Canine brucellosis due to Brucella canis: *description of the disease and control measures*

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Keywords
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Summary
Brucellosis is a contagious disease caused by bacteria of the genus *Brucella*, which can affect different animal species. Dogs may occasionally be infected with *B. abortus, B. melitensis* or *B. suis*, or by the endemic form of the disease, caused by *B. canis*. Among the brucellosis-affecting domestic animals, that of the dog is certainly the least frequent, but also the least studied. Canine brucellosis due to *B. canis* represents the dog-specific brucellosis, both because it is the main susceptible animal species, and because it constitutes its fundamental reservoir of infection. The disease can also affect humans, although its course does not assume the characteristics of severity typical of the infection determined by the 'classical' species of the genus *Brucella*. In Italy, there are frequent imports of dogs from countries where the disease is present, often with non-controlled movements and without sanitary controls. Considering that the zoonotic potential of the disease can be favored by the close cohabitation between man and dog, which occurs especially in urban environments, canine brucellosis has to be regarded as a public health problem susceptible to introduction and spread in the Italian territory.

Introduction

Brucellosis is a contagious disease caused by bacteria of the genus *Brucella*, which can affect different animal species. Dogs may occasionally be infected with *B. abortus*, *B. melitensis* or *B. suis*, or be affected by the endemic form of the disease, caused by *B. canis*. Among the brucellosis-affecting domestic animals, that of the dog is certainly the least frequent, but also the least studied.

Canine brucellosis due to *B. abortus* or *B. melitensis* is uncommon in dogs living in close contact with infected ruminants. In these cases, it only represents an epiphenomenon of the infection circulating in the affected farm, and the dog does not play the role of disease reservoir. The same applies for dog brucellosis due to *B. suis*, which is rarely detected in dogs and, in any case, always in connection with a coexisting infection in pig farms.

Canine brucellosis due to *B. canis* represents the dog-specific brucellosis. Dogs in fact are the main susceptible animal species, and the fundamental reservoir of infection.

The disease can also affect humans, although its course does not assume the characteristics of severity typical of the infection due to the 'classical' species of the genus *Brucella*; however, in countries where the disease is present (particularly in the American continent) it is considered an authentic zoonosis.

In Italy, there are frequent imports of dogs from countries where the disease is present, often with non-controlled movements and without sanitary control. Considering that the zoonotic potential of the disease can be favored by the close cohabitation between man and dog, which occurs especially in urban environments, canine brucellosis has to be regarded as a public health problem susceptible to introduction and spread across the Italian territory.

Decription of the disease

Etiology

Brucellae are Gram-negative coccobacilli or short rods measuring from 0.6 to 1.5 µm long and from 0.5 to 0.7 µm wide. They are usually arranged singly, and less frequently in pairs or in small groups. The morphology of *Brucella* is fairly constant, except in old cultures where pleomorphic forms may be evident. Bacteria of the genus *Brucella* are normally non-motile. They do not form spores, or pili; true capsules are not produced. They are aerobes and asporigenic.

With regards to *B. canis*, it has no biovar, does not require carbon dioxide to grow in first isolation, and grows in presence of thionin but not with basic fuchsin. In first isolation, colonies of *B. canis* always occur in phase R (rough) or M (mucoid), while their existence in phase S (smooth) has never been reported. *B. canis* does not agglutinate in the presence of *Brucella* A- and M- monospecific antisera, while it agglutinates with specific antisera towards the R antigen of *B. ovis* (REO 198 strain). Furthermore, cross-reactions with surface antigens of other species of the genus *Brucella* in a non-smooth phase are also possible. *B. canis* does not produce H_2S, does not oxidize the substrate based on L-Asparagine or D-Xylose, does not reduce nitrates to nitrites, and does not show lysis in the reaction with different phages, except with the phage R/C (Corbel and Brinkley-Morgan 1989).

Epidemiology

Geographical distribution

B. canis was first reported in 1966 during an investigation on several outbreaks of abortion and infertility in dogs occurring in different areas of the United States of America (Carmichael 1966). Subsequently, *B. canis* was isolated in different countries of the world, while in others its presence has been suspected based on serological positivity. Recently, Hensel and colleagues (Hensel *et al.* 2018) have collected the information available from the international literature regarding serological investigations carried out for canine brucellosis due to *B. canis* worldwide (Figure 1). These studies show that there is a wide level of variability in serum positivity (from 1% to 28%), depending on the country studied and the sample taken (Hensel *et al.* 2018).

Seroprevalence is strongly influenced by the type of serological test used and the interpretation of its results, due to the fact that *B. canis* shares antigen determinants with all R-phase (rough) *Brucellae* and with microorganisms of other genera not related to the genus *Brucella* (Carmichael 1990). Furthermore, apart from the true disease prevalence in the area under study, the wide spread of high level of seroprevalence recorded could also be attributed to the sampling design and the algorithm used for the interpretation of test results (Hensel *et al.* 2018).

In Italy, several surveys reported the presence of seropositivity at various levels and in different geographical areas (Tolari and Pizzirani 1978, Colella *et al.* 1980, Ciuchini *et al.* 1982, Vesco *et al.* 1987, Valente *et al.* 1991, Buonavoglia *et al.* 1992, Prosperi *et al.* 1994, Casalinuovo *et al.* 1996). The only clinical case described in scientific literature dates to 2008, when Corrente and colleagues (Corrente *et al.* 2010) detected *B. canis* by PCR in a half-breed dog suffering from chronic prostatitis and discospondylitis. In 2020, *B. canis* has been detected and isolated for the first time in a commercial breeding kennel (De Massis *et al.* 2021).

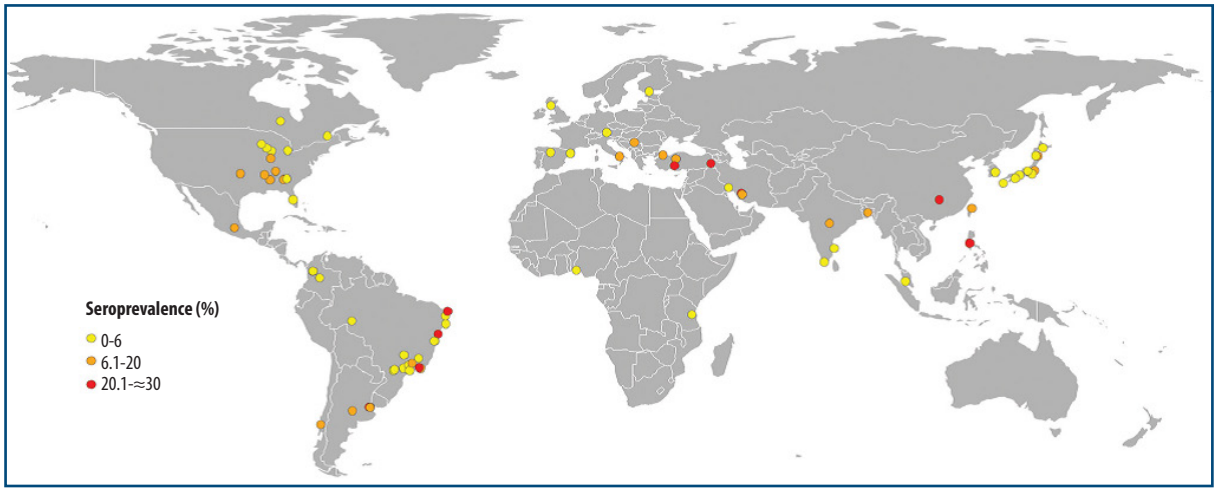

Figure 1. *Location and outcome of serological investigations for the detection of antibodies against* B. canis *recorded in the international scientific literature.* Each point represents a published study; the color of the point represents the identified source (from Hensel *et al.* 2018)

Susceptible species

Unlike S-phase (smooth) *Brucellae*, which are able to infect different animal species, *B. canis* has a limited range of possible hosts. Dogs and wild canids are thought to be the only significant hosts for *B. canis*. Cattle, sheep and swine were found to be highly resistant to the infection. Cats appear to be moderately sensitive and, following oral exposure to *B. canis*, can develop bacteremia with low antibody titers. Guinea pigs, mice, rats, and non-human primates are also susce ptible to experimental infection. (Carmichael 1990). The rabbit is more sensitive than other laboratory species, developing orchitis and peritoneal abscesses following high doses inoculated through intraperitoneal route (Carmichael and Bruner 1968).

