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Determining the status of *Brucella canis* in dogs in the Maputo region of Mozambique using various techniques

By

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Contents

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Summary

Determining the status of *Brucella canis* in dogs in the Maputo region of Mozambique using various techniques

by

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Brucella canis causes canine brucellosis in dogs inducing mainly contagious abortion. Diagnosis of *B. canis* is based on bacterial isolation that is time-consuming and inconsistent; serological tests (more than one test) that is ambiguous and lacks specificity; and PCR that may lack sensitivity as bacteraemia may not be constant. Since bacteraemia of *B. canis* develops 7-30 days after infection, often resulting in a sustained bacteraemia, PCR was investigated for the detection of *B. canis* in whole blood of dogs. The PCR sensitivity was validated to detect 3.8 fg *Brucella* DNA mixed with dog DNA as well as 1 x 10^2 cfu/ml *B. canis* in dog blood (mock infection) using primers (ITS66 and ITS279) that amplifies the 16S-23S ribosomal DNA intergenic spacer (ITS) region. The PCR assay for the detection of *B. canis* in whole blood samples was compared with bacterial isolation, serological tests, which include the rapid slide agglutination test (RSAT), 2-mercaptoethanol RSAT (2ME-RSAT) and imunochromatographic assay (ICA). These techniques were used to test 56 dog samples obtained from the Michangulene and Mafavuca villages at the municipality of Changalane, in District of Namaacha in Maputo, Mozambique for *B. canis*. No *B. canis* was isolated from dog blood using the classical

microbiology isolation and PCR. A sample was only presumed positive if both the 2ME-RSAT and ICA tested positive. None of the samples in this study tested positive using this criterion for serological testing. Results of this study indicated that *B. canis* was not present in the 56 dogs sampled in the Maputo region of Mozambique using bacteriology, PCR and serological tests (RSAT, 2ME-RSAT and ICA). Due to the discrepancy between serological tests we cannot conclude that *B. canis* is not present in the Maputo region of Mozambique. In future the accuracy of the serological tests, bacteriology and PCR assay should be assessed using experimentally infected *B. canis* dogs over a period followed by a surveillance study in Mozambique that includes urine, semen and blood samples collected from dogs.

GENERAL INTRODUCTION

Canine brucellosis is a disease affecting dogs and various members of the family Canidae. The disease is caused by bacteria belonging to the genus *Brucella*. Classification of *Brucella* species is based on its preferred host and pathogenicity, but phenotypic laboratory tests though time consuming, can be used to differentiate species and biovars in this genus. Brucellosis infection in dogs is mostly caused by *B. canis*.

Brucella canis was identified by Carmichael in 1966 as being the causative agent of abortion among Beagle dogs in the USA. Since then, many other countries have reported *B. canis* in dogs. Canine brucellosis occurs worldwide, but seems to be of great concern in central and southern America where it was reported to have an economic impact, mainly for the commercial breeders. The disease was also reported in South America, Europe, Asia and South Africa. Despite various investigations and prevalence studies, little data is available on the current status of the infection and prevalence, especially in developing countries.

Canine brucellosis is described as being a disease causing contagious abortion in dogs. The general clinical signes of the disease in dogs are reproductive failure (infertility or sterility) and abortion. Other clinical manifestations include testicular abnormalities, epididymitis and lymph node enlargement. Clinical symptoms of canine brucellosis are usually unapparent and many infected dogs, may go unnoticed. Transmission of *B. canis* commonly occurs by direct contact between dogs, e.g. by ingesting infected material or during mating. Aborted material and uterine discharges are the main sources of the organism and the most important vehicle for the spreading of bacteria. Transmission by urine in male dogs is higher since greater numbers of bacteria are found in male dog urine than in female dog urine. *Brucella canis* is transmissible to humans, making the disease a zoonosis. However, brucellosis in humans caused by *B. canis* is usually a mild disease. The infection is acquired by humans through contact with

secretions from the reproductive tract, such as aborted fetuses, urine, or vaginal secretions from infected dogs.

Bacteriological isolation of *B. canis* remains the gold standard (most reliable diagnostic method) but is time consuming and the absence of brucellae does not rule out brucellosis. Since clinical diagnosis such as abortion in females and infertility in males are not specific for the disease and clinical signs are often inapparent or non-specific, serological tests are most often used to define the brucellosis status. Currently, the traditional rapid slide agglutination test (RSAT), tube agglutination test (TAT), complement fixation test (CFT) and agar gel immunodiffusion test are the serological methods most commonly used. Nevertheless, these serological tests are non-specific since they cross-react with other Gram-negative bacteria. Recently, enzyme-linked immunosorbent assay (ELISA) and immunochromatographic assays (ICA) have been proposed but still require standardization. As conventional diagnostic methods have their limitations, there has been increased interest in developing rapid and accurate methods for detecting *Brucella spp*. PCR of the *Brucella* specific 16S-23S rDNA intergenic spacer region have been used to arbiter the brucellae infection status of dogs.

Thus far no suitable vaccine to prevent dog brucellosis is available. The prevention of the disease is mainly achieved by precluding the infection from being introduced in a kennel and / or interrupting the transmission from infected to healthy dogs through castration and sterilization of infected dogs followed by antibiotic treatment. The treatment of canine brucellosis in dogs is long and expensive and often yields unsuccessful results. Administration of antibiotic combinations is the most successful treatment.

With the globalization of the economy, the trading of animals and animal products has become regular among bordering regions. Animal and animal product movement increases the risk of spread of diseases and therefore poses a threat

to animal and / or public health. Furthermore it might contribute to the introduction of exotic diseases in countries. The improvement of the national staff and laboratorial proficiency are relevant actions that need to be taken to enable epidemiological control of emerging diseases and maintenance of global heath.

No official records on *B. canis* occurrence in Mozambique were found, although some clinicians refer to abortion in bitches. The compulsory testing of animals that are required for international trading make it relevant to establish suitable tests for diagnosis of the disease. Additionally, due to the risk of transmission to humans, dogs suspected of brucellosis should be tested immediately. In view of this, the present research project is designed to focuse on canine brucellosis in Mozambique. The project aims to clarify the *B. canis* infection situation in Mozambique.

Objectives:

The Maputo region in Mozambique was selected as study area. *Bacteriology*, serological tests and PCR were used to determine whether dogs in the Maputo region were infected with *B. canis*. The serological tests used in this study included the RSAT and ICA. The PCR assay using the 16S-23S ribosomal DNA (rDNA) ITS region for the detection of *B. canis* in whole blood from dogs were also verified in the present study.

CHAPTER 1: LITERATURE REVIEW

Etiology

Members of the genus *Brucella* are the causative agents of brucellosis. The disease is also known as undulant fever, contagious abortion and Malta fever (Moreno and Moriyó 2006). Brucellae are pathogenic both to humans and animals. Despite the fact that *Brucella* organisms are found in a broad range of animals, the bacterial species and strains have shown a strong tendency to remain in their preferred host, although they may infect secondary hosts (Greene and Carmichael 2006; Moreno and Moriyó 2006).

Ten species are found in the genus *Brucella*, primarily classified by their preferred host and pathogenicity. *Brucella abortus* (8 biovars) is pathogenic to cattle, and *B. canis* is pathogenic to dogs. *Brucella melitensis* infects goats and sheep; rodents are susceptible to *B. neotomae*, whereas sheep are infected by *B. ovis*. *Brucella suis* and its biovars infect pigs, hares, rodents and wild ungulates, swine and reindeer (Corbel and Brinley-Morgan 1984). *Brucella pinnipedialis* and *B. ceti* were isolated from marine mammals and their preferred host is seals and cetaceans, respectively (Foster *et al.* 2007). *Brucella microti* was isolated from the common vole *Microtus arvalis* (Scholz *et al.* 2008). The tenth species, *B. inopinata* is a single isolate from a human breast implant infection (Scholz *et al.* 2010)

Most of the *Brucella* species can infect humans causing mild to serious diseases (Young 1983; Carmichael and Shin 1996; Greene and Carmichael 2006). Transmission to humans is primarily from livestock and therefore a risk to consumers of contaminated animal products, farmers and other occupational exposure (Young 1995; Corbel 1997). Canine brucellosis is caused primarily by *B. canis* (Carmichael and Kenney 1968; 1970; Mateu-de-Antonio and Martin 1995; Wanke 2004), however other *Brucella* spp., (e.g. *B. abortus* and *B. suis*) have occasionally been reported to cause canine infections (Shin and

Carmichael 1999; Greene and Carmichael 2006). Canine brucellosis in humans occurs rarely and is a mild disease (Carmichael and Kenney 1968; Young 1983; 1985; Carmichael and Greene 1990; Wanke 2004; Greene and Carmichael 2006; Moreno and Moriyó 2006). Therefore humans seem to be relatively resistant. Only people in contact with very high numbers of bacteria, such as dog breeders or those in research or diagnostic laboratories, are considered to be at risk of *B. canis* infection (Carmichael and Bruner 1968; Carmichael and Kenney 1970; Lucero et *al.* 2005a).

Historical overview of *Brucella canis*

Brucella canis was first recognized in the late 1960s (Carmichael and Bruner 1968; Carmichael and Kenney 1968; Carmichael 1976) following initial reports of a *Brucella* like organism associated with more than 200 abortions in Beagles (Carmichael and Kenney 1968). Epidemics of infectious abortions caused by the *Brucella* like organism where reported since 1964 in dogs in the USA (Hall 1971). During 1966-1969 several investigators reported isolation of similar bacteria from increased incidence of abortions, epididymitis, and reproductive failures in dogs in the USA (Moore and Bennett 1967; Carmichael and Bruner 1968; Carmichael and Kenney 1968; 1970; Moore and Gupta 1970; Spink and Morisset 1970). Leland Carmichael and other researchers independently, isolated the organism from placental and foetal tissues and vaginal discharges (Carmichael 1966; Moore and Bennett 1967; Carmichael and Kenney 1968). Jones *et al.* (1968) indicated that the canine organism resembled *B. suis* biotype 3 based on growth characteristics as well as metabolic tests. However the canine organisms did not oxidize erythritol like *B. suis*. Since the organism is similar to *B. s*uis biotype 3 based on biochemical and metabolic tests, but differ antigenically from biotype 3 the name *B. suis* biotype 5 was suggested (Jones *et al.* 1968). However, Carmichael and Burner (1968) suggested the name *B. canis* and Jones *et al.* (1968) indicated they favoured the designation *B. canis* since the organism was not identical to any of the known *Brucella* species.

The name *B. canis* was chosen because it has characteristics typical of the genus *Brucella*, and was mainly isolated only from dogs (Carmichael 1976). Carmichael (1976) indicated that the Subcommittee of Taxonomy of *Brucella* in 1970 accepted *B. canis* as a separate species, with the reference strain designated RM6/66. Its taxonomic position as a member of the genus *Brucella* was based on DNA homology studies where similar polynucleotide homologies where found between *B. canis* and the established members of the genus *Brucella* (Verger et *al.* 1985, Alton et *al.* 1988).The agent was also isolated from laboratory workers (Carmichael 1976) and from owners of infected dogs (Munford et *al.* 1975; Ramacciotti 1978; Wanke 2004; Greene and Carmichael 2006).

Aetiology

Unlike the smooth *Brucella* organisms that infect several domestic animal species, the susceptible host range of *B. canis* is limited; the disease has been observed naturally only in dogs and in wild *Canidae* (Greene and Carmichael 2006). Natural infections with the three "classical" *Brucella* species (*B. abortus, B. suis* and *B. melitensis*) also may occur in dogs (Wanke 2004). However, such infections are sporadic, and epizootics do not occur. Cats, rabbits and non-human primates can be infected experimentally (Greene and Carmichael 2006). Nevertheless, cats are relatively resistant, showing only a transient bacteraemia (Carmichael and Kenney 1970; Greene and Carmichael 2006). Stray and feral dogs are deemed as the predominant reservoirs in the environment (Flores-Castro and Segura 1976; Hollett 2006).

Epidemiology of canine brucellosis

Most infections of *B. canis* were originally associated with the Beagle breed; however, this could be attributed the use of this breed as research animals and their popularity as purebred animals (Carmichael 1976; Hollett 2006). Outbreaks affecting large commercial or research breeding kennels (Beagle) and packs of field dogs were especially common in the USA (Carmichael 1966; Wooley *et al.*

1977; Gordon *et al.* 1985; Wanke 2004; Akan *et al.* 2005). However, since its initial identification in Beagles it has been reported in several breeds such as Labrador Retrievers, Cocker Spaniels, German Shepherds, Boston Terriers, Poodles and many more, (Carmichael 1976; Myers *et al.* 1974; Wanke 2004; Hollett 2006), as well as, any sexually mature, reproductively active and mixed breed dogs (Carmichael and Kenney 1968; Myers *et al.* 1974; Blankenship and Sanford 1975; Carmichael 1976; Hollett 2006).

