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Multiple locus variable number of tandem repeat analysis (MLVA) of isolates of *Brucella melitensis* isolated in the Republic of Croatia

Željko Cvetnić[1]*, Maja Zdelar-Tuk[1], Sanja Duvnjak[1], Ivana Račić[1], Mario Škrivanko[2], and Silvio Špičić[1]

[1]*Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases, Croatian Veterinary Institute, Zagreb, Croatia*

[2]*Laboratory for Diagnostics, Regional Veterinary Institute, Vinkovci*

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ABSTRACT

In the period from 2009 to 2013, bacteriological testing was conducted on 336 sheep, goat and cattle samples. Using classical bacteriological and molecular procedures, *B. melitensis* was confirmed in 14 (4.2 %) samples. *Brucella* was isolated in the Karlovac, Lika-Senj and Split-Dalmatia Counties. *Brucella* isolates were genotyped using the MLVA method and compared with isolates from neighbouring Bosnia and Herzegovina (BH). A total of 14 isolates (strains) originating from Croatia and 25 from BH were analysed. Complete matches between Croatian and BH isolates were found in two genotype groups. Overall, the MLVA analysis indicated that the Croatian and BH genotypes of *B. melitensis* from animals were highly homogenous. The Hunter Gaston diversity index (HGDI) showed that diversity was found among the 16 tested loci for 5 loci of panel 2B (Bruce 04, 07, 09, 16, 30). The discriminating loci are optimal for the use in epidemiological investigation of *B. melitensis* infections in these two countries.

Key words: multiple locus variable number of tandem repeat analysis (MLVA), *B. melitensis*, ruminants, Croatia

Introduction

Brucellosis is a chronic infectious disease that affects various species of animals and humans (zoonosis). Brucella infections result in economic losses, and the euthanasia of infected flocks. As the most important causative agents of the disease the species *Brucella*

*Corresponding author:

Željko Cvetnić, PhD, Laboratory for Bacterial Zoonoses and Molecular Diagnostics of Bacterial Diseases, Croatian Veterinary Institute, Savska cesta 143, 10 000 Zagreb, Croatia, Phone: +385 1 612 3688; E-mail: cvetnic@veinst.hr

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(*B.) abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis, B. neotome* and *B. microti* have been recognized. The first three species are the most significant, and within these species there are a number of biovars (VERGER et al., 1987; SCHOLZ et al., 2008). The species *B. ceti* has been isolated and described, usually from dolphins, and *B. pinnipedialis* from seals (FOSTER et al., 2007). *B. inopinata* has been isolated from infected breast implants in women with clinical signs of brucellosis (DE et al. 2008; SCHOLZ et al., 2010). Brucellosis is considered one of the most dangerous zoonoses, and humans are most often infected with the species *B. melitensis*, less often with *B. abortus* and *B. suis,* and rarely with the species *B. canis,* though the species *B. ceti* and *B. pinnipedialis* can also rarely cause human disease (SOHN et al., 2003, McDONALD et al., 2006).

The genus *Brucella* is highly homogenous and shows little genetic diversity (CHAIN et al., 2005; SRIRANGANATHAN et al., 2009). In recent years, multiple locus variable number of tandem repeat analysis (MLVA) has stood out as the molecular technique capable of differentiation at the strain level, making it useful for epidemiology (LE FLECHE et al., 2006). The method has proven to be very effective, practical and multipurpose. It has been used to prove the sources of infection, in distinguishing new infections from recurring infections, and distinguishing vaccination or natural infection (GARCIA-YOLDI et al., 2007; KATTAR et al., 2008).

The objective of this paper was to determine the causative agents of brucellosis in herds of cattle, and flocks of sheep and goats, during the study period from 2009 to 2013, and to identify the species and biovars using bacteriological and molecular techniques in Croatia. The MLVA method was used to genotype isolates of *B. melitensis* in Croatia and neighbouring Bosnia and Herzegovina (BH), to determine their differences and the suitability of this method for epidemiological research.

