Detection of *Brucella canis* in a dog in Italy

Marialaura Corrente[1], Delia Franchini[2], Nicola Decaro[1], Grazia Greco[1], Maria D'Abramo[1],
Maria Fiorella Greco[1], Francesca Latronico[1], Antonio Crovace[2], Vito Martella[1]

[1]*Department of Public Health, Faculty of Veterinary Medicine, University of Bari, Italy;*
[2]*Department of Emergency and Organ Transplantation, Division of Veterinary Surgery, Faculty of Veterinary Medicine,*
University of Bari, Italy

―――― **SUMMARY** ――――

Brucella spp. is a worldwide zoonotic pathogen. Infection by *Brucella canis* in dogs is endemic in the Southern USA and in Central and South America, but it appears sporadically in other parts of the world, including Europe. Tissue samples from a dog with chronic prostatitis, discospondylitis and locomotor problems were subjected to clinical and laboratory examinations. *B. canis* was detected by PCR in biological fluids and tissues of the animal, while antibodies to *B. canis* were found in the serum, providing additional strong evidence for the circulation of *B. canis* in Italy.

KEY WORDS: Brucella canis, Dog, Real-time PCR, Zoonosis, Italy

INTRODUCTION

Infection by *Brucella* spp is diffused worldwide in several animal species and in humans. The most clinically relevant *Brucella* species, *Brucella abortus, B. melitensis, B. canis* and *B. suis,* tend to be host-adapted, although infections of other animal species, including humans, may occur sporadically (Michaux and Charachon, 2002). Canine brucellosis, due to *B. canis,* was first recognized in 1966 in USA from episodes of abortion and reproductive failure in kennels (Carmichael, 1966). Since then, the disease has been reported in several countries (Carmichael and Shin, 1996). Canine brucellosis is endemic in the Southern USA and in Central and South America with seroprevalence rates of nearly 30% (Flores-Castro and Segura, 1975; Carmichael, 1990). In Europe, canine brucellosis is observed sporadically. *B. canis* has been identified in the United Kingdom in a dog imported from Spain (Dunne *et al.,* 2002) and in a male dog in Germany (Nöckler *et al.,*

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Corresponding author
Prof. Marialaura Corrente
Department of Public Health
Faculty of Veterinary Medicine
University of Bari, Italy
E-mail: m.corrente@veterinaria.uniba.it

2003). However, indirect serological evidence suggests that the infection is present in canine populations in several European Countries, Italy included (Ebani *et al.,* 2003; Wanke, 2004).
The infection may be transmitted in dogs either by the venereal or oral route and may cause late abortion in females, and epididymitis and prostatitis in males, leading to infertility and economic losses in kennels (Wanke, 2004). Moreover, extra-genital signs (lymphadenitis, diskospondylitis) or asymptomatic infections may occur (Carmichael and Kenney, 1970). Diagnosis of *B. canis* infection is usually accomplished by isolation of the pathogen from whole blood, semen, vaginal secretions, urine and lymphoid tissues (Greene and Carmichael, 2005). However, cultivation is time-consuming and fastidious, as members of the genus *Brucella* do not grow easily. In addition, negative cultures do not rule out the infection since the pathogen may be absent from the cultured samples or obscured by antibiotic treatments (Wanke, 2004). Accordingly, serologic and biomolecular diagnostic techniques have been developed to overcome the limits of bacterial isolation. Several serological assays are available, based on specific antigens obtained from *B. canis* or *B. ovis*, such as rapid slide agglutination (RSAT) (Carmichael and Joubert, 1987), rapid slide agglutination with 2-mercaptoethanol

(2ME-RSAT) (Badakhsh *et al.,* 1982) and agar gel immudiffusion (AGID) (Carmichael, 1990). The serological assays are practical and rapid, but the results in terms of specificity and sensitivity are often discordant and must to be confirmed by direct diagnosis (Keid *et al.,* 2009). For detection of *Brucella* spp., polymerase chain reaction (PCR) appears to be more rapid and sensitive than bacterial cultivation since it does not depend on bacterial viability and it is not affected by other bacterial contaminants (Bricker, 2002). Identification and characterization in PCR of *Brucella* at the genus level may be performed using primers targeting highly conserved regions such as Bcsp31 (Baily *et al.,* 1992) or 16S-rRNA (Romero *et al.,* 1995). For characterization of the various species and/or biovars of *Brucella,* several PCR-based strategies have been set up, using highly specific oligonucleotides (Greene and Carmichael, 2005; Imaoka *et al.,* 2007).

