New Brucella variant isolated from Croatian cattle

Spicic, Silvio; Zdelar-Tuk, Maja; Ponsart, Claire; Hendriksen, Rene S.; Reil, Irena; Girault, Guillaume; Leekitcharoenphon, Pimlapas; Rukavina, Vesna; Rubin, Martina; Freddi, Luca

Total number of authors:
11

Published in:
BMC Veterinary Research

Link to article, DOI:
10.1186/s12917-021-02833-w

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

BMC Veterinary Research

CASE REPORT Open Access

New *Brucella* variant isolated from Croatian cattle

Silvio Spicic[1*], Maja Zdelar-Tuk[1], Claire Ponsart[2], Rene S. Hendriksen[3], Irena Reil[1], Guillaume Girault[2], Pimlapas Leekitcharoenphon[3], Vesna Rukavina[4], Martina Rubin[5], Luca Freddi[2] and Sanja Duvnjak[1]

Abstract

Background: A novel *Brucella* strain closely related to *Brucella* (*B.*) *melitensis* biovar (bv) 3 was found in Croatian cattle during testing within a brucellosis eradication programme.

Case presentation: Standardised serological, brucellin skin test, bacteriological and molecular diagnostic screening for *Brucella* infection led to positive detection in one dairy cattle herd. Three isolates from that herd were identified to species level using the Bruce ladder method. Initially, two strains were typed as *B. melitensis* and one as *B. abortus*, but multiplex PCR based on IS*711* and the Suis ladder showed that all of them to belong to *B. melitensis,* and the combination of whole-genome and multi-locus sequencing as well as Multi-Locus Variable numbers of tandem repeats Analysis (MLVA) highlighted a strong proximity within the phylogenetic branch of *B. melitensis* strains previously isolated from Croatia, Albania, Kosovo and Bosnia and Herzegovina. Two isolates were determined to be *B. melitensis* bv. 3, while the third showed a unique phylogenetic profile, growth profile on dyes and bacteriophage typing results. This isolate contained the 609-bp *omp31* sequence, but not the 723-bp *omp31* sequence present in the two isolates of *B. melitensis* bv. 3.

Conclusions: Identification of a novel *Brucella* variant in this geographic region is predictable given the historic endemicity of brucellosis. The emergence of a new variant may reflect a combination of high prevalence among domestic ruminants and humans as well as weak eradication strategies. The zoonotic potential, reservoirs and transmission pathways of this and other *Brucella* variants should be explored.

Keywords: *Brucella melitensis*, Variant, Cattle, Eradication, Croatia

Background

Brucellosis in cattle, which can be caused by *B.abortus*, *B. melitensis* and *B. suis* [1], can significantly impact productivity on beef and dairy farms, and it poses zoonotic risk to humans, in whom infection can cause severe illness. The last reported *B. abortus* infections in cattle in Croatia occurred in 1964, while *B. melitensis* infections in cattle were reported in 2008 in herds kept with infected sheep [2] and in 2019 in herds kept with infected goats [3]. Since 2011, Croatia has conducted a brucellosis eradication programme in cattle according to European Directive 64/432/EC. All animals older than 12 months are tested annually using the Rose Bengal test (RBT), and positive animals are further tested using complement fixation (CFT), as well as indirect and competitive ELISAs. Depending on the epidemiological situation, seropositive animals are tested using a brucellin skin test, tissues and organs (head, mammary and genital lymph nodes, uterus, spleen, udder, fetal membranes) and stomach contents, spleen and lung from foetuses are collected at slaughterhouse for bacteriological testing.

Within this eradication programme, potential *Brucella* isolates are identified to genus and species levels using a

* Correspondence: spicic@veinst.hr
[1]Department of Bacteriology and Parasitology, Laboratory for Bacterial Zoonosis and Molecular Diagnostics of Bacterial Diseases, Croatian Veterinary Institute, Savska street 143, Zagreb, Croatia
Full list of author information is available at the end of the article

combination of classical biotyping, multiplex PCR and molecular genotyping methods, which can include Multi-Locus Variable number of tandem repeats Analysis (MLVA), Multi-Locus Sequence Typing (MLST) [1, 4–7] and Whole-Genome Sequencing (WGS).

Here we describe the identification of a dairy cow herd with brucellosis within the framework of the Croatian eradication programme. The disease was attributed to infection with *B. melitensis* bv. 3, except in one cow infected with a *B. melitensis* variant difficult to identify using standard classical and molecular methods. The emergence of this novel strain points to ongoing *Brucella* evolution in the western Balkans area, which may be due to the appearance of new reservoirs or vectors forced strain mutation and poor efficacy of eradication measures. Future studies should explore new reservoirs and zoonotic significance for this and other potential new *Brucella* variants in the region.