Materials and methods

Bacteriology examination.

Material sources. In the period from 2009 to 2013, samples of sheep, goat and cattle materials were submitted to the Laboratory for bacterial zoonoses and molecular diagnostics of bacterial diseases for bacteriological testing for brucellosis. The material originated from animals who had miscarried or who had shown a positive serological reaction to brucellosis, and after bacteriological testing infection with B. melitensis was confirmed. Samples of lymph nodes (parotid, submandibular, retropharyngeal, portal, subiliac, mesenteric, supramammary), liver, spleen and reproductive organs (uterus and testes) of the animals or aborted foetuses were taken.

During the study period, bacteriological testing was performed on samples originating from 336 cattle, sheep and goats from 17 counties and the City of Zagreb. In 2009, 59 samples were submitted from 11 counties, in 2010 there were 103 samples from 13

counties, in 2011 there were 56 samples from 12 counties, in 2012 there were 47 samples from 12 counties and in 2013 there were 71 samples from 14 counties.

Bacteriological analysis. Several grams of processed materials (testes, uterus, lymph nodes, aborted foetuses) were inoculated onto selective agars and blood agar, *Brucella* agar and modified Farrell selective agar. Plates with inoculated material were incubated at a temperature of 37 °C and with the addition of 5 - 10 % CO_2. Colony growth was observed at daily intervals, and was typically visible after 3 - 7 days. Isolates were identified on the basis of colony morphology (i.e. small, convex, transparent and rough (R), production of H_2S, growth on substrates with the addition of 20 μg/mL thionine and basic fuschin and agglutination with monospecific antisera) (ALTON et al., 1988; CORBEL et al., 1983).

Molecular identification of Brucella sp. The isolation of DNA from isolates was conducted using the QIAcube system (QIAGEN, Hilden, Germany). *Brucella* sp. DNA in isolates was proven by amplification of the part of the gene that codes for the synthesis of the BCSP-31 protein (SERPE et al., 1999).

The Bruce-ladder test was used to determine which species of *Brucella* was present, including the vaccine 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 characteristic mutations, insertions and deletions in their genomes (LOPEZ-GONI et al., 2008). The amplification products were analysed by capillary electrophoresis on the QIAxcel System (QIAGEN, Hilden, Germany) with 100 - 3000 bp size marker (QIAGEN, Hilden, Germany). A total of 14 strains of *B. melitensis* were examined from Croatia: 1 cattle strain from the Karlovac County (137), 6 goat strains from the Split-Dalmatia County (133, 134, 135, 180, 181, 182) and 7 sheep strains from the Lika-Senj County (162, 171, 172, 256) and the Split-Dalmatia County (173, 174, 175). A total of 25 strains from our laboratory archives originating from BH were analysed: 17 cattle (158, 159, 160, 161, 183, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249), 4 sheep (164, 165, 184, 185), 2 goat (217, 218) and 2 of unknown origin (138, 139).

Molecular typing and data analysis. For molecular typing, MLVA was used, which examines the number of tandem repeats of microsatellites at 16 different gene loci. Loci are divided into three panels: panel 1 includes the loci: Bruce 06, 08, 11, 12, 42, 43, 45 and 55, and they are used to differentiate strains into species, while Panel 2A (Bruce 18, 19, 21) and Panel 2B (Bruce 04, 07, 09, 16, 30) allow differentiation to the strain level, as they are more variable. The strain *B. melitensis* 16M was used in the investigation as the reference strain. Test procedures were performed according to LE FLECHE et al. (2006) and AL DAHOUK et al. (2007). The number of tandem repeats was calculated according to the size of the amplified product. The results were displayed in the form of a sixteen digit code (as per LE FLECHE et al., 2006). Group analysis was based on the categorical coefficient and the unweighted pair group with mathematical mean (UPGMA) methods.

Genetic diversity was calculated using the Hunter-Gaston diversity index (HGDI) (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). The value of the HGDI is the measure of the variation of the number of repeats at each locus, and can range from 0.0 (no diversity) to 1.0 (complete diversity).