Humans can get *B. canis* infection through direct contact with infected dogs, with their reproductive secretions, or with their blood (Lawaczeck *et al.* 2011, Lucero *et al.* 2010). Clinical disease has been described.

Transmission

Canine brucellosis due to *B. canis* is especially common among stray dogs, in shelter kennels, in commercial breeding kennels, or in places where they live in large groups (Carmichael and Joubert 1988). When *B. canis* is introduced into a kennel, the spread is rapid (Carmichael and Bruner 1968). *B. canis* can be transmitted from infected to healthy adult males after a few weeks or months of cohabitation. Conversely, it is not transmitted between non-sexually mature males or females (Carmichael and Joubert 1988).

A number of natural pathways of transmission of canine brucellosis have been observed, but the most common is the contact with placenta, fetal tissues and vaginal discharges resulting from abortion due to *B. canis* infection. Moreover, transmission is also possible during estrus or mating (Carmichael 1990). Vaginal discharge can contain more than 10^{10} microorganisms per ml, and elimination by this route can continue for several weeks after abortion (Carmichael and Joubert 1988). Other body secretions contain lower concentrations of *B. canis* and are less important for the spread of infection.

Most puppies become infected in the uterus. Infected mothers or their milk also represent a potential source of infection for those which survive infection.

Seminal fluid and urine of dogs harboring *B. canis* in the prostate and epididymis have been demonstrated as an important source for disease spreading. During the first 6-8 weeks of infection, the concentration of *B. canis* isolated from semen of infected males is high; the elimination of the microorganism in low concentrations for a period exceeding 60 weeks was also observed (George *et al.* 1979). Under experimental conditions, the isolation of *B. canis* from prostate and epididymis was possible up to more than two months after the end of bacteremia, and the venereal transmission of the disease continued for at least two years in clinically normal appearing dogs. Even after castration, males may remain a source of infection because the bacterium can persist in the prostate and lymphatic tissues (Carmichael 2012).

Urinary elimination begins a few weeks after the onset of bacteremia and continues for at least three months. In the urine of infected males, concentrations of microorganisms from 10^3 to 10^6 Colony Forming Units (CFU)/ml have been found, while for females this number is lower (Serikawa and Muraguchi 1979).

The bacteria have also been isolated from saliva, nasal and ocular secretions of dogs (Carmichael and Joubert 1988, Moore 1969). Unlike what occurs in the male, it seems that in females urine is not important for the transmission of the disease, due to the low number of microorganisms present (Carmichael 1990). The fact that the male eliminates the microorganism with urine in greater quantities is probably related to the specific localization of *B. canis* in the prostate and epididymitis or contamination with seminal fluid (Carmichael and Joubert 1988). Despite this evidence, according to Henesel and colleagues (Hensel *et al.* 2018), the role of urine as a means of transmission of canine brucellosis from *B. canis* has not yet been fully clarified (Hensel *et al.* 2018).

Pathogenesis and pathological lesions

Although the pathogenesis of *B. canis* infection has not yet been fully elucidated, it is likely it follows the general pattern of *Brucella* infection common to other animal species. The route of entry of the pathogen are the conjunctival, oral, and genital mucous membranes (Carmichael 1990). In the dog, the minimum oral infectious dose is around 2×10^6 CFU, while the minimum infectious dose via venereal route has not been determined yet, although it is assumed to be slightly lower (Carmichael and Joubert 1988). The infectious dose via conjunctival route is between 10^4 and 10^5 CFU (Serikawa and Muraguchi 1979).

After oral infection, the incubation period is variable, however a bacteremia associated with leukocytes can normally be detected one or two weeks after infection (Carmichael and Joubert 1988).

A characteristic feature of the infection, also significant from a diagnostic point of view, is the prolonged bacteremia. Commonly, it is possible to

find in the blood the presence of a number of bacteria even more than 10^3 per ml, starting 2-3 weeks after infection. Bacteremia commonly persists for at least one or two years. In some cases, it has also been observed for a period of five years (Carmichael *et al.* 1984). The bacteremia, constant in the acute phase of the disease, becomes intermittent in the chronic phase (Alton *et al.* 1988). *B. canis* can be isolated for several months from spleen, lymph nodes, bone marrow, prostate, and epididymis even when the bacteria cannot be found in the blood any more (Carmichael 1990).

No specific lesions due to *B. canis* infection have been described; lymph node hypertrophy and splenomegaly can be observed in infected adults and surviving puppies. Acute or chronic inflammatory lesions (Carmichael 1990) can also be found in the genital system. Microscopically, a generalised lymphoreticular hyperplasia affecting all lymphoid organs is costantly observed despite the bacteremia (Serikawa and Muraguchi 1979). In chronic bacteremia, lymph node and splenic sinuses can show infiltration of plasma cells and macrophages containing phagocytised bacteria. In all organs of the urogenital system, a widespread lymphocytic infiltration can be observed at the submucosa level, which mainly affects the prostate, epididymis, renal pelvis and uterus (Carmichael 2012). Subacute or chronic endometritis, granulomatous prostatitis, and testicular atrophy and fibrosis are common in chronically infected dogs. Other lesions may involve the kidney, with ialine thickening of the glomerulus basal membrane, with poor cell infiltration. Other described alterations include focal hepatic necrosis, myocarditis, and meningoencephalitis (Carmichael 1990). Alterations at the eye level are represented by granulomatous iridociclitis and exudative retinitis, characterized by widespread infiltration of lymphocytes, plasma cells and neutrophils (Saegusa *et al.* 1978). Aborted fetuses show subcutaneous edema, congestion, and hemorrhages in the subcutaneous areas of the abdominal region (Carmichael 2012). Placental annexes might contain foci of coagulative necrosis in the chorionic villi, and a large number of bacteria within epithelial trophoblastic cells (Feldman and Nelson 1996).

Symptomatology

Although *B. canis* infection is systemic, infected adult dogs rarely show obvious symptoms (Carmichael 1990). Apart from rare cases, the disease evolves in the absence of fever (Carmichael and Bruner 1968). There are no pathognomonic symptoms (Hensel *et al.* 2018).

A common symptom for both males and females is a unilateral or bilateral lymph node enlargement at sites of bacterial entry (usually retropharyngeal lymph nodes for the oral route and superficial inguinal lymph nodes for the vaginal route). The lymphadenopathy is the only detectable symptom in the non-pregnant adult female, while prostatitis and epididymitis can be observed in the adult male.

In pregnant females, the main symptom is late abortion, which generally occurs between the 45th and 55th day of gestation (Carmichael 2012). The abortion is followed by a yellow to brown odorless vaginal discharge, that persists from one to six weeks (Carmichael 1968).

Cases of embryonic resorption or abortion from the 10th to the 20th day of mating have also been described. They often go unnoticed in practice, as they are normally considered as an infertile mating (Carmichael 1990).

In exceptional cases, pregnancy can be brought to the end, with simultaneous birth of both live and dead puppies (Feldman and Nelson 1996). Most live-born puppies die within a few hours or days; those that survive normally show a generalized increase in lymph node volume, which is the main symptom until they reach sexual maturity (Carmichael 1990). These puppies may be bacteriemic but apparently healthy (Carmichael 2012). In addition, as a systemic manifestation of *B. canis* infection, surviving puppies usually show persistent hyperglobulinemia, and some of them may have transient fever, leukocytosis, or convulsions (Carmichael 2012). It is possible that apparently healthy puppies born from infected mothers spread *B. canis* to other dogs and humans (Dentinger *et al.* 2012).

As with brucellosis affecting other animal species, *B. canis* infection in dogs does not interfere with the normal oestrus cycle. Actually, it has been found that more than 85% of females which have had abortions because of *B. canis* infection can have normal gestations with regular births, while the remaining females may still experience reproductive problems, which can also occur intermittently. More rarely, infected females can have abortions more than four consecutive times, or have more than three unsuccessful mating (Carmichael 1990). These animals represent a reservoir of infection for the still healthy dogs inside the kennel (Carmichael 1968, Carmichael 2012).