In May 2008, a dog WITH chronic prostatitis and discospondylitis was seen at the Hospital of the Faculty of Veterinary Medicine of Bari. *B. canis* was detected by PCR in biological fluids and tissues of the animal, while antibodies to *B. canis* were found in the serum, providing additional strong evidence for the circulation of *B. canis* in Italy.

METHODS

Clinical case
A 6-year old, mixed-breed, intact male dog was presented to the Hospital of the Faculty of Veterinary Medicine (Bari, Italy). Clinical signs included stiffness, a decreased level of physical activity, lethargy, depressed pelvic limb reflexes and faecal incontinence. The dog had a history of chronic prostatitis, and had been treated with amoxicillin (22 mg/kg BID for 10 days) without any improvement.

Lymph node enlargement and testicular atrophy were also observed. Radiographs and CT scan of the lumbosacral spine revealed discospondylitis at the lumbosacral space, with disk extrusion. Prostatic ultrasonography with fine-needle aspiration biopsy was performed. At ultrasonography, the prostate gland was hyperechoic with parenchymal cavities, irregular and asymmetric silhouette. Cytological examinations revealed

granulomatous inflammation with intracellular gram-negative coccobacilli.

The dog was subjected to decompressive surgery by removal of the inter-vertebral disk and was administered dihydrostreptomycin, 20 mg/kg per day, for two weeks. Notwithstanding, the condition worsened substantially with the animal becoming paraplegic, requiring euthanasia.

Microbiological analysis
A fragment of the inter-vertebral disk, heparinised blood and urine collected by cistocentesis from the dog were subjected to bacteriological investigations. Five ml of urine and 5 ml of blood were inoculated into Tryptose soy broth (TSB) supplemented with 7% of equine serum. The samples were also streaked on Columbia blood agar and MacConkey agar and all media were incubated in aerobic, microaerophilic and anaerobic conditions at 37°C. Because of the small sample size, the disk was split into two portions. One was used for inoculation into TSB and incubated in aerobic conditions; the second portion was analyzed by PCR. Subcultures in Tryptose soy agar with 7 % equine serum of all the TSB-cultured samples were made after 3, 5, 7 and 14 days of incubations. The subcultures were monitored daily for 1 month before being discarded. All the media and reagents were purchased from Liofilchem (Teramo, Italy).

PCR assays
DNA extraction
Nucleic acids were extracted from all the samples using the DNeasy Blood & Tissue Kit (QIAGEN S.p.A., Milan, Italy), following the manufacturer's instructions. DNA extraction was made either from the whole blood and from the buffy coat obtained by using a specific reagent (Cederlane, Hornby, Canada).

Real-time PCR for Brucella spp. quantification
A real-time PCR assay targeting the BCsp31 gene of *Brucella* spp. was used to evaluate the bacterial DNA load in the samples, with certain modifications (Probert *et al.,* 2004). Absolute quantification was achieved by constructing a standard curve using plasmid DNA. To generate the standard DNA, a 630-bp fragment of the BCsp31 gene of *Brucella melitensis* strain 16 M was amplified by PCR using primers BCsp31P-F (5'-GAGCTTT

GCGGTTGCACA-3') and BCsp31P-R (5'-AGATC GGAACGAGCGAAATA-3') and cloned into PCR blunt vector-TOPO (Invitrogen srl, Milan, Italy). The reaction was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City CA) with iTaq™ Supermix added with ROX (Bio-Rad Laboratories Srl, Milan, Italy).

PCR specific for B. canis
For characterization of *B. canis*, a combined PCR protocol (Imaoka *et al.*, 2007) was used, with 4 sets of primers targeting the genes BCSP31, *omp*2 and *omp*31. The protocol used is able to characterize simultaneously *B. abortus, B. melitensis, B. canis* and *B. suis*. For amplification, the Accuprime ™ Taq DNA polymerase was used (Accuprime™ SupermixII, Invitrogen, Milan, Italy).

Serological investigations
Rapid slide agglutination test (RSAT)
The dog's serum was screened for the presence of antibodies against *B. canis* by the RSAT, using a less mucoid strain of *B. canis* (*B. canis* M-) as antigen (Carmichael and Joubert, 1987). As a positive control, antiserum from a dog with confirmed infection by *B. canis* was used. The sera were pre-treated with 2-mercaptoethanol (Sigma, Milan, Italy) in order to improve the specificity of the test (Badakhsh *et al.*, 1982).

Complement fixation test (CFT)
The serum of the suspect dog was tested by a CFT kit specific for rough strains of *Brucella* spp. following the manufacturer's instructions (Diesse diagnostica Senese s.p.a., Siena, Italy). The antigen of the CFT assay is obtained from the rough strain *B. melitensis* B 115 (Adone *et al.*, 2008).

