doi: 10.3325/cmj.2010.51.320

Serological, Bacteriological, and Molecular Diagnosis of Brucellosis in Domestic Animals in Croatia

Silvio Špičić, Maja Zdelar-Tuk, Ivana Račić, Sanja Duvnjak, Željko Cvetnić

Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases, Croatian Veterinary Institute, Zagreb, Croatia

Aim To present the surveillance data on *Brucella melitensis*, *B. suis,* and *B. ovis* infection in cattle, sheep, goats, and swine in Croatia obtained in 2008 by serological, bacteriological, and molecular methods for diagnostics of brucellosis in domestic animals.

Methods We serologically tested 42 785 cattle serums, 22 686 sheep and goat serums, and 28 520 swine serums using the Rose Bengal test, complement fixation test, and various immunosorbent assays. We also tested 10 173 ram blood samples for *B. ovis* infection using the complement fixation test. Bacteriological examination was conducted on 214 samples collected from 34 serologically positive animals. Different molecular methods were employed in the identification and typing of 20 isolates from the samples.

Results *B. melitensis* biovar (bv.) 3 was confirmed with different identification methods in 2 flocks in 2 Croatian counties and *B. suis* bv. 2 in 3 flocks in 3 counties. *B. melitensis* in cows was confirmed for the first time in Croatia. Infection with *B. ovis* was serologically confirmed in 202 rams in 12 counties.

Conclusions In 2008, the size of the brucellosis-affected area in Croatia and the efficiency of detection and prevention of brucellosis in sheep, goats, and swine were satisfactory. Infection with *B. melitensis* in cattle was confirmed for the first time and possible links for infection in humans were detected. More efficient measures for suppression and control of ovine epididymitis are required and a new strategy may be necessary for complete eradication of this disease.

Received: February 9, 2010

Accepted: June 9, 2010

Correspondence to:
Silvio Špičić
Croatian Veterinary Institute
Savska cesta 143
10000 Zagreb, Croatia
spicic@veinst.hr

Brucellosis is a chronic infectious disease caused by bacteria of the genus *Brucella* that affects animals and humans. Each species of Brucella has their preferred host: *B. abortus* infects cattle, *B. metitensis* sheep and goats, *B. suis* swine, *B. canis* dogs, and *B. ovis* sheep, although they can also infect other animals (1). Brucellosis in sheep and goats is endemic in the Mediterranean region but is spread throughout Asia, Africa, and Central and South America (2,3). Along with tuberculosis and rabies, brucellosis is the most important bacterial zoonosis and remains an important public health and economic concern.

With the exception of *B. ovis* and *B. neotomae,* all *Brucella* species can cause infections in humans. New *Brucella* species pathogenic for humans – *B. ceti* and *B. pinnipedialis* – have recently been discovered in marine mammals (4). Infection is transmitted to humans though direct contact with the infected animals or by consuming infected milk or fresh cheese (1).

In Croatia, brucellosis in domestic animals is controlled in accordance with the annual order issued by the Ministry of Agriculture. Serological blood examination of all male breeding animals is mandatory twice per year, and all cases of abortion must be reported and tested for brucellosis. On large cattle and pig farms, 20% of breeding animals must be tested annually. Castration of seropositive rams without the obligation of bacteriological testing is required as an eradication measure for *B. ovis* infection.

Bovine brucellosis (*B. abortus*) was eradicated in Croatia in 1964, while brucellosis in sheep and goats has occurred sporadically in the recent years, limited to 1-2 sheep flocks per year. All of the occurrences have resulted from epizooty originating in the neighboring country of Bosnia and Herzegovina (BH) (5,6). Swine brucellosis has been detected in swine and wild boars during regular controls (7,8) and *B. suis* isolates were determined as biovars (bv.) 1, 2, or 3 (7-11).

B. ovis in rams and sheep causes either clinical or subclinical disease and is not pathogenic for humans (12). According to simulation models, *B. ovis* infection causes significant economic losses in flocks with no control measures, but there is no exact confirmation of the extent of such losses (13,14). Eradication is possible, but requires considerable resources.

