCN 94-18	bru0209	*B.abortus* bv. *3*	cattle	France (87)	Le Flèche *et al.* (2006)
BCCN 94-19	bru0211	*B.abortus* bv. *3*	cattle	France (87)	Le Flèche *et al.* (2006)
BCCN 95-7	bru0212	*B.abortus* bv. *3*	cattle	France (87)	Le Flèche *et al.* (2006)
BCCN 95-34	bru0213	*B.abortus* bv. *3*	cattle	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN 95-31	bru0214	*B.abortus* bv. *4*	cattle	Italia (Sicilia)	Le Flèche *et al.* (2006)
BCCN 80-133	bru0215	*B.abortus* bv. *9*	dog	Belgium	Le Flèche *et al.* (2006)
BCCN 87-46	bru0217	*B.abortus* bv. *9*	cattle	France (65)	Le Flèche *et al.* (2006)
BCCN 99-98	bru0218	*B.abortus* bv. *7*	cattle	Mongolia	Le Flèche *et al.* (2006)
BCCN V5	bru0219	*B.abortus*	cattle	United States	Le Flèche *et al.* (2006)
BCCN R22	bru0220	*B.ovis*	sheep	United States	Le Flèche *et al.* (2006)
BCCN 97-41	bru0221	*B.ovis*	sheep	Argentina	Le Flèche *et al.* (2006)
BCCN 98-46	bru0223	*B.ovis*	sheep	Argentina	Le Flèche *et al.* (2006)
bru0240	BfR 1	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0241	BfR 2	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0242	BfR 3	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0243	BfR 4	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0244	BfR 5	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0245	BfR 6	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0247	BfR 7	*B.melitensis* bv. *2*	human	Turkey	Le Flèche *et al.* (2006)
bru0248	BfR 8	*B.melitensis* bv. *1*	human	Turkey	Le Flèche *et al.* (2006)
bru0250	BfR 10	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0251	BfR 11	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0252	BfR 12	*B.melitensis* bv. *2*	human	Greece	Al Dahouk *et al.* (2007)
bru0254	BfR 13	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0256	BfR 15	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0257	BfR 16	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0259	BfR I	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0261	BfR II	*B.melitensis* bv. *1*	human	Unknown	Al Dahouk *et al.* (2007)
bru0262	BfR III	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0263	BfR IV	*B.melitensis* bv. *2*	human	Lebanon	Al Dahouk *et al.* (2007)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
bru0266	BfR VII	*B.melitensis* bv. *2*	human	Syria	Al Dahouk *et al.* (2007)
bru0269	BfR IX	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0270	BfR X	*B.melitensis* bv. *2*	human	Bosnia	Al Dahouk *et al.* (2007)
bru0272	BfR XII	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0274	BfR XIV	*B.melitensis* bv. *2*	human	Unknown	Al Dahouk *et al.* (2007)
bru0301	BfR 1	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0302	BfR 2	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0304	BfR 4	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0305	BfR 5	*B.melitensis* bv. *2*	human	Germany (Düsseldorf)	Al Dahouk *et al.* (2007)
bru0308	BfR 7	*B.abortus* bv. *6*	cattle	Tchad	Le Flèche *et al.* (2006)
bru0309	BfR 8	*B.abortus* bv. *6*	cattle	Tchad	Le Flèche *et al.* (2006)
bru0310	BfR 9	*B.suis* bv. *2*	wild boar	Germany (Halle)	Le Flèche *et al.* (2006)
bru0314	BfR 12	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0315	BfR 13	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0316	BfR 14	*B.melitensis* bv. *1*	human	Germany (München, lab)	Al Dahouk *et al.* (2007)
bru0319	BfR 16	*B.melitensis* bv. *1*	human	Germany (Stuttgart, lab)	Al Dahouk *et al.* (2007)
bru0320	BfR 17	*B.melitensis* bv. *1*	human	Germany (Berlin)	Al Dahouk *et al.* (2007)
bru0321	BfR 18	*B.melitensis* bv. *1*	human	Turkey	Al Dahouk *et al.* (2007)
bru0322	BfR 19	*B.melitensis* bv. *1*	human	Germany (Mannheim)	Al Dahouk *et al.* (2007)
bru0323	BfR 20	*B.melitensis* bv. *1*	human	Pakistan	Al Dahouk *et al.* (2007)
bru0327	BfR 23	*B.melitensis* bv. *1*	goat	United Arab Emirates	Al Dahouk *et al.* (2007)
bru0329	BfR 25	*B.melitensis* bv. *1*	goat	United Arab Emirates	Al Dahouk *et al.* (2007)
bru0332	BfR 27	*B.melitensis* bv. *1*	human	Unknown	Al Dahouk *et al.* (2007)
bru0333	BfR 28	*B.melitensis* bv. *1*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0334	BfR 29	*B.melitensis* bv. *2*	human	Unknown	Al Dahouk *et al.* (2007)
bru0335	BfR 30	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0337	BfR 31	*B.melitensis* bv. *2*	human	Germany (München)	Al Dahouk *et al.* (2007)
bru0339	BfR 33	*B.melitensis* bv. *2*	human	Germany (Ulm)	Al Dahouk *et al.* (2007)
bru0340	BfR 34	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0341	BfR 35	*B.melitensis* bv. *2*	human	Germany (München)	Al Dahouk *et al.* (2007)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
bru0343	BfR 36	*B.melitensis* bv. *2*	human	Germany (Berlin)	Al Dahouk *et al.* (2007)
bru0344	BfR 37	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0345	BfR 38	*B.melitensis* bv. *3*	human	Germany (Berlin)	Al Dahouk *et al.* (2007)
bru0346	BfR 39	*B.melitensis* bv. *2*	human	Portugal	Al Dahouk *et al.* (2007)
bru0347	BfR 40	*B.melitensis* bv. *2*	human	Germany (München)	Al Dahouk *et al.* (2007)
bru0350	BfR 42	*B.melitensis* bv. *2*	human	Unknown	Al Dahouk *et al.* (2007)
bru0351	BfR 43	*B.melitensis* bv. *2*	unknown	Unknown	Al Dahouk *et al.* (2007)
bru0352	BfR 44	*B.melitensis* bv. *2*	unknown	Unknown	Al Dahouk *et al.* (2007)
bru0353	BfR 45	*B.melitensis* bv. *2*	human	Unknown	Al Dahouk *et al.* (2007)
bru0355	BfR 46	*B.melitensis* bv. *2*	unknown	Germany (Bonn)	Al Dahouk *et al.* (2007)
bru0356	BfR 47	*B.melitensis* bv. *2*	human	Turkey	Al Dahouk *et al.* (2007)
bru0359	BfR 50	*B.melitensis* bv. *2*	sheep	Syria (Aleppo)	Al Dahouk *et al.* (2007)
bru0361	BfR 51	*B.melitensis* bv. *2*	sheep	Syria (Meskane)	Al Dahouk *et al.* (2007)
bru0362	BfR 52	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0363	BfR 53	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0364	BfR 54	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0367	BfR 56	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0368	BfR 57	*B.melitensis* bv. *2*	human	Switzerland (Bern)	Al Dahouk *et al.* (2007)
bru0369	BfR 58	*B.melitensis* bv. *2*	human	Italy	Al Dahouk *et al.* (2007)
bru0370	BfR 59	*B.melitensis* bv. *2*	human	Italy	Al Dahouk *et al.* (2007)
bru0371	BfR 60	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0373	BfR 61	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0374	BfR 62	*B.melitensis* bv. *3*	human	Irak	Al Dahouk *et al.* (2007)
bru0375	BfR 63	*B.melitensis* bv. *3*	human	Greece	Al Dahouk *et al.* (2007)
bru0376	BfR 64	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0377	BfR 65	*B.melitensis* bv. *3*	human	Unknown	Al Dahouk *et al.* (2007)
bru0379	BfR 66	*B.melitensis* bv. *3*	human	Germany (Bonn)	Al Dahouk *et al.* (2007)
bru0380	BfR 67	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0381	BfR 68	*B.melitensis* bv. *3*	human	Germany (Urlaub, Tyrol)	Al Dahouk *et al.* (2007)
bru0383	BfR 70	*B.melitensis* bv. *3*	human	Italy	Al Dahouk *et al.* (2007)

