

First detection of *Brucella canis* infections in a breeding kennel in Austria

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SUMMARY

Brucella canis occurs almost worldwide and is a potential danger to the health of dogs and humans. The pathogen was detected in the placenta and fetuses of a Standard Poodle by direct culture and immunohistochemistry. Further, Brucellae were also isolated from the blood samples of two asymptomatic female Medium Poodles. The isolates were identified as *B. canis* by conventional microbiological methods and a novel Bruce-ladder multiplex PCR. Genotyping was performed by multiple locus variable number tandem repeats analysis (MLVA).

KEY WORDS: *Brucella canis*, Dog, Immunohistochemistry, Isolation, PCR, MLVA, Zoonosis, Austria.

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Brucella canis is a gram-negative, facultative intracellular bacterial agent first isolated in 1966 as the causative agent of abortion in Beagle breeding kennels in the USA (Carmichael, 1966). The pathogen occurs almost worldwide and is a potential danger to the health of dogs and humans (Lucero *et al.*, 2010). In Europe, few clinical cases in dogs or outbreaks in kennels have been described (Dunne *et al.*, 2002; Nöckler *et al.*, 2003; Corrente *et al.*, 2010; Gyuranecz *et al.*, 2011; Holst *et al.*, 2012). The main symptoms suggestive of canine brucellosis are late abortions in bitches, prostatitis and epididymitis in males, infertility, lymphadenitis, uveitis and spondylodiscitis. Generally, infected dogs are apparently healthy and show no fever, but intermittent bacteremia may persist in untreated dogs for years (Hollett, 2006). Since there is no fail-safe antiobiotic treatment, infected dogs must be removed from the kennel and no longer used for breeding (Wanke, 2004). Neutering

in combination with antibiotic therapy reduces the risk of transmission to other animals and humans, but treatment is expensive and cures are difficult to achieve (Shin and Carmichael, 1999; Brennan *et al.*, 2008). In Austria, *B. canis* was first isolated in 2003 from an infected eye of a mongrel dog imported from Greece (own unpublished data). Here we report the first detection of canine brucellosis in a breeding kennel.

A female Standard Poodle was presented with clear signs of iritis in the left eye at a veterinary clinic. The eye was miotic and showed marked pigment formation and adhesions between the iris and the anterior lens capsule. Blood samples were sent to a veterinary diagnostic laboratory, where a high *B. canis* serum agglutination test (SAT) - titer (1:400) was observed. In February 2010, during the ultrasound imaging of the pregnant Standard Poodle, one fetus showed retarded growth and no more heart activity. In a subsequent examination all fetuses were shown to be dead and a Porro cesarean section was performed.

The surgically removed uterus including placenta and fetuses was examined by necropsy and organ specimens were prepared for histological ex-

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amination using 7.5% neutral buffered formaldehyde solution (Merck, Darmstadt, Germany) and embedded in paraffin. The uterus contained five macerated fetuses 5 to 11 cm in length. The fetal placenta was slightly oedematous and showed green-black discoloration. For light microscopic evaluation, 3-4 μm thick sections were stained with hematoxylin and eosin. In the histological examination, subacute purulent placentitis was found (Figure 1). In the fetal lungs foci of purulent alveolitis were found, as well as emboli of placental trophoblast cells in some pulmonary vessels. No other histological alterations, as necrotic and inflammatory lesions in heart, liver and kidney or any malformations, were observed. Immunohistochemical detection of *Brucella* antigen was performed using avidin-biotin complex immunoperoxidase (ABC) technique according to the manufacturer (Vectastain ABC Kit, Vector Laboratories, USA). The primary antibody was a polyclonal rabbit antiserum (anti-Brucella Positive Control Serum (AMS), BD, USA) at a dilution of 1:1000 as described by Pérez *et al.* (1998). *Brucella* antigen was visualized using the chromogen 3-amino-9-ethylcarbazole (AEC Peroxidase Substrate Kit, Vector Laboratories, USA). Immunohistochemically, abundant Brucellae were detected in the placental and pulmonary lesions. The bacteria presented both intra and extracellularly as cloudy and dusty red-brown reaction product (Figure 2). The intrauterine death of the Poodle puppies could be clearly attributed to *Brucella* infection, as large quantities of Brucellae were detected in placental and pulmonary lesions. The pathomorphological findings and the intralésional antigen distribution are consistent with those of the literature (Gyuranecz *et al.*, 2011). Pathomorphologically, there was no evidence of virus (e.g. canine herpesvirus and parvovirus) associated lesions, such as inclusion bodies, necrosis, nonpurulent inflammation and malformations.

Blood samples from seven clinically healthy dogs of the breeding kennel (3 Medium Poodles, 2 Labrador Retrievers, 2 Golden Retrievers) were subjected to bacteriological examination and serological tests. Liquid enrichment culture of the non-sterile blood samples was done using BBL Brucella Broth (BD) with Oxoid Modified Brucella Selective Supplement (2,500 IU Polymixin B, 12,500 IU Bacitracin, 25 mg

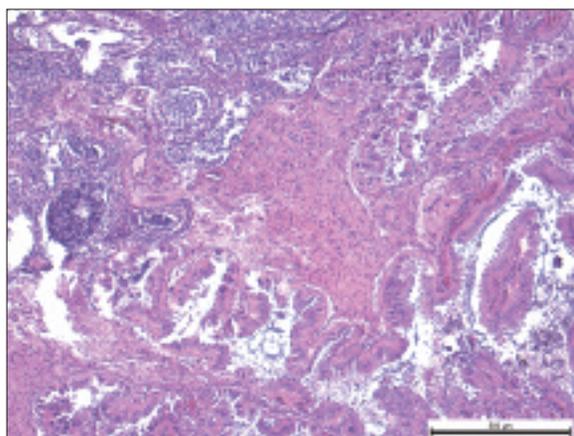


FIGURE 1 - Purulent placentitis. Microphotograph. H.E., Bar = 500 μm .