The aim of this study was to determine the effectiveness of the existing programs for diagnosis and control of brucellosis in domestic animals in order to prevent transmission of disease to humans and to reduce economic losses in animal production. This article describes the spread of brucellosis caused by *B. melitensis*, ***B. suis,*** and ***B. ovis*** in cattle, sheep, goats, and swine in the Republic of Croatia in 2008, as determined using different diagnostic methods.

METHODS

Serology

Serum samples. During 2008, 42 785 cattle, 22 686 sheep and goat, and 28 520 swine blood samples were tested with Rose Bengal Test (RBT), ELISA, and complement fixation tests (CFT) for brucellosis **(***B. abortus*, *B. melitensis,* and *B. suis*) at the Croatian Veterinary Institute in Zagreb. Sheep and goat samples from Split-Dalmatia county were tested by RBT at the Veterinary Institute of Split and positive sera were re-tested by other methods at the Croatian Veterinary Institute in Zagreb. A total of 10 173 ram blood samples were tested for *B. ovis* infection. All together, 133 700 different serological tests were conducted (Table 1).

Serological tests. Serological methods prescribed in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals from 2008 were employed (3). RBT (Institut Pourquier, Montpellier, France) was used as a screening method for the presence of smooth *Brucella* species – *B. abortus*, *B. melitensis,* and *B. suis.* The ELISA tests Chekit (Bommeli, Bern, Switzerland), Brucella-Ab ELISA, and c-ELISA (SVANOVIR, Svanova Biotech AB, Uppsala, Sweden) were used as confirmatory tests for brucellosis in cattle. CFT (Institut Pourquier) was used as a confirmatory test for *B. melitensis* infection in sheep and goats. Sera giving a titer of 20 international CF units (ICFU) per mL or more were considered positive (3). The ELISA kit Ingezim Brucella Porcina (Ingenasa, Madrid, Spain) was used to confirm *B. suis* infection in swine. Test results were interpreted according to manufacturer's recommendations. All animals from flocks where positive reactors were found and animals that were introduced into flocks for the first time were subjected to confirmatory tests (CFT, immunosorbent assays). The CFT with *B. ovis* antigen (Veterinary Laboratories Agency, Waybridge, UK) was used for detection of *B. ovis* infection. Sera giving a titer of 50 ICFU/mL or more were considered positive (12).

Bacteriological examination

Tissue and lymph node samples were taken from 3 cows and 8 goats and sheep from Karlovac and

Split-Dalmatia counties (2 flocks), in which brucellosis had been serologically confirmed. Tissue samples were taken from 23 pigs serologically positive for brucellosis from Osijek-Baranja (9 pigs, 1 flock), Sisak-Moslavina (12 pigs, 2 flocks), and Križevci-Koprivnica Counties (2 pigs, 1 flock). The material for bacteriological tests was not taken from all animals that were serologically positive, but only from a few animals from each flock. As a measure to eradicate *B. ovis* infection, castration of rams was required without the obligation of bacteriological testing. Examined samples from each animal included the reproductive organs (testes, uterus), lymph nodes (supramammary, inguinal, mandibular, mesenterial), liver, and spleen. In total, 214 samples were bacteriologically tested. Several grams of tissue (testis, uterus, or lymph node) were homogenized and inoculated on blood agar, Brucella agar (Brucella medium base, Oxoid CM0169, Oxoid Ltd, Basingstoke, UK), and Farrell's selective growth medium (15). Inoculated plates were incubated at 37°C in normal atmospheric conditions and with the addition of 10% CO_2. Colony growth was checked daily and was usually observed after 2-4 days. Colonies were identified based on morphology (small, translucent, con-

TABLE 1. Results of serological testing of animal blood samples for brucellosis in Croatia in 2008*