Brucellosis caused by *B. canis* is widely distributed around the world. Review articles by Wanke (2004) and Hollett (2006) cited worldwide reports from Mexico, Brazil, Argentina, Chile, Germany, Spain, Italy, Czechoslovakia, Poland, France, Britain, Europe, Brazil, various states in the USA, Canada, Japan, China, India, Philippines, Korea, Japan, Turkey, Malaysia, Taiwan and Nigeria. In 2005, *B. canis* was isolated from dogs in South Africa (Gous *et al.* 2005). In spite of the diagnosis of *B. canis* in various parts of the world, its prevalence and exact global distribution is still unknown (Carmichael and Shin 1996; Moreno and Moriyó 2006).

The prevalence of *B. canis* infection varies according to the animal's age, housing conditions, breed, and geographic location. Pet dogs in suburban environments have a lower prevalence compared with stray dogs in economical depressed areas, which may reflect increased population density and uncontrolled breeding of dogs (Alton *et al.* 1988; Greene and Carmichael 2006). Carmichael (1990) in turn stated that high prevalence's are probably attained in countries possessing a large population of dogs, mainly in low-income countries where most dogs are found roaming freely around urban and suburban areas.

Infection and transmission

The infection of *B. canis* in dogs is characterized by abortions in females (Carmichael 1990; Mateu-de-Antonio and Martin 1995; Greene and Carmichael 2006); testical atrophy, epididymitis and infertile males (Carmichael 1990; Akan

et al. 2005); postatitis and orchitis (Mareu-de-Antonio and Martin 1995) and generalized lymphadenomegaly in both sexes (Akan *et al.* 2005; Greene and Carmichael 2006). The bacteraemia in dogs appears 2-4 weeks after initial infection (Wanke 2004; Hollett 2006; Greene and Carmichael 2006) and may last for one year or more (Hall 1971; Carmichael 1976; Wanke 2004; Greene and Carmichael 2006; Hollett 2006). Many dogs may not be bacteraemic, but they can still be infectious (Moore and Kakuk 1969; Zoha and Carmichael 1982). After cessation of bacteraemia the organism tends to locate in target reproductive tissues and may be released in a continuous or periodic way for months or years (Hollett 2006).

Venereal transmission of *B. canis* is common (Carmichael 1966 ; Carmichael 1976; Carmichael and Joubert 1988; Walker 1999) since large numbers of the organism are shed in genital secretions (Carmichael and Kenney 1970; Hollett 2006). Carmichael and Greene (1990) indicated up to 10^{10} per ml of organism are discharged through vaginal secretions (Wanke 2004) and spread by this route may continue for 4-6 weeks after abortion (Carmichael 1976). This makes the aborting bitch a high risk for distributing infection in kennels (Hollett 2006). The male prostate and epididymides are the effective organs for emission of the organisms and play an important role in the dissemination of the disease if the male remains actively breeding. Males harbour the organism in the epididymis and prostate gland (in addition to usual sites like the spleen and lymphatic tissue) and from some dogs the organism has been isolated beyond two months after cessation of the bacteraemia (Carmichael 1976). The concentration of the bacteria in initial semen sampling is higher during the first 2 months post infection (PI). After that the output became sporadic and scarce with the dog showing no apparent illness (Hollett 2006). Asymptomatic dogs harbour the bacteria for lengthy intervals (Moore and Kakuk 1969; Carmichael and Shin 1996).

Urine is another vehicle for disseminating *B. canis* to other dogs (Walker 1999; Wanke 2004). Although both sexes excrete bacteria in urine, the concentration in male urine is higher, reaching 10^3-10^6 bacteria per ml of urine (Greene and Carmichael 2006). For this reason, urine from a male animal is more dangerous as a source of infection. Excretion of bacteria through urine starts a few weeks after the onset of bacteraemia and continues for at least three months (Greene and Carmichael 2006). Furthermore, milk of infected lactating bitches (Hollett 2006) is also potentially infectious to nursing pups. Moreover, cages, equipment and people in contact with infected dogs have been reported as sources of infection (Johnson and Walker 1992). Blood transfusions, vaginoscopy, artificial insemination, and contaminated syringes are artificial vehicles for transmission of the bacteria (Hollett 2006).

Canine brucellosis is of particular importance to dog breeders since infection with *B. canis* usually ends a dog's reproductive career (Alton *et al.* 1988). Due to its economical impact on animal health it remains as an important disease, mainly in countries where *B. canis* is endemic (Keid *et al.* 2004; Brower *et al.* 2007; Watarai *et al.* 2007) and the risk to the human population (Keid *et al.* 2004; Watarai *et al.* 2007). The disease is insidious, and many dogs do not have prominent clinical signs (Alton *et al.* 1988; Greene and Carmichael 2006). Thus, apparently infected animals are important sources of transmission (Zoha and Carmichael 1982).

Characteristics of *Brucella canis*

Morphology and growth

Brucella canis is a small, Gram-negative, aerobic, coccobacillary organism. It has a rough colonial morphology and can be differentiated based on biochemical and antigenic reactions from other members of the genus *Brucella* (Greene and Carmichael 2006). *Brucella canis* grows luxuriantly on enriched media such as brucella broth (Albimi). Other media that support abundant growth are 5% sheep blood agar, trypticase-soy agar, tryptose medium, and Thayer-Martin medium.

Growth is somewhat slow, requiring 2-3 days for colonies to mature, and it is inhibited by CO_2. After several days of incubation, the translucent colonies become very mucoid (ropy in broth). Mature colonies are generally 1.0-1.5 mm in diameter (Jones *et al.* 1968; Alton *et al.* 1988; Carter *et al.* 1995). *Brucella canis* is described as being relatively short-lived outside the dog and is readily inactivated by common disinfectants (Carmichael and Shin 1996).

Antigenic properties

Brucella canis has similar biochemical reactions to *B. suis*, but it is antigenically similar to *B. ovis*. *Brucella canis* and *B. ovis* are both rough (R) *Brucella* strains, expressing rough lipopolysacharides (R-LPS). Lipopolysaccarides has three domains namely the lipid A, core oligosaccharide and the O-antigen or O-side chain (Gomes Cardoso *et al.* 2006). The R-LPS has a reduced or absent O-antigen compared to S-LPS with a 96-100 glycosyl subunit O-antigen (Carmichael and Shin 1996; Edmonds *et al.* 2002; Gomes Cardoso *et al.* 2006). *Brucella canis* and *B. ovis* share surface R-antigens, which can be used for the diagnosis of both canine and ovine brucellosis (Myers *et al.* 1974; López *et al.* 2005). The *B. canis* antigen cross-reacts with other R-*Brucella* such as *B. ovis* or *B. abortus* RB51 or 45/20, and with LPS moieties of certain bacterial species such as *Bordetella bronchiseptica*, *Actinobacillus equuli* and certain mucoid strains of *Pseudomonas* spp. and *Staphylococcus epididymidis* (Carmichael and Bruner 1968; Zoha and Carmichael 1982; Mateu-de-Antonio *et al.* 1993; Carmichael and Shin 1996; Nielsen *et al.* 2004). Myers *et al.* (1972) and Diaz and Jones (1973) demonstrated that hot saline-extracted antigen complexes from rough phase brucella react strongly with sera from animals infected with other bacteria possessing the “rough cell wall antigen” and only weakly with smooth (S) *Brucella* surface antigens. Later hot saline-extracted antigen was reported to cause cross reaction with *B. melitensis* infected animals since it contains a number of surface proteins that may cause the cross reaction with S- *Brucella* as well as R-LPS (Nielsen *et al.* 2004).

The natural mucoidiness of *B. canis* observed in broth after several days or on solid medium is a unique property (Carmichael and Bruner 1968; Carmichael and Kenney 1970; Alton *et al.* 1988). Since the growth of wild-type *B. canis* is naturally mucoid (M+), either on liquid or solid media, and tend to autoagglutinate and sometimes cause activation of complement in the absence of antibody, the reproduction of stable antigen for diagnostic tests has been a formidable problem (Serikawa et *al.* 1989; Carmichael and Shin 1996; Nielsen *et al.* 2004). Methods have been described for the use of either the pathogenic strain of *B. canis* (RM 6/66) (George and Carmichael 1974); *B. ovis* (Myers *et al.* 1974; George and Carmichael 1978; Alton *et al.* 1988) or *B. canis* less mucoid (M-) variant (Alton *et al.* 1988; Shin and Carmichael 1999) as sources of antigen for *B. canis* serodiagnosis. In general, published work in this area shows that tests using antigens made from *B. canis* (M-) lead to better results (Wanke 2004).

Pathophysiology

Brucella is a facultative intracellular parasite. Thus, both the pathogenesis of brucellosis (Schurig *et al.* 2002; Seleem *et al.* 2008) as well as the nature of the protective immunity is closely related to this property (Schurig *et al.* 2002). Different to other pathogenic bacteria, no classical virulence factors, such as exotoxins, cytolysins, capsules, fimbria, flagella, plasmids, lysogenic phages, resistant forms, antigenic variation, endotoxic LPS or apoptotic inducers have been described in *Brucella* organisms. Instead, they have molecular determinants as true virulence elements (Young 1995; Pappas et *al.* 2005; Moreno and Moriyó 2006). Those determinants allow them to invade, survive intracellularly and reach their replicating niche in professional and non-professional phagocytes (Corbel 1997; Pappas *et al.* 2005; Moreno and Moriyó 2006). The LPS O-side chain plays a role in virulence. Certain strains carry mutations in the genes involved in O-side chain biosynthesis. These mutants R-strains are generally less virulent than the wild type S strain. (Young 1995; Gomes Cardoso *et al.* 2006). Naturally R-strains (*B. canis* and *B. ovis*) are naturally virulent (Corbel 1997; Gomes Cardoso *et al.* 2006).

The mechanism by which *B. canis* binds cells or penetrates cells has not been studied extensively, however, it is likely that the pathogenesis of *B. canis* infection in dogs is similar to other animal species naturally infected with brucellae (Carmichael and Kenney 1970; Carmichael 1990; Young 1995; Corbel 1997). Young (1995) in turn, states that it may involve a potent, superoxide dismutase enzyme. R-*Brucella* with the exception of *B. canis* and *B. ovis* induce necrosis in macrophages. This is in contrast to S-*Brucella,* which inhibits host cell apoptosis, favouring bacterial intracellular survival by escaping host immune surveillance (Pei *et al.* 2006, Seleem *et al.* 2008). Nevertheless, the mechanisms and virulence factors that mediate macrophage cell death are not fully understood (Seleem *et al.* 2008). As with other pathogenic organisms, *Brucella* organisms have probably developed specialized structures that allow them access to host cytoplasmatic compartments and replication (Moreno and Moriyó 2006; Seleem *et al.* 2008). *Brucella* tends to localize within organs of the reticulo-endothelial system. This may explain some of the clinical manifestations of systemic brucellosis, such as lymphadenomegaly, hepatosplenomegaly, and the propensity for involvement of the skeletal system. Brucellosis infection in a susceptible animal depends on the nutritional and immunological status of the host, the size and route of the inoculum, and the species of *Brucella* causing the infection (Young 1995).

The general pathological changes following infection by *Brucella* is summarized in Figure 1. The bacteria gain entry into the animal after attaching to the exposed mucous membrane. The predominant routes of infection are the cells of mucosal surfaces of the mouth, vagina and the conjunctiva. However, transmission through respiratory and gastrointestinal tracts, as well as those membranes covering the prepuces may also occur, provided that sufficient numbers of organisms are introduced (Greene and Carmichael 2006). The minimum oral and conjunctival infectious doses for dogs are about 10^6 and 10^4 to 10^5 organisms, respectively (Carmichael 1990; Greene and Carmichael 2006).