Case presentation

During routine annual testing within the national eradication programme, a dairy cow herd (12 cows, 7 heifers and 4 calves) with brucellosis was identified in October 2018 on a farm in the Croatian village of Katinovac (45°14′30.6″N 15°55′31.8″E), close to the northern border with Bosnia and Herzegovina. At the time of testing, animals were healthy and showed no clinical signs of brucellosis. Other animal species were not present on farm. A total of 19 animals aged 1 year and older were tested using the RBT. A total of 10 animals were identified positive by RBT and indirect ELISA, and 7 of these animals were also positive by the CFT and competitive ELISA. In November 2018, 19 animals older than 1 year were tested on the brucellin skin test. Positive reaction on brucellin was found in 5 previously seropositive cows, which were sent to the slaughterhouse, where samples were collected and analysed bacteriologically. During the post mortem inspection visible lesions were not recorded.

Epidemiological investigation showed that the Croatian herd had been brucellosis-free since 2013, and no new animals had been introduced since 2008. Since 2016, the farm practised artificial insemination with no recent history of abortions. The farm owner denied contact with other sheep or cattle herds and indicated that the herd was kept on pastures bordering Bosnia and Herzegovina. Their water source was the river Glina, a natural borderline in this area.

Brucella strains were isolated from milk and various tissues from three cows that were found without any clinical symptoms. The strains were classically biotyped as described [4] based on CO_2 requirement, H_2S production, oxidase and urease activity, growth on dyes, lysis by phages and agglutination with monospecific sera (Tables 1 and 2). This biotyping was performed at the Croatian National Reference Laboratory (Zagreb) and European Reference Laboratory (Maisons-Alfort, France). All three isolates could grow without CO_2, they produced H_2S and expressed oxidase, and they hydrolysed urea. They did not grow on basic fuchsin medium, and they triggered agglutination of anti-A and anti-M sera. These findings are consistent with *B. melitensis* bv.3. However, isolate 7 was lysed by Tbilisi phages at 10^4 routine test dilution (RTD).

Species was determined using multiplex PCR based on the Bruce ladder [8] and the "AMOS" method [9], followed by the Suis ladder [10] and another PCR based on detection of the *omp*31 gene [11]. Isolates 6 and 11 gave results consistent with *B.melitensis,* but isolate 7 lacked *omp*31 gene sequences tested in the Bruce ladder, suggesting that it was *B.abortus*.

To confirm the identification of isolates 6 and 11 as well as to complete the identification of isolate 7, we performed MLVA based on 16 loci [5, 6] in the following order: *panel 1, Bruce*06 - *Bruce*08 - *Bruce*11 - *Bruce*12 - *Bruce*42 - *Bruce*43- *Bruce*45 - *Bruce*55; *panel 2a, Bruce*18 - *Bruce*19 - *Bruce*21; and *panel 2b, Bruce*04 - *Bruce*07 - *Bruce*09 - *Bruce*16 - *Bruce*30. *B. melitensis* 16M was used as the reference strain for comparison and verification of test quality. In addition, MLST was performed based on 9 loci [7]: *gap - aroA - glk - dnaK - gyrB - trpE - cobQ - int-hyp* (*orf1*)*-omp25*.

Moreover, isolates 6 and 7 were subjected to whole-genome shotgun sequencing using the Illumina NexteraXT system (protocol 150,319,425,031,942, revision C), which has been deposited in DDBJ/ENA/GenBank under accession

Table 1 Results of classical biotyping tests of *Brucella* isolates

Strain	CO_2	H_2S	Growth on		Lysis with			Agglutination
			thionin	fuschin	Tbilisi phages RTD/10^4RTD	Weybridge phages	Izatnagar1 phages	
B.melitensis 16M	–	–	+	+	–/–	–	–	M+
B.abortus 544	+	+	–	+	+/+	+	+	A+
Isolate 6	–	–	+	–	–/–	+	+	M+, A+
Isolate 7	–	–	+	–	–/+	+	+	M+, A+
Isolate 11	–	–	+	–	–/–	+	+	M+, A+