RESULTS

Microbiological analysis
All the dog samples tested negative, with no bacterial growth after 30 days of incubations, both in primary cultures and subcultures.

PCR assays
Real-time PCR for Brucella spp. quantification
All tissue samples from the dog, except whole blood, tested positive to the real-time PCR spe-

cific for *Brucella* spp. In particular, the intervertebral disk and the urine tested positive, with a threshold value of 1.35×10^2 copies ml^{-1} and 2.18×10^2 copies ml^{-1}, respectively. The buffy coat tested positive (1.52×10^2 copies ml^{-1}).

PCR specific for B. canis
When tested by the combinatorial *Brucella*-typing PCR, the intervertebral disk, urine and buffy coat samples yielded an amplification pattern specific for *B. canis*.

Serological investigations
Specific antibodies to *B. canis* were detected by RSAT. Low antibody titers (1: 8) were also detected by CFT.

DISCUSSION

B. canis is a potential zoonotic agent that infects almost exclusively dogs and wild *Canidae*. The infection is particularly suspected in male dogs with epididymitis, infertility or diskospondylitis. Occasionally, *B. canis* may also produce focal infections in various organs, especially in the osteoarticular, lymphoid and genital systems (Greene and Carmichael, 2005).
The dog was presented to the Veterinary Hospital because of locomotor problems caused by discospondylitis, as revealed by radiography and CT images. The animal's anamnesis also disclosed prostatitis. However, since the animal had never mated, possible infertility problems likely went undiagnosed. By bacteriological investigations, all the samples collected from the animal (blood, urine and a fragment of the intervertebral disk) tested negative.
However, the samples tested positive by using a real-time PCR for *Brucella* spp. and a combinatorial PCR able to identify *B. canis*. In addition, brucellar antibodies were found in the serum of the animal by RSAT and CFT.
The observed inconsistencies were not surprising. Hemoculture is considered the 'gold standard' for diagnosis of brucellosis, particularly for canine brucellosis. However, it has been shown that the hemocultures may yield false negative results (Romero *et al.*, 1995). Disagreement concerning diagnostic tests have been also observed in human chronic and relapsing brucellosis, as

brucellae may be nonviable, intramacrophage-located or present in low numbers (Nimri, 2003). In addition, antibiotic treatments started after the suspected diagnosis may affect the results of bacteriological investigations (Queipo-Ortuño *et al.*, 1997; Morata *et al.*, 2001).

Moreover, during the late phase of infection, bacteremia ceases, but the circulating antibodies may still be detectable. In all those cases, the PCR techniques have shown better sensitivity than conventional cultures, especially for non blood samples (Morata *et al.*, 2001). Several PCR protocols for the diagnosis of canine brucellosis are described in the bibliography (Keid *et al.*, 2007a; 2007b).

In the case described here, failure of the bacteriological investigations may be accounted for by previous antibiotic treatment and/or by chronic focal localization within granulomas. It is interesting to note that brucellar DNA was detected in the buffy coat, but not in the whole blood, suggesting intra-macrophage localization of the pathogen. In addition, the brucellar load was low, as determined by real time PCR.

Antibodies against rough strains of *Brucella* spp. were shown by RSAT and CFT. Those serological tests used for diagnosis of canine brucellosis may be affected by low specificity and sensitivity (Carmichael and Shin, 1996). The need for biomolecular or cultural assays to confirm the serology and avoid false positive results is especially important in countries where *B. canis* has never, or rarely, been reported.

In this case the source of infection by *B. canis* could not be established. The dog was kept in a small yard, but in summers the owners spent their holidays camping. In that period, the dog may have had contact with other dogs. As the animal had signs of chronic infection, the beginning of the infection could be not traced.

Human infection by *B. canis* is considered uncommon and clinical signs are mild or, in some cases, atypical (Lucero *et al.*, 2005). Human infections are not readily diagnosed because specific serological tests are not available in most laboratories. Accordingly, human exposure to *B. canis* is likely underrated. Currently, the Italian Veterinary Regulations do not provide specific restrictive measures for infections by *B. canis*. However, infection by *Brucella* spp. is included in the list of the zoonosis requiring surveillance (Italian Legislative Decree no. 191, April 4, 2006). As precautionary measure, the animal was put in quarantine at home. Despite decompressive surgery and specific antibiotic treatment, the general condition of the dog worsened and it was humanely euthanized.

CONCLUSION

We report a case of canine brucellosis that was diagnosed using serological and biomolecular assays but not with standard cultural methods. Real-time PCR proved to be a useful diagnostic tool being specific and sensitive and can be applied reliably even in focal or relapsing forms of brucellosis. Due to the potential zoonotic risks, *B. canis* should always be included in diagnostic algorithms of canine diseases.

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