Part 1. Veterinary medicine

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FIRST REPORT OF CANINE BRUCELLOSIS IN UKRAINE: PATHOGEN ISOLATION AND CHARACTERIZATION

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Summary. For the first time in Ukraine we confirmed canine brucellosis caused by *Brucella canis*. The bacterium was isolated from testicles of three-year-old male Labrador retriever with orchitis and epididymitis. Initially blood serum sample was positive in cCFT, AGID and LFIA. In addition to the pathogen isolation and identification by biochemical test and PCR, the antimicrobial susceptibility test was performed that showed sensitive of *B. canis* to the commonly used antibiotics, which should be taken into account for the further therapy

Keywords: antibiotics, Bruce-ladder PCR, *Brucella canis*, orchitis, epididymitis, serology

Introduction. Canine brucellosis is a zoonotic disease mainly caused by *Brucella* (*B.*) *canis*, and sporadically by *B. melitensis*, *B. suis*, and *B. abortus* (Hensel, Negron and Arenas-Gamboa, 2018). *B. canis* can be transmitted through urogenital secretions of infected animals (Kang et al., 2011), and is particularly associated with the reproductive disorders (abortion in females, epididymitis and prostatitis in male dogs), discospondylitis and uveitis (Gyuranecz et al., 2013), leading to significant economic losses for breeding dogs in infected kennels. It is known that *B. canis* can persist in an animal even after long-term antibiotic treatment. Humans are susceptible to *B. canis* infection (Krueger et al., 2014).

Routine diagnostics of the disease based on the serological investigations, such as rapid slide agglutination test (RSAT) with and without 2-mercaptoethanol, tube agglutination test (TAT), complement fixation test (CFT), agar gel immunodiffusion (AGID) and ELISA with rough antigens (*B. canis* or *B. ovis*) (Hollet et al., 2006). As the definitive diagnosis of the infection pathogen isolation and the polymerase chain reaction (PCR) are recommended (Kang et al., 2014).

Canine brucellosis remains endemic in many regions of the world, with predominance in Central and South America (Lucero et al., 2008), in Asia and southern USA (Hubbard, Wang and Smith, 2018; Whitten et al., 2019; Jamil et al., 2019). Various cases have been also described in Europe (Holst et al., 2012; Egloff et al., 2018; Buhmann et al., 2019), but no data are available regarding Ukraine, where *B. canis* infection may be frequent due to the big population of stray dogs.

Aim of the study. In this article we report the first confirmed case of canine brucellosis in Ukraine.

Materials and methods. *Sampling.* In July 2020, three-year-old male Labrador retriever with obviously enlarged testicle was observed in the veterinary clinic in Volnovakha (Donetsk Region). Due to suspicion of brucellosis blood, serum blood, and urine samples were taken for the further studies. After primary samples studies surgically removed testicles were sent to the laboratory for the pathogen isolation.

Serological tests. The cold modification of complement fixation test (cCFT), agar gel immunodiffusion (AGID) with *B. ovis*-antigen and Rose Bengal test (RBT) were performed to detect *Brucella* antibodies in blood samples (Alton et al., 1988). The reference serum against *B. canis* was obtained from ANSES and was used as the positive sample. Additionally, samples were studied by lateral flow immunoassay (LFIA) using commercial kit 'Antigen Rapid C. Brucella Ab Test Kit' (BioNote Inc., South Korea).

Bacteriological studies. Blood, urine samples and testicles were plated on defibrinated sheep blood agar (5%) and tryptic soy agar. Plates were incubated at 37°C up to 10 days. Colonies of isolate were tested by agglutination with acriflavine, crystal violet staining, agglutination with monospecific sera against A and M antigens, hydrolysis of urea, oxidase test, H_2S production, and growth in the presence of CO_2. Growth on tryptic soya agar containing basic fuchsin (20 μg/mL) and thionin (20 μg/mL). A bacterial suspension was prepared from pure and fresh colonies and the tube turbidity adjusted to the 0.5 McFarland turbidity standards. The suspensions were spread onto *Brucella* agar plates and incubated at 37°C. Disk diffusion susceptibility tests were performed for 13 antibiotics: streptomycin (30 μg per disk), gentamicin