Natamycin, 2.5 mg Nalidixic acid, 50,000 IU Nystatin and 10 mg Vancomycin to 500 ml of medium). Fetal placenta and fetal lungs from the surgically removed uterus of the Standard Poodle and vaginal swabs from five clinically healthy female dogs were cultivated directly using 10% sheep blood Columbia agar (BD) with Oxoid Modified Brucella Selective Supplement as described for the Brucella Broth. The solid media were incubated for five days, the liquid cultures for five weeks, with final plating on 10% sheep blood Columbia agar. The cultures were incubated at 37°C in ambient air. Slow growth of

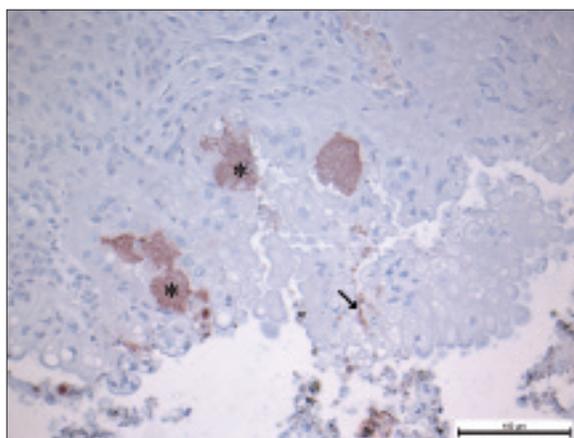


FIGURE 2 - Immunohistochemically abundant Brucellae presented both intra- (*) and extracellularly (→) as cloudy and dusty red-brown reaction product in the placenta. Microphotograph. ABC-technique, Bar = 100 μm .

non-haemolytic, round, convex, glossy, rough colonies was observed among the cultures of fetal placenta and fetal lungs from the surgically removed uterus of the Standard Poodle, the cultures of blood and vaginal swab from an asymptomatic female Medium Poodle, and the culture of blood from another clinically healthy female Medium Poodle. Phenotyping of the organisms was done as described previously (Alton *et al.*, 1988). Modified Ziehl-Neelsen staining showed red rods. Slide agglutination with anti-rough *Brucella* serum and rapid urea-splitting on a slope of Christensen's medium indicated *B. canis*. The novel Bruce-ladder multiplex PCR assay for the discrimination between *B. suis* and *B. canis* confirmed the identity of all 3 Poodle isolates as *B. canis* (López-Goñi *et al.*, 2011). Using original INgene Bruce-ladder V and INgene Bruce-ladder suis multiplex conventional PCR (Ingenasa, Madrid, Spain), the isolates were misidentified as *B. suis* biovar 4. It is known that some *B. canis* strains are identified erroneously as *B. suis* by the original Bruce-ladder PCR (López-Goñi *et al.*, 2008).

The serological investigation of the blood samples was carried out as micro-agglutination test with *B. canis* antigen Salt-ME (NVSL Ames, Iowa). Among seven investigated asymptomatic dogs, one bacteremic Medium Poodle showed a titer (1:20) in the micro-agglutination test. The other bacteremic Medium Poodle tested negative. The low or absent serum titer of the Medium Poodles with proven bacteremia may suggest a chronic stage of infection. If serological methods are used, there is a known risk that infections will remain undetected (Wanke, 2004). Blood cultures should always be performed because of the long periods of bacteremia (Shin and Carmichael, 1999).

Genotyping was performed by a published protocol for multiple locus variable number tandem repeats analysis (MLVA-16) (Le Flèche *et al.*, 2006). Sequence analysis was performed using Genetic Analyzer 3130 and the GeneMapper Software Ver. 4.0 (Applied Biosystems, Darmstadt, Germany). The Poodle isolates showed the same genotype, but were different from other *B. canis* genotypes in two of the most stable loci bruce08 and bruce12 (panel 1). To date, this genotype was not known among more than 700 *Brucella* isolates available at the data-

base (<http://minisatellites.u-psud.fr/>). Based on this very limited database for *B. canis*, it seems that the reported outbreak has to be seen as an isolated one. The differences from other known genotypes of *B. canis* in Europe are plainly visible, since they are in the most stable loci of the MLVA16 genotype. The first Austrian isolate, which was cultured 2003 from an infected eye of a mongrel dog imported from Greece, has the MLVA8 and MLVA11 genotypes 3 and 26, respectively. The MLVA16 genotype is closely related to the *B. canis* reference strain RM6/66 to which only two of the most variable loci in panel 2B are different in one repeat each (bruce04 and bruce09). For more exhaustive epidemiological research, it is necessary to genotype more *B. canis* isolates from all over the world to improve the quality of the database.

This report demonstrates that the detection of canine brucellosis in breeding kennels may require various samples and laboratory techniques. If infections go undetected because of false-negative test results or unrecognized symptoms, the risk of pathogen spread and transmission to humans remains. In breeding dogs with uveitis, disorders of pregnancy, infertility or other symptoms that could indicate brucellosis so that *B. canis* infection should be considered.

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