A Antiserum A, *M* Antiserum M, *RTD* Routine test dilution

Table 2 Molecular identification and genotyping of *Brucella* isolates

Method(s)	Reference	Result
PCR AMOS	Bricker &Halling, 1993	Isolates 6, 7, 11: *Brucella melitensis*
Bruce ladder	Lopez-Goni et al. 2008	Isolate 6: *Brucella melitensis* Isolate 7: *Brucella abortus* Isolate 11:*Brucella melitensis*
PCR omp31	Vizcaino et al., 1997	Isolate 6:positive Isolate 7: negative Isolate 11: positive
Suis ladder	Lopez-Goni et al., 2011	Unique pattern for all *B. melitensis* strains
MLVA 16 (Bruce06–08 - 11 - 12 - 42 - 43- 45 - 55, 18–19 - 21, 04–07 - 09 - 16 - 30)	La Fleche et al., 2006; Al Dahouk et al., 2007	Isolate 6: 1–5–3-13-3-2-3-2-4-41-8-5-4-3-7-6 Isolate 7: genotype (1–5–3-13-2-2-3-2-4-41-8-5-4-3-7-6), consistent with *B. melitensis*
MLST 9 (gap - aroA - glk - dnaK - gyrB - trpE - cobQ - int-hyp (orf1)-omp25)	Whatmore et al., 2007	Isolates 6 and 7: sequence type 8, 3–2–3-2-1-5-3-8-2; consistent with *B. melitensis*
WGS/wgSNP	Illumina NexteraXT guide (no. 150319425031942), following protocol revision C	DDBJ/ENA/GenBank under accession numbers CVI_6 ChI CP058599, CVI_6 ChII CP058600, CVI_7 ChI CP058597, and CVi_7 ChII CP058598

numbers CVI_6 ChI CP058599/CVI_6 ChII CP058600 and CVI_7 ChI CP058597/CVI_7 ChII CP058598. A phylogenetic tree was generated using Bioumerics 7.6.3 (Applied Maths, BioMérieux). A set of *B. melitensis* genomes was retrieved from public databases (NCBI and PATRIC) and numbered in table (see Additional file 1). Sequencing reads were simulated for each genome using ART and all reads were mapped against a chimeric genome of *B. melitensis* 16M genome. Eight *B. abortus* genomes were used as outgroup. The SNPs obtained were then filtered (20X of absolute coverage, 10 bp inter-SNP distance, ambiguous and unreliable bases were removed, repeated elements removed) and a maximum parsimony tree was generated from these SNPs. The tree is represented with a logarithmic scale (see Additional files 2 and 3). This sequencing also revealed that isolate 7 contained a 609-bp *omp*31 sequence also present in isolate 6, but not the 723-bp *omp*31 sequence present in isolate 6. Based on classical and molecular methods, we assigned *Brucella* isolates 6 and 11 as *B. melitensis* bv. 3, while isolate 7 appeared to be a novel *B. melitensis* variant.

Discussion and conclusions

Human brucellosis, considered one of the most dangerous zoonoses, is most often caused by *B. melitensis* and less often by *B. abortus* or *B. suis*. The disease is endemic to the Mediterranean in general and the Balkan peninsula in particular [12–16]. Nevertheless, bovine brucellosis cases are sporadic and infrequent in Croatia, with the only recent reports limited to instances of transmission from sheep and goats on the same farms [2, 3]. The present case is the first recent report of brucellosis in Croatia that cannot definitively be attributed to contacts with other infected animal species.

This work highlights the need for continuing vigilance and research into potential *Brucella* reservoirs and spreading pathways. The disease in the present report may easily have come from Bosnia and Herzegovina, because herds on both sides of the border often share pastures, and illegal migrations are common which is documented throughout complete border line between countries [2, 3, 17]. Bosnia and Herzegovina has conducted a vaccination programme to control brucellosis in small ruminants since 2009, yet incidence of the disease remains high in animals and humans [18], and has even been increasing since 2012 [3]. This lack of efficacy is likely due largely to non-compliance with vaccination programmes [18], which can also foster the emergence of new *Brucella* strains [19].