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Strain[a]	Other number[a]	*Brucella* species	Host	Country	Reference
bru0385	BfR 71	*B.melitensis* bv. *3*	human	Greece	Al Dahouk *et al.* (2007)
bru0386	BfR 72	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0387	BfR 73	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0388	BfR 74	*B.melitensis* bv. *3*	human	Italy	Al Dahouk *et al.* (2007)
bru0389	BfR 75	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0392	BfR 77	*B.melitensis* bv. *3*	human	Spain	Al Dahouk *et al.* (2007)
bru0394	BfR 79	*B.melitensis* bv. *3*	human	Germany (Heidelberg)	Al Dahouk *et al.* (2007)
bru0395	BfR 80	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0397	BfR 81	*B.melitensis* bv. *3*	human	Unknown	Al Dahouk *et al.* (2007)
bru0398	BfR 82	*B.melitensis* bv. *3*	human	Kazakhstan	Al Dahouk *et al.* (2007)
bru0399	BfR 83	*B.melitensis* bv. *3*	human	Italy	Al Dahouk *et al.* (2007)
bru0400	BfR 84	*B.melitensis* bv. *3*	human	Germany (Würzburg)	Al Dahouk *et al.* (2007)
bru0401	BfR 85	*B.melitensis* bv. *3*	human	Turkey	Al Dahouk *et al.* (2007)
bru0403	BfR 86	*B.melitensis* bv. *3*	human	Germany (Gelsenkirchen)	Al Dahouk *et al.* (2007)
bru0404	BfR 87	*B.melitensis* bv. *3*	human	Germany (Ulm)	Al Dahouk *et al.* (2007)
bru0405	BfR 88	*B.melitensis* bv. *3*	human	Germany (Leipzig)	Al Dahouk *et al.* (2007)