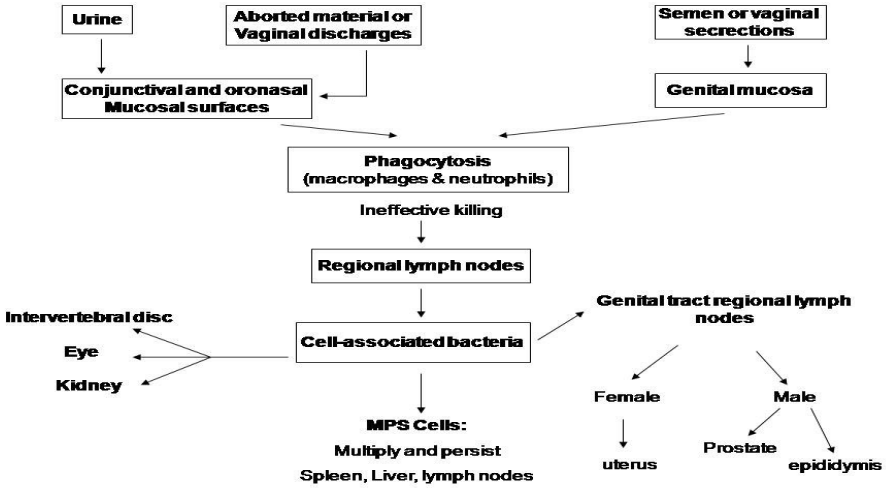

Fig.1 Patogenesis of the infection by *B. canis* [From Greene, C.E. and Carmichael, L.E. 2006. Canine brucellosis, In Greene, C.E. (eds.) *Infectious diseases of dog and cat*, 3rd ed. Canada : Saunders]

At the site of embedment, the bacteria are probably ingested by macrophages and other resident phagocytic cells. The virulence increase if more bacteria invade the tissue and phagocytosis continues. Thereafter, these intracellular bacteria cause a systemic infection. Intracellular bacteria are most prominent in organs such as lymph nodes, spleen and reproductive (steroid-dependent) tissues occurring as intracellular inclusions where the organisms initiate replicate. The genital or steroid-dependent tissue includes prostate, testicle, and epididymides in the males and fetus, gravid uterus, and placenta in the females (Carmichael and Kenney 1968; Greene and Carmichael 2006; Hollett 2006). Similarly to other blood-born bacteria, *B. canis* is likely to be localized in non-reproductive tissues such as in the circulation of the intervertebral disk and kidney where they cause diskospondylitis and glomerulopathy, respectively (Greene and Carmichael 2006; Hollett 2006). Other tissues that may become involved include the eye (anterior uveitis) (Wanke 2004; Greene and Carmichael 2006; Hollett 2006) and meninges (meningoencephalitis) (Greene and Carmichael 2006) as result of the invasion of the central nervous system (Young 1995).

The bacteraemia due to canine brucellosis is leukocyte-associated and is likely to develop within 7–30 days (Wanke 2004; Greene and Carmichael 2006; Hollett 2006) from initial exposure. After initial exposure the organisms persist for 6 months and longer, and then occur sporadically for up to five years (Carmichael 1990; Wanke 2004; Hollett 2006). This long period of sustained bacteraemia is common and significant for diagnoses of the disease. Greater than 10^3 bacteria per ml of blood are common found after week 1-2 month post infection (Greene and Carmichael 2006). Following cessation of the bacteraemia, organisms may persist as already mention for many months in the spleen, lymph nodes, bone marrow and reproductive tract (prostate gland and epididymides) of infected dogs (Carmichael and Bruner 1968; Carmichael 1976; Greene and Carmichael 2006).

Common histopathological signs include a generalized lymphoreticular hyperplasia and hyperglobulinemia (Greene and Carmichael 2006), and a granulomatous response in skin tests and organs (Greene and Carmichael 2006; Hollett 2006). A spermatic granuloma is generated from the cellular damage of the epididymides and testes (Greene and Carmichael 2006). As a result the semen consists of sperm with abnormal morphology and there is agglutination or absence of sperm (George *et al.* 1979; George and Carmichael 1984). Furthermore, there is leakage of the antigenic material into the surrounding tunica. This action induces antisperm agglutinating antibodies and delayed-type hypersensitivity responses against sperm (George et *al.* 1979; Greene and Carmichael 2006). These immunoreactions are produced against spermatozoa but are not related to the humoral responses against *B. canis*. Instead, they contribute to the reproductive failure seen in most infected male dogs (Greene and Carmichael 2006; Hollett 2006). After abortion the placenta may show multifocal coagulative necrosis of the villi, necrotizing arteritis, massive bacterial invasion of the trophoblastic epithelial cells (Hollett 2006).

Dogs may recover naturally from the infection within one year to more than five years post infection and are immune to reinfection, suggesting that protective immunity is cell mediated (Carmichael and Kenney 1970; Moore and Gupta 1970; Greene and Carmichael 2006). These dogs have low or negative agglutination titres. Natural recovery from *B. canis* infection appears to be essential to maintain the immunity at protective levels (Greene and Carmichael 2006).

Immunology

The immune response to *B. canis* is complex (Carmichael 1976), and have not been described, but most likely will follow the pattern of natural *Brucella* infections in other animal species (Carmichael and Kenney 1968). The antibody response to *B. abortus* will be used as a model since it has been studied thoroughly (Nielsen 2002). The antibody response to *B. abortus* in cattle consists of an early IgM isotype response depending on either the entrance route and as well as the health status of the animal (Beh 1974; Nielsen and Duncan 1990; Nielsen 2002; He 2006). Since IgM antibodies are mainly produced during exposure to organisms other than *Brucella* spp. or environmental antigens, serological tests that measure IgM results in false positives and a low assay sensitivity (Corbel 1985; Nielsen and Duncan 1990; Nielsen 2002). Soon after the IgM response, IgG1 is produced at high levels in *Brucella*-exposed cattle and later followed by little amounts of IgG2 and IgA antibodies (Nielsen and Duncan 1990; Nielsen 2002; Pappas *et al.* 2005). As IgG2 and IgA antibodies accumulate later after infection and are present in small amounts, the main isotype for testing is IgG1 and is the most useful (Nielsen 2002).

Cell-mediated immune response (CMI) appears to be the most important defense mechanism in response to infection by *B. canis* (Carmichael and Kenney 1970; Corbel 1997; Greene and Carmichael 2006; He 2006). Pavlov *et al.* (1982); Araya *et al.* (1989); Oliveira *et al.* (1996) and He (2006) indicated in their studies that both CD4+ and CD8+ T-cells are involved in immunity against brucellosis. As

products of CMI, cytokine molecules work in parallel with other signals arising from direct cell-to-cell contact, playing an important role in every function of the immune response (Zhan and Cheers 1995; Roitt *et al.* 1998; He 2006). Interferon-gamma (IFNγ) is an important cytokine that activates macrophages in general, and regulates antigen presenting cell (APC) function in many cell types. It is produced by activated T-cells (Th1 cells) and natural killer cells. Interferon-gamma (IFNγ) production is positively regulated by interleukin-2IL-2, a T-cell growth factor released by macrophages and dendritic cells (Roitt *et al.* 1998; He 2006).

Disease patterns in dogs

Canine brucellosis has been observed naturally in dogs and wild canids.(Carmichael and Kenney 1968; Carmichael 1976). Therefore the causal agent, *B. canis* has a limited host range (Carmichael 1990). Canine brucellosis is a disease that affects the reticuloendothelial system, reproductive organs and fetus. If pathological signs are present they are detected accordingly (Moreno and Moriyó 2006). Despite systemic infection, dogs are rarely observed ill. Fever is uncommon since this bacterium lacks the LPS O-side chain to produce endotoxins (Carmichael and Kenney 1970; Carmichael 1976; Hollett 2006). Most infections are not diagnosed by routine and physical examination. However, enlarged lymph nodes, prostatitis and epididymitis are among the prevailing signs detectable on physical examination (Carmichael 1976; Wanke 2004; Hollett 2006) It may vary from asymptomatic to mild infection. There are no clinical signs pathognomonic for canine brucellosis, but the infection should always be suspected in dogs examined for reproductive failure or infertility (Carmichael and Kenney 1970; Mateu-de-Antonio and Martin 1995; Wanke 2004; Hollett 2006).

The morbidity of *B. canis* is high but mortality is low in the affected animals (Hollett 2006). Clinical sns may be subtle or vague such as dry lusterless coats, loss of vigor, and decreased exercise tolerance (Greene and Carmichael 2006). Overt clinical signs usually include reproductive abnormalities in sexually mature

animals. For the female it may include infertility, apparent failure to conceive, early embryonic death, fetal resorption, failure to whelp and, late-term abortion that might go unnoticed by the owner (Moore and Gupta 1970; Greene and Carmichael 2006; Hollett 2006). Conception failures may also occur at any time after breeding. Early embryonic death and resorption, or abortion 10-20 days after mating, or near to term should be suspected if a bitch fails to conceive after an apparently succeialssful mating. Although aborted fetuses might not be found because the bitches usually ingest the expelled mater. Undetected abortions due to canine brucellosis can therefore be overlooked in bitches failing to conceive (Carmichael and Kenney 1970; Shin and Carmichael 1999; Greene and Carmichael 2006).

Reproductive disorders, including abortions (about 75% of the cases) (Shin and Carmichael 1999) and premature birth (Watarai et *al.* 2007) are the main problems (complaints) in pregnant animals with canine brucellosis. Bitches usually abort in the last trimester (after 45-60 days) of gestation but show little evidence of infection. Following abortion there is characteristic brown or greenish-grey vaginal discharge that persists for 1-6 weeks. Brucellosis should be suspected under any circumstance when apparently health bitches abort two weeks before term (Greene and Carmichael 2006). The female may subsequently deliver a normal litter or give birth to living but partially autolyzed, and stillborn pups (Carmichael and Kenney 1970; Hollett 2006). The pups, which are not dead when aborted, die within a few hours or days whereas surviving pups are infected and bactaeremic for several months or longer (Hollett 2006). The surviving pups may show generalized enlargement of peripheral lymph nodes as the primary clinical manifestation of disease until they reach sexual maturity. Such puppies may also have persistent hyperglobulinaemia and may present with transient fever, leukocytosis, or seizures as the systemic manifestations of their infections (Greene and Carmichael 2006).

Brucella infections (including *B. canis)* do not interfere with normal estrus cycles and breeding. Up to 85% of bitches that abort may have normal litters during the next pregnancy (Carmichael and Greene 1990; Shin and Carmichael 1999; Keid et al. 2007a; Hollett 2006; Greene and Carmichael 2006). However, some infected females will experience intermittent reproductive failure (Greene and Carmichael 2006). Rarely, bitches may abort up to four times in succession and have as many as three consecutive unsuccessful matings (Carmichael and Kenney 1968).

Possible signs of canine brucellosis in males include painful scrotal enlargement or testicular atrophy with decreased spermatogenesis, moist scrotal dermatitis, loss of libido, reluctance to breed, poor semen quality and high percentage of morphological abnormal sperm (Shin and Carmichael 1999; Greene and Carmichael 2006). Males appear to be in good health but may present an enlarged scrotum due to accumulation of serosanguineous fluid in the tunica. Scrotal dermatitis may also occur as the result of constant licking of the painful epididymides (Carmichael and Kenney 1970; Schoeb and Morton 1978; George *et al.* 1979; George and Carmichael 1984; Hollett 2006) and secondary infection with non-hemolytic staphylococci (Carmichael and Bruner 1968; Carmichael and Kenney 1970). The major cause of testicular swelling is enlargement of the tail of epididymis. Orchitis and primary testicular enlargement are rarely apparent. In fact, chronically infected males usually develop unilateral or bilateral testicular atrophy. Abnormal semen and testicular degeneration may also be seen in males (Alton *et al.* 1988). These include oligozoospermia, teratozoospermia or azoospermia in the ejaculate (Carmichael 1976; Hollett 2006).

Clinical signs other than reproductive abnormalities have also been reported from dog infected with *B. canis* (Greene and Carmichael 2006; Hollett 2006). Besides the splenomegaly which may accompany the diffuse enlargement of lymph nodes in some dogs, the liver may become enlarged. The spleen shows a firm and nodular consistency as a result of a granulomatous reaction (Hollett 2006).

Diskospondylitis of the thoracic and / or lumbar vertebrae may also be visualized by radiography (Carmichael 1976; Hollett 2006). Dogs with diskospondylitis may suffer fro spinal pain, paresis and ataxia. Lameness may be due to osteomyelitis or polyarthritis (Greene and Carmichael 2006). Ocular lesions including anterior uveitis, secondary glaucoma, hyphaema, retinal detachment, chorioretinitis, optic neuritis vitreal haze, enophthalmitis with secondary glaucoma or phthisis bulbi, and corneal edema with opacificattion; as well as diskospondylitis, polyarthritis, glomerulonephritis and osteomyelitis are often associated with canine brucellosis (Taul *et al.* 1967; Carmichael 1976; Keid *et al.* 2004; Greene and Carmichael 2006). Meningoencephalitis has also been reported but neurobrucellosis in dogs is rare. As previously described, the disease may go unnoticed in some infected dogs (Hollett 2006), making them important sources of infection (Keid *et al.* 2004).