In infected adult males, epididymitis, unilateral or bilateral testicular atrophy and scrotal dermatitis are normally found. Palpation of the scrotum or testicle generally does not cause acute pain. Generally, there is a decrease in the volume of ejaculate without loss of *libido* by the subject; however, it is possible to notice some suffering of the subject at the time of ejaculation. The seminal liquid of infected males

has a considerable number of sperm abnormalities and inflammatory cells, especially during the first three months of infection. Chronic infection can lead to bilateral testicular atrophy with consequent aspermia (Carmichael 2012).

In addition to symptoms affecting the reproductive sphere, although less frequently, *B. canis* infection can cause disorders in other organs as well. In some subjects, for example, generalized lymphadenopathy may be accompanied by splenomegaly (Carmichael 2012). A well-known and described clinical manifestation of *B. canis* infection is discospondylitis (Henderson *et al.* 1974). This can arise in apparently healthy dogs or those which have had history of reproductive disorders and have been treated with antibiotics (Kervin *et al.* 1992, Hurov *et al.* 1978). Dogs initially experience pain in the spinal cord, then, if compression of the spinal cord increases, they present paresis and ataxia (Kornegay 1983). The incidence of discospondylitis is higher in males than in females, probably due to the localization of *B. canis* in the prostate, which can cause intermittent bacteremia even in castrated males (Carmichael and Joubert 1988, Kerwin *et al.* 1992, Hurov *et al.* 1978).

Cases of osteomyelitis affecting the appendicular skeleton and resulting in lameness on the affected limb have also been described (Smeak *et al.* 1987). Although the onset of meningoencephalitis has only been observed as a consequence of an experimental infection, some authors have detected the presence of behavioral changes, anisocoria, ataxia, hyperesthesia and circle movements in dogs with *B. canis* infection, in which such neurological signs started three weeks after mating (Carmichael 2012). In some dogs with chronic *B. canis* infection, the onset of recurrent anterior uveitis with corneal edema has also been described (Saegusa *et al.* 1978, Reike and Rhodes 1975). Other manifestations of *B. canis* infection may be represented by polyarthritis (Carmichael 2012).

Diagnosis

Clinical diagnosis

The clinical signs may only allow a suspicion of *B. canis* infection. Since *B. canis* infection is one of the most common causes of reproductive disorders in dogs, it should be ruled out before investigating other causes of infertility or abortion (Carmichael 2012). However, if reproductive failure is not observed, canine brucellosis can be difficult to diagnose.

The hematological and biochemical values remain generally unchanged or in any case they don't show characteristic changes. A hyperglobulinemia affecting the fractions β and γ can be indicative of *B. canis* chronic bacteriemia. Urine biochemical tests are generally normal, despite the possible presence of bacteriuria.

In cases of meningoencephalitis, analysis of cerebrospinal fluid reveals the presence of neutrophils and an increase in protein concentration, changes that are not evident if only discospondylitis is present. Radiological findings of intervertebral disc infection should be followed by serological testing for *B. canis* and, where possible, bacteriological confirmation (Carmichael 2012).

Indeed, the examination of the seminal fluid may be of greater help in the presumptive diagnosis of the disease. Sperm abnormalities which may affect more than 90% of spermatozoa, start being present 5 weeks after infection and become evident from the 8th week onwards even after 20 weeks (George *et al.* 1979). In any case, the sperm disorders induced by *B. canis* infection are not specific, so the detection of an abnormal spermogram is not indicative of brucellosis (Berthelot and Garin-Bastuji 1993).

Laboratory diagnosis

Direct methods

Isolation

The isolation of the bacterium represents the only proof of *B. canis* infection. It is not difficult to isolate *B. canis* since the microorganism grows in the culture media normally used for all other species of the genus *Brucella* (Nicoletti and Chase 1987b).

Isolation of *B. canis* is possible from blood, fresh samples (vaginal discharged material, placental and fetal tissues, urine, semen, milk) and samples taken from necropsy (lymph nodes, spleen, prostate, epididymis, uterus, bone marrow, eye, intervertebral discs) (Feldman and Nelson 1996).

Blood culture is the method of choice for confirming *B. canis* infection, provided that they have not been treated with antibiotics (Carmichael 2012). In dogs experimentally infected per *os*, bacteremia begins 7 days after exposure, while blood culture begins to provide positive results from 2-3 weeks after exposure. If in the meantime the subject has not been treated with antibiotics, the positivity to blood culture usually persists for at least 6 months, and may last up to 2 years (Carmichael 1990). In this regard, the international scientific literature also reports cases of experimentally infected dogs that remained positive to the blood culture for a period of 5.5 years (Carmichael *et al.* 1984).

To avoid the excessive development of contaminants, blood samples should be sowed on selective media (Carmichael 2012). Blood samples should be collected in a sodium citrate or sterile lithium heparin blood collection tube, stored at refrigeration temperature (not frozen) and submitted within 24 hours at the laboratory, where they can be cultured on a Farrell's medium or on a modified Thayer-Martin medium (Carmichael and Greene 2006, CFSPH 2010, GDA 2020).

At necropsy lymph nodes and spleen are the recommended organs for culturing the bacterium. It is also possible to collect samples from the eye in case of uveitis or the intervertebral disc in case of discospondylitis (Feldman and Nelson 1996). In all these cases, the negativity of the culture does not exclude the presence of infection (Carmichael 1990).

Unfortunately, the chances of growing this microorganism in culture depend on several factors, including *B. canis* concentration in the collected sample, the intermittent germ elimination from infected subjects, due to the quality of samples, the sample handling, the forms of the microorganism, or how culture media have beeen used (Carmichael and Greene 2006). Therefore, a negative culture cannot exclude the presence of infection, since the low sensitivity of the test can lead to relatively high number of false negatives. Although culture is to be considered inappropriate as a screening test, it still represents the ideal test for confirming infection. The test can give positive results as early as 2-4 weeks after infection and the dog can remain positive for several years (Hollett 2006).

Isolating the bacterium from samples is the best method for the diagnosis of early infection in dogs that have not received antibiotic treatment.

Expected results of the culture according to the time of sampling and the material examined are summarized in Table I.

Polymerase chain reaction (PCR)

Several PCR primers have been used to detect *B. canis* DNA in whole blood, vaginal secretions and semen. Due to its high sensitivity and specificity, PCR can be used as a rapid screening test or as confirmation test for serum positive dogs (Keid *et al.* 2007, Kauffman *et al.* 2014, Kang *et al.* 2014). There is a good correlation between PCR test results and those obtained with the 2ME-RSAT test (Mol *et al.* 2020). The matrix of choice is the whole blood taken with anticoagulant sodium citrate (light blue cap tubes). PCR testing on serum has little diagnostic value as it provides unsatisfactory results in terms of sensitivity, much lower than those of blood culture, whole blood PCR, RSAT and 2ME-RSAT (Keid *et al.* 2010).

Given the potential impact that a positive or negative result can have on individual dogs, on the dog population of origin, on a single customer or on a kennel operator, quality control and assurance of the *B. canis* diagnostic tests are fundamental, in particular for PCR (Cosford 2018).

Assuming that the accuracy of the results can be improved, the advantages of PCR include: the possibility of detecting the species and (sometimes) the *Brucella* biovar involved in the infection, the high sensitivity and specificity, the minimum requirements needed in terms of biological containment, the relatively short time to obtain the results, and the possibility of performing a genetic fingerprinting which is useful for epidemiological investigations aimed at controlling the disease (Yu and Nielsen 2010).

Most of the PCR assays reported in the literature are based on the identification of the *Brucella* genus. In the past, multiplex PCRs have also been set up to distinguish between some *Brucella* species,

Table I. *Possible results of the culture of* B. canis *in relation to the time at which it is performed and the material under examination (adapted from Feldman & Nelson 1996).*

Culture material	Optimal moment of cultivation	Expected results
Post-abortion discharges	When present	+ + +
Placenta	When present	+ +
Fetus	When present	Possible negativity
Seminal fluid	3-11 weeks PI	+ + +
	12-60 weeks PI	Reduction in number
	> 60 weeks PI	-
Blood	5-30 weeks PI	100% +
	After 30 weeks PI	Intermittently
	6-12 months PI	>80% +
	28-48 months PI	50-80% +
	48-58 months PI	25-50% +
	> 58 months PI	< 25% +
Epididymis	35-60 weeks PI	50-100% +
	> 100 weeks PI	-
Urine	8-30 + weeks PI	Emission of more microorganisms in males than in females
Prostate	Up to 64 weeks PI	Usually +
Lymph nodes, bone marrow and spleen	During bacteremia	Usually +
	During the non-bacteriemic phase phase	+/-
Eye	In case of uveitis	+ + +
Intervertebral disks	In case of discospondylitis	+/-

PI = Post-infection; + = Positivity; - = Negativity.

including *B. canis* (López-Goñi *et al.* 2011). In recent years, specific PCRs for *B. canis* (Kaden *et al.* 2014, Kauffman *et al.* 2014, Kang *et al.* 2014, Boeri *et al.* 2018) have also been developed. The performance of these assays however needs to be completed in order to assess sensitivity and specificity. Until then, data from PCR trials should only be used in conjunction with clinical and serological information (Cosford 2018).

