

MELITOCOCCOSIS IN THE REPUBLIC OF CROATIA

Željko Cvetnić¹, Silvio Špičić¹, Tomislav Kiš², Maja Zdelar-Tuk¹, Sanja Duvnjak¹, Ivana Račić¹,
Miroslav Benić¹, Boris Habrun¹, Irena Reil¹ & Zvonimir Šostar³

¹Croatian Veterinary Institute Zagreb (Department of Bacteriology and Parasitology), Zagreb, Croatia

²Ministry of Agriculture - Directorate for Veterinary Medicine and Food Safety, Zagreb, Croatia

³Andrija Stampar Teaching Institute of Public Health, Zagreb, Croatia

SUMMARY

Background: Melitococcosis is one of the most widespread zoonoses worldwide. In the period from 2009 to 2013, comprehensive melitococcosis testing was conducted in the Republic of Croatia.

Methods and results: During the testing, the Rose Bengal test was applied to 344019 blood samples of sheep and goats, and positive reactions were confirmed in 1143 (0.3%) of samples. The complement fixation test (confirmatory test) was conducted on 43428 samples, with positive reactions confirmed in 768 (1.8%) of samples. The organs and tissues of 336 sheep and goats were inspected bacteriologically, and *Brucella* sp. was isolated in 15 (4.5%) of samples. Positive serological and bacteriological reactions were confirmed in the Karlovac, Lika-Senj and Split-Dalmatia Counties. Bacteriological and molecular techniques (Bru-up/Bru-low and Bruce-Ladder) in isolates proved the presence of *Brucella melitensis* biovar 3.

Conclusion: On the basis of this study, it can be concluded that Croatia has a favourable situation concerning the infection of ruminants with *B. melitensis*, and that ongoing controls of the disease are necessary.

Key words: melitococcosis - *Brucella melitensis* – ruminants - serological and bacteriological tests - Republic of Croatia

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INTRODUCTION

Melitococcosis or brucellosis is a chronic infectious disease of sheep and goats caused by the species *Brucella* (B.) *melitensis*. It is one of the most widespread zoonoses worldwide. Its appearance has a great influence on human and animal health, economic development and the agriculture and tourism of that country. It is particularly widespread in sheep and goats in the Mediterranean region. It is considered to be one of the most dangerous diseases transmitted from animals to humans. According to the Center for Disease Control and Prevention (CDC; Atlanta, USA), brucellosis falls within the B category of diseases due to its potential for use as a biological weapon (Saleem et al. 2010). According to the assessment of the World Health Organization (WHO), some 500000 cases of brucellosis in humans is reported each year, though this figure may be up to 25 times higher in reality (Pappas et al. 2006, Bosilkovski 2013).

In Croatia, limited cases of infection appear in sheep and goat flocks, and such cases were recorded in 2004, 2005, 2008 and 2010 (Cvetnic et al. 2006, Spicic et al. 2010, 2013). The distribution of brucellosis caused by *B. melitensis* in Bosnia-Herzegovina indicates the constant threat of the spread of the disease into Croatia (Dautovic-Krkic 2006, Velic & Bajrovic 2006, Zvizdic et al. 2006, Punda-Polic & Cvetnic 2006). In Serbia, this disease appears sporadically, and usually in the southern parts of the country (Zutic et al. 2013). In Slovenia, the disease has been eradicated since 1951 (Krt & Socan 2013). In Italy, Spain, Greece, Turkey and some Balkan countries (Macedonia, Albania and Kosovo), the disease

is present, and various brucellosis eradication programmes are ongoing (Pappas et al. 2006, Godfroid & Kasbahrer 2002, Taleski et al. 2002, Pappas 2010).

This paper gives an overview of the distribution of melitococcosis in the Republic of Croatia in the period from 2009 to 2013. Bacteriological and molecular techniques were used to prove and confirm the species *Brucella* sp.

SUBJECT AND METHODS

Serological research

In the investigated period from 2009 to 2013, a total of 344019 blood samples of sheep and goats were tested serologically using the Rose Bengal test (RBT) for brucellosis (*B. melitensis*) at the Croatian Veterinary Institute. In addition, 43428 samples were tested using the complement fixation test (CFT). The blood samples of sheep and goats were collected from the territories of 20 counties and the City of Zagreb (Table 1).