(10 μg per disk), rifampicin (5 μg per disk), tetracycline (30 μg per disk), doxycycline (30 μg per disk), ceftazidime (30 μg per disk), ampicillin (10 μg per disk), kanamycin (30 μg per disk), ciprofloxacin (5 μg per disk), gatifloxacin (5 μg per disk), azithromycin (15 μg per disk), sulfadiazine (300 μg per disk), and meropenem (10 μg per disk). The results of antimicrobial test were assessed within 48 h of incubation.

DNA extraction and PCR conditions. Obtained colonies were boiled for 10 min at 90°C. DNA extraction from blood, urea and testicles was realized using Qiagen DNA extraction kit (Germany) according to the manufacturer's instructions. A genus detection protocol based on *IS711* gene amplification by Real-Time TaqMan PCR assay (Hinić et al., 2008) and species identification protocols the Bruce-ladder PCR (López-Goñi et al. 2008) were performed. As the positive control *B. abortus* 99, *B. ovis* 63/290, *B. melitensis* REV-1, and *B. suis* 1330 were applied.

Results. As the first step, suspected dog was tested serologically. The reason for these investigations was an enlarged left testicle and epididymis. However, the dog was in an overall good general condition. As owner mentioned the dog was not imported from abroad, never bred, kept alone in the family with and regularly vaccination and deworming. RBT was negative whereas AGID with R-antigen (*B. ovis*) was clearly positive. cCFT results demonstrated positive reaction at the serum dilution of 1:40. LFIA also was positive. According to the bacteriological studies no *Brucella* colonies were grown from the urine and blood specimens.

After one week a serum sample was taken for additional testing. It was found increasing of antibody titer to 1:160 in cCFT. It was recommended to provide a surgical castration with the further bacteriological investigation of the normal and affected testicles. Castration executed and both testicles were cultured on defibrinated sheep blood agar (5%) and *Brucella* agar, in aerobic conditions.

In parallel homogenized testicular tissue samples were tested by real-time PCR with the aim to amplify *IS711* specific region for *Brucella* spp. Both samples were positive with the Ct value 25 and 27 (Fig. 1).