Cosford (Cosford 2018) has recently reviewed the state-of-the art of the different researches available to date on PCR for *B. canis* (Table II).

Indirect methods

Several serological methods are available for detecting antibodies against *B. canis*. The Rapid Slide Agglutination Test (RSAT), the 2-Mercaptoethanol-Rapid Slide Agglutination Test (2ME-RSAT), and the Tube Agglutination Tests (TAT, and 2ME-TAT) represent, in countries where the disease is present, the tests most widely used in the field. The Agar Gel Immuno-Diffusion test (AGID), that uses either cell-wall antigens (AGIDcwa), or cytoplasmic antigens (AGIDcpa), is used as confirmatory test. Other available tests include indirect fluorescent antibody assay (IFA), Complement Fixation Test (CFT) and immunoenzymatic tests (Enzyme-Linked Immunosorbent Assay, ELISA). They, however, have only been used in experimental studies (Carmichael and Shin 1996, Ebani *et al.* 2003, Carmichael and Greene 2006, Hollett 2006, Keid *et al.* 2009).

It is important to note that *B. canis* does not have smooth-phase cell wall antigens such as *B. abortus*, *B. melitensis* or *B. suis*, but only rough antigens (Carmichael and Bruner 1968), like *B. ovis*. Therefore, serological tests aimed at detecting antibodies against *B. canis* should be based on a wall antigen of *B. canis* or *B. ovis* (complete cross-reaction), or on a cytoplasmic antigen of *B. abortus* or *B. canis*. Whichever test or antigen is used, *B. canis* antibodies in the blood are usually detectable not earlier than 5-8 weeks after infection (Carmichael 1990). The results then may be negative in the first 3-4 weeks of infection, although the dog may be bacteremic as early as two weeks after infection (Carmichael and Greene 2006). When the bacteremia ceases, the antibody titer starts descreasing. However, level of detectable antibodies can be found for further 4-6 months. After that period, serological tests have negative or doubtful results (Carmichael 1990). The antibody titer may fluctuate even in the presence of persistent bacteremia and its value does not reflect the disease progress. Furthermore, a possible decrease of this value does not necessarily indicate the efficacy of the therapy (Feldman and Nelson 1996).

The characteristics of the main serological tests for *B. canis* antibodies have been summarized by Carmichael and Shin (Carmichael and Shin 1996, Table III), and by Cosford (Cosford 2018, Table IV).

Rapid slide agglutination test (RSAT and 2ME-RSAT)

RSAT in the original technique elaborated by George and Carmichael (George and Carmichael 1978) uses a heat-inactivated *B. ovis* or *B. canis*, colored with Rose Bengal. Ebani and colleagues (Ebani *et al.* 2003) performed the test with an antigen from heat-inactivated and Rose Bengal-stained *B. ovis* strain 63/290 prepared according to Alton and colleagues (Alton *et al.* 1988). Currently, the strain recommended by the OIE for the production of

Table II. *PCR assays for the detection of* Brucella canis *in dogs from Cosford, edited (Cosford 2018).*

16S-23S rDNA interpace region (44)	***Brucella* genus**	**Whole blood**	**100%**	**100%**
16S-23S rDNA interpace region (45)[a]	*Brucella* genus	Vaginal swabs	Not available	Not available
16S-23S rDNA interpace region (46)[a]	*Brucella* genus	Semen	Not available	Not available
16S-23S rRNA sequence (47)	*Brucella* genus	Whole blood	100%	100%
16S-23S rRNA interpace region (48)	*Brucella* genus	Inguinal lynph nodes	100%	100%
16S-23S rRNA interpace region (49)	*Brucella* genus	Whole blood Serum	Whole blood: 97.14% Serum: 25.71%	
Intergenic Spacer IS711 823)[b]	*Brucella* genus	Males: proputial swabs, semen or urine Female: vaginals swabs	Not available	Not available
Gene fragment on chromosome 1 (23)[b]	*Brucella canis* specie-specific	As intermediately above	Not available	Not available
B. canis outer membrane protein 25 DNA quantitative PCR (50)	*Brucella canis* specie-specific	Vaginal swabs Whole blood	Vaginal swabs: 92.31% Whole blood: 16.67%	
BCAN_B0548-0549 region in chromosome 2 of *Brucella canis* (51)[c]	*Brucella canis* specie-specific	Whole blood Buffy coats	Not available	Not available

[a]PCR on vaginal swabs and semen in these studies correlates with blood PCR and blood coltures assessed by a Kappa-coefficient and the Mc Nemar test.
[b]Both *Brucella canis* genus-based and *Brucella canis* specific PCRs used in Swedish outbreak investigation.
[c]*Brucella canis* inoculated samples; PCR on Buffy coat separated from whole blood was approximately 100 times more sensitive that from whole blood.

B. ovis antigen is *B. ovis* REO 198 (OIE 2018). RSAT is a quick, easy to perform and read test, commercially available (*e.g.* D-Tec CB, Synbiotics, San Diego CA, USA). Positive reactions are detectable as early as 3-4 weeks after the onset of infection (Hollett 2006). The test has a sensitivity (defined as the probability of the test not to give rise to false negative reactions) of 99% (Carmichael 1990). On the contrary, specificity (defined as the probability of the test not to give rise to false positive reactions) is rather limited, counting false positive rates that commonly range from 20% up to even 50% (Carmichael and Shin 1996). False positive results would apparently be produced by cross-reactions between the antigen used and specific antibodies, possibly present in the tested serum. Possible cross-reactions have been observed against bacteria, such as *Pseudomonas* spp., *Bordetella* spp., *Streptococcus* spp. and, more generally, some *Enterobacteriaceae* (Carmichael 2012).

In order to reduce the incidence of false positivity, the RSAT test was then modified to include the addition of 2-mercaptoethanol (2ME-RSAT) to the test serum before mixing with the antigen. 2-mercaptoethanol reduces the incidence of false positivity substantially because it inactivates the less specific IgM antibodies (Badakhsh *et al.* 1982). The

Table III. *PCR assays for the detection of* Brucella canis *in dogs (Cosford 2018).*

Test	Nature of the antigen	Positivity limits[a]	Comments
2ME-RSAT	Cell wall	From 5-8 Sept. PI up to 3 months after the cessation of the bacteremia (followed by variable results)	High sensitivity (99%); low specificity (50%-80%); quick and easy performing.
TAT	Cell wall	Similar to 2ME-RSAT.	False positivity as in 2ME-RSAT; semi-quantitative; titles above 1:200 are indicative of infection in progress.
2ME-TAT	Cell wall	Similar to 2ME-RSAT.	Slightly higher specificity than TAT; longer laboratory process.
$AGID_{cpa}$	Cell wall	Similar to 2ME-RSAT, possibility of detection 1-2 weeks before.	higher Sensitivity than RSAT; permanence of frequent non-specific reactions; complexity of execution; difficulties of interpretation
$AGID_{cwa}$	Cytoplasm	From 8-12 weeks to at least 12 months after the end of the bacteremia, up to 36 months	Greater specificity (97%); but less sensitivity; reveals chronic cases negative to other tests; reveals infections from other *Brucellae*.
ELISA	Cell wall Cytoplasm	Unknown, believed to be similar to TAT	Technique in experimental phase; high specificity when using wall antigens of *B. canis* in phase M or cytoplasmic antigens.
IFA	Cell wall	Unknown	Unpublished data; appears to have less sensitivity than 2ME-TAT.