	Cattle		Sheep and goats		Rams (*B. ovis*)	Pigs (*B. suis*)	
County	RBT	ELISA (i-ELISA, c-ELISA)	RBT	CFT	CFT	RBT	ELISA
Bjelovar-Bilogora	8371	1855	1351	19	1323 (58+, 4.4%)	828	
Brod-Posavina	2591	299	430		350 (1+, 0.3%)	1623	94
Istria	449		618		403 (9+, 2.2%)		
Karlovac	2258 (3+, 0.1%)	11 (3+, 27.2%)	11 995 (367+, 3.1%)	9487 (367+, 3.9%)	1257 (13+, 1%)	131	27
Koprivnica-Križevci	1907	2243	79	1	642	1378 (21+, 1.5%)	1398 (21+, 1.5%)
Krapina-Zagorje	940	380	72	9	20	197	36
Lika-Senj	262		623	203	613 (15+, 2.4%)		
Međimurje	2667	131	542		11	1336	166
Osijek-Baranja	6418	4833	1362	22	1352 (3+, 0.2%)	14 056 (25+, 0.2%)	1297 (22+, 1.7%)
Požega-Slavonija	1745	80	890	2	950 (6+, 0.6%)	607	
Primorje-Gorski Kotar	58	22	86		70 (3+, 4.3%)		
Sisak-Moslavina	5294	1311	632	83	638 (3+, 0.5%)	1064 (135+, 12.7%)	442 (135+, 30.5%)
Split-Dalmacija	15	6	776 (3+, 0.4%)	1266 (3+, 0.2%)		3	
Šibenik-Knin	227		629				3
Varaždin	1344	4	457	2	15	536	9
Virovitica-Podravina	2257	844	881	1	1044 (56+, 5.3%)	3529	379
Vukovar-Srijem	1483 (1+, 0.1%)	1201	362		449	316	19
Zadar	676		527		753 (23+, 3.1%)		
Zagreb	3823	899	374	42	283 (12+, 4.2%)	2916	410
Total	42 785 (4+, 0.01%)	14 119 (3+)	22 686 (370+, 1,6%)	11 137 (370+, 3.3%)	10 173 (202+, 2%)	28 520 (181+, 0.6%)	4280 (178+, 4.2%)

*The table shows the total number of test performed with the number and percentage of positive tests (+) in brackets. Abbreviations: RBT – Rose Bengal tests; CFT – complement fixation test.

vex, smooth), ability to grow in a 10% CO_2 atmosphere, H_2S production, and growth on media supplemented with 20 µg/mL of thionin and basic fuchsine (13-16).

Molecular identification

After *Brucella* sp. was isolated bacteriologically, further analysis of isolates was conducted by polymerase chain reaction (PCR). In total, 20 isolates from cattle, sheep, and swine were analyzed (Table 2). Biovar isolates identified as *B. melitensis* after molecular testing were agglutinated using the monospecific anti-*Brucella* A, M, and R serums (Veterinary Laboratories Agency, Newcastle upon Tyne, UK).

Genomic DNA isolation

Bacterial cultures (1-3 colonies) were suspended in 50 µL of water (Molecular Biology Reagent, W4502, Sigma, Mannheim, Germany), heated to 99°C for 20 minutes, and centrifuged at 14 000 g for 1 minute. Supernatant was used as DNA template for PCR reactions.

Molecular typing of Brucella species by multiplex PCR (Bruce-ladder)

Multiplex PCR (Bruce-ladder), modified from Garcia-Yoldi et al (17), was used for the identification and differentiation of *Brucella* species. The assay was carried out in a 20-µL reaction mixture containing 10 µL of QIAGEN Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 5 µL of RNase-Free Water (Qiagen), 0.4 µM of BMEI0998f and BMEI0997r primers (Invitrogen, Paisley, UK), 0.1 µM of each of the other primers described by Garcia-Yoldi et al (16), and 2 µL of DNA. Thermal cycling was performed with a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA). After initial denaturation (95°C/15 minutes), the PCR profile was as follows: 35 cycles of denaturation (95°C/30 seconds), annealing (64°C/45 seconds), and extension (72°C/3 minutes), with a final extension step (72°C/10 minutes). The expected sizes of the amplification products for *B. melitensis* were 1682, 1072, 794, 587, 450, and 152 bp. *B. suis* bv. 1-5 showed an additional 272-bp fragment.