Laboratory diagnosis

Canine brucellosis is a disease often without overt clinical manifestations, making laboratory diagnosis essential (Zoha and Carmichael 1982; Wanke 2004). Bacteriology and serological tests are the primary diagnostic tests used for canine brucellosis (Carmichael 1976; Hollett 2006; Watarai et *al.* 2007). Bacteriological characterization is the golden standard where the causative agent (*B. canis*) is isolated and identified from the blood, vaginal discharges, milk or semen from infected dogs, infected placental or fetal tissues (Carmichael and Bruner 1968; Zoha and Carmichael 1982; Shin and Carmichael 1999). However, serological tests are the most commonly used diagnostic assys to evaluate the status of dogs before breeding or whenever brucellosis is suspected. Due to false positive results obtained in diagnostic serology (Carmichael *et al.* 1984a; Alton *et al.* 1988; Shin and Carmichael 1999) and chronic cases that may give negative results, there is a need to complement diagnostics with bacteriological studies (Wanke 2004). Bacteriological studies are the only method that has been considered specific and provides a definitive diagnosis of brucellosis in a dog. Blood cultures are useful for the isolation of *B. canis* because of the prolonged

bacteraemia that occurs. Isolation and identification of *B. canis* are regarded as the gold standard method of diagnosing the disease especially when serological results are ambiguous (Carmichael 1976; Baldi *et al.* 1997). Zoha and Carmichael (1982) as well as Alton *et al.* (1988) in turn argue that intermittent periods of abacteraemia may occur, therefore if a bacterial culture is not obtained it cannot be used as a criterion to exclude canine brucellosis. Wanke (2004) reported that clinical data and anamnesis must be used in conjunction with serology and bacteriology to reach a definitive diagnosis.

The most significant diagnostic feature in canine brucellosis is the long period of sustained bacteraemia. It may last for periods longer than 1-2years (Carmichael 1976; Zoha and Carmichael 1982). Carmichael and Joubert (1987) reported that experimentally infected or untreated dogs remained positive on blood cultures for approximately five years. However, intermittent periods of abactaeremia may occur of variable duration following the initial bacteraemic phase of the disease (Zoha and Carmichael 1982; Alton *et al.* 1988; Greene and Carmichael 2006). *Brucella canis* may therefore be sequestered in organs such as the testis, prostate gland, and bone marrow for several months to a year after the bacteraemia has ceased. For this reason canine brucellosis cannot be excluded based on a negative blood culture (Carmichael 1976; Zoha and Carmichael 1982; Alton *et al.* 1988; Lucero *et al.* 2002; Hollett 2006). Urine samples have also cultured positive for the organism even in animals in which no bacteria were found in blood (Wanke 2004). Due to negative blood cultures, diagnostics of canine brucellosis is usually based on bacteriological characterization and serological tests (Carmichael 1976; Hollett 2006; Watarai *et al.* 2007). However, diagnostics is further complicated since serology can give false positive results (Carmichael *et al*. 1984a; Alton *et al.* 1988; Shin and Carmichael 1999; Lucero *et al.* 2002) and chronic cases can give negative () results. Thus, clinical signs and case history must be combined with bacteriological isolation and serological tests to ensure a definitive diagnostics (Wanke 2004).

Serodiagnosis

Several methods have been developed for the serodiagnosis of brucellosis (Nielsen 2002). *Brucella canis* presents viscous growth on solid media, therefore preparing stable, non-aggregating, antigens for diagnostic tests is difficult (Zoha and Carmichael 1981). Since *B. canis* and *B. ovis* are both R-strains that cross-react, *B. ovis* antigen has been employed for detection of canine serum antibodies against *B. canis* (Carmichael and Bruner 1968; Jones *et al.* 1968; Meyer 1969). Later the antigen *B. canis* (M- variant) has been proposed for serodiagnosis of *B. canis* infection in dogs using a rapid screening agglutination test (RSAT) (Carmichael and Joubert 1987) and an indirect enzyme-linked immunoassay (iELISA) (Lucero *et al.* 2002).

Various authors (Myers *et al.* 1974; Zoha and Carmichael 1982; Carmichael and Joubert 1987; Keid *et al.* 2009) argued that the variability in the values of sensitivity and specificity of the serological tests found in serological studies may be the result of different protocols for antigen production. In these studies differences occurred in the properties of cell wall antigens obtained from *B. ovis*, wild type *B. canis* strains and the less virulent (M-) *B. canis* strains. These findings suggest that the *Brucella* strains used to produce the antigens may also have different impacts upon the performance of the serological tests. Antigens used in the serological tests can be LPS of the bacterial cell wall of *B. ovis,* the pathogenic strain of *B. canis* (RM6/66) and the less mucoid strain (M-) of *B. canis* (Myers *et al.* 1974; George and Carmichael 1978; Wanke 2004). Wanke (2004) indicated that tests using the cell wall antigens of *B. canis* (RM6/66) and *B. ovis* lead to more false positive reactions compared to those using the M- strain antigen (Carmichael and Joubert 1987). Precipitation tests and primary binding assays (including the ELISA) can also be prepared with antigens that only have cytoplasmic proteins to *B. canis* and *B. abortus* (Baldi *et al.* 1997). Antibodies against LPS emerge earlier and vanish shortly after bacteraemia while antibodies against cytoplasmic proteins tend to persist 6-12 months and thus allow detection of chronic cases (Zoha and Carmichael 1982).

Agglutination tests

Serological diagnosis of canine brucellosis is usually performed by agglutination tests using the RSAT (George and Carmichael 1974; Zoha and Carmichael 1982; Wanke 2004), and TAT (Carmichael and Joubert 1967; George and Carmichael 1974; Myers *et al.* 1974; Carmichael 1976; Flores-Castro and Carmichael 1978; Shin and Carmichael 1999; Watarai *et al.* 2007). As mentioned previously, antigens used in these tests can be LPS of the bacterial wall of *B. ovis*, the pathogenic strain of *B. canis* or the less mucoid strain (M-) of *B. canis* (George and Carmichael 1978; Badakhsh *et al.* 1982; Carmichael and Joubert 1987). Agglutination tests detect antibodies directed to bacterial cell wall antigens, from which the rough lipopolysaccharide (R-LPS) is the most important (Carmichael and Joubert 1987). Both tests can be used with or without 2-mercaptoethanol (2-ME). The 2-ME removes naturally occurring IgM agglutinins responsible for some of the cross-reactivity and therefore increases the test specificity (George and Carmichael 1978; Bosu and Prescott 1980). Nevertheless, false-positive reactions may still occur (Flores-Castro and Carmichael 1978; Carmichael and Joubert 1987; Johnson and Walker 1992; Wanke 2004). The non-specific reactions could be due to the use of whole bacterial cell antigens in the TAT and RSAT (Corbel 1985; Watarai *et al.* 2007).

The tube agglutination test described above has the following disadvantages which limits its widespread use: (i) Since dog blood haemolyses readily, many serum samples are unsatisfactory for the tube agglutination test; (ii) The test is not sensitive enough to detect the low antibody titers of some chronically infected dogs; (iii) The prozone phenomenon is seen with some sera; and (iv) The procedure requires a 48 hours incubation period. The RSAT was developed to provide a presumptive diagnosis rapidly and has been found to be accurate in identifying non-infected dogs. False positive reactions are common since the IgM cross-reacts with other bacteria including steptococci, staphylococci and *Pseudomonas* (Carmichael 1990).

RSAT and 2ME-RSAT

George and Carmichael (1978) developed a rapid plate test with a rose bengal stained antigen (Alton *et al.* 1988). The RSAT is a rapid diagnostic test, accurately identifying non-infected dogs, however false positive reactions are common due to shared determinants between the surface antigens of *B. canis* and certain other Gram-negative bacteria (Carmichael *et al.* 1984a). The RSAT has been modified to include rapid reaction of test sera with 2ME (0.2M) prior to adding test antigen, which improved the specificity but did not eliminate false positive reactions (Carmichael *et al.* 1984a). This test has been commercialized as canine brucellosis antibody test kit and is manufactured by Synbiotics Corporation, USA.

Precipitation tests

Precipitin tests include the agar gel immunodiffusion test (AGID) or complement fixation test (CFT). The CFT is occasionally used in the serodiagnoses of dog brucellosis (Myers *et al.* 1972; Nielsen 2002; Wanke 2004) and has been reported to have a good correlation with TAT, but due to anti-complement activity of canine sera it is not routinely used (Wanke 2004).

The AGID employs crude LPS cell wall extracts as antigen extracted with various methods like hot saline, or sodium desoxychocolate (SDC), or cytoplasmic proteins extracted by sonication or pressure disruption (George and Carmichael 1974; Myers *et al.* 1974; Flores-Castro and Carmichael 1978; Carmichael *et al.* 1989; Watarai *et al.* 2007). The AGID for *Brucella* spp. was development since the agglutinin tests could not distinguish between antibodies of vaccine strains and natural *Brucella* infections (Myers *et al.* 1972; Zoha and Carmichael 1982; Carmichael 1990; Nielsen 2002). The AGID test has been extensively used with a hot saline extracted antigen. This assay has been shown to be as accurate as the CFT But the AGID as well as CFT are difficult to standardize, slow and require large amounts of reagents (Nielsen *et al.* 2005).

Primary binding assay

Problems with the agglutination tests resulted in the development of the primary binding assays (Nielsen 2002) such as ELISA and fluorescence polarization assay (FPA). Many authors have recommended the use of ELISA for the detection of anti-*B. canis* antibodies in dogs, but variable results have been reported due to the use of differently prepared antigens. Barrouin-Melo *et al.* (2007), using *B. canis* surface antigens, obtained equivalent specificity (91%) and sensitivity (95%) when compared with AGID. Mateu-de-Antonio et *al.* (1993) obtained 95.6% specificity and 93.8% sensitivity in an ELISA test employing as antigen an extract prepared from a less mucoid variant (M-) of *B. canis*. A capture ELISA test based on the detection of serum antibodies against cytoplasmic proteins (p18) (Baldi *et al.* 1994; 1997) and recombinant cytoplasmic proteins (p15, p17 and p39) (Letesson *et al.* 1997) were developed for the diagnosis of canine and cattle brucellosis. The capture ELISA has showed a high correlation with the 2ME-RSAT test and appears as sensitive and specific as the indirect ELISA previously described (Baldi *et al.* 1994), which employed a whole extract of cytoplasmic proteins of *Brucella*. The most recent validation of an ELISA method for the serological diagnosis of canine brucellosis was by de Oliveira *et al.* (2010) where a heat soluble bacteria extract from wild type *B. canis* had high sensitivity (91.18%) and specificity (100%). Although ELISA has been commonly applied to the serological diagnosis of infectious diseases including classical brucella, few studies applied ELISA to detect *B. canis* (Baldi *et al.* 1997; Lucero *et al.* 2002; de Oliveira *et al.* 2010).

Immunochromatographic assay (ICA)

The Anigen Rapid C. *Brucella* Ab Test Kit is an ICA, manufactured by Bionote Inc, Korea (previously known as Anigen Inc.). This ICA qualitatively detects *B. canis* antibody in whole blood, plasma, or serum. The test uses *B. canis* antigens as both capture and detector materials to identify *B. canis* antibodies in specimens with a high degree of accuracy. The ICA is the simplified version of the ELISA (Lucero *et al.* 2002; Kim *et al.* 2007). Kim *et al.* (2007) evaluated ICA,

haemoculture and 2ME-RSAT and found the sensitivity and specificity of ICA comparable with hemoculture and 2ME-RSAT. The ICA is a one-step testing procedure that provides a quick and accurate tool for serodiagnosis of canine brucellosis (Kim *et al.* 2007). However the Anigen Rapid C. *Brucella* Ab Test Kit has the following limitations: (i) it will only indicate the presence of antibody to *B. canis*; (ii) if the test result is negative and clinical signs persist, additional testing using other clinical methods is recommended. Therefore a negative result does not at any time preclude the possibility of canine brucellosis.

Bacterial isolation

Serological tests can result in false seropositive and seronegative results (as indicated above). Therefore, bacteriological methods should always be used to confirm *B. canis* infection in dogs. Blood samples are the preferred specimens for the detection of the organism in dogs due to; (i) the prolonged period of bacteraemia characteristic of canine brucellosis; (ii) the low possibility of contamination using blood samples (venal puncture less prone to contamination); and (iii) haemoculture allows early stage detection compared to serology when antibody levels are not yet detectable (Carmichael and Kenney 1970; Johnson and Walker 1992; Carmichael and Shin 1996; Keid *et al.* 2004). Isolation of organisms from the urine may be successful in dogs that were negative to haemoculture (Carmichael and Greene 1990; Wanke 2004, Hollett 2006). 10 UFC/mL to 10^5 UFC/mL of bacteria are shed through urine and the bacteria are detectable between eight to thirth weeks post infection (Carmichael and Greene 1990). However isolation from male urine will be easier and consistent since it has been reported that culture from urine is more reliable and easier to carry out in males since female urine is often contaminated with other micro-organisms (Wanke 2004). Nevertheless, the success of the *Brucella* isolation is dependent on, provided that the animal had not received antibiotic therapy, the viability of the bacteria as well as on the phase of the infection. Thus, negative results do not rule out the possibility of infection (Carmichael 1976; Johnson and Walker 1992; Carmichael and Shin 1996).