[a]Times are approximate; RSAT = Rapid slide agglutination test; ME = Test with 2-mercaptoethanol; TAT = Tube agglutination test; AGID = Agar gel immunodiffusion test; ELISA = Immunoenzymatic test; IFA = Immunofluorescence assay; PI = Post-infection; + = Positive; - = Negative.

Table IV. *Comparison of traditional serological tests for the diagnosis of canine brucellosis.* From Cosford, edited (Cosford 2018).

Test	Antigen	Sensitivity	Specificity	How to use the test
RSAT	*B. ovis* (M-) strain *B. canis*	Moderate to high	Low to moderate	Screening test
		older studies suggest high	older studies suggest 40%-50%	
		newer studies suggest 70.58%	newer studies suggest 83.34%	
2-ME-RSAT	(M-) Strain of *B. canis*	Lower than RSAT (31.76% vs. 70.58%)	Higher than RSAT (100% vs. 83.34%)	Confirmatory test
TAT	*B. canis*	High	Low	Screening test
IFA	Anti-canine immunoglobulin (Ig)G directed against antibodies to *B. canis*	Unknown	Unknown	Screening test
$AGID_{cwa}$	Lipopolysaccharide antigen from the cell wall of *B. canis*	High	Lower than $AGID_{cpa}$	Screening test
$AGID_{cpa}$	LPS-free soluble, internal cytoplasmic proteins extracted from *B. canis* or *B. abortus*	Low	High	Confirmatory test
		52.94 sensitivity	100%	
		47.06 false negatives		

RSAT test has been further modified by replacing the *B. ovis* antigen with an antigen derived from *B. canis* in phase M (mucoid) (Carmichael and Joubert 1988). This resulted in a reduction of the rate of false positivity to about 10%.

Therefore, since false negativity to this test is rare, it can be used as screening test to identify and separate negative subjects (Wanke 2004).

Tube agglutination test (TAT)

The technique has been described by Carmichael and Kenney (Carmichael and Kenney 1968) and Alton and colleagues (Alton *et al.* 1975). The TAT is able to detect antibodies to *B. canis* in dogs tested positive for RSAT or 2ME-RSAT. The test begins to provide positive results already 2-4 weeks after exposure (Hollett 2006). TAT consists in the addition of a fixed dose of heat inactivated *B. canis* antigen to different test serum dilutions. It is able to determine the antibody titer (Feldman and Nelson 1996). The test is sensitive but not very specific, allowing false positive results (Hollett 2006). As for RSAT, the addition of 2-mercaptoethanol (2ME-TAT) reduces false positive reactions (Carmichael 1990). Although more data on the reliability of this test are needed, the serum samples are considered negative when the agglutinating titer is less than 1:50 and doubtful when agglutinating titers are between 1:50 and 1:200. Titers above 1:200 are considered as positive. However, blood culture is always required to confirm the infection (Fredrickson and Barton 1974, Rhoades and Mesfin 1980, Flores-Castro and Carmichael 1977, Henderson *et al.* 1974, Carmichael and Shin 1996). There is a good correlation between TAT titer ≥ 1:200 and the isolation of the microorganism by blood culture (Hollett 2006).

In the United States and in countries where the disease is present, 2ME-TAT is no longer used in laboratories. They prefer to use 2ME-RSAT which has the same diagnostic accuracy, is easier to perform, standardizable and capable of giving comparable results between laboratories (Carmichael and Shin 1996). Being however a semi-quantitative test, 2ME-TAT is still used in kennels where brucellosis, due to *B. canis* infection, has been diagnosed. It allows to indirectly evaluate the response to antibiotic therapy, through the decrease of the agglutination antibody titer (Carmichael and Shin 1996), although this correlation has not yet been sufficiently demonstrated (Nicoletti and Chase 1987a).

Agar gel immunodiffusion test (AGID)

AGID can employ two different types of antigens: a *B. canis* cell wall antigen (AGIDcwa) or antigenic proteins extracted from cytoplasm of *B. canis* or other species of the genus *Brucella* (AGIDcpa), *B. abortus* in particular (Carmichael and Shin 1996). The technique has been described by Zoha and Carmichael (Zoha and Carmichael 1982).

The use of cytoplasmic-derived antigen further increases the specificity of the test, lowering the percentage of false positivity to 3% (Zoha and Carmichael 1982). Conversely, the same test performed using the cell wall antigen, suffers from the same false positivity problems as the agglutination tests previously described (Carmichael 2012). To produce the antigen, Ebani and colleagues (Ebani *et al.* 2003) used a hot saline extract (HSE) of *B. canis* strain RM6/66. The lipopolysaccharide wall antigen is less specific than the cytoplasmic antigen. Therefore, AGIDcwa has a high sensitivity but it still has the probability to give false positive results. Positive results appear 8-12 weeks after infection and can remain for 3-4 years (Hollett 2006).

The assay which uses cpa antigen is more specific but less sensitive since it can react with antibodies against other *Brucella* species (*e.g. B. canis*, *B. abortus*, *B. suis*) (Hollett 2006). The disadvantage when using cytoplasmic-derived antigen is the long period of time necessary for the test to become positive, which is around 8-12 weeks after the exposure. This makes the test not indicated for revealing the early stages of the infection. However, the assay remains positive for a longer period, about 12 months after the cessation of bacteremia. So this test can be more useful in detecting chronic infections (Carmichael 2012). In the literature, positive results to this test have been reported to persist more than 5 years after infection (Hollett 2016).

AGIDcpa test is the most effective technique in kennels infected with *B. canis*. Provided that the definitive diagnosis of *B. canis* infection always requires confirmation by blood culture, AGIDcpa test it can be used as a confirmatory test for those sera resulting positive to the agglutination tests (Carmichael and Shin 1996, Cosford *et al.* 2018).

A limited number of veterinary diagnostic laboratories are capable of carrying out AGID (Hollett 2006) due to the difficulties of antigen preparation and purification, as well as the requirement of specialized personnel (Feldman and Nelson 1996, Hollett 2006).

Indirect Immunofluorescence Assay (IFA)

This test can be considered as alternative when RSAT and TAT are not avalaible (Weber and Hussein 1976). However, results from the Cornell University's Diagnostic Laboratory indicate a high rate of false positive reactions with the IFA test (Wanke 2004). and, since the sensitivity of the IFA is not yet fully known, there is also a probability that infected dogs

may not be detected when tested (Carmichael and Greene 2006).

Immunoenzymatic test (ELISA)

Data on the performance of the ELISA for the detection of *B. canis* antibodies have recently been summarized by Cosford (Cosford 2018) (Table V).

These tests have been developed by using either cell wall of *B. canis* (M– and RM 6/66), or cytoplasm of *B. abortus*, as antigens (Baldi *et al.* 1997). The cytoplasmic antigens, common to all strains of the genus *Brucella*, have the advantage of not showing cross-reactivity with bacteria belonging to genera other than *Brucella* spp. However, it cross reacts with all bacteria of the genus *Brucella*. ELISAs which use cell wall antigens of *Brucella* strains in phase M– are highly specific but not highly sensitive (Serikawa *et al.* 1989, Mateau de Antonio *et al.* 1993). High false positive rates were instead observed when the ELISAs which use the RM 6/66 strain as antigen, were employed (Mateau de Antonio *et al.* 1993, Ebani *et al.* 2003).

Complement fixation test (CFT)

The Complement Fixation test was described by Alton and colleagues (Alton *et al.* 1975), and Weber and Krauss (Weber and Krauss 1977). Altough showing a good correlation with TAT (Weber and Krauss 1977), CFT is not used on a routine basis since dog serum often is anticomplementary (Alton *et al.* 1975, Weber and Krauss 1977, Ebani *et al.* 2003). The antigen used by Ebani and colleagues (Ebani *et al.* 2003) was a HSE of *B. ovis* strain 63/290.

Table V. *Comparison of ELISA tests for the diagnosis of canine brucellosis.* From Cosford, edited (Cosford 2018).