We were unable to identify isolate 7 using classical microbiological methods [4] which are based on phenotype. This suggests that classical methods may not be well suited for characterising new *B. melitensis* strains in brucellosis-endemic regions. In fact, we were able to unambiguously identify the three isolates only by combining MLVA, MLST and whole-genome sequencing. These techniques showed our strains to be phylogenetically related to strains circulating in Croatia as well as Bosnia and Herzegovina [15, 17]. In particular, MLVA typing allowed us to assign a unique 16-digit code to the novel isolate 7, based on differences from the *Bruce*42 locus. Isolates 6 and 7 were assigned to the previously reported sequence type 8, related to *B. melitensis* strains circulating in Turkey, Kosovo and Macedonia. The two strains CVI_6 and CVI_7 clustered together in a subclade comprising 4 others strains (F9/05 from Turkey, BwIM_XXX_12 from unknown origin, F8/01–155 from Kosovo and BwIM_ALB_46 from Albania). Interestingly, this subclade contains two strains from the Balkan and one from Turkey. Moreover, in a recent paper [20], a strain from Serbia is clustered with the strain of Albania. The 2 strains isolated in this study seem to belong to a clade composed by strains that circulate in the Balkan

area (see Additional files 2 and 3). Cross-contamination at the borders with animals can be a reason.

Our findings highlight the need for continuing, even enhanced, efforts to surveillance brucellosis in domestic animals and to research potential *Brucella* reservoirs and transmission pathways to ensure timely detection of zoonotic threats.

Abbreviations

NCBI: The National Center for Biotechnology Information advances science and health by providing access to biomedical and genomic information (https://www.ncbi.nlm.nih.gov/); PATRIC: The Pathosystems Resource Integration Center is the all-bacterial Bioinformatics Resource Center (BRC) (http://www.patricbrc.org).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-021-02833-w.

Additional file 1. Table with strains metadata.

Additional file 2. Textual description of phylogenetic tree.

Additional file 3. Figure Phylogenetic Tree.

Acknowledgements

We thank Mrs. Silvija Drašković, Mrs. Marijana Novosel and Mr. Filip Radač from Croatian Veterinary Institute, NRL for Brucellosis for technical help.

Authors' contributions

MZT, SS, CP, IR and LF conducted serological and bacteriological analysis and interpreted data. SD, RSH, GG, PL performed the molecular examination of the strains including genotyping with result interpretation. VR, MR and SS conducted epidemiological analysis. All authors drafted and participated in writing of manuscript, read and approved the final manuscript.

Funding

This study was supported by Croatian Ministry of Agriculture and Croatian Veterinary Institute during collection, analysis, and interpretation of results within the framework of the Croatian eradication programme.

Availability of data and materials

All data generated during this study are included in this published article. The datasets of WGS genomes generated during the current study are available in the DDBJ/ENA/GenBank repository under accession numbers CP058599-CP058600 and CP058597-CP058598.

Declarations

Ethics approval and consent to participate

All investigations were conducted as a part of regular disease-prevention-control activities and approved by Croatian Veterinary Directorate.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Bacteriology and Parasitology, Laboratory for Bacterial Zoonosis and Molecular Diagnostics of Bacterial Diseases, Croatian Veterinary Institute, Savska street 143, Zagreb, Croatia. [2]French Agency for Food, Environmental & Occupational Health Safety (ANSES), Bacterial Zoonoses Unit - Animal Health Laboratory, National & OIE/FAO Animal Brucellosis Reference Laboratory, EU Reference Laboratory for Brucellosis, 14 rue Pierre et Marie Curie, Maisons-Alfort, Cedex, France. [3]Technical University of Denmark, National Food Institute, Research Group for Genomic Epidemiology, Kemitorvet, Building 204, 2800 Kgs. Lyngby, Denmark. [4]Ministry of Agriculture, Veterinary and Food Safety Directorate, Veterinary Office Sisak, Branch Office Glina, Trg bana Josipa Jelačića 2, 44 400 Glina, Croatia. [5]Ministry of Agriculture, Veterinary and Food Safety Directorate, Department for Veterinary Epidemiology, Planinska 2a, 10000 Zagreb, Croatia.

Received: 14 October 2020 Accepted: 11 March 2021
Published online: 20 March 2021