Commonly used media include serum dextrose, tryptose, and *Brucella* (albimi) agars or broth; trypticase soy, horse or cow blood agar, and brain hearth infusion broth (Moore and Gupta 1970; Alton *et al.* 1988). Contamination may impair growth of *Brucella* in culture. Therefore isolation attempts should be made using media containing actidione (30mg/l), bacitracin (7500 U/l), and polymixin B (1800 U/l) or selective media are used both with and without the incorporation of ethyl violet (1:800000) (Walker 1999). Inoculated plates are incubated at 37°C aerobically for a minimum of 10 to 21 days (Moore and Bennett 1967; Alton *et al.* 1988; Quinn *et al.* 1994). Growth on solid media may only be visible after 48 hours of subculture from broth, and mature colonies (1.0-1.5 mm in size) develop after 3-4 days. Colonies become very mucoid after several days of incubation, especially when the media pH is bellow 6.8 (Zoha and Carmichael 1981). In broth, turbidity is first detected after 2-3 days, and a ropy sediment forms after several days of growth. Incubation should be done aerobically at 35 to 37°C since CO_2 is inhibitory (Zoha and Carmichael 1981).

PCR

As described previously, microbiological culture is time consuming and can yield false-negative results. In addition, some samples may be heavily contaminated with other microorganisms, which may impair growth of *Brucella* (Johnson and Walker 1992; Wanke 2004). PCR is a good alternative to overcoming some of the major drawbacks of bacteriological methods for the direct diagnosis of brucellosis since it is a rapid and sensitive technique to detect *Brucella* DNA. Furthermore, it does not depend on bacteria viability, nor is it affected by the presence of contaminants (Bricker 2002). Although PCR appears to be faster, more sensitive and more specific than traditional bacteriology tests, it is considered to be only a confirmatory test due to problems such as inhibitors and sensitivity (bacteraemia may not be constant) (Queipo-Ortuno *et al.* 1997, Elfaki *et al.* 2005; Keid *et al.* 2007a).

A number of different factors can influence the outcome of PCR amplification. High DNA concentrations have been reported to interfere with PCR amplification (Morata *et al.* 1998; Zerva *et al.* 2001; Navarro *et al.* 2002). Morata *et al.* (1998) found that concentrations higher than 4 µg total DNA in the PCR reaction can inhibit the reaction. It has been speculated that high concentration of host DNA can also influence the PCR (Navarro *et al.* 2002). Furthermore other compounds such as haemoglobulin, heparin and EDTA anticoagulants can be potential PCR inhibitors. Fredricks and Relman (1998) reported that commercial blood culturing media are known to contain PCR-inhibiting substances, like polyanetholesulfonate (SPS), thus limiting the sensitivity of PCR assays.

Numerous PCR-based methods for direct detection of *Brucella* have been developed like the 16S ribosomal DNA (rDNA) (Herman and De Ridder 1992), rRNA operon (Navarro *et al.* 2002), 16S-23S rDNA intergenic spacer (ITS) region (Fox *et al.* 1998), BCSP31 / 31 kDa omp (Baily *et al.* 1992) and omp2a and omp2b genes (Leal-Klevezas *et al.* 1995). The 16S rDNA and rRNA operon gene cross-react with *Ochrobactrum anthropi* whereas the others including the 16S-23S rDNA ITS region had no false positive results with *O. anthropi* (Cortez *et al.* 2001). Despite the high level of conservation among *Brucella* species, extensive efforts to design more specific PCR assays capable of differentiating *Brucella* organisms at species and/ or biovar level have been expended. The AMOSPCR assay designed with highly specific primers in a multiplex PCR reaction was based on the differences of the genetic element IS*711* also known as IS*6501* (Halling *et al.* 1993; Bricker and Halling 1995). This assay differentiate *B. abortus* (biovars 1, 2 and 4), *B. melitensis* (biovars 1, 2 and 3), *B. ovis* and *B. suis* biovar 1. Another multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains S19 and RB51 of *B. abortus and B. melitensis* Rev1 was based on various genes that enabled species and strain differentiation (Garcia-Yoldi *et al.* 2006). Multiple-locus variable-number tandem analysis (MLVA) has become a powerful tool for *Brucella* species identification as

well as further differentiation of strains associated with a local outbreak or investigation (Le Fleche *et al.*, 2006).

Detailed methods for the PCR for detection of *Brucella spp*. DNA in blood (Queipo-Ortuno *et al.* 1997; Keid *et al.* 2007a), semen (Keid *et al.* 2007b) and vaginal swabs (Keid *et al.,* 2007c) in dogs, have already been published. Keid *et al.* (2007a) used the 16S-23S ITS rDNA because ITS sequences have been reported to be relatively identical within the *Brucella* genus. This author designed a primer pair, ITS66 and ITS279, potentially capable of amplifying genetic sequences from *Brucella* spp. without cross-reaction with other closely related organisms (Keid *et al.,* 2007a). The DNA extracted from whole-blood of naïve dogs were spiked with decreasing amounts of *B. canis* DNA and tested by PCR. In this study the PCR could detect 3.8 fg *Brucella* DNA in the presence or absence of 450 ng dog DNA. As inferred from the molecular mass of the *Brucella* genome, 3.8 fg *B. canis* DNA corresponds to two bacterial cells in dog blood (Keid *et al.* 2007a). Furthermore the ability of the PCR to amplify *Brucella* DNA from naturally infected dogs was evaluated and compared to blood culture and serodiagnosis using RSAT and 2ME-RSAT. The PCR and blood culture showed good concordance in their ability to detect *Brucella*-infected dogs. However the molecular method, PCR, allows for faster diagnosis. The authors found that a positive result in 2ME-RSAT, blood culture or PCR strongly imply infection in the animal and concluded that PCR should be used as confirmatory test for positive RSAT results (Keid *et al.* 2007a). Keid *et al.* (2009) compared the RSAT, 2ME-RSAT, AGID, microbiological culture and PCR for the diagnosis of canine brucellosis. They observed a significant proportion of false-negative results from the serological tests that are most commonly used for canine brucellosis diagnosis. They indicated that direct methods like blood culture and PCR is essential to improve diagnosis of canine brucellosis.

Prevention and eradication

Unsuccessful attempts have been made to produce a suitable vaccine to prevent canine brucellosis. The level of the immunity achieved by the studied vaccines as well as the interference of the vaccine antibodies in the serodiagnosis has discouraged the development of vaccine against dog brucellosis (Shin and Carmichael 1999). Thus, prevention of the disease in a kennel is based on eliminating the infected dogs in kennels (Moore and Bennett 1967; Currier *et al.* 1982; Johnson and Walker 1992; Shin and Carmichael 1999; Hollett 2006). It has been demonstrated that the eradication of *B. canis* infection in dogs can be best achieved by systematic testing of dogs and removal strategy or castration of reactors (Flores-Castro and Carmichael 1981, Mateu-de-Antonio and Martín 1995; Shin and Carmichael 1999; Hollett 2006).

No treatment thus far reported ensure complete elimination of *B. canis* infection (Carmichael 1966; Carmichael and Kenney 1968; Wanke 2004). Antimicrobial therapy is expensive and has not always been successful probably because *Brucella* is a facultative intracellular parasite that localizes in the prostatic tissue and lymph nodes where many antibiotics do not reach therapeutic levels (Carmichael and Kenney 1968; Moore and Gupta 1970; Flores-Castro and Carmichael 1981). Furthermore, single antibiotic therapy is not efficacious to eliminate the infection. The most successful and practical treatment results were obtained when using a combination of two antibiotics (Mateu-de-Antonio and Martín 1995; Wanke 2004; Hollett 2006) namely a tetracycline drug (e.g., tetracycline hydrochloride, doxycycline, minocycline) and streptomycin administered during the first three months of infection (Shin and Carmichael 1999).

Lack of quarantine and preventive measures have contributed to the spread of the disease in kennels and also around the world (Shin and Carmichael 1999). Measures that need to be implemented once canine brucellosis is diagnosed until complete eradication of the disease include: (i) quarantine of the infected

kennels; (ii) all dogs in the kennel must be submitted to serological tests and haemoculture; (iii) the source of the infection must be identified; (iv) all the reactors must be taken away from the unit to avoid the spread of the disease; (v) the animals must be physically isolated as much as possible; (vi) the healthy dogs must be treated with tetracycline and streptomycin and they should be monitored for one month so that the new positives may be eliminated from the group until no more positives are detected for three consecutive months; (vii) vigorous measures of hygiene must be applied to eliminate the bacteria in the kennel using quaternary ammonia and iodides disinfectants to clean out the unit; (viii) follow-up testing every three months for a year until the colony is negative on two successive tests (Carmichael and Joubert 1968; Shin and Carmichael 1999; Wanke 2004; Hollett 2006).

Control among pet dogs is difficult. Some control strategies include sanitation and removal of seropositive animals. The infected dogs are then castrated and submitted to treatment. Although the treatment with antibiotics have not been proven effective, the chance to achieve success is greater in early infections. Continue serological testing is recommended for 3 months post-treatment. Euthanasia should also be considered due to the uncertain results and the expense of the antimicrobials (Shin and Carmichael 1999; Hollett 2006).

Public health significance

Transmission of *Brucella canis* to humans is rare (Carmichael 1990). Currently, only a few cases of human infections (approximately 40) have been reported worldwide (Ying *et al.* 1999; Shin and Carmichael 1999; Piampiano *et al.* 2000; Lucero *et al.* 2005b; 2008). The true prevalenc of illness is unknown (Shin and Carmichael 1999; Wanke 2004) since cases are misdiagnosed or not reported (Corbel 1997; Shin and Carmichael 1999; Lucero *et al.* 2005b; 2009). Human brucellosis has long been recognized to be an occupation-related disease affecting primarily laboratory workers or animal technicians, kennel personnel, dog owners and few cases where the sources of infection were unknown

(Carmichael 1976; Carmichael and Shin 1996; Wanke 2004; Hollett 2006). Common inoculation routes include cuts and abrasions in the skin or via the conjunctiva, inhalation of infected aerosols, and via oral (Young 1995). Unlike dogs, infected people respond rapidly to antibiotic therapy (Ramacciotti 1980; Shin and Carmichael 1999).

Susceptibility to infection depends upon various factors, including the nutritional and immune status of the host, the size and the route of the inoculum, and the species of *Brucella* involved (Young 1995). The symptoms of *B. canis* infection are relatively mild compared to brucellosis caused by *B. melitensis, B. suis* and *B. abortus* (Corbel 1997; Greene and Carmichael 2006). *Brucella canis* has been demonstrated to cause hepatosplenomegaly (Tosi and Nelson 1982; Rousseau 1985; Schoenemann *et al.* 1986; Lucero *et al.* 2005b), intermittent fever and bacteraemia for 4 months in humans (Carmichael 1976; Lucero *et al.* 2005b; Hollett 2006). Some infected individuals were asymptomatic (Carmichael 1976) and others developed non-specific signs such as headache and weakness (Hollett 2006). The disease has a greater impact on immunosuppressed individuals, children and pregnant women.

CHAPTER 2: MATERIALS AND METHODS

Animals, clinical examination and sampling

Dogs in the villages of Michangulene and Mafavuca, at the municipality of Changalane, District of Namaacha, in Maputo were sampled from June 2007 to July 2008 during outreach programs organized by the Veterinary Faculty of the University of Eduardo Mondlane in Maputo, Mozambique. The outreach programs were done specifically for *Brucella canis* survey. Dogs were submitted for clinical examination and the dog owners (were applicable) were asked to answer a questionnaire (see Appendix 1) to record general information and clinical data such as: age, sex, bread, habits (free living or enclosed), abortions or conception failure, whelping of dead puppies, neonatal death, vaginal discharge, orchitis, epididymitis, lymph nodes enlargement and uveitis. Canine brucellosis would be suspected if an animal presented with at least one of the clinical signs (Keid *et al.* 2004). Samples were taken from 56 adult dogs of either sex from several breeds that were stray, free living (has an owner but are not enclosed by a fence in a village) or enclosed dogs. The study also included collecting blood from a non-infected dog that was used in the analytical sensitivity evaluation of the PCR in this study. The non-infected dog blood was previously tested and was brucellosis free by the Rapid Slide agglutination Test.

Decontamination of the skin before sampling was done on all dogs in order to avoid contamination of the cultures. The skin over the jugular vein was disinfected by application of tincture of iodine. After few minutes (to permit the disinfectant to work in skin surface), a total of 10 ml of blood was collected from each animal by jugular vein puncture. Of this, 4 ml were collectedinto a 5 ml blood tube without anticoagulant and left to coagulate at room temperature . On arrival at the Microbiology laboratory of the Faculty of Veterinary Science, Eduardo Mondlane University, tubes with coagulated blood samples were then centrifuged (at 1500 rpm/ 10 min.) to get serum specimens. The sera were stored into 500 µl tubes for two days at -20℃ prior to testing for antibodies to *B. canis*

using RSAT, 2ME-RSAT and ICA. Whole blood samples (6 ml) were withdrawn directly into the sodium citrate blood tube. These tubes were immediately cooledand transported (Alton *et al.* 1988) to the Microbiology laboratory of the Faculty of Veterinary Science, Eduardo Mondlane University, for bacterial examination and PCR. One ml of whole blood was stored at –20ºC for DNA extraction and PCR (Keid *et al.* 2007a).