Antigens	Sensitivity	Specificity
Lipopolysaccharides-free cytoplasmic proteins of *B. abortus*	92%	96.7%
Hot-saline extract of *B. canis* containing outer membrane antigens	92%	94.3%
Luminaze synthase of *Brucella* spp.	81%	96.7%
18kDa cytoplasmic protein of *B. canis*	87%[a]	98%[b]
Bacterial whole cell extract from wild isolate of *B. canis* used as solid phase antigen	95%	91%
Heat soluble bacterial extract from wild isolate *B. canis* antigen[c]	91.18%	100%
M-strain *B. canis* antigen	100%	98.8%
B. ovis strain #11 antigen	100%	98.8%
B. abortus RB51 strain antigen	100%	98.8%

[a]Real sensitivity not reported as percentage was calculated considering 26/30 known cases that tested positivie with this ELISA.
[b]Specificity not reported as percentage was calculated from the data set as 2/103 animal tested falsely positive with this ELISA in the healthy population.
[c]Heat soluble extract were more useful than ultrasonic homogenates of bacteria isolates to generate candidate capture antigens, a sonicated antigens were associated with more cross reactivity, and, therefore, false positives in both ELISA and Western Blotting.

Immunoblotting (IB)

Ebani and colleagues (Ebani *et al.* 2003) explored the performances of Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) using *B. ovis* and *B. canis* HSE. The results of the study suggest that immunoblotting may be the most specific serodiagnostic method for detecting antibodies to *B. canis*. The authors had no false positive reactions with any of the evaluated sera. Serum samples positive to AGID or CFT were all negative to immunoblotting test. Immunogenic bands were evidenced with both *B. canis* and *B. ovis* HSE antigens only when positive control sera were tested. On the basis of the results obtained, the authors recommended to use the immunoblotting test as a confirmatory test. However, because of the intensive labor and time required for running the test, IB is not performed in laboratories on a routine basis (Ebani *et al.* 2003).

Interpretation of diagnostic tests

The different serological tests available have different levels of sensitivity and specificity depending on the stage of the disease and the type of method and antigen used. Medical history and clinical data, where available, should always be used in conjunction with the laboratory results to achieve a definitive diagnosis (Wanke 2004).

False negative results may occur following sampling carried out prior to seroconversion or due to low titers of circulating antibodies in some chronically infected subjects (Carmichael and Greene 2006).

False positive results, on the other hand, are the major problem when using these serological tests. These drawbacks can depend on specific and non-specific cross reactions with surface antigens of other microorganisms, such as *Pseudomonas aeruginosa, Bordetella bronchiseptica, Actinobacillus equuli, Streptococcus* spp., *Staphylococcus* spp., *Moraxella*-type microorganisms and Gram-negative bacteria (Carmichael and Greene 2006, Hollett 2006, CFSPH 2020, Yu and Nielsen 2010). These tests can be used as screening tests but positive results should be confirmed by a high-specific confirmatory test such as 2ME-RSAT or AGIDcpa (Carmichael and Greene 2006, Hollett 2006, Keid *et al.* 2009, Cosford 2018).

It has been reported that agglutinating antibodies may not protect the dog from infection (Pollock 1979) or from bacteriemia (Serikawa and Muraguchi 1979, CDC 1977).

Therapy

Treatment of canine brucellosis due to *B. canis* is possible, although the results are often disappointing, because of the intracellular localization of the bacterium for long periods, and its ability to generate episodic bacteremia (Carmichael 1990). For this reason, although *in vitro B. canis* is sensitive to different antibiotics, often the therapy is not effective and relapses of infection are common.

Among the various proposed therapeutic protocols, the scheme which combines tetracycline (25 mg/Kg t.i.d., PO) for four weeks, and dihydrostreptomycin (10 mg/Kg b.i.d., IM) in the first and last week of therapy gave the best results (Carmichael 1996). This experimental therapeutic scheme led to serological negativization of 94% of treated animals within two months after treatment (Nicoletti 1991).

The therapy is less successful in males than in females, probably due to the greater difficulty encountered in eliminating foci of infection from the male genital tract, especially from the prostate (Carmichael 1990). Despite the treatment, male subjects can still develop irreversible sterility and this, along with the difficulty of eliminating the infection from the prostate, suggests that these subjects should be excluded from reproduction anyway (Carmichael 2012).

The variability of the results obtained by the various authors in the application of experimental therapeutic protocols (Flores-Castro and Carmichael 1981, Zoha and Walsh 1982, Nicoletti and Chase 1987a, Nicoletti, 1991) also depends on the criterion adopted to define recovery (*i.e.* negativization vs bacteriological or serological examinations, respectively) (Carmichael and Shin 1996). In any case, in light of the fact that the infection can reoccur even in weeks or months after the end of therapy, it is recommended to carry out serological monitoring of the dogs for at least three months after completing the therapeutic cycle, repeating it in case serological positivity is found (Carmichael 1996). In addition, sterilization of the subject is recommended (Carmichael 1990).

The clinician should inform the owner about the problems associated to the therapy, its costs, length, and possible failures (Feldman and Nelson 1996).

Prophylaxis

Indirect prophylaxis

As far as indirect prophylaxis is concerned, all attempts to produce an efficacious vacine have not provided encouraging results. On the other hand, the existence of a vaccine for some aspects does not seem to be desirable, since it would interfere with the serological tests carried out to identify infected subjects (Carmichael 1990).

Direct prophylaxis

Management of kennels free from B. canis *and prevention of spreading between kennels*

Since a valid immunological product is not available, the prevention of canine brucellosis due to *B. canis*, in territories where the disease is present, should be based on the classic sanitary measures of direct prophylaxis, based on regular serological testing of the animals hosted in the kennel.

Strict biosecurity measures should be implemented, together with adequate kennel management and strict environmental controls (cleaning, disinfection, ensuring adequate temperature and humidity). It is necessary to take special care to properly clean and disinfect on a daily basis the delivering sites and the spaces where the newborn puppies are housed (USDA 2015).

Quarantine

Kennels free from *B. canis* should keep any newly introduced dog in quarantine, subjecting them to at least two specific serological tests carried out at four weeks of distance, admitting them to be in contact with the others only if both tests are negative (Carmichael 1996). The best way to keep brucellosis out of the kennel is then to isolate and test all incoming dogs, and testing them negative before placing into the kennel. This is best achieved by isolating newly purchased dogs in a separate building or facility, away from the rest of the population, for a minimum of eight weeks. All incoming dogs should be tested for *B. canis* on arrival and again after eight weeks. Only after having obtained two negative screening tests on all dogs of the isolation facility, they can be safely transferred in contact with the rest of the kennel population. If during this eight-week isolation period a newly introduced and isolated dog results positive to *B. canis* serology, it should be immediately removed from the facility. The eight-week isolation period will then restart for the remaining dogs in isolation. The quarantine isolation approach combined with laboratory tests has been shown to be the safest way to introduce new dogs into an established reproductive population without the fear of introducing brucellosis or other infectious agents (USDA 2015).

Control of breeders

B. canis infection is of major relevance for breeders

as it is normally a problem that forces to end their reproductive career (Carmichael 1990). In breeding kennels located in areas where the disease is endemic, annual serological testing of breeding dogs should be carried out, combined with a further check to be performed at least three weeks before each mating (Feldman and Nelson 1996).

Ideally, breeding dogs should never leave the breeding facilities except to be visited by a veterinarian for the necessary care (*e.g.* caesarean section, serious injuries or illnesses). It is advisable to keep the breeding dog population sheltered in the kennel, and to avoid sending females out for mating. These subjects could pose a risk of introduction of the disease; therefore, any dog that leaves the structure for mating or for any reason other than a caesarean section should be tested 90 days after its return to confirm its negative status. It is best to isolate these subjects from the rest of the kennel population upon their return, although doing so may not be practical. It may in fact require isolation during gestation and delivery, which may be problematic in some facilities (USDA 2015).

An alternative approach to genetic improvement of the breeding kennel population could be the use of artificial insemination (AI) on breeding females, using semen obtained from external breeding dogs proven negative for *B. canis* (at least eight weeks prior to semen collection). If male breeding dogs are subjected to outdoor mating, the safest approach would be to offer this service only through the use of AI, using semen collected in the kennel and then shipped to the requesting kennel without the female being introduced into the premises or the male leaving them (USDA 2015).

Control of other introductions

Stray dogs should be prevented from entering breeding kennels. Likewise, contact with animals or groups of animals of unknown or doubtful health status should be avoided, particularly in the case of competitions or mating (Carmichael 1996).

Visitors (including the customers themselves) should not have visited any other breeding kennel on the same day; they should wear clean clothing, disinfect their shoes, wear disposable protective shoe covers and wash their hands properly. Ideally, visitors should not touch or handle dogs or equipment. One solution could be to provide direct contactless access using a video display of the mother and father, as well as the litter during the period of parental care. This would eliminate most of the direct risk of transmission of the disease until the puppy is moved to its new home (USDA 2015).