Results

Results of the bacteriology survey. During the research period from 2009 to 2013, tissue and organ samples from 336 sheep, goats and cattle were tested bacteriologically, and *Brucella* was isolated from 14 samples. It was detected in the Karlovac County (1 isolate), the Lika-Senj County (4 isolates) and the Split-Dalmatia County (9 isolates). During 2009, 5 isolates were isolated (Karlovac County and Split-Dalmatia County), in 2010 there were 8 isolates (Lika-Senj County and Split-Dalmatia County), in 2013 there was 1 isolate in the Lika-Senj County. No strains of it were isolated from the materials delivered in 2011 or 2012 (Table 1).

Results of molecular identification of Brucella species. Following the isolation and identification of *Brucella* sp. using classical bacteriological procedures, identification was carried out using the polymerase chain reaction (PCR) method. All isolates obtained from the different animal species and humans were proven to be members of the genus *Brucella* spp. The Multiplex PCR (Bruce-ladder) was used for the identification and differentiation of *Brucella* species. According to the amplicon sizes of 1682, 1071, 794, 587, 450 and 152 bp isolates from sheep (strain numbers 162, 171, 172, 173, 174, 175, 256), goats (strain numbers 133, 134, 135, 180, 181, 182) and cattle (strain number 137) were typed as *B. melitensis*.

Results of Molecular Typing and Data Analysis. Brucella melitensis isolates from sheep (7), goats (6) and a cow (1) from Croatia and sheep, goat and cattle isolates from Bosnia and Herzegovina (25) were used to determine the epidemiological value of the MLVA-16 method. Furthermore, we considered similarities and differences, possible sources and directions of infection spread, and the regional specificities of *Brucella melitensis* strains. 16 different gene loci, divided into Panel 1 (Bruce 06, 08, 11, 12, 42, 43, 45 and 55), panel 2A (Bruce18 and 21) and Panel 2B loci (Bruce 04, 07, 09, 16, 19, 30) were investigated. For comparison of strains at the species level, it was sufficient to use Panels 1 and 2A, while differences found on Panel 2B loci were sufficient to compare isolates at the strain level. Among the 25 isolates from Bosnia and Herzegovina and 14 isolates from Croatia, heterogeneity was found in only 5 loci (Bruce 04, 07, 09, 16, 30) of Panel 2B. Based on all 16 loci, a total of 14 unique genotypes were confirmed for the isolates from BIH, two of which (no. 138 and 245) were identical to Croatian isolates (no. 133-135). Also, 5 completely unique genotypes were confirmed among the Croatian isolates (Fig. 1). Infection cases caused by *B. melitensis* in Croatia were primarily situated in the border area with BIH. Significant diversity was found for five loci (Bruce 04, 07,