Bacteriological study

Isolation of *Brucella* organisms from blood with sodium citrate was done as previously describe by Alton *et al.* (1988). Each specimen (5 ml of blood) was tranfered assepticaly to a 75 ml Columbia blood agar and broth flasks immediately after arrival at the laboratory. The flasks were incubated at 37℃, aerobically in upright position and examined every three days up to 10-21 days Any colonies ressembling those of *Brucella* that appeared on the solid media were subcultured on Columbia blood agar and incubated at 37℃ for 5 days for bacterial identification. The genus identification of the grown cultures was based on colonial characteristics, microscopic appearance and biochemical tests according to Quinn *et al.* (1994).

Cultures

Brucella spp. DNA (*B. abortus, B. suis, B. melitensis*) and isolates from nine related bacterial species (*Escherichia coli, Yersinia enterocolitica, Salmonella typhimurium, Streptococcus pneumoniae, Enterococcus faecalis, Spaphylococcus aureus, Pseudomonas aeruginosa, Bordetella bronchiseptica and Vibrio cholerae*) were obtained from the Microbiology laboratory, Department of Veterinary Tropical Diseases, of the Faculty of Veterinary Science, University of Pretoria, South Africa for inclusion in this study. A South African *B. canis* isolate was kindly obtained from the Agriculture Research Council-Onderstepoort Veterinary Institute (ARC-OVI), Onderstepoort, South Africa and used as a positive control for PCR as well as in the evaluation of analytical PCR sensitivity studies to detect *B. canis*. The *B. canis* isolate was grown on Columbia blood

agar at 37℃ for 3 days and the nine related bacterial species were grown on Nutrient agar at 37℃ for 24 hours.

PCR

DNA extraction from whole blood

Two months after collection of whole blood samples, blood tubes were thawed at room temperature. DNA samples were extracted, aliquoted and stored at -20ºC until tested. For the extraction of *Brucella* DNA from blood samples, the samples were pre-treated as reported by Keid *et al.* (2007a), by using 1 ml of whole blood collected in sodium citrate anticoagulant. The whole blood (1 ml) was thawed by leaving it to equilibrate to room temperature. The buffy-coat fraction from whole blood was collected by centrifugation of whole blood at 2500 x g for 10 minutes at room temperature, and total DNA was then purified according to the manufacturer's instructions (Qiagen, Germany). The manufacturer herindicates that the buffy-coat fraction yield approximately 5-10 times more DNA than an equivalent volume of whole blood (Qiagen, Germany). The DNA was extracted from the 56 dog samples as well as *B. canis* spiked dog blood samples and a non-infected dog sample (see below for detail).

DNA extraction from bacterial cultures

Brucella canis and the nine closely related bacteria were grown on Columbia blood agar and nutrient agar, respectively. Bacterial colonies were lifted from plates using sterile disposable loops and diluted in 8 ml sterile saline. The DNA was then extracted using the Qiagen DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions**.**

Analytical sensitivity and specificity

Analytical sensitivity evaluation of *B. canis* in dog blood (spiked dog blood)

The analytical sensitivity of the PCR using primers ITS66 and ITS279 that amplify the 16S-23S rDNA ITS region was evaluated by using serial dilutions of *B. canis*. A concentrated suspension from the *B. canis* culture was prepared by lifting the

B. canis colonies from the plate using a sterile disposable loop and diluting it in saline. The original undiluted *B. canis* suspension was estimated and diluted to $1x10^6$ cfu/ml. To verify the dilution accuracy, a 0.1 ml suspension of each dilution from 1.0×10^0 to 1.0×10^6 cfu/ml was inoculated onto Columbia blood agar plates and incubated at 37ºC for 5 days, and the colonies grown on the plates were counted. To enable calculation of the analytical sensitivity of the PCR primers, decreasing amounts of bacterial cells were added to non-infected dog blood. The spiked dog blood (mock-infected dog blood) samples were subjected to DNA extraction and PCR amplification. A negative control was included by using only DNA from non-infected dog blood.

Analytical sensitivity evaluation of PCR *B. canis* DNA

In order to study the influence of canine DNA on PCR amplification of *Brucella* DNA, different amounts of *B. canis* DNA (38 pg, 380 fg, 38 fg and 3.8 fg) were added to non-infected dog blood DNA. This was done by extracting DNA from the *B. canis* culture as well as non-infectious dog blood. Concentrations of the DNA extracts obtained from the culture and non-infected dog blood were determined spectrophotometrically. Suspensions of *B. canis* DNA in TE buffer were prepared with the following concentrations: 38 ng/µl, 3.8 ng/µl 38 pg/µl, 380 fg/µl, 38 fg/µl and 3.8 fg/µl. One microlitre of each dilution was mixed with 1.65 µl of DNA from a non-infected dog (38 ng/µl) and used as template in the PCR. A PCR reaction negative control was performed using all PCR reaction components but containing no *B. canis* DNA. An experimental negative control was carried out using only DNA from non-infected dog blood. Positive PCR controls were performed using genomic DNA from *B. canis*.

Evaluation of the analytical specificity

The analytical specificity of the PCR using primers ITS66 and ITS279 was assessed by testing DNA of *Escherichia coli, Yersinia enterocolitica, Salmonella typhimurium, Streptococcus pneumoniae, Enterococcus faecalis, Spaphylococcus aureus, Pseudomonas aeruginosa, Bordetella bronchiseptica and Vibrio cholerae*.

PCR amplification

PCR amplification was performed as described previously (Keid *et al.* 2007a) using the primers directed to the 16S-23S rDNA interspace region of *Brucella spp*. (ITS66: ACATAGATCGCAGGCCAGTCA and ITS279: AGATACCGACGCAAACGCTAC). PCR reactions (containing a total volume of 25 µl) consisted of the following reagents: 1× reaction buffer (JMR Holdings, UK), 2mM MgCl2, 0.25 mM of each dNTP, 0.5 µM of each primer and 0.1 U ExSel High Fidelity DNA *Taq* Polymerase. The DNA template that was added consisted of 2.5 µl DNA extracted from blood samples (spiked dog blood) and the DNA amount of *B. canis* and non-infected dog blood DNA as indicated above. Cycling conditions were as follows: 1 cycle of 95 ℃ for 2 min; 35 cycles of 95℃ for 30 s; 62℃ for 30 s and 70℃ for 30 s with a final elongation step at 70℃ for 5 min. The negative control consisted of sterile water and the positive control was DNA of South African *B. canis* (ARC-OVI). The PCR samples were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 mg/ml), and bands were visualized under UV light.

Serological studies

RSAT

Agglutination and immunochromatographic tests were applied to the sera samples. RSAT screening test was performed as indicated by the canine brucellosis antibody test kit of Synbiotics Corporation, USA. Briefly, 25 µl (one drop) of serum dilution was mixed with 25 µl of antigen on a card provided in the kit. The card was gentle rocked for 10-15 sec and placed on a flat surface. The

results were read macroscopically for no longer than two minutes, including a standard serum control. The antigen used in the kit was from the M- *B. canis* strain. The negative and positive controls were used that was supplied by the manufacturer. The 2-Mercaptoethanol (2ME) -RSAT was performed if the card test scored positive following the instructions of the manufacturer.

2ME-RSAT

Briefly, 2 drops of 2ME were added to a tube containing 2 drops of serum to be tested, and mixed well. A drop of this mixture was placed on a card. A drop of antigen was then added as abovementioned to the serum solution and mixed well with stir stick. The presence of agglutination was observed for not longer than 2 min. Both tests (the RSAT and 2 ME-RSAT) was scored negative when agglutination was absent and positive when agglutination was observed. The result was doubtful if a sample was positive by RSAT and negative by 2 ME-RSAT.

ICA

The Anigen Rapid C. *Brucella* Ab Test Kit (Bionote, Korea) is the immunochromatographic assay that was applied to detect *B. canis* antibodies in serum of suspected dogs. The test procedures were performed following the instructions of the manufacturer. Briefly, the flow assay was performed by the addition of one drop of serum directly onto the sample application pad in the sample well of the plastic assay device. Following the addition of 4 drops of diluent, the result is read 10 min later by visual inspection for staining of the antigen and control lines in the test window of the device. The control line should always appear if the procedure is performed properly and the test reagents of control line are working. The assay was scored negative when no staining of the antigen line was observed and positive when a distinct purple “test line” was visible. The negative and positive controls supplied by the manufacturer were used.

Statistical analysis

Cohen's kappa value was used to evaluate the agreement between 2ME-RSAT and ICA. Landis and Koch (1977) reported that the kappa value of 0.01–0.2 indicated slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–0.99 almost perfect agreement.

CHAPTER 3: RESULTS

Evaluation of analytical sensitivity and specificity

In this study the analytical sensitivity of the PCR assay that amplifies the 16S-23S rDNA ITS region of mock *Brucella* infected dog blood (spiked dog blood) as well as different *Brucella* DNA concentrations in non-infected dog blood DNA, as previously described by Keid et *al.* (2007a; b), were verified. The PCR assay in this study detected 10^2 cfu/ml *Brucella* cells in non-infected dog blood (Fig. 1). Furthermore, the PCR assay also detected 3.8 fg of *B. canis* DNA mixed with 63 ng of non-infected dog blood DNA (Fig. 2). A 214 bp fragment unique to *Brucella* spp., which is amplified by primers ITS66 and ITS279, was obtained form all *Brucella* strains as well as from spiked *B. canis* blood.

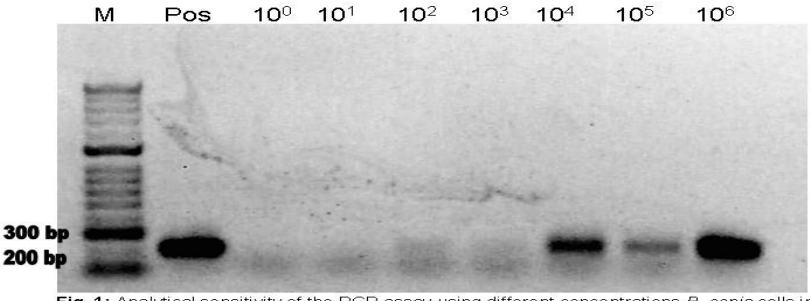

Fig. 1: Analytical sensitivity of the PCR assay using different concentrations *B. canis* cells in non-infected dog blood (mock infected dog blood). M, Hyperladder II (Bioline, U. K.); Pos, Positive control with 214 bp fragment of *B. canis*; PCR assay of different concentrations of *B. canis* in dog blood (1×10^0 to 1×10^6 cfu/ml).

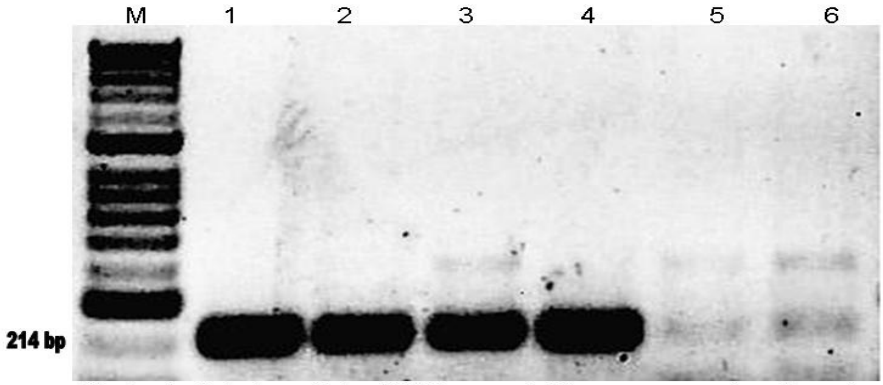

Fig. 2: Analytical sensitivity of PCR assay of different concentrations *B. canis* DNA mixed with 63 ng non-infected dog blood DNA. M, Gene'Oruler 100 bp + 500 bp (Fermentas, USA); Lane 1-6, 28 ng, 2.8 ng, 380 pg, 380 fg, 38 fg and 3.8 fg of *B. canis* DNA with 63 ng non-infected dog blood DNA.

The analytical specificity of the PCR assay that amplifies the 16S-23S rDNA ITS region (with primers ITS66 and ITS279) was also verified by including closely related bacterial species, namely *Escherichia coli, Yersinia enterocolitica, Salmonella typhimurium, Streptococcus pneumoniae, Enterococcus faecalis, Spaphylococcus aureus, Pseudomonas aeruginosa, Bordetella bronchiseptica and Vibrio cholerae*. None of the closely related bacterial species amplified the 214 bp fragment (Fig 3), showing a good specificity of the primers used.