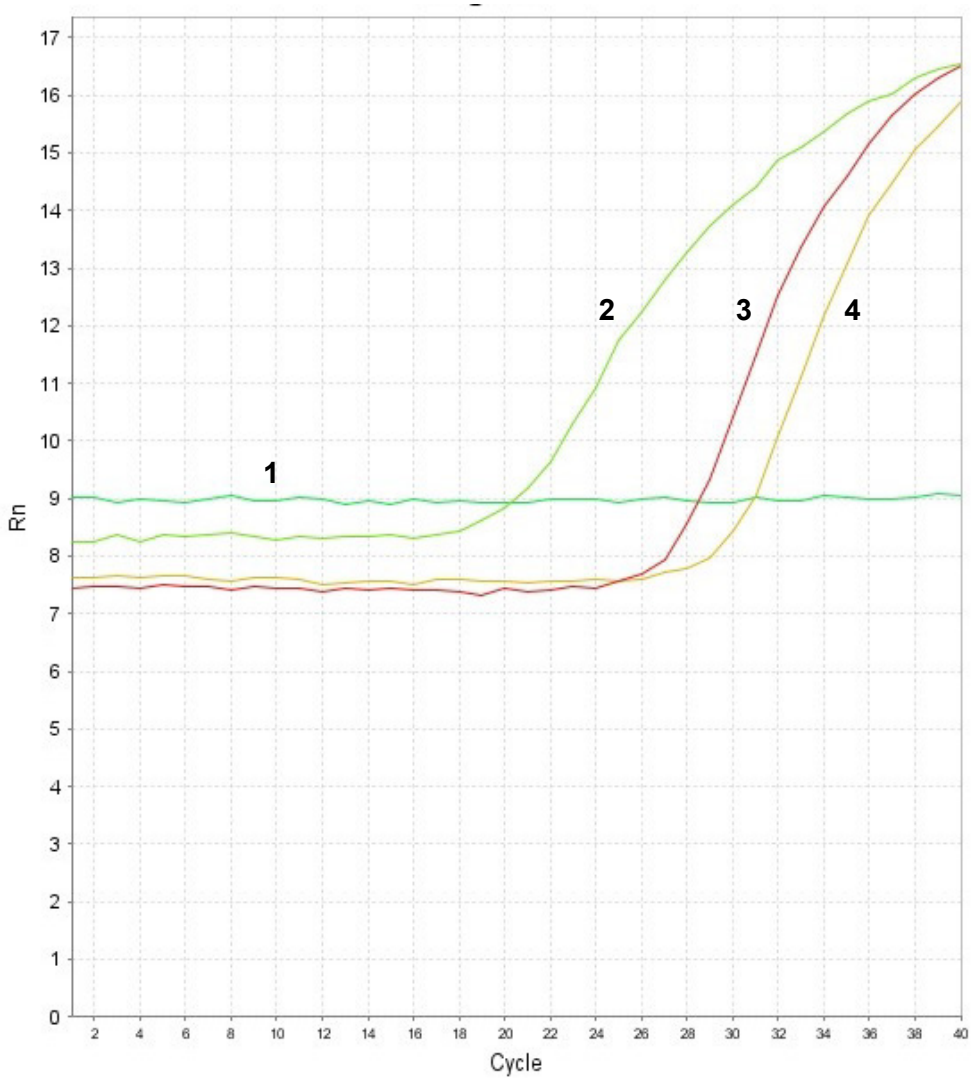

Figure 1. Amplification curves for *IS711* real-time PCR: 1 — negative sample, 2 — positive sample (*B. abortus* 99), 3 — homogenized tissue from the left testicle, 4 — homogenized tissue from the right testicle

After 48 h of incubation the colonies of Gram-negative coccobacilli were appeared in all plates. Obtained culture was characterized as *B. canis* by the following tests: oxidase and urease positive, not produced H_2S, resistant to thionin and basic fuchsin. Autoagglutination with acriflavine and crystal violet staining were positive. No agglutination with monospecific sera against A and M antigens were observed. Growth in the presence of CO_2 was moderate. *In vitro* antimicrobial susceptibility testing of the isolate was provided (Table 1).

Most of the tested drugs, except ceftazidime and sulfadiazine, are effective against obtained *B. canis* isolate. However, treatment should include combination of the different antibiotics with long period of their administration. Finally, obtained *Brucella* isolate was used for identification by providing Bruce-ladder assay (Fig. 2). In the sample positive PCR reaction was obtained with seven visible amplicons, corresponds to both *B. canis* and *B. suis* profiles.

Discussion. *B. canis* causes canine brucellosis that is zoonotic disease responsible mainly for abortions in bitches and orchitis in male dogs. The prevalence of the disease is variable according to the region and diagnostic methods used. In the present study, initially infection was found serologically and lately confirmed by culture isolation following PCR identification. However, the source of the infection for this pet dog is unknown. *B. canis* transmission realizes usually through natural mating, during oronasal contact with infected dogs, and inhalation of aerosolized material or ingestion of contaminated tissue or fluid (Greene and Carmichael, 2012; Hollett, 2006). Other authors reported vertical transmission of the pathogen from infected bitches to puppies and the disease development can occur some period of time later (Carmichael and Kenney, 1970; Gyuranecz et al., 2011; Holst et al., 2012). On the other hand, the high density of stray dog population in Ukraine and particular in Donetsk Region seems as potential harbor of *B. canis* infection for pet dogs. Infected animal can shed pathogen by urine, vaginal discharges and semen up to two years (Greene and Carmichael, 2012). Thus, control of stray dog population is an important measure which may minimize not only infection spreading in kennels but also a risk of possible zoonotic impact.