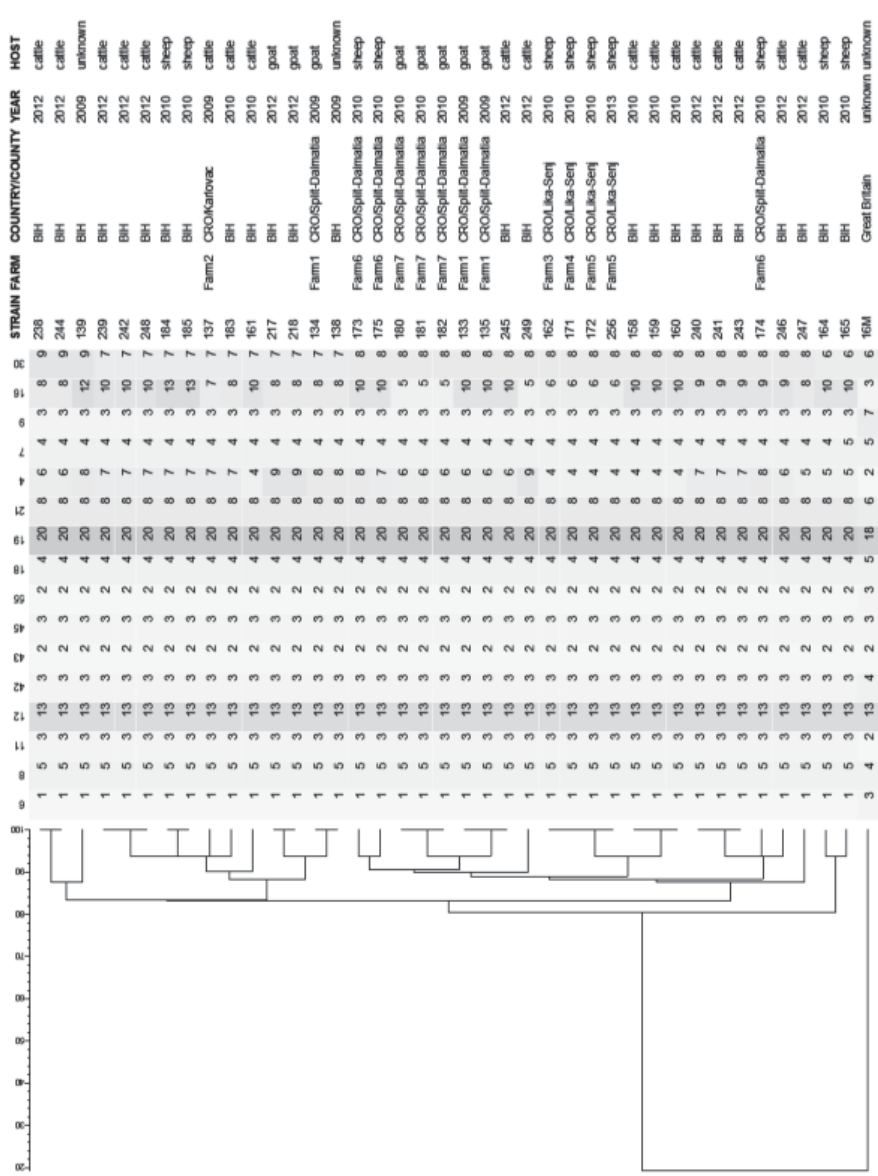

Fig. 1. Dendrogram of clustered MLVA-panel genotypes (UPGMA algorithm)
The MLVA genotyping results were analysed using the unweighted pair group method with arithmetic mean (UPGMA) algorithm incorporated in Bionumerics 7.1 (Applied Maths, Belgium). CRO - Croatia; BIH - Bosnia and Herzegovina

Table 1. Overview of the number of bacteriological tests and isolates during the study period

Year	2009	2010	2011	2012	2013	
County	Bacteriological tests (isolates)	Bacteriological tests (isolates)	Bacteriological tests (isolates)	Bacteriological tests (isolates)	Bacteriological tests (isolates)	Total tests (isolates)
Bjelovar-Bilogora	17	2	2	5	0	26
Brod-Posavina	0	0	0	0	0	0
Dubrovnik-Neretva	0	1	0	0	0	1
Istria	0	1	0	0	10	11
Karlovac	6 (1)	4	8	1	3	22 (1)
Koprivnica-Križevci	0	0	0	0	0	0
Krapina-Zagorje	1	0	4	2	0	7
Lika-Senj	5	24 (3)	5	2	15 (1)	51 (4)
Međimurje	0	0	0	0	0	0
Osijek-Baranja	1	0	0	0	1	2
Požega-Slavonia	0	8	1	0	2	11
Primorje-Gorski Kotar	6	2	5	0	2	15
Sisak-Moslavina	3	1	2	11	2	19
Split-Dalmatia	10 (4)	25 (5)	0	5	1	41 (9)
Šibenik-Knin	4	18	7	4	7	40
Varaždin	0	0	0	2	0	2
Virovitica-Podravina	1	1	2	0	2	6
Vukovar-Srijem	0	0	2	2	5	9
Zadar	0	7	1	7	15	30
Zagreb	5	9	17	4	4	39
City of Zagreb	0	0	0	2	2	4
Total	59 (5)	103 (8)	56 (0)	47 (0)	71 (1)	336 (14)

09, 16, 30) belonging to Panel 2B (Table 2). The HGDI range was between 0.827 for locus Bruce 04 and 0.050 for Bruce 09.