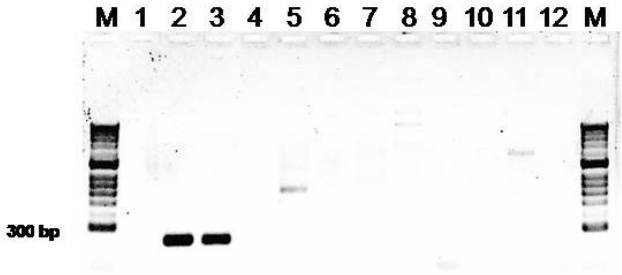

Fig. 3: Analytical specificity of the PCR assay using closely related bacteria. M: GeneoRuler 1000 bp + 500 bp (Fermentas, USA); Lane 1: Negative control (water); Lane 2-3: *B. melitensis* and *B. canis*; Lane 4-12: *Bordetella bronchiseptica, Vibrio cholerae, Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae, Enterococcus faecalis, Yersinia enterocolitica, Staphylococcus aureus.*

Survey of B. canis in dog blood in Maputo region

After the analytical sensitivity and specificity of the PCR assay was verified a survey of *B. canis* in dog blood from the Maputo region in Mozambique using PCR, bacteriology and serology was conducted. During the survey 56 dogs were examined and sampled during the outreach program in the villages of Michangulene and Mafavuca at the municipality of Changalane, District of Namaacha in Maputo, Mozambique. Of the 56 dogs, five female dogs (14, 21, 83, 89 and 97) showed at least one of the clinical signs suggestive of brucellosis (see Appendix 1). Of this five, only two (89 and 97) had reported past abortion or fetal death, three (14, 21 and 83) had reported infertility of which one (14) had a vaginal discharge. None of the dogs had orchitis, epididymitis and uveitis detected during the clinical examination and all the dogs were free living (Appendix 1).

PCR

No PCR product of 214 bp was obtained that indicated *B. canis* infection in whole blood collected from the 56 sampled dogs (Fig 4 lanes 2-9 indicated that dog samples 13, 69, 85, 86, 87, 96, 92, 97 did not amplify 214 bp fragment). The *B. canis* DNA from culture produced 214 bp fragment (Fig. 4).

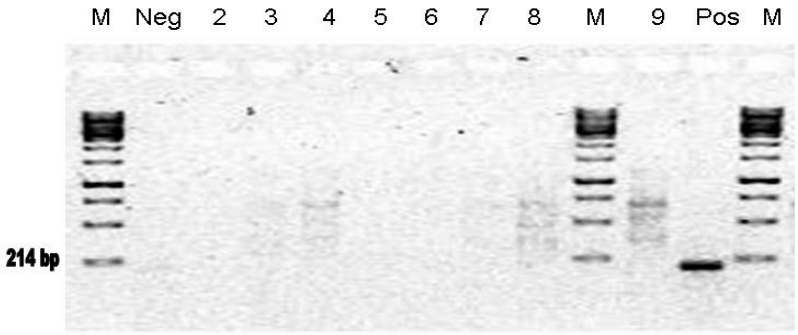

Fig 4. Ethidium bromide-stained 2% Agarose gel PCR amplification of template from dog blood samples. M: Geneoruler 100bp+500bp (Fermentas, USA); Negative control; lanes 2-9: 63ng/ml of genomic DNA of suspected seropositive dogs of *B. canis*; M: Geneoruler 100bp+500bp (Fermentas, USA); Positive control with 214 bp fragment of *B. canis*; M: Geneoruler 100bp+500bp (Fermentas, USA).

Blood culture

Whole blood was collected from the 56 dogs to establish bacterial culture from the blood. No *B. canis* organism was isolated from the blood. However, *Staphylococcus sp.* and *Klebsiella oxytoca* were identified from 85, 86 and 96, whereas *Enterobacter cloacae* (a possible contaminant since *E. cloacae* does not occur in blood) was identified from dog 92.

Serology tests

Serological tests were performed to determine whether *B. canis* specific antibodies occurred in the 56 sera samples using RSAT, 2ME-RSAT and ICA (Table 1). With the RSAT, 12 (21%) dogs were seropositive, whereas six (11%) were positive with 2ME-RSAT. None of the dogs that were 2ME-RSAT positive showed any clinical signs compatible with brucellosis. When the same 56 samples were tested by ICA, only four (7%) were positive (Table 1). Only one (sample 97) of the four dogs that showed a positive result with the ICA had past history of abortions. This bitch was positive by RSAT and ICA, but the 2ME-RSAT result was doubtful (Table 1). None of the samples were seropositive with both 2ME-RSAT and ICA test. The kappa value between 2ME-RSAT and ICA was -0.65.

Table 1. Serological results of RSAT, 2 ME-RSAT and ICA assay using 56 serum samples from dogs in the Maputo region in Mozambique.

Serum no.	*B. canis* antigen		
	RSAT	2ME-RSAT	ICA
1	-		-
2	-		-
3	-		-
4	-		-
5	-		-
6	-		-
7	-		-
8	-		-
9	-		-
10	-		-
11	-		-
12	-		-
13	+	+	-
14	-		-
15	-		-
16	-		-
17	-		-
18	-		-
19	-		-
20	-		-
21	-		-
22	-		-
23	-		-
24	-		-
69	+	+	-
71	-		-
72	-		-
73	-		+
76	-		-
77	-		-

79	-		-
80	-		-
81	-		-
82	-		-
83	+	D	-
84	+	D	-
85	+	+	-
86	+	+	-
87	+	+	-
88	+	+	-
89	-		-
90	+	D	-
91	-		-
92	+	-	+
93	-		-
94	-		-
95	-		-
96	+	D	+
97	+	D	+
98	D		-
99	D		-
100	D		-
101	D		-
102	D		-
103	D		-
104	D		-

RSAT: rapid slide agglutination test

2ME-RSAT: 2-mercapto ethanol RSAT

ICA: immunochromatographic assay

(+): positive result

(-): negative result

D: doubtful result/ not clear.

CHAPTER 4: DISCUSSION AND CONCLUSION

In this study, we first verified the sensitivity and specificity of the PCR assay that amplify the 16S-23S rDNA ITS region for the detection of canine brucellosis in the blood of dogs. Once the sensitivity and specificity of the PCR methods was verified for the detection of *B. canis* in whole blood of dogs, we continued to use PCR, haemoculture and serological tests (RSAT, 2ME-RSAT and ICA) to determine whether *B. canis* can be detected in 56 dogs in the Maputo region of Mozambique. Results of this study indicated that *B. canis* was not present in the tested dogs using PCR and haemoculture. Furthermore, no correlation was obtained with the RSAT and ICA serological tests. Therefore *B. canis* was not detected in the sampled dogs in this study using various techniques in the Maputo region of Mozambique.

The primers ITS66 and ITS279 were used since it was reported to be specific for *Brucella* detection (Keid *et al.* 2007a). Keid *et al.* (2007a) found that the PCR assay was able to amplify the 16S-23S rDNA ITS region unique to *Brucella* from mock *B. canis* infected dog blood sample containing as little as 1.0×10^{0} cfu/ml. In our study, the sensitivity of this primer pair had a detection limit of 10^{2} cfu/ml (Chapter 3, Fig. 1). The difference observed between our study and Keid *et al.* (2007a) could be due to sample preparation and use of different DNA extraction methods (Bricker *et al.* 2002; Navarro *et al.* 2002). O'Leary *et al.* (2006) indicated that the method of bacterial DNA extraction from host samples may be crucial in the ability of the PCR assay to detect the bacterium since *Brucella* may be present at very low numbers in the sample. A different extraction method was used in our study (commercial DNA extraction kit from Qiagen, Germany) compared to the extraction methods used by Keid *et al.* (2007a; b; c). Furthermore a recent study indicated that commercial extraction kits vary in their recovery of *Brucella* DNA from serum (Queipo-Ortuno *et al.* 1997). Zerva *et al.* (2001) in their studies demonstrated that serum specimens should be used preferentially over whole blood because inhibitors were often detected in whole

blood, thus, decreasing the recovery of *Brucella* DNA by PCR. Furthermore the PCR reagents used in our study and by Keid *et al.* (2007a; b; c) were from different manufacturers. The variation in results between our study and Keid *et al.* (2007a) is not uncommon considering the complexity of PCR methods and the differences between procedures (Navarro *et al.* 2002).

The sensitivity of the PCR assay method for the detection of *Brucella* DNA in non-infected dog blood DNA was also verified. This is important since dog blood samples are used for diagnosis of canine brucellosis. We obtained the same results where we detected up to 3.8fg *Brucella* DNA (Chapter 3, Fig. 2) than those by Keid *et al. (*2007a).

Keid *et al.* (2007a; b) identified the ITS66 and ITS279 primers that are potentially capable of amplifying 214 bp of the 16S-23S rDNA ITS region of only the six 'classical' *Brucella* spp. namely, *B. abortus, B. canis, B. melitensis, B. neotomae, B. ovis* and *B. suis* and its biovars (Greene and Carmichael 2006), and none of the closely related organisms (*Escherichia coli, Yersinia enterocolitica, Salmonella typhimurium, Streptococcus pneumoniae, Enterococcus faecalis, Spaphylococcus aureus, Pseudomonas aeruginosa, Bordetella bronchiseptica and Vibrio cholera*) searched using homology searches in BLAST. Various other PCR assays used for the detection of *Brucella* spp., like the 16S rRNA and rRNA operon gene (Romero *et al.* 1995; Cetinkaya *et al.* 1999) amplified *O. anthropi* and is therefore non-specific. Keid *et al.* (2007a; b) tested *Brucella* spp. and *O. anthropi* with the 16S-23S rDNA ITS *Brucella* specificity PCR assay and determined that it was specific for *Brucella*. In our study various other closely related bacteria namely *E. coli, Y. enterocolitica, S. typhymurium, S. pneumoniae, E. faecalis, S. aureus, P. aeruginosa, B.bronchiseptica and V. cholerae* was also included to evaluate the specificity of the PCR assay using ITS66 and ITS279 primers. No PCR product of 214 bp was observed with any of the closely related bacteria (Chapter 3, Fig. 3), especially LPS-rich outer membrane organisms such as *Y. enterocolitica*, *E. coli* and *V. cholerae* that may

results in false positive serological results due to cross-reactions with antigen. The primers ITS66 and ITS279 did amplify a 214 bp fragment for *B. canis* and *B. melitensis* DNA.

In order to investigate *Brucella canis* infection in dogs a survey was conducted in the Maputo area. Blood samples were collected from 56 dogs and tested using serological (RSAT, 2ME-RSAT and ICA) methods, bacteriology and PCR. Between 7% (4 out of 56 samples tested by ICA) and 11% (6 out of 56 samples tested by 2ME-RSAT) of the dogs tested were seropositive to *B. canis* infection (Chapter 3, Table 1).

A PCR based on primers ITS66 and ITS279 and haemoculture did not detect *Brucella* DNA from 56 dog whole blood samples in the Maputo region of Mozambique. Agglutination methods like RSAT has lowered specificities and therefore false positive reactions are common (Baldi *et al.* 1997; Lucero *et al.* 2002). These false-positive results are attributable to non-specific binding with other pathogens (Carmichael 1976; Badakhsh *et al.* 1982; Flores-Castro and Carmichael 1978; Carmichael and Joubert 1987; Carmichael and Greene 1990; Johnson and Walker 1992). Treatment with 2-ME in agglutination tests increase the specificity by 50% since non-specific agglutinins (IgM) reported to occur in the sera of normal dogs are removed from canine sera making the 2ME-RSAT more specific (Badakhsh *et al.* 1982; Gordon *et al.* 1985; Akan *et al.* 2005). Nevertheless, false-positive reactions may still occur (Wanke 2004). In our study, 12 of the 56 dogs that tested seropositive with RSAT were decreased 50% with the 2ME-RSAT (six seropositive dogs). Another serological test, ICA, was used and seropositives with the ICA and 2ME-RSAT would be presumed positive. With the ICA test four of 56 dogs tested positive but there was no correlation between the ICA and 2ME-RSAT tests and all samples were presumed negative. This conclusion is verified by Keid *et al* (2007a; b; c) that reported the PCR assay is more sensitive than blood culture the 2ME-RSAT. Furthermore the authors reported that RSAT positives should be confirmed by blood cultures.

Kim *et al.* (2007) found higher positive results with 2ME-RSAT and ICA than haemoculture. We also suggest expanding the sampling to urine, serum and blood followed by serological tests, bacteriology and PCR, since bacterial count has been found to be high especially in male dogs, but the problem is urine is not easy to collect especially during outreach programmes. Blood samples are collected for serology. This is 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) since this sample provide the maximum of target bacteria with presence of PCR inhibitors like anticoagulants, haemoglobin, host DNA or other substances present in whole blood (Navarro *et al*. 1999; 2002; Ilhan *et al*. 2008). Greater sensitivity than whole blood was reported in serum samples (Zerva *et al*. 2001; Elfaki *et al.* 2005) despite the fact that in DNA in serum is presumable lower (Queipo-Ortuno *et al*. 2008). O'Leary *et al*. (2006) found that whole blood is not a good template for the detection of *B. abortus* DNA in cattle. They suggested that using the buffy coat (white cell pellet) might be a better template since bacteria are taken up by marcophages 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).