The serological methods prescribed in the OIE Manual of Standards for diagnostic test and vaccines, 2009 were used for the serological diagnosis of brucellosis. The Rose Bengal test was used as a screening test to detect brucellosis (*B. melitensis*) in sheep and goats, and the complement fixation test was used as a confirmation test. For the RBT, an antigen produced at the Croatian Veterinary Institute Zagreb was used, and for the CFT, we used an antigen produced at the Institute Pourquier Montpellier - France. The results were interpreted according to the manufacturer's instructions or the test instructions.

Table 1. Results of serological testing of blood samples of sheep and goats for brucellosis in the period from 2009 to 2013

Year County	2009		2010		2011		2012		2013	
	RBT (+)	CFT (+)	RBT (+)	CFT (+)	RBT (+)	CFT (+)	RBT (+)	CFT (+)	RBT (+)	CFT (+)
Bjelovar-Bilogora	2357 (1)	71	903 (8)	56	1083 (2)	6	15655 (64)	107	17140 (101)	133
Brod-Posavina	338	0	350	0	140	0	291	0	213	0
Dubrovnik-Neretva	393	0	84	0	130	0	51	0	24	0
Istria	937 (1)	0	474 (1)	1	508	0	7181	0	7399	0
Karlovac	25651 (19)	24818 (5)	6547 (9)	6019	550	0	2538	0	2874 (7)	7
Koprivnica-Križevci	491	24	501 (8)	12	198	1	2091 (4)	6	1655 (13)	16
Krapina-Zagorje	120	0	127 (1)	1	58 (1)	1	227	0	317	2
Lika-Senj	407	24	4368 (283)	3975 (342)	5986 (8)	5486 (1)	3990	0	4285 (73)	107 (79)
Međimurje	690	148	245	0	86	0	3155 (7)	15	3019 (6)	15
Osijek-Baranja	1824	0	1543	0	542 (2)	5	1709 (1)	1	2474 (3)	4
Požega-Slavonia	1080	0	416	19	646	0	4199 (1)	1	3337 (8)	8
Primorje-Gorski Kotar	1351	1	750	0	967	0	1286 (1)	1	1651	0
Sisak-Moslavina	1086 (6)	517	384	149	472	5	892 (3)	3	796 (6)	6
Split-Dalmatia	33962 (258)	657 (243)	35136 (114)	223 (98)	10516 (13)	15	3347 (6)	9	9558 (17)	20
Šibenik-Knin	2571	1	4179 (1)	2	1434	0	2008 (1)	1	1863	0
Varaždin	761	0	269	0	168	0	4987 (24)	46	4605 (6)	5
Virovitica-Podravina	1391	0	506	0	487	0	8156 (1)	3	7710 (6)	7
Vukovar-Srijem	490	1	718 (3)	198	346 (3)	93	338 (2)	2	239	0
Zadar	2524	8	380	0	1482	0	23332 (11)	16	22920 (55)	67
Zagreb	924	1	311	0	259 (7)	3	544	0	742	0
City of Zagreb	0	0	0	0	0	0	801	0	949	0
Total	79348 (284)	26271 (248)	58064 (420)	10643 (440)	26059 (36)	5612 (1)	86778 (102)	211 (0)	93770 (301)	691 (79)

RBT – Rose Bengal test, CFT - complement fixation test, + - number of positive samples

Bacteriological testing

During the investigated period, animals that were serological positive for brucellosis were brought in for slaughter. Samples of lymph nodes (parotid, submandibular, retropharyngeal, portal, subiliac, mesenteric, supramammary), liver, spleen and reproductive organs (uterus and testes) were taken from available animals or from aborted fetuses delivered to the laboratory for

bacteriological testing. Samples for bacteriological testing were taken from 26 sheep, goats or cow from Bjelovar-Bilogora County, 1 from Dubrovnik-Neretva County, 11 from Istria County, 22 from Karlovac County, 7 from Krapina-Zagorje County, 51 from Lika-Senj County, 2 from Osijek-Baranja County, 11 from Požega-Slavonia County, 15 from Primorje-Gorski Kotar County, 19 from Sisak-Moslavina County, 41 from Split-Dalmatia County, 40 from Šibenik-Knin County,

2 from Varazdin County, 6 from Virovitica-Podravina County, 9 from Vukovar-Srijem County, 30 from Zadar County, 39 from Zagreb County and 4 samples from the area of the City of Zagreb. In this period, no samples were submitted from Međimurje County, Brod-Posavina County and Koprivnica-Krizevci County. During the survey period, samples from 336 sheep and goats from 16 counties and the City of Zagreb were analysed.