There is no totally effective antibiotic for the eradication of canine brucellosis therefore we provided *in vitro* antibiotic sensitivity of the obtained *B. canis* isolate by disk diffusion test with the aim to select optimal drug for therapy. It was shown high activity of doxycycline that is agreed with previously study (Mateu-de-Antonio and Martín, 1995) and it was recommended a long-term administration in combination with other antibiotics. A successful treatment regimen of therapy with tetracycline and streptomycin was also previously described (Greene and Carmichael, 2012).

The isolation of *B. canis* was correlated with serological positive results in cCFT, AGID, and LFIA. Due to molecular identification of the pathogen, we provided Bruce-ladder multiplex PCR as recommended by the OIE. The result showed the same profile as *B. suis,* that was previously reported (López-Goñi et al., 2011). However, differentiations in colony morphology and biochemical tests gave possibility to identify the isolate as *B. canis*. For the deep characterization and comparing with other strains, a full genome sequencing of the isolate needs to be provided with the identification of different markers.

Table 1 — Antibiotic sensitivity of *B. canis* isolate obtained by disk diffusion test

Antibiotic	Concentration, μg/disk	Range, mm	Antimicrobial sensitivity		
			Resistant	Intermediate	Sensitive
Streptomycin	30	35	≤ 20	21–24	≥ 25
Gentamicin	10	19	≤ 14	15–19	≥ 20
Rifampicin	5	19	≤ 16	17–19	≥ 20
Tetracycline	30	35	≤ 20	21–25	≥ 26
Doxycycline	30	38	≤ 14	15–18	≥ 19
Ceftazidime	30	0	≤ 20	21–24	≥ 25
Ampicillin	10	30	≤ 19	—	≥ 20
Kanamycin	30	26	≤ 15	16–19	≥ 20
Ciprofloxacin	5	32	≤ 19	20–24	≥ 25
Gatifloxacin	5	34	—	—	—
Sulfadiazine	300	0	—	—	—
Azithromycin	15	32	—	—	—
Meropenem	10	32	—	—	—

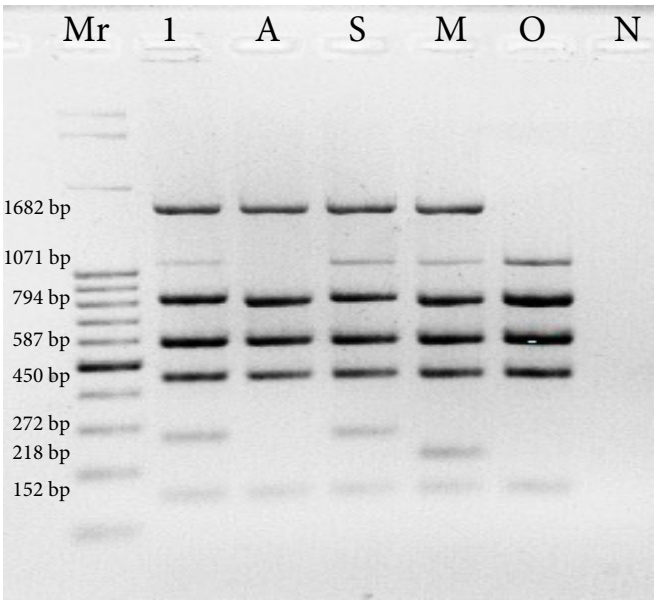

Figure 2. The Bruce-ladder PCR result of the obtained *Brucella* isolate: 1 — *Brucella* isolate, Mr — marker (Thermo Scientific, USA); Controls: A — *B. abortus* 99, S — *B. suis* 1330, M — *B. melitensis* REV-1, O — *B. ovis* 63/290, N — negative control (distilled water)

Conclusions. Our findings confirmed the circulation of *B. canis* in Ukraine, which could lead to significant economic losses also in commercial kennels. More investigations including a higher number of samples and other geographical locations of the country are needed to elaborate an effective measure for controlling of canine brucellosis among stray dogs and disease outbreak in kennels.

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