Dogs can be declared negative when the agglutination tests are negative, especially with the RSAT, which seldom have false negative results when used correctly (Gordon *et al.* 1985; Alton *et al.* 1988). Serological test results with RSAT positive, 2ME-RSAT negative and ICA negative combination is most probably due to non-specific binding in the RSAT which is eliminated by the 2ME-RSAT and ICA tests (Wanke 2004; Kim *et al.* 2007). Samples 96 and 97 tested positive for RSAT and ICA, but the 2ME-RSAT was doubtful. These results might

be due to blood being slightly haemolysed, leading to erroneous interpretation of the RSAT results, which might be reflected by the doubtful RSAT results (Table 1). Alton *et al.* (1988) described the tendency of dog sera to readily haemolyse causing interference with the reading of agglutination tests and therefore adding to the complication in testing dog sera. Dog 92 was RSAT positive, 2ME-RSAT negative and ICA positive. In these cases it would be advisable that the tests should be repeated (30 days after first sampling) to prove that the animal is not in the early stages of *B. canis* infection. Furthermore, resampling the dogs is advisable due to the large discrepancy between serological tests as well as PCR and bacteriology in this study (Gordon *et al.* 1985; Nielsen 2002). However, locating the same dogs for resampling in our study is difficult to impossible as sampling occurred during a community outreach. The precise locality of the owner and/or dogs in the community is not known making resampling difficult to impossible. However, we do not preclude the possibility of false positive reactions since these results were not confirmed as positives by both serological tests, PCR and bacteriology Keid *et al.* (2007a). Carmichael (1976); Zoha and Carmichael (1982); Carmichael and Joubert (1987) indicated that false positive reactions in serological results may be interpreted as non-specific binding antibodies to *B. canis*. Dogs that have low titres may be infected but abacteraemic (these dogs should be in intermittent periods of bacteraemia or in chronic phase of infection) (Carmichael 1976; Carmichael and Joubert 1987; Carmichael and Greene 1990). These dogs might harbour *B. canis* in organs such as spleen, lymph nodes, prostate gland and epididymis (Moore and Kakuk 1969).

The inconsistency between the serological test results of ICA and 2ME-RSAT might be due to the difference of the nature of the antigen used in the two tests. Various other authors also suggested that the variability seen in sensitivity and specificity of serological tests may result from the use of different protocols for antigen production (Myers *et al.* 1974; Zoha and Carmichael 1982; Carmichael and Joubert 1987; Mateu-de Antonio *et al.* 1994). According to these authors,

there have been reported differences in the properties of cell the wall antigens obtained from *B. ovis*, a wild type *B. canis* strain and the less virulent (M-) *B. canis* strain. These findings suggest that the *Brucella* strains used to produce the antigens may also have different impacts upon the performance of the serological tests. Accordingly to the manufacturers of the test kits (Chalange S; Cho J., personal communication) used in our study, the RSAT were prepared using cell wall antigen from a less mucoid strain (M-) of *B. canis,* whereas the ICA used antigen composed of the cell wall antigen (LPS) and some contents of cytoplasmic protein. Tests based on the detection of anti-*Brucella* envelope antibodies are prone to false positive reactions (Carmichael *et al.* 1984a; Corbel 1985; Watarai *et al.* 2007) whereas the use of internal antigens has been proposed as a possible solution to eliminate the cross-reactivity problems in the serodiagnosis of canine brucellosis (Zoha and Carmichael 1982; Diaz and Moriyon 1989; Baldi *et al.* 1994). This supports in theory the suggestion by Kim *et al.* (2007) that the ICA is more sensitive and specific than the 2-ME RSAT to detect *B. canis* from blood samples. The RSAT, with and without 2-mercaptoethanol (2ME-RSAT and RSAT, respectively) that employs a less mucoid strain (M-) of *B. canis* is considered the most widely used serological test to diagnose of canine brucellosis (Carmichael and Joubert 1987; Carmichael *et al.* 1984b). Nevertheless, we suggest that the ICA test should be used with care as a screening test for the detection of *Brucella* infection in dogs, as this method has not been standardized (Carmichael and Shin 1996).

Attempts to isolate *B. canis* were unsuccessful in the present study. Organisms such as *Staphylococcus* spp. and *Klebsiella oxytoca* that were isolated from 85, 86 and 96 could have resulted in positive serological results (Table 1). The presence of these bacteria in the blood samples of the dogs could be due to skin contamination during blood sample collection. This may explain the (false) positive results obtained with the serologic tests since *B. canis* surface antigens are also common to other bacteria species such as *P. aeruginosa*, *Staphylococcus* sp. and *B. bronchiseptica* (Carmichael and Kenney 1968;

Carmichael 1976; Carmichael *et al.* 1984a; b; Alton *et al.* 1988; Baldi *et al.* 1997). In future the blood collection method will be improved to eliminate skin contamination. Accordingly to Alton *et al.* (1988), the skin over the vein should be shaved and defatted by rubbing with ether-soaked swab before disinfection with tincture of iodine.

Although blood culture is considered the gold standard for *Brucella* diagnosis, PCR seems to be a promising technique to arbiter the disease status of dogs especially in cases where it is not possible to isolate *B. canis.* This is supported by Keid *et al.* (2007a; b; c) who concluded from results of their studies that PCR could be used as a confirmatory test to diagnose canine brucellosis. We however, suggest that PCR should be used with care as a screening test because several PCR assays fail to detect Brucella DNA sequences due to existence of PCR-inhibitory components in the clinical samples. Additionally, the sample pretreatment and extraction methods, the different primers pairs used and the amounts of host DNA applied are cited among possible factors that may interfere with the outcome of PCR. Practitioners conducting PCR assessments for diagnosis of brucellosis in dogs should have detailed knowledge of all parameters that may compromise the sensitivity of PCR (Morata *et al.* 1998; Casanas *et al.* 2001; Navarro *et al*. 2002; Gee *et al.* 2004; O´Leary *et al.* 2006; Queipo-Ortuno *et al.* 2008). However we agree that once these potential problems are overcome, the PCR assay will sufficiently identify the Brucella spp

All the dogs sampled in the Maputo region of Mozambique were negative for the presence of *B. canis* using PCR, haemoculture and interpretation of results of serological tests by both 2ME-RSAT and ICA. Furthermore the kappa value between 2ME-RSAT and ICA was less than 0.2 which indicates no agreement between the 2ME-RSAT and ICA according to Landis and Koch (1977). This is in contrast to the kappa value found by Kim *et al.* (2007) between the 2ME-RSAT and ICA that indicated almost perfect agreement according to Landis and Koch (1977). Due to the large discrepancy between serological tests as in this study

we can conclude that *B. canis* is not present in the Maputo region of Mozambique. We recommend future research of brucellosis on urine, semen and blood samples using of experimental infected dogs. This suggested study should include assessment and/ or validation of serological tests, haemoculture and PCR. Caution should be taken when selecting the method(s) and sample(s) in order to prevent failures to diagnose the disease. Knowledge acquired from above mentioned study will enable a surveillance of dogs using methods and samples that will accurately reflect the prevalence of *B. canis* in the Maputo region of Mozambique using various techniques.

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Appendix 1:

Results of *Brucella canis* survey of dogs in the Michangulene and Mafavuca villages of Maputo, Mozambique.

ID of dog	Information obtained from owners were possible					Results obtained from clinical examination		
	Sex*	Age**	Abortions/ stillbirths ***	Inferti-lity****	Free-living habits *****	Vaginal discharge	Abnormalities of testicle/ epididymis	Neck pain
1	M	3yr	N/A	N/A	Yes	N/A	No	No
2	F	4yr	No	No	Yes	No	N/A	No
3	F	2yr	No	No	Yes	No	N/A	No
4	M	8m	N/A	N/A	Yes	N/A	No	No
5	F	8m	No	No	Yes	No	N/A	No
6	F	-1yr	No	No	Yes	No	N/A	No
7	M	3yr	N/A	N/A	Yes	N/A	No	No
8	F	+1yr	No	No	Yes	No	N/A	No
9	F	3yr	No	No	Yes	No	N/A	No
10	M	5yr	N/A	N/A	Yes	N/A	No	No
11	M	2yr	N/A	N/A	Yes	N/A	No, but had Skin infection	No
12	M	8m	N/A	N/A	Yes	N/A	No	No
13	F	+1yr	No	No	Yes	No	N/A	No

ID of dog	Information obtained from owners were possible					Results obtained from clinical examination		
	Sex*	Age**	Abortions/ stillbirths ***	Inferti-lity****	Free-living habits *****	Vaginal discharge	Abnormalities of testicle/ epididymis	Neck pain
14	F	+1yr	No	Yes	Yes	Yes, yelow-white	N/A	No
15	M	+1yr	N/A	N/A	Yes	N/A	No	No
16	M	1yr	N/A	N/A	Yes	N/A	No	No
17	M	1yr	N/A	N/A	Yes	N/A	No	No
18	M	9m	N/A	N/A	Yes	N/A	No	No
19	M	1yr	N/A	N/A	Yes	N/A	No	No
20	M	8m	N/A	N/A	Yes	N/A	No	No
21	F	2yr	No	Yes	Yes	No	N/A	No
22	M	3yr	N/A	N/A	Yes	N/A	No	No
23	M	3yr	N/A	N/A	Yes	N/A	No	No
24	M	+1yr	N/A	N/A	Yes	N/A	No	No
69	F	9m	No	No	Yes	No	N/A	No
71	M	+1yr	N/A	N/A	Yes	N/A	No	No
72	F	3yr	No	No	Yes	No	N/A	No
73	F	4yr	No	No	Yes	No	N/A	No
76	F	3yr	No	No	Yes	No	N/A	No

ID of dog	Information obtained from owners were possible					Results obtained from clinical examination		
	Sex*	Age**	Abortions/ stillbirths ***	Inferti-lity****	Free-living habits *****	Vaginal discharge	Abnormalities of testicle/ epididymis	Neck pain
77	M	+2yr	N/A	N/A	Yes	N/A	No	No
79	F	1yr	No	No	Yes	No	N/A	No
80	M	+2yr	N/A	N/A	Yes	N/A	No	No
81	M	3yr	N/A	N/A	Yes	N/A	No	No
82	M	+1yr	N/A	N/A	Yes	N/A	No	No
83	F	2yr	No	Yes	Yes	No	N/A	No
84	F	+1yr	No	No	Yes	No	N/A	No
85	M	+1yr	N/A	N/A	Yes	N/A	No	No
86	M	+1yr	N/A	N/A	Yes	N/A	No	No
87	F	3yr	No	No	Yes	No	N/A	No
88	F	9m	No	No	Yes	No	N/A	No
89	F	2yr	Yes, frequently	No	Yes	No	N/A	No
90	F	3yr	No	No	Yes	No	N/A	No
91	M	2yr	N/A	N/A	Yes	N/A	No	No
92	M	+1yr	N/A	N/A	Yes	N/A	No	No
93	F	-1yr	No	No	Yes	No	N/A	No

ID of dog	Information obtained from owners were possible					Results obtained from clinical examination		
	Sex*	Age**	Abortions/ stillbirths ***	Inferti-lity****	Free-living habits *****	Vaginal discharge	Abnormalities of testicle/ epididymis	Neck pain
94	M	2yr	N/A	N/A	Yes	N/A	No	No
95	M	2yr	N/A	N/A	Yes	N/A	No	No
96	M	-1yr	N/A	N/A	Yes	N/A	No	No
97	F	+1yr	Yes	No	Yes	No	No	No
98	M	8m	N/A	N/A	Yes	N/A	No	No
99	M	9m	N/A	N/A	Yes	N/A	No	No
100	M	2yr	N/A	N/A	Yes	N/A	No	No
101	M	-1yr	N/A	N/A	Yes	N/A	No	No
102	F	2yr	No	No	Yes	No	N/A	No
103	F	2yr	No	No	Yes	No	N/A	No
104	M	8m	N/A	N/A	Yes	N/A	No	No

* M: male dog and F: female dog.

** m: indicate months, yr: age in years, -yr: less than a year, +yr: more than a year, N/A: Not applicable

*** Information from owner that indicated whether female dog aborted at some stage or whether the dog had problems in becoming pregnant.

**** Information obtained from owner that indicated whether dog was thought to be pregnant but later the owner noticed it was no longer pregnant.

***** Information obtained from owner indicating whether dog roamed freely and fed with other dogs of the area.