Isolation and disinfection at delivery

It would also be appropriate to isolate females at the time of delivery, as well as the regular disinfection of kennel premises, particularly the delivering rooms (Berthelot and Garin-Bastuji 1993).

Management of infected kennels

In case the infection is identified in a kennel, the entire existing population should be confined (prohibition of entry/exit of animals) and subjected to serological tests. All the dogs present should be considered suspected cases.

Protection of operators

In order to reduce the likelihood of exposure to *B. canis*, personnel who work in infected kennels should wear disposable protective gloves when assisting delivery, including handling newborn puppies, placenta, fetal membranes or possible contact with urine or vaginal secretions. Extreme care should be taken when handling miscarriage, including dead or partially developed puppies, their fetal membranes and placentas. Protective gloves should also be used during assistance for insemination, both natural and artificial. To prevent *B. canis* infection during cleaning and disinfection of premises or handling of animals in quarantine and isolation situations, appropriate use of personal protective equipment (*i.e.* respiratory and ocular protection) would be advisable in addition to the simple use of gloves. It is also recommended that veterinarians, staff, owners, and laboratory personnel be careful when collecting and handling blood, serum, fluids or tissues for laboratory testing (USDA 2015).

Census of animals present

Before starting the checks, a thorough survey of the correspondence of the kennel register and the animals really hosted should be carried out. Subsequently, a register of controls should be set up where animals are distinguished by sex, race, and age, taking care to specify for each one of them the date of birth, so that it would be possible to easily identify the subjects to be monitored.

Animal tracing

It is necessary to identify the source of entry of the infection and its possible origin. Similarly, all animals having left the infected premise should be tracked. The animals and the sheltering kennel traced in this way should be subject to the same provisions as those adopted in the premise of origin.

Controls on animals

Once brucellosis is diagnosed, the only way to regain the freedom from the disease is to remove all positive dogs from the kennel.

All dogs over six weeks of age should be serologically tested with a first screening test (*e.g.* RSAT, 2ME-RSAT or TAT). Dogs to be tested must not have received any antibiotic treatment in the three months prior to sampling (Hollett *et al.* 2006). Dogs tested negative to the first screening test should undergo a second screening test to be carried out at least 4 weeks later. Dogs that tested negative to this second test can be considered as not infected with *B. canis*.

Dogs that test positive to the first serological test should be classified as suspected of *B. canis* infection (USDA 2015). They should be isolated and subjected to a confirmation test (*e.g.* AGIDcwa) to be carried out not earlier than 4 weeks. The dogs should remain isolated until the second response is obtained. If the second test is also positive, these dogs should be considered suspected of infection. They should be removed from the colony. To confirm or definitively rule out brucellosis, blood culture (Feldman and Nelson 1996), or a third diagnostic test (*e.g.* AGIDcpa) could be performed eight weeks after the second test (USDA 2015).

Puppies born to positive mothers or in any case younger than 6 weeks at the time of control should undergo three blood cultures carried out every 24 hours. Puppies with positive blood culture should be removed from the plant. Puppies over six weeks at the time of control must follow the same diagnostic protocol as adults (Hollett 2006).

In order to reach the status of kennel free from *B. canis*, the tests should be repeated with the remaining dogs every four weeks, until there are two consecutive negative tests on the entire population of the kennel (USDA 2015, Carmichael 1996).

Isolation of positive animals – ban on mating and sale

Dogs infected or suspected of infection should be kept physically isolated from negative dogs. However, this may not be feasible or sufficient to prevent the spread of the disease, even if strict hygiene measures are maintained (Carmichael and Joubert 1988). For this reason, it is recommended to remove from the plant all animals infected (Wanke 2004).

In kennels infected or suspected to be infected with *B. canis*, it is recommended to put in place isolation procedures for all positive dogs, and to stop any trade or exchange of positive subjects or puppies born to positive mothers. In addition to this, it is strongly recommended to stop all sales or transfer of all dogs present, for whatever reason, until the kennel reaches the *B. canis*-free status.

Depending on the epidemiological situation of the kennel and on the problems that may be present in relation to the welfare of the hosted dogs, official veterinarians should verify whether a suspicious or infected kennel should be subject to a strict quarantine (which means a ban on the sale or transfer of any subject until the achievement of the *B. canis*-free status) or to a partial quarantine, in which it is forbidden to sell or transfer only positive animals and puppies born to positive mothers.

The private veterinarians responsible for the kennel should closely link up with the respective official veterinarians to agree on how to manage quarantine in relation to the suitability of the facilities, periodic serological checks, the removal and isolation of positive animals and compliance with the rules on animal welfare.

Destruction of fetal membranes and aborted fetuses

Females should deliver in separate rooms that are properly washable and disinfectable. The placenta and fetal membranes should be removed and destroyed. Similarly, aborted fetuses or puppies that die before weaning should be removed and destroyed.

Cleaning and disinfection

B. canis does not survive for long periods in the environment, and it is normally sensitive to common disinfectants, such as ammonium quaternary salts and iodophores (Carmichael 1990) or to direct sunlight (USDA 2015). *Brucella* is also sensitive to 1%, sodium hypochlorite, to 70% ethanol, alcohol/iodine solutions, glutaraldehyde and formaldehyde (Hollett 2006). In the presence of organic debris, *B. canis* remains stable in the environment for up to two months (proper cleaning and disinfection are therefore essential). *B. canis* is resistant to drying in the presence of organic debris. It can withstand freezing and can survive in water, dust and soil. The combination of organic debris, high humidity, low temperatures and little or no sunlight promotes the organism's survival (this corresponds in most kennels to winter conditions).

An important and often neglected part of kennels management is the correct cleaning and disinfection of environments. When dealing with brucellosis or other diseases, cleaning and disinfection serve to limit the spread of infections and are fundamental components for their prevention. Proper cleaning and disinfection needs time and should be carried

out correctly in order for a kennel to be considered truly disinfected. It is important to remember that a clean kennel is not always a disinfected kennel.

The kennel consultant veterinarian should be sure that operators thoroughly understand the entire cleaning and disinfection process, including the correct dilution and storage of detergents and disinfectants, as well as the fact that compliance with the expected contact times and proper rinsing are absolutely necessary. The frequency required for cleaning and disinfection (daily or weekly) should be thoroughly discussed with the operators. The structure should always be cleaned and disinfected following an order of susceptibility of animals to the disease, starting first of all from the areas of the kennel that host the most sensitive animals (puppies and lactating females), followed by the areas that house healthy adults and finally the areas that house animals in poor health or in isolation (USDA 2015).

Treatment and relocation of positive animals

In countries where the disease is present, although the infection does not expose the dog to a lethal risk, in the view that antibiotic therapy does not guarantee the bacteriological recovery of infected dogs and that they may represent a source of infection both for other dogs and for humans, euthanasia is recommended. Possibly an attempt of therapy may be reserved exclusively for high-value breeding animals (Carmichael 1996). However, although cases of partially successful treatments have been reported, no treatment has been shown 100% effective, and puppies born to mothers with chronic brucellosis, if they survive, are often infected. For this reason, it is essential that dogs positives to diagnostic tests are not maintained as breeders, even if they have a high genetic value (Wanke 2004).

If the owner does not intend to choose euthanasia, an attempt of therapy could be recommended by providing from the beginning the necessary information on the zoonotic potential of the disease, especially underlining that even a sterilized dog subjected to specific therapeutic treatment could still be a source of contagion for humans (Feldman and Nelson 1996).

It is important to emphasize that canine brucellosis due to *B. canis* is currently not considered a curable disease in dogs. Attempts at therapeutic treatment led to very disappointing results, with relapses commonly occurring. The attempt at treatment can also mask diagnostic test results, and has been shown to be an important contributing factor to the spread of the disease. The impact of this evidence for dog breeding kennel owners is that animals infected with *B. canis* must be removed from the breeding population (USDA 2015).

Because of the zoonotic potential of *Brucella*, dogs that have been confirmed positive for brucellosis should not be given for adoption.