[a] ZW: Zimbabwe *Brucella i*solates from obtained from CVL; REF indicate reference strains; ATCC: American type culture collection; BCCN *Brucella* culture collection, Nouzilly, France; BfR: Federal Institute for Risk Assessment, Berlin , Germany; bru: *Brucella* number at Universite Paris, Orsay France
[b] *Brucella* species determine using growth characteristics and biochemical profiles (phages lyses were not included).

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Appendix Table 3: The copy number repeats at each locus indicated for each strain from Zimbabwe and some reference strains.

	Copy number repeats at each locus															
	Panel 1								Panel 2A			Panel 2B				
Strain	Bruce06	Bruce08	Bruce11	Bruce12	Bruce42	Bruce43	Bruce45	Bruce55	Bruce18	Bruce19	Bruce21	Bruce30	Bruce04	Bruce07	Bruce09	Bruce16
ZW001	2	3	6	9	4	2	5	2	5	18	6	4	7	7	6	5
ZW201	2	3	6	8	4	2	5	2	5	18	4	3	6	7	6	5
ZW248	4	6	4	10	2	3	3	3	6	20	5	5	3	6	3	3
ZW052	4	6	4	11	2	3	3	3	5	20	5	6	3	5	3	2
ZW053	4	6	4	11	2	3	3	3	5	20	5	6	3	5	3	2
ZW323	4	6	4	11	2	3	3	3	6	20	5	5	3	4	3	5
ZW283	4	6	4	11	2	3	3	3	6	20	5	6	3	4	3	5
ZW002	3	6	2	8	1	1	4	2	3	4	5	2	6	9	6	8
ZW600	3	4	6	14	4	3	5	2	8	23	9	3	7	6	11	8
ZW040	3	6	4	11	2	3	3	2	9	21	7	5	6	4	5	3
ZW048	3	6	4	14	3	3	5	7	9	22	8	4	3	4	6	3
ZW105	3	5	2	14	4	3	3	3	8	18	6	6	2	5	11	4
ZW043	3	6	3	14	2	3	2	3	9	20	6	3	5	5	3	4
ZW045	3	6	6	14	2	3	2	3	9	20	6	3	5	5	3	3
ZW046	3	6	6	14	1	2	3	3	9	20	7	4	5	7	14	8
ZW047	3	5	9	6	6	2	5	2	8	20	8	5	9	4	19	2
ZW050	2	5	6	6	3	3	5	2	7	19	8	4	7	6	6	3
ZW004	2	3	5	10	4	1	3	2	4	19	9	3	8	6	6	5
ZW011	2	3	5	10	4	1	5	2	3	18	10	3	6	6	6	5
ZW005	3	5	2	10	1	1	5	2	3	4	9	2	5	7	6	7
ZW100	2	3	9	11	3	1	5	2	5	20						
ZW103	2	5	9		3	1	5	2	5	20	9	7	6	10.5	7	3
REF 16M*	3	4	2	13	4	2	3	3	5	18	6	6	2	3	8	3
REF 544*	4	5	4	12	2	2	3	3	5	21	8	5	3	5	3	4
REF 86/8/59*	4	5	4	12	2	1	3	3	6	21	8	5	3	4	3	3
REF 292*	4	5	4	12	2	2	3	2	6	21	8	5	3	4	3	3
REF 1330*	2	3	6	10	4	1	5	2	4	19	9	3	6	6	5	5
ZW377	2	3	8	10	3	1	5	2	4	20	9	3	7	6	9	6
REF BOW 63/290*	3	5	2	10	1	1	5	2	3	4	9	2	6	7	7	6
REF RM 6/66*	2	3	9	11	3	1	5	2	5	20	9	3	8	6	7	5

* Indicate results from reference strains DNA included in this study as controls

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