Molecular typing of *B. suis* isolates (INgene Bruce-ladder Suis, Ingenasa)

The INgene Bruce-ladder Suis kit (Ingenasa) was used for the identification of *Brucella suis* biotypes. The kit allows detection and differentiation of *B. suis* bv. 1-5. The assay was carried out in a reaction mixture containing 25 µL of Reagent A, 25 µL of Reagent B, and 1 µL of DNA. The mixture was processed in a GeneAmp® PCR System 2700 (Applied Biosystems), with an initial denaturation step at 95°C/7 minutes, followed by 30 cycles of denaturation (95°C/35 seconds), annealing (63°C/45 seconds), and extension (72°C/1 minute), and a final extension step (72°C/6 minutes). The expected sizes of the amplification products for *B. suis* bv. 1 were 197 and 425 bp; for bv. 2 278 and 548 bp; for bv. 3 197 and 302 bp; for bv. 4 197 and 611 bp; and for bv. 5 197, 278, and 611 bp.

Amplification products were separated on 2% agarose gels and stained with ethidium bromide. Visualization was conducted using a UV transilluminator and a BioCapt Document System camera (Vilbert Lourmat, Marne La Vallee, France).

RESULTS

Serological results

Three cows and 370 goats and sheep from 3 neighboring flocks in the Karlovac County had positive reaction on ELISA and CFT. Some cattle and sheep with positive reaction were from the same flock. In a single flock from Split-Dalmatia County, 3 sheep blood samples showed a posi-

TABLE 2. Isolates of *Brucella* sp. typed by molecular methods*

	Swine		Sheep		Cattle	
County	No. of isolates/ flocks	Strain ID	No. of isolates/ flocks	Strain ID	No. of isolates/ flocks	Strain ID
Karlovac	–	–	5/1	KS1-5	3/1	KC1-3
Sisak-Moslavina	4/2	S1-4	–	–	–	–
Split-Dalmatia	–	–	3/1	SS 1-3	–	–
Osijek-Baranja	5/1	OS1-5	–	–	–	–

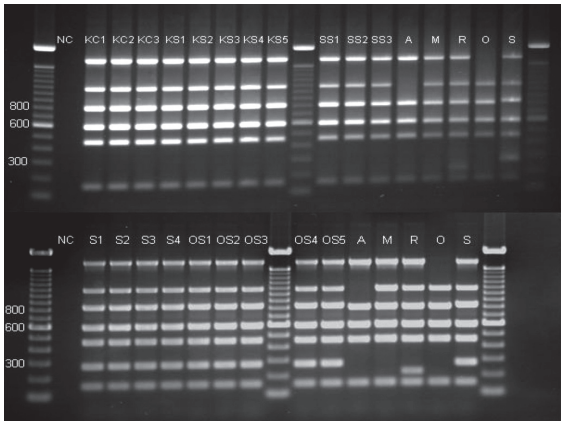
*S1-4 – swine isolates from 2 flocks in Sisak-Moslavina County; OS1-5 – swine isolates from 1 flock in Osijek-Baranja County; KS1-5 – sheep isolates from 1 flock in Karlovac County; SS1-3 – sheep isolates from 1 flock in Split-Dalmatia County; KC1-3 – cattle isolates from 1 flock in Karlovac County; ID – identification number.

tive reaction on CFT. In 178 swine blood samples from 4 flocks in 3 counties, positive reactions were detected with RBT and ELISA. Positive reactions for *B. ovis* infection were detected in 202 ram serum samples from 12 counties (Table 1).

Results of the bacteriological examination

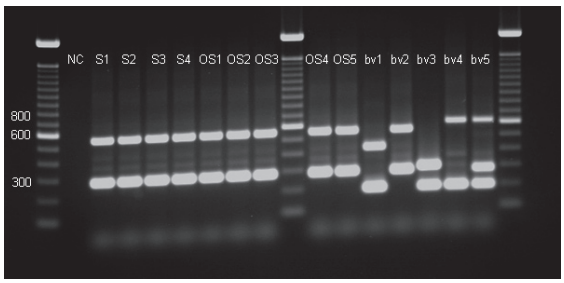
Samples from 34 animals that were serologically positive on brucellosis were bacteriologically examined. *Brucella* sp. was confirmed in 3 cows (sample KC 1-3), 8 sheep (sample KS1-5, SS1-3), and 9 swine (samples S1-4, OS1-5). Bacterial colonies became visible after 2-4 days and all isolated strains grew without added CO_2.