The decision regarding the possible adoption of *B. canis* positive dogs can only be taken with the authorization of the competent health authorities both in the areas of origin and destination (USDA 2015). If a decision to authorize the adoption of *B. canis* positive dogs is taken, they should be subject to ovarian-hysterectomy or castration, as well as to appropriate long-term antibiotic therapy, with appropriate supervision by an official veterinarian. This should include periodic lifetime laboratory tests for *B. canis*. Canine brucellosis is considered a potentially long-life infection; even after undergoing surgical spaying and long-term use of antibiotics, both male and female dogs can continue to eliminate the bacterium intermittently. New owners should be made aware of the potential risk these dogs may pose over the course of their lives regarding infection of humans, other dogs and other susceptible animal species they could come in contact (USDA 2015).

Training and information for operators

Official or private veterinarians managing dog breeding kennels infected from *B. canis* should discuss in depth with the staff and the owners of the facility the potential for legal liability (beyond damaging the reputation of the kennel) that would inevitably accompany cases of zoonosis from *B. canis* as a result from the sale of infected puppies or adult dogs. These puppies or adult dogs commonly come in contact with children, the elderly or other who may be immunocompromised (USDA 2015). A recent example was recorded in New York City in 2012 involving a 3-year-old girl. It was the first documented case of transmission of *B. canis* from a puppy to a child in the United States (Dentinger *et al.* 2015).

Public health aspects

B. canis can cause disease in humans, which can acquire infection through direct contact with infected dogs, their reproductive secretions, or their blood (Lawaczeck *et al.* 2011, Lucero *et al.* 2010).

Sources of infection

A potential source of spread of *B. canis* are breeding kennels, both for the nature of the disease, and for the fact that the animals are housed in close contact with each other, and for the constant movement of dogs for reproduction or for sale (Brower *et al.* 2007). Recent outbreaks in kennels in the USA, Hungary,

Sweden and Colombia highlight the link between outbreaks and the inter-regional or international movement of breeding dogs (Kaden *et al.* 2014, Castrillón-Salazar *et al.* 2013, Gyuranecz *et al.* 2011, Brower *et al.* 2007). Uncontrolled handling of puppies or non-spayed dogs is a known risk factor for the spread of infectious diseases, and this has led to human infection with *B. canis* (Dentinger *et al.* 2015, Brower *et al.* 2007).

Compared to owned dogs, stray dogs are more likely to be not spayed, and may have a higher level of seropositivity to *B. canis* (Flores-Castro and Segura 1976, Brown *et al.* 1976). A high incidence of canine brucellosis in stray dog populations may cause a spillover in the human population, especially in areas with a large number of non-spayed stray dogs, as these dogs are brought into shelter kennels or placed in other types of premises awaiting adoption. In the United States, 30% of pet dogs are adopted from animal shelters, and testing for *B. canis* is not a standard procedure before adoption (Brower *et al.* 2007). However, there is no evidence of a direct link between the number of fertile stray dogs in an area and the potential for exposure to humans.

Another potential source of *B. canis* infection may be represented by laboratory accidents. *Brucella* spp. is considered a high-risk pathogen and it requires manipulation in a specialized laboratory at biosecurity level 3 (BSL 3), which if not used may result in laboratory acquired exposure (Yagupsky and Baron 2005). Dentinger and colleagues (Dentinger *et al.* 2012) described an incident in which 31 laboratory technicians were exposed to *B. canis* after handling an unknown gram-negative bacterium on the bench. No one has fallen ill with clinical disease, even those classified as having had high-risk exposures (according to CDC guidelines) or who have declined post-exposure prophylaxis (5 out of 21 of those at high risk) (Dentinger *et al.* 2012). A case of laboratory-acquired exposure has been documented in a technician who used oral pipetting to re-suspend an M-phase strain of *B. canis*; the technician showed symptoms despite this particular strain being considered non-virulent in dogs (Wallach *et al.* 2004).

Categories at risk

Laboratory staff, veterinarians and animal keepers are the categories at greater risk of exposure to *B. canis* (Lucero *et al.* 2010, Marzetti *et al.* 2013, Krueger *et al.* 2014).

In addition to these, several reports in the literature highlight pet dog owners as possible categories at risk (Swenson *et al.* 1972, Munford *et al.* 1975, Lucero *et al.* 2010, Dentinger *et al.* 2012, Tosi and Nelson 1982). In particular, children and immunosuppressed people would have a higher risk of contracting the disease (Dentinger *et al.* 2012, Marzetti *et al.* 2013, Tosi and Nelson 1982, Lucero *et al.* 2010). Three cases have been reported in children under 4 years of age (Dentinger *et al.* 2012, Marzetti *et al.* 2013, Tosi and Nelson 1982). In one of the reports, Dentinger and colleagues (Dentinger *et al.* 2012) described the transmission of *B. canis* to a child by an infected puppy that had been purchased from a pet store and had been believed healthy during the preliminary veterinary visit. The child showed fever and *B. canis* infection was diagnosed with blood culture. The isolated strains from the child and the puppy were sent to the Centers for Disease Control and Prevention (CDC, USA) where the two strains showed a close genetic similarity, suggesting that the puppy had been the source of infection. However, clinical signs did not develop in four adults belonging to the same family, all exposed to the puppy. Several recent reports of *B. canis* infection in patients with HIV infection also highlight this population as at risk (Lawaczeck *et al.* 2011, Lucero *et al.* 2010, Moreno *et al.* 1998). These cases of *B. canis* infection have been linked to the owning of non-spayed dogs that had a history of reproductive failure and a following diagnosis of *B. canis* infection by serology and blood culture (Lawaczeck *et al.* 2011, Moreno *et al.* 1998).

Symptomatology

Symptoms of *B. canis* infection in humans are generally similar to those of brucellosis caused by other *Brucella* species (*e.g. B. abortus* or *B. melitensis*) (USDA 2015). Symptoms are often non-specific and may include one or more of the following: fever (often periodic and nocturnal), fatigue, headache, weakness, general malaise, nausea, chills, sweating, loss of weight, hepatomegaly, splenomegaly and lymphadenopathy (Swenson *et al.* 1972, USDA 2015). Endocarditis, meningitis, arthritis and visceral abscesses can represent further complications, however rare (Carmichael 2012).

Although there are reports in the literature that the course of the disease would still be less severe when compared to the infection caused by the 'classical' species of the genus *Brucella* (Swenson *et al.* 1972, Polt *et al.* 1982), however, severe manifestations have been also described. These include septic arthritis, aortic valve vegetation, osteomyelitis, epidural abscess, pleural effusion, oral lesions, lower limb aneurysms and culture-negative endocarditis (NASPHV 2012).

Diagnosis

The diagnosis of infection in humans, as well as in dogs, is based on serological examination followed by blood culture. However, in humans, diagnosis

is often complicated due to non-specific signs and symptoms and it is therefore associated with a low rate of disease suspicion by many physicians. If the disease is placed in a differential diagnosis, blood culture is the only test available to confirm *B. canis* infection in humans. However, confirmation is not straightforward, due to intermittent and low-level bacteremia (Rumley and Chapman 1986). Regarding serology, human antibodies to *B. canis* react with the same antigens used in dog serological tests, while they do not react with the *B. abortus*-derived antigens (*Brucella abortus* strain 99, Weybridge or *B. abortus* strain 1119-3, USDA), which are used in routine tests for the diagnosis of human brucellosis caused by smooth strains (Carmichael 1990). Therefore, even if the physician may suspect *B. canis* infection on the basis of clinical findings, the diagnosis may not be supported by serological tests available on the market, as these are aimed at detecting antibodies produced against *Brucella* in a smooth phase and do not detect antibodies against *B. canis* (Lucero *et al.* 2005). Serological tests for the detection of *B. canis* infection developed on dogs have been adapted for use in humans, but test results should be interpreted with caution (Hensel *et al.* 2018).

Therapy

Unlike canine brucellosis, the disease in humans can be quickly and effectively treated with tetracycline therapy administered for two to three weeks (Carmichael 1990).

Prophylaxis

In countries where the disease is present, it is recommended that veterinarians always inform the owners of infected animals about the potential zoonotic risk of cohabitation with their pets and to use the outmost caution and hygiene when visiting dogs suspected of infection, especially the female dogs who have aborted in the recent past (Carmichael 2012).

Similarly, caution is recommended in the laboratory when handling samples to be subjected to diagnostic tests for *B. canis* (Carmichael 2012).

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