References

1. Manual of Diagnostic Tests and Vaccinesfor Terrestrial Animals 2019 (OIE): Brucellosis (*Brucella abortus, B. melitensis and B. suis*) (infection with *B. abortus, B. melitensis and B. suis*): https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.04_BRUCELLO-SIS.pdf. Accessed 31 Dec 2019.
2. Spicic S, Zdelar-Tuk M, Racic I, Duvnjak S, Cvetnic Z. Serological, bacteriological, and molecular diagnosis of brucellosis in domestic animals in Croatia. Croat Med J. 2010;51(4):320–6. https://doi.org/10.3325/cmj.2010.51.320.
3. World Animal Health Information System (WAHIS) - Version: 2: https://www.oie.int/wahis_2/public/wahid.php/countrymapinteractive. Accessed 31 Dec 2019.
4. Alton GG, Jones LM, Angus RD, Verger JM. Techniques for the brucellosis laboratory. Paris: Institut National de la Recherche Agronomique (INRA); 1988.
5. Le Flèche P, Jacques I, Grayon M, et al. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiol. 2006;6(1):9. https://doi.org/10.1186/1471-2180-6-9.
6. Al Dahouk S, Flèche PL, Nöckler K, et al. Evaluation of *Brucella* MLVA typing for human brucellosis. J Microbiol Methods. 2007;69(1):137–45. https://doi.org/10.1016/j.mimet.2006.12.015.
7. Whatmore AM, Perrett LL, MacMillan AP. Characterization of the genetic diversity of *Brucella* by multilocus sequencing. BMC Microbiol. 2007;7(1):34. https://doi.org/10.1186/1471-2180-7-34.
8. López-Goñi I, García-Yoldi D, Marín CM, et al. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. J Clin Microbiol. 2008;46(10):3484–7. https://doi.org/10.1128/JCM.00837-08.
9. Bricker BJ, Ewalt DR, MacMillan AP, Foster G, Brew S. Molecular characterization of *Brucella* strains isolated from marine mammals. J Clin Microbiol. 2000;38(3):1258–62. https://doi.org/10.1128/JCM.38.3.1258-1262.2000.
10. López-Goñi I, García-Yoldi D, Marín CM, de Miguel MJ, Barquero-Calvo E, Guzmán-Verri C, Albert D, Garin-Bastuji B. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. Vet Microbiol. 2011;154(1–2):152–5. https://doi.org/10.1016/j.vetmic.2011.06.035.
11. Vizcaíno N, Verger JM, Grayon M, Zygmunt MS, Cloeckaert A. DNA polymorphism at the *omp*-31 locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers. Microbiology. 1997;143(9):2913–21. https://doi.org/10.1099/00221287-143-9-2913.
12. Krkic-Dautovic S, Mehanic S, Ferhatovic M, Cavaljuga S. Brucellosis epidemiological and clinical aspects (is brucellosis a major public health problem in Bosnia and Herzegovina?). Bosn J Basic Med Sci. 2006;6(2):11–5. https://doi.org/10.17305/bjbms.2006.3162.
13. Pappas G. The changing *Brucella* ecology: novel reservoirs, new threats. Int J Antimicrob Agents. 2010;36(S1):S8–S11. https://doi.org/10.1016/j.ijantimicag.2010.06.013.
14. Arapovic J, Spicic S, Ostojic M, et al. Epidemiological, clinical and molecular characterization of human brucellosis in Bosnia and Herzegovina - an ongoing brucellosis outbreak. Acta Med Acad. 2018;47(1):50–60. https://doi.org/10.5644/ama2006-124.214.
15. Duvnjak S, Racic I, Spicic S, Zdelar-Tuk M, Reil I, Cvetnic Z. Molecular epidemiology of *Brucella melitensis* strains causing outbreaks in Croatia and Bosnia and Herzegovina. Acta Vet Hung. 2018;66(2). https://doi.org/10.1556/004.2018.017.
16. Wareth G, et al. Brucellosis in the Mediterranean countries: history, prevalence, distribution, current situation and attempts at surveillance and control, Technical Series, vol. 12; 2019. ISBN 978–92–95115-00-2
17. Cvetnic Z, Zdelar-Tuk M, Duvnjak S, Racic I, Skrivanko M, Spicic S. Multiple locus variable number of tandem repeat analysis (MLVA) of isolates of *Brucella melitensis* isolated in the Republic of Croatia. Vet Arhiv. 2015;85:481–92.

18. Seric-Haracic S, Fejzic N, Saljic E, Hadzijunuzovic-Alagic D, Salman M. The scenario tree epidemiological model in estimation effects of *B. melitensis* rev. 1 vaccination on disease prevalence. Turk J Vet Anim Sci. 2018;42(5): 416–4229. https://doi.org/10.3906/vet-1710-67.
19. Moreno E. Retrospective and prospective perspectives on zoonotic brucellosis. Front Microbiol. 2014;5:213. Published 2014 May 13. https://doi.org/10.3389/fmicb.2014.00213.
20. Georgi E, Walter MC, Pfalzgraf MT, Northoff BH, Holdt LM, Scholz HC, Zoeller L, Zange S, Antwerpen MH. Whole genome sequencing of *Brucella melitensis i*solated from 57 patients in Germany reveals high diversity in strains from Middle East. PLoS One. 2017;12(4):e0175425. https://doi.org/10.1371/journal.pone.0175425.

Publisher's Note