Figure 1.



Identification and differentiation of *Brucella* species by multiplex polymerase chain reaction (Bruce-ladder). Samples KC1-KC3 – cattle from Karlovac County; samples KS1-KS5 – sheep from Karlovac County; samples SS1-SS3 – sheep from Split-Dalmatia County; samples S1-S4 – swine from Sisak-Moslavina County; samples OS1-OS5 – swine from Osijek-Baranja County; A – *B. abortus*; M – *B melitensis*; R – *B melitensis* Rev1; O – *B. ovis*; S – *B. suis*; NC – negative control. 100 bp ladder (Invitrogen, Carlsbad, CA, USA) was used as a size standard.

Figure 2.



Differentiation of *Brucella suis* biovars by INgene Bruce-ladder Suis. Samples S1-S4 – swine from Sisak-Moslavina County; samples OS1-OS5 – swine from Osijek-Baranja County; bv1-bv5 – positive controls for *Brucella suis* biovars 1-5; NC – negative control. 100 bp ladder (Invitrogen, Carlsbad, CA, USA) was used as a size standard.

Results of molecular identification

Isolates from cattle (sample KC1-3) and sheep (samples KS1-5, SS1-3) were typed as *B. melitensis*. All isolates of *B. melitensis* were agglutinated with the monospecific anti-*Brucella* A and M serums, which is characteristic for *B. melitensis* bv. 3. The amplification of swine isolates (samples S1-4, OS1-5) by Bruce-ladder gave a positive result for *B. suis* bv. 1-5 (Figure 1).

The INgene Bruce-ladder Suis showed that all swine strains (samples S1-4, OS1-5) belonged to *B. suis* bv. 2 (Figure 2).

DISCUSSION

Our results showed that brucellosis had low prevalence among cattle, sheep, and swine in Croatia in 2008, demonstrating that the existing brucellosis control program provides permanent disease control of some species of domestic animals.

There were 370 positive reactions in sheep and goats in 4 flocks in 2 Croatian counties. Due to the epizootiologic connection of brucellosis in cattle, sheep, and goats in 3 flocks in Karlovac County, it is probable that they share the same origin of infection. Following euthanasia of the infected animals, infection with *B. melitensis* bv. 3 was bacteriologically confirmed. Preferred animal reservoirs for *B. melitensis* are sheep and goats, but cases of infection in cattle have also been described. *B. melitensis* can be transmitted by cow's milk and can cause a serious public health problem (1). Infection was diagnosed in owners of the flocks, their family members, and 2 veterinarians in the Karlovac County (personal communication with the local veterinary authorities). It is probable that the source of infection in flock owners and family members were milk and cheese from the infected cows, and that the veterinarians were infected during veterinary intervention in the treatment of postpartal complications in cows. The sheep on these farms were semi-extensively kept for the production of lambs, so the contact between sheep and cattle was possible only during the winter and the owners did not consume sheep's milk. Therefore, it is not likely that sheep were the source of infection in humans. This has been the first record of *B. melitensis* infection in cattle in Croatia since 1964, when bovine brucellosis (*B. abortus)* was eradicated.

In the neighboring BH, brucellosis in humans, sheep, goats and cattle is an important concern and traditionally there has been no control of communication between farmers (flocks) from both sides of the border. According to Dautović-Krkić (18), 245 human infections were reported between 2000 and 2005, with the number of reports reaching 335 in 2007. Brucellosis was found in animals in all counties of BH (18-20).

In uninfected swine flocks, infection occurs following the introduction of infected animals. Another potential route of transmission in swine kept at pasture is through direct and indirect contact with infected wild boars (7,8). All 3 cases of swine brucellosis that we recorded in 2008 occurred on small farms with inadequate husbandry practices, especially for preventing contact with wild boars or pigs reared in semi-extensive conditions. In 2008, 178 swine from 3 counties were found to be serologically positive to *B. suis* infection by both RBT and ELISA. Twenty-three samples from 4 flocks in 3 counties were bacteriologically tested for brucellosis. Infection with *B. suis* bv. 2 was confirmed in 1 flock in Osijek-Baranja County and 2 flocks in Sisak-Moslavina County. This biovar has previously been found in wild boars and pigs in Croatia and other European countries (7,9,21,22). In Croatia, there is a long tradition of livestock keeping and many people come to contact with potentially infected pigs. However, *B. suis* infection in humans has never been reported. *B. suis* bv. 2 is a non-zoonotic agent and in Croatia isolates of bv. 3 (based on biochemical characteristics) and of bv.1 (based on molecular characteristics) from swine, wild boar, and horse have never been reported as causes of human brucellosis (7-11). According to these findings, we believe that Croatian isolates of *B. suis* have no zoonotic potential.