Table 2. HGDI values for Croatian and BIH *B.melitensis* strains isolated from 2009 to 2013.

Locus	HGDI	CI	K	Max (pi)
Bruce04	0.827	0.781-0.852	7	0.275
Bruce16	0.818	0.745-0.891	9	0.350
Bruce30	0.612	0.502-0.721	4	0.550
Bruce07	0.097	0.000-0.219	2	0.950
Bruce09	0.050	0.000-0.142	2	0.975

HGDI: Hunter Gaston Diversity Index, ranges from 0.0 (no diversity) to 1.0 (complete diversity); CI: Confidence Interval, expressed as 95 % upper & lower boundaries; K: Number of different repeats present at this locus in this sample set; max (pi): fraction of samples that have the most frequent repeat number in this locus (range 0.0 to 1.0)

Discussion

In order to prevent and to combat epidemics and epizootics of brucellosis in humans and animals in the earliest stages, constant supervision of healthy animals is required. In humans, brucellosis is most often seen in persons professionally linked with animal husbandry (livestock breeders, veterinarians, farmers) or after consuming unpasteurised milk. In the Republic of Croatia, controls of herds and flocks for brucellosis has been in place for decades, and in recent years controls have intensified due to the programme to achieve the status of brucellosis-free bovine herds (from *B. abortus*). In 2014, controls began of the entire national flocks of sheep and goats for brucellosis caused by the species *B. melitensis*. This control programme includes all sheep and goat farms in the Republic of Croatia, and all animals will be subjected to serological testing. In earlier years (1991, 2004, 2005, 2008 and 2010), limited outbreaks and infections of humans with *B. melitensis* were proven, primarily in the southern Croatian counties (mostly the Split-Dalmatia County) or those bordering with Bosnia and Herzegovina (Karlovac, Lika-Senj Counties) (CVETNIĆ et al., 2001, 2006, 2008; ŠPIČIĆ et al., 2010, 2013). Epidemiological and epizootiological surveillance has always led to Bosnia and Herzegovina, where epidemics of brucellosis in humans have been reported in the past decade, which encompassed the entire country, affecting many people (ZVIZDIĆ et al., 2006; PUNDA-POLIĆ and CVETNIĆ, 2006).

In this study, we obtained 14 isolates of *B. melitensis* from three regions of the Republic of Croatia. Bru-up/Bru-low PCR was used to prove the presence of the amplicon of 443 bp, which is evidence for the presence of the gene for the external membrane antigen BCSP-31 characteristic for *Brucella* sp. The Bruce-ladder multiplex PCR, which is relatively new and very simple to use, was employed to differentiate the strains, and it

was established that all the isolated strains belonged to the species *B. melitensis* (LOPEZ-GONI et al., 2008). Furthermore, we genotyped obtained strains using MLVA. In this case, the MLVA analysis, using a set of 8 loci of Panel 1 and 4 loci of Panel 2A, did not confirm diversity. Panel 1 allows for primary determination of species and potential grouping of strains in non-endemic regions, while in endemic regions, Panel 2 is essential for strain typing since as is more polymorphic (LE FLECHE et al., 2006; AL DAHOUK et al., 2007).