Several grams of delivered and examined materials (testes, uterus, placenta, aborted fetuses and lymph nodes) were inoculated on selective agar and on blood agar, Brucella agar and modified selective Farell agar. Agars with inoculated materials were incubated at normal atmosphere conditions at a temperature of 37°C with the addition of 5–10% CO₂. Colony growth was observed daily, and was usually visible after 3–7 days. Isolates were identified on the basis of colony morphology (small, convex, transparent, rough (R), growth with CO₂, production of H₂S, growth on medium with the addition of 20µg/ml thionine and basic fuchsin and agglutination with monospecific antisera (Alton et al. 1988, Corbell et al. 1983, OIE Manual 2009).

Molecular analysis of *Brucella* sp.

The isolation of DNA from isolates was conducted using QIAcube system (QIAGEN, Hilden, Germany).

Brucella sp. in isolates was proven by amplification of the part of the gene that codes for the synthesis of the BCSP-31 protein (Bricker & Halling 1994, Serpe et al. 1999). The size of the amplification product was 443 bp (base pairs). We used the primers BRU-UP (GGG CAA GGT GGA AGA TTT) and BRU-LOW (CGG CAA GGG TCG GTG TTT) (Invitrogen, USA).

The Bruce Ladder test was used to prove which *Brucella* species was present, including the referent strains (*B. abortus* S19, *B. abortus* RB51 and *B. melitensis* Rev1). Eight pairs of primers were used per reaction mixture. Members of individual species were differentiated on the basis of the characteristic mutations, insertions and deletions in their genomes (Lopez-Goni et al. 2008). The amplification products were analysed using capillary electrophoresis on the QIAxcel system (QIAGEN, Hilden, Germany) with 100-3000 bp size marker (QIAGEN, Hilden, Germany).

Statistical analysis was performed using the statistical program Stata 13.1 (Stata Corp., USA). Numerical data for the seroconversion tested using the Rose Bengal test were compared among years and among regions (Slavonia and Baranja, central Croatia and Lika, Istria, Primorje and Dalmatia) using the chi-squared and Fisher exact tests.