Ram epididymitis is caused by a non-zoonotic agent and is spread worldwide (12). In sheep flocks in Croatia, the disease was first confirmed in 2002 and again in 2003 (23). The disease eradication program in sheep flocks was based on slaughtering or castration of positive serological reactors (rams). In addition, owners were recommended to keep young and old rams separate and to test them before introduction to breeding. These measures related only to rams are not sufficient to eradicate the disease, because infected ewes also play an important role in the transmission of the disease by excreting *B. ovis* in vaginal discharges and milk (12). During 2008, 202 seropositive rams were found in 12 counties. In addition to control of rams, sheep should also be included in the disease control program from the earliest stages (12,13). However, under this program, complete eradication of the disease in some areas was not accomplished.

The current brucellosis control program in Croatia allows rapid detection of the disease and in 2008 the size of the brucellosis-affected area in Croatia was small. The most important measures for prevention of the *B. melitensis* infection in sheep and cattle would be to educate farmers on the characteristics of the disease and control measures, to prevent the uncontrolled circulation of the animals, and adapt the programs for disease eradication to each particular situation. This is the first time that infection with *B. melitensis* in cattle was confirmed in Croatia and links for possible human infection were detected. Croatia has active surveillance programs and infrastructure for rapid and reliable diagnosis. Therefore, with an appropriate political support, it could continue to effectively control brucellosis in domestic animals. Complete eradication of brucellosis is also influenced by factors such as extensive farming, uncontrolled movement of flocks, contact with wildlife, and the ability of farmers and the government to apply measures of eradication.

References

1 Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: a re-emerging zoonosis. Vet Microbiol. 2010;140:392-8. Medline:19604656 doi:10.1016/j.vetmic.2009.06.021

2 Taleski V, Zerva L, Kantardjiev T, Cvetnic Z, Erski-Biljic M, Nikolovski B, et al. An overview of the epidemiology and epizootology of brucellosis in selected countries of Central and Southeast Europe. Vet Microbiol. 2002;90:147-55. Medline:12414140 doi:10.1016/S0378-1135(02)00250-X

3 Caprine and ovine brucellosis. In: Manual of diagnostic tests and vaccines for terrestrial animals. Paris: Office international des epizooties; 2009. p. 1-10.

4 Foster G, Osterman BS, Godfroid J, Jacques I, Cloeckaert A. Brucella ceti sp. nov. and Brucella pinnipedialis sp. nov. for Brucella strains with cetaceans and seals as their preferred hosts. Int J Syst Evol Microbiol. 2007;57:2688-93. Medline:17978241 doi:10.1099/ijs.0.65269-0

5 Cvetnić Ž, Gašpar A, Listeš E, Punda Polić V, Špičić S, Marjanović S, et al. Epizootiology of brucellosis in sheep and goats in the Southern Croatia [in Croatian] Veterinarska stanica. 2006;37:69-75.

6 Punda Polic V, Cvetnic Z. Human brucellosis in Croatia. Lancet Infect Dis. 2006;6:540-1. Medline:16931403 doi:10.1016/S1473-3099(06)70557-6

7 Cvetnic Z, Mitak M, Ocepek M, Lojkic M, Terzic S, Jemersic L, et al. Wild boars (Sus scrofa) as reservoirs of Brucella suis biovar 2 in Croatia. Acta Vet Hung. 2003;51:465-73. Medline:14680058

doi:10.1556/AVet.51.2003.4.4

8 Cvetnic Z, Spicic S, Toncic J, Majnaric D, Benic M, Albert D, et al. Brucella suis infection in domestic pigs and wild boar in Croatia. Rev Sci Tech. 2009;28:1057-67. Medline:20462164