Complete matching between the Croatian and BH isolates was found in two cases. In both cases, these were strains isolated from a Croatian farm in 2009 and BH strains isolated in 2009 and 2012. From this, it could be concluded that the infection was present on the Croatian farm with two different strains, or one could suspect *in vivo* (within-host) or *in vitro* (during culturing) mutation. Laboratory contamination is excluded due to the different times of culturing the particular strains. Both strains are present in BH, and epidemiological links for cattle infection in BH are not known (strain 245 from 2012). In this study, heterogeneity was found for 5 loci on Panel 2B (Bruce 04, 07, 09, 16, 30). Analysing the CRO and BH genotypes of *B. melitensis* in the period from 2009 to 2013, with a consensus of 88 % according to the UPGMA algorithm, high overall homology was found. The terrestrial border between Croatia and BH is over 1000 km long and the use of common pasture and illegal migrations of animals is not uncommon. In 2009, BH started a vaccination program for sheep and goats with the Rev1 vaccine, but without cattle control. The genotypes found in this study, originating from both countries, show the closely related genotypes for cattle, sheep and goat strains (e.g. strains 246, 174 and 240, 241, 243).

The single-locus and double-locus variants found in the Croatian and BH strains may reflect the microevolution of shared *B. melitensis* strains. JIANG et al. (2011) found similar results for human B. *melitensis* strains in China. MICK et al. (2014) highlighted certain loci instabilities in a study on 65 human and animal isolates, particularly on 25 epidemiologically connected strains from 2012. Recently, GYURANECZ et al. (2013) described cases of mutated individual MLVA loci *in vivo* (within-host) during a *B. canis* outbreak in an animal and in animals in the same flock. An abbreviated genotyping scheme restricted to panel 2B could be used as a short-term epidemiological tool in a small region of endemicity, as it allows for the discrimination of distinct outbreaks, clustering the strains known as epidemiologically related and identifying a common infectious source (KATTAR et al., 2008; MICK et al., 2014).

Comparing previous studies, the highest HGDI values were also found for panel 2B loci. In the case of French strains from humans and animals isolated from 1989 to 2012, the highest diversity was found for locus 16 (0.865) (MICK et al., 2014). VALDEZATE et al. (2010) found a diversity of 0.727 for locus 7 for a panel of 108 Spanish human strains, while JIANG et al. (2013) found a diversity of 0.826 for locus 16 in China. In this study of

39 strains, the highest HGDI value was found for locus 4 (0.827), followed by locus 16 (0.818), and locus 30 (0.612). To establish the epidemiological relationship of strains in an outbreak or connected outbreaks, it is necessary to carry out classical epidemiological investigations, to avoid excessive culturing and, where possible, to conduct direct MLVA testing on samples. As a scheme for rapid laboratory species identification and genotype characterisation of *B. melitensis* in Croatia and BIH, we were highly satisfied with the use of the Bruce-ladder method and MLVA panel 2B loci for rapid epidemiological investigation.

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SAŽETAK

U razdoblju od 2009. do 2013. u Hrvatskoj je izvršena bakteriološka pretraga uzoraka ukupno 336 ovaca, koza i goveda. Klasičnim bakteriološkim i molekularnim postupcima *B. melitensis* je potvrđena u 14 (4,2%) izolata. Brucele su izdvojene u Karlovačkoj, Ličko-senjskoj i Splitsko-dalmatinskoj županiji. Analiza broja uzastopnih ponavljanja na više lokusa (engl. multiple locus variable number of tandem repeat analysis, MLVA) primijenjena je radi genotipizacije i usporedbe s izolatima iz susjedne Bosne i Hercegovine. Ukupno je analizirano 14 izolata podrijetlom iz Hrvatske i 25 iz susjedne Bosne i Hercegovine. U dva slučaja genotipovi

izolata iz Hrvatske u potpunosti su se poklapali s genotipovima u Bosni i Hercegovini. Korištenjem metode MLVA utvrđen je visok stupanj homogenosti izolata u ove dvije zemlje. Od korištenih 16 MLVA lokusa raznolikost je utvrđena samo na 5 lokusa panela 2B (Bruce 04, 07, 09, 16, 30) što je potvrđeno računanjem HGDI indeksa. Korištenje ovih 5 razlikovnih lokusa optimalno je za provođenje epidemioloških istraživanja u Hrvatskoj i Bosni i Hercegovini.

Ključne riječi: MLVA, *B. melitensis*, preživači, Hrvatska