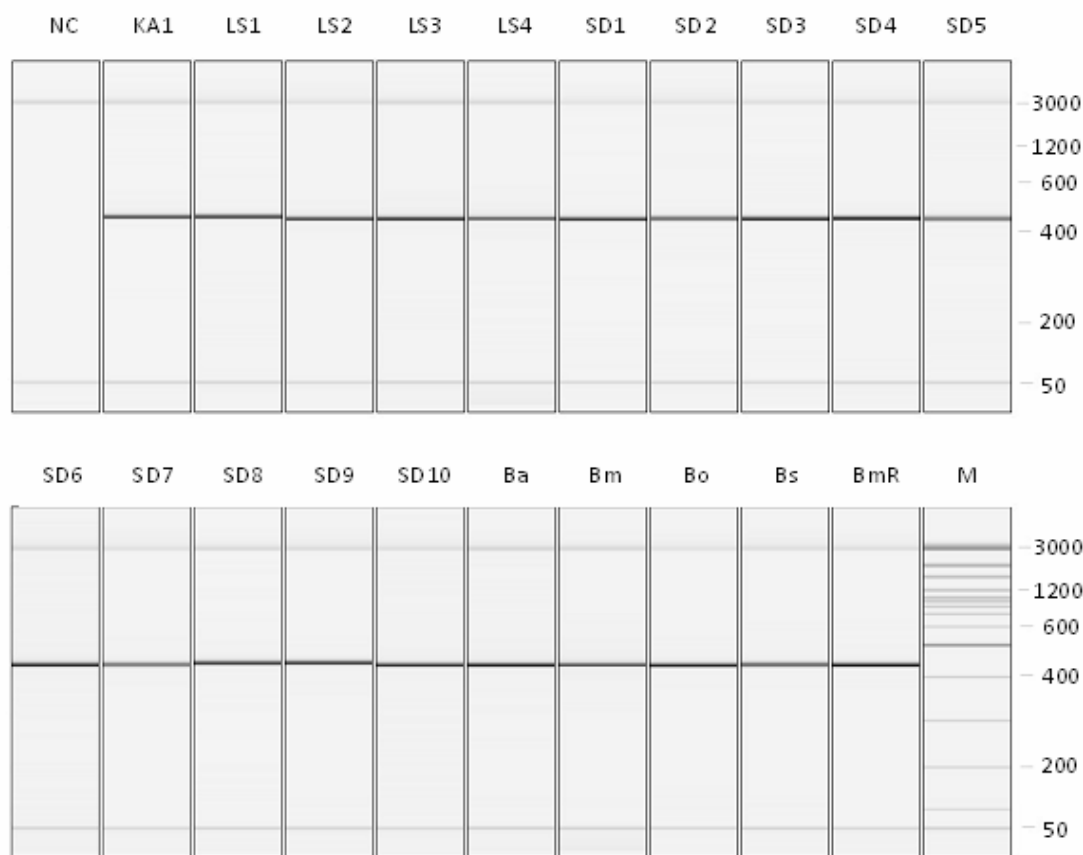


Figure 1. Identification of *Brucella* genus using Bru-up/Bru-low PCR method. NC – negative control, KA1 – *Brucella melitensis* isolate from Karlovac County, LS1- LS4 - *Brucella melitensis* isolates from Lika-Senj County, SD1-SD10 - *Brucella melitensis* isolates from Split-Dalmatia County, control strains (Ba – *Brucella abortus* 544, Bm – *Brucella melitensis* 16M, Bo – *Brucella ovis* 63/290, Bs – *Brucella suis* 1330, BmR – *Brucella melitensis* Rev1), M – size marker 50 – 3000 bp (Qiagen)

RESULTS

Serological tests

During the survey period from 2009 to 2013, the RBT screening test was applied to 344019 blood samples, with positive reactions confirmed in 1143 (0.3%) of blood samples of sheep and goat. The CFT method was used to test 43428 samples, with positive reactions confirmed in 768 (1.8%) of blood samples. Positive reactions were most often found in Karlovac, Lika-Senj and Split-Dalmatia Counties (Table 1).

The observed differences in the incidence of positive results of the Rose Bengal test between individual regions were statistically significant in all years ($p < 0.0001$) except 2011 ($p = 0.09$). The observed differences in the incidence of positive results of the Rose Bengal test between years were statistically significant ($p < 0.0001$).

Organ and tissue samples of 336 sheep and goats were examined bacteriologically, and brucellosis was isolated in 15 (4.5%) of samples. Positive bacteriological analysis was confirmed in Karlovac County (1 isolate),

Lika-Senj County (4 isolates) and in Split-Dalmatia (10 isolates). During 2009, 5 isolates were isolated (Karlovac, Split-Dalmatia Counties) and in 2010, 9 isolates were obtained (Lika-Senj and Split-Dalmatia Counties), while in 2013, only 1 isolate was found (Lika-Senj County). In 2011 and 2012, no brucellosis isolates were isolated from the tested samples.

Following the isolation and identification of *Brucella* sp. using classical bacteriological procedures, identification using the polymerase chain reaction (PCR) method was carried out. All 15 isolates obtained from sheep, goats and one bovine sample were proven to belong to the genus *Brucella* sp., i.e. the presence of the gene for the protein BCSP-31 was proven (Figure 1).

Upon confirming that all investigated samples belonged to the genus *Brucella*, the Bruce ladder test, a multiplex PCR assay with eight primers, was used to confirm that all 15 isolates belonged to the species *B. melitensis*. Samples were differentiated on the basis of the combination, presence or absence of amplicons of different sizes: 1682, 450, (1320), 1071, 794, 587, 272, 218 and 152 bp (Figure 2).