9 Cvetnic Z, Spicic S, Curic S, Jukic B, Lojkic M, Albert D, et al. Isolation of Brucella suis biovar 3 from horses in Croatia. Vet Rec. 2005;156:584-5. Medline:15866906

10 Ferrao-Beck L, Cardoso R, Munoz PM, de Miguel MJ, Albert D, Ferreira AC, et al. Development of a multiplex PCR assay for polymorphism analysis of Brucella suis biovars causing brucellosis in swine. Vet Microbiol. 2006;115:269-77. Medline:16530357 doi:10.1016/j.vetmic.2006.02.002

11 Fretin D, Whatmore AM, Al Dahouk S, Neubauer H, Garin-Bastuji B, Albert D, et al. Brucella suis identification and biovar typing by real-time PCR. Vet Microbiol. 2008;131:376-85. Medline:18499359 doi:10.1016/j.vetmic.2008.04.003

12 Ovine epididymitis (Brucella ovis). In: Manual of diagnostic tests and vaccines for terrestrial animals. Paris: Office international des epizooties; 2009. p. 1-9.

13 Blasco JM, Marin CM. Ovine epididymitis: Etiology and bacteriological diagnostic [in Spanish]. Ovis. 1990;8:15-22.

14 Krt B. Evaluation of individual serological methods during diagnostic of ovine brucellosis – infection with Brucella ovis [master's thesis in Slovenian]. Ljubljana: Veterinary Faculty University of Ljubljana; 1992.

15 Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory. Paris: Inra; 1988.

16 Corbel MJ, Gill KPW, Thomas EL. Methods for the identification of Brucella. London: Ministry of Agriculture, Fisheries and Food; 1983.

17 Garcia-Yoldi D, Marin CM, De Miguel PM, Munoz PM, Vizmanos JL, Lopez-Goni I. Multiplex PCR assay for the identification and differentiation of all Brucella species and the vaccine strains Brucella abortus S19 and RB51 and Brucella melitensis Rev1. Clin Chem. 2006;52:779-81. Medline:16595839 doi:10.1373/clinchem.2005.062596

18 Dautović-Krkić S. Human brucellosis in BiH, clinical and epidemiological aspects [In Croatian]. In: Bruceloza javno-zdravstveni problem Bosne i Hercegovine i regiona. Proceedings of the Twentieth Infective Diseases Symposium; 2006 Feb 23-25; Sarajevo, Bosnia and Herzegovina; Sarajevo: Clinic for infection diseases, Clinical Center, University of Sarajevo; 2006.

19 Cvetnić Ž, Velić R, Špičić S, Duvnjak S, Zdelar-Tuk M, Majnarić D et al. The spread of brucellosis in Croatia and the current state in Bosnia and Herzegovina [in Croatian]. Infektološki glasnik. 2008;28:117-23.

20 Velić R, Bajrović T. Brucellosis: some epizootiological characteristics in Federation of Bosnia and Herzegovina [in Croatian]. In: Bruceloza javno-zdravstveni problem Bosne i Hercegovine i regiona. Proceedings of the Twentieth Infective Diseases Symposium; 2006 Feb 23-25; Sarajevo, Bosnia and Herzegovina; Sarajevo: Clinic for infection diseases, Clinical Center, University of Sarajevo; 2006.

21 Godfroid J, Michel P, Uytterhaiegen L, De Smedt C, Rasseneur F, Boelaert F, et al. Brucellosis caused by Brucella suis biovar 2 in wild boar (Sus scrofa) in Belgium [in French]. Ann Med Vet. 1994;138:263-8.

22 Garin-Bastuji B, Hars J, Calvez D, Thiebaud M, Artois M. Brucellosis in domestic pigs and wild boar caused by Brucella suis biovar 2 in France [in French]. Epidémiologie & Santé Animale. 2000;38:1-5.

23 Spicic S, Marjanovic S, Zdelar-Tuk M, Cvetnic Z. First evidence of Brucella ovis infection in Republic of Croatia. Dtsch Tierarztl Wochenschr. 2009;116:209-13. Medline:19537042