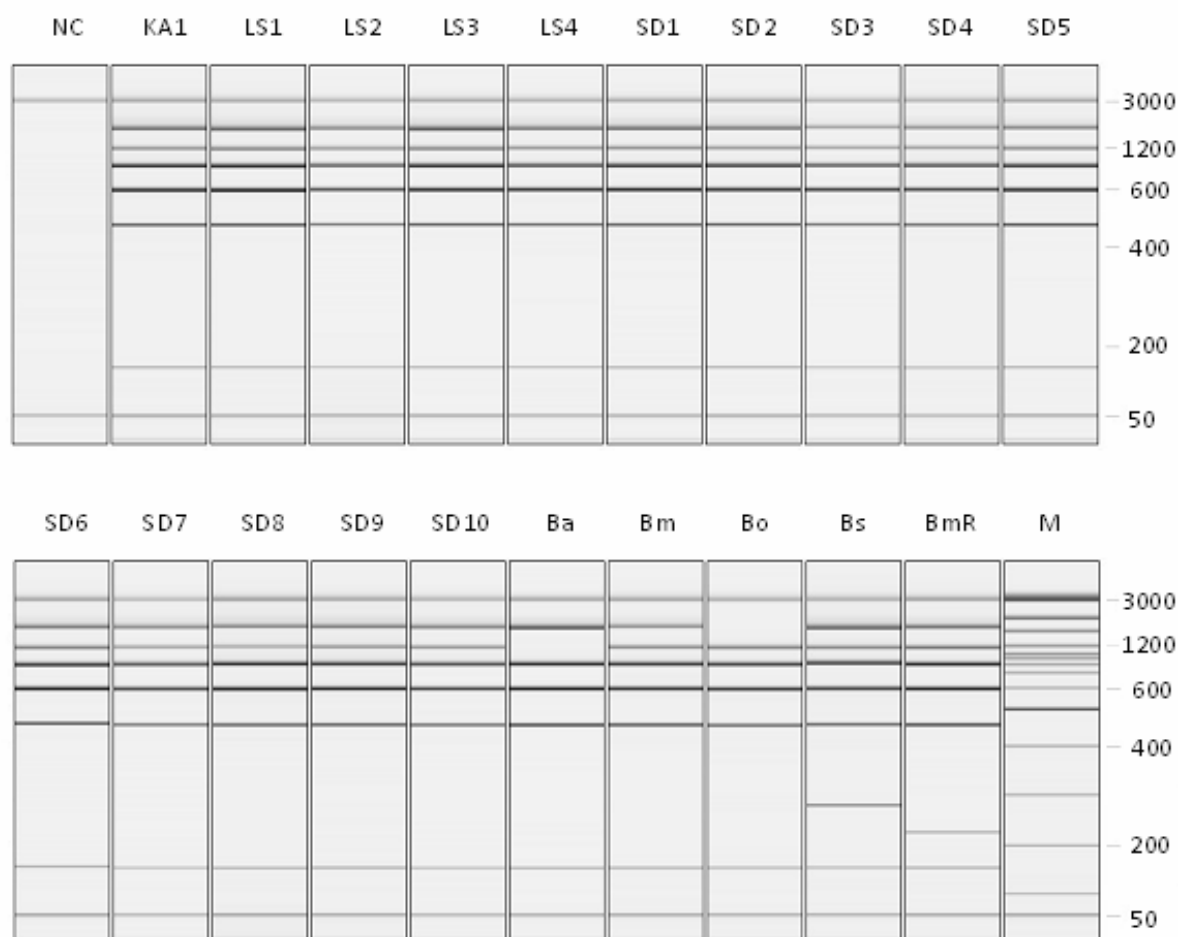


Figure 2. Identification of *Brucella* species using Bruce-ladder multiplex PCR method²¹. NC – negative control, KA1 – *Brucella melitensis* isolate from Karlovac County, LS1- LS4 - *Brucella melitensis* isolates from Lika-Senj County, SD1-SD10 - *Brucella melitensis* isolates from Split-Dalmatia County, control strains (Ba – *Brucella abortus* 544, Bm – *Brucella melitensis* 16M, Bo – *Brucella ovis* 63/290, Bs – *Brucella suis* 1330, BmR – *Brucella melitensis* Rev1), M – size marker 50 – 3000 bp (Qiagen)

DISCUSSION

In recent years, systematic work has virtually succeeded in eradicating this disease in Croatia; however, despite all efforts, it still survives in varying intensity. In order to prevent and stop outbreak of this disease in humans and animals from the very early stages, constant supervision over animal health is imperative. Brucellosis in humans is first found in people professionally tied to livestock (breeders, veterinarians, farmers) than in those consuming the products (milk, cheese) of infected animals.

During a survey from the period of 2009 to 2013, the RBT screening test was used to test 344019 blood samples, and positive reactions were confirmed in 1143 (0.3%) of blood samples of sheep and goats. The CFT method was applied to 43428 samples, and positive reactions were obtained in 768 (1.8%) of blood samples. Positive serological reactions and bacteriological tests were found in Karlovac, Lika-Senj and Split-Dalmatia Counties. Croatia is one of the rare Mediterranean countries with a favourable status with the appearance of melitococcosis. All cases of the appearance of the disease to date have appeared in flocks and people living in areas directly along the border with Bosnia-Herzegovina (BiH), and it is believed that the illegal import of animals from BiH is the main source of infection with *B. melitensis* (Cvetnic et al. 2006). This was also evident from earlier studies by Spicic et al. (2010), where melitococcosis appeared in the same bordering counties (Karlovac, Lika-Senj and Split-Dalmatia) (Spicic et al. 2010). In the literature, cases of illegal imports of infected animals have been reported in various areas, from Albania into north-western Greece, from Turkey into southern Bulgaria, and from Mexico into the southern USA (Pappas et al. 2006, Russo et al. 2009).

Several larger outbreaks of melitococcosis have occurred in Croatia. The first was described in Istria in 1947, when more than 300 people became infected with melitococcosis. The last known case of the disease in a human was reported in 1954, and in sheep and goats in 1961 (Karlovic 2000, Terlevic 2006). In 1990, an outbreak of melitococcosis was reported in Istria, and in 1991 and 1992 in the Varazdin and Bjelovar regions (Cvetnic et al. 2001). According to the data of the Croatian Public Health Institute, 7 humans were reported to have clinical symptoms of brucellosis in 1990, 17 patients in 1991, 12 patients in 1992 and 4 patients in 1993. During 2004, an outbreak caused by *B. melitensis* was reported in Split-Dalmatia County. Positive reactions were confirmed in 372 sheep and goats and 5 dogs in 5 flocks, and veterinary measures were employed to destroy 1567 sheep and goats. During the outbreak, clinical symptoms of brucellosis were confirmed in 4 people. Later, a case of brucellosis in a human was confirmed in Dubrovnik-Neretva County (Metkovic), and brucellosis was recorded in several sheep and goat flocks during 2005 (Cvetnic et al. 2006,

Punda-Polic & Cvetnic 2006). Pappas (2010) described the spread of the disease on the Balkan Peninsula, and stated that melitococcosis is present in Greece, Turkey, Macedonia, Albania, Kosovo and southern Serbia, and it later spread to Bosnia-Herzegovina, and from Bosnia-Herzegovina into Croatia (Pappas 2010). Dautovic-Krkic (2005) stated that in the period from 2000 to 2005, there were 245 cases of brucellosis reported in people in Bosnia-Herzegovina (Dautovic-Krkic 2006). Velic & Bajrovic (2005) and Cvetnic et al. (2008) stated that cases of brucellosis in animals were recorded in all cantons of the Federation of Bosnia-Herzegovina, with 335 cases of human infections (Velic & Bajrovic 2006, Cvetnic et al. 2008).

The European Union strategy for the control and eradication of the disease includes testing and slaughter in low incidences countries (Croatia). In countries with moderate incidences, also vaccination of replacement females is carried out, while in cases of high prevalence, massive vaccinations are implemented (Greece, certain regions of Spain and Portugal, Bosnia-Herzegovina). Through the implementation of joint policies of control and eradication of the disease, there has been a reduction of infections in humans, from about 4000 in 1999 to 400 in 2011.

Every country has its own legislation on the control of the disease, trade in livestock, marking, etc., and all of these regulations are more or less based on the legislation of the European Union. However, the strategy to eradicate the disease differs between countries, based largely on the situation with brucellosis in the country, the opportunities, and other socio-political and financial circumstances that are important for the implementation of comprehensive measures to control and eradicate brucellosis. There are constant threats, including the fact that it is always possible the disease will emerge in a country resulting from a reduction in supervision due to an underestimation of the incidence of the disease. Terminations of programs to vaccinate sheep and goats result in flare ups of the disease as do weak border controls and imports of infected animals into disease-free countries. Ultimately, wild ruminants also represent sources of brucellosis. For example, France has officially been a brucellosis-free country in ruminants since 2005; however, brucellosis caused by the species *B. melitensis* was proven in alpine ibex (*Capra ibex*) in the French Alps. Subsequent research and molecular testing proved that the strain in humans and goats and domesticated ruminants was identical to that in the ibex (Mick et al. 2014).

Based on this survey, it can be concluded that Croatia has a favourable situation with regard to the infection of humans with *B. melitensis*, although there is the ongoing threat of the entry of infections from the territory of BiH into Croatia, which has been proven in this study. The appropriate strategies have proven to be efficient, and constant supervision and control of the disease is mandatory even in disease-free regions, and also in the Republic of Croatia.

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Conflict of interest : None to declare.

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Correspondence:

Prof. Željko Cvetnić, VMD, PhD
Croatian Veterinary Institute
Savska cesta 143, 10000 Zagreb, Croatia
E-mail: cvetnic@veinst.hr