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Abstract: Brucella melitensis is a major human and animal pathogen, with a wide host range that includes all domestic ruminant species, although small ruminants are its preferred hosts. Outbreaks in cattle due to B. melitensis have become a worldwide emerging problem particularly difficult to control due to the lack of knowledge on the epidemiology in this host species and of an effective vaccine. However, combination of molecular tools and strict biosecurity measures can help to solve these difficulties and eventually eradicate the disease from infected herds. In the present report, management of an outbreak in Spain involving four farms, more than 2000 cattle and several human cases is described. Application of Multiple Locus VNTR Analysis (MLVA) allowed identifying the most likely source of infection. Stamping out and test-and-cull strategies were applied depending on the infection level of each herd, successfully controlling the outbreak without the need of other extraordinary measures.

*Manuscript

1 **MANAGEMENT OF AN OUTBREAK OF BRUCELLOSIS DUE TO *B.***
2 ***melitensis* IN DAIRY CATTLE IN SPAIN**

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24

25 **ABSTRACT**

26 *Brucella melitensis* is a major human and animal pathogen, with a wide host range
27 that includes all domestic ruminant species, although small ruminants are its preferred
28 hosts. Outbreaks in cattle due to *B. melitensis* have become a worldwide emerging
29 problem particularly difficult to control due to the lack of knowledge on the
30 epidemiology in this host species and of an effective vaccine. However, combination of
31 molecular tools and strict biosecurity measures can help to solve these difficulties and
32 eventually eradicate the disease from infected herds. In the present report, management
33 of an outbreak in Spain involving four farms, more than 2000 cattle and several human
34 cases is described. Application of Multiple Locus VNTR Analysis (MLVA) allowed
35 identifying the most likely source of infection. Stamping out and test-and-slaughter
36 strategies were applied, proving their usefulness to control the outbreak depending on
37 infection level, and without the need of other alternative measures.

38 Keywords: brucellosis, *B. melitensis*, cattle, outbreak, control, zoonosis.

39 *B. melitensis*, causative agent of small ruminant brucellosis, is acquiring an
40 increasing importance in bovine as an emergent pathogen (Corbel, 1997; Kahler, 2000).
41 *B. melitensis* is also the main causative agent of human brucellosis, one of the most
42 serious zoonoses all over the world (EFSA, 2007; OIE, 2008). The main source of
43 infection for the population is consumption of unpasteurized milk and milk products,
44 but other ways of infection such as respiratory or conjunctival routes have been
45 described.

46 In several countries *B. melitensis* is the most frequently isolated species from all
47 domestic ruminants (Refai, 2002). Outbreaks in cattle are often attributed to the
48 presence of infected sheep and goat flocks in the surrounding area (Benkirane, 2006;
49 Samaha et al., 2008). *B. melitensis* infection in cattle poses a serious problem to both
50 farmers and veterinarians, because of the lack of information regarding several aspects
51 of its epidemiology, such as cattle-to-cattle transmission or within-herd persistence of
52 infection (Bercovich, 1998). Control of *B. melitensis* infection in cattle is impeded due
53 to difficulties on the implementation of vaccination strategies: although cross-species
54 protection between *Brucella* species has been demonstrated using recombinant RB51
55 vaccine in mice (Vemulapalli et al., 2004), it has not been proven in cattle yet; *B.*
56 *melitensis* vaccine (Rev1) have been applied before in cattle in developing countries
57 (Denes, 1997) and its administration in this host species has been authorized by the
58 European Union (Anon., 2002), but to our knowledge its usefulness to control *B.*
59 *melitensis* outbreaks in cattle in developed countries has not been fully assessed. Thus,
60 slaughtering of all exposed animals is one of the available options to control a *B.*
61 *melitensis* outbreak in a cattle herd. Alternatively, if the infection level is low and the
62 outbreak is detected at an early stage, the disease might be controlled and eventually
63 eradicated through the implementation of very strict management procedures (Hamdy et

64 al., 2008). This paper describes the management of an outbreak of brucellosis due to *B.*
65 *melitensis* involving human cases and four cattle farms (A, B, C and D) in the north east
66 of Spain using serological techniques, bacteriology and molecular characterization
67 tools. Strategies followed depending on the epidemiological status of every premise are
68 detailed in order to identify their potential usefulness.

69 *Farms management system*: farms A, B and C were large dairy farms (>550
70 animals) that shared feed and milk transport and the veterinary services located in an
71 area were no brucellosis vaccination had been performed since 2002. All three farms
72 routinely send new born heifers to farm D (916 animals at the beginning of the
73 outbreak), where they were served. These animals were kept in farm D for most of the
74 pregnancy and approximately one week before calving the pregnant cows were sent
75 back to their farms of origin. Before moving back, all animals were segregated within
76 the previous 30 days before transportation to a separated pen ("pre-movement yard")
77 and subjected to *Brucella* testing using Rose Bengale Test (RBT) (OIE, 2008). The pre-
78 movement yard would hold approximately 18 animals.

79 *Outbreak*: On September 14th 2007 brucellosis was confirmed in a man that
80 worked in farm A. Almost at the same time, routine tests performed as part of the
81 Spanish brucellosis eradication program detected three positive reactors at the
82 complement fixation test (CFT) with titers equal or above 20 international complement
83 fixation test units (ICFTU)/ml (OIE, 2008) in the same farm (Table 1), located in an
84 area where no bovine brucellosis had been detected for more than 6 years. Positive
85 animals were slaughtered and sampled for bacteriology, and serological analyses were
86 repeated on day 19 post-detection (p.d.) of the outbreak, revealing 53 reactors at CFT
87 and/or RBT (Table 1). Epidemiologically connected farms B, C and D were also tested;
88 in farm D only two positive reactors were found; both have just arrived four days before

89 from farms A and B respectively, and consequently infection in their farms of origin
90 was the most likely hypothesis. No more positive animals were detected in this farm on
91 subsequent analysis. In farms B and C 131 and 23 reactors were detected in the first
92 analysis (Table 1). Once the extent of the outbreak was determined, periodical testing of
93 all animals from the four farms were performed (Table 1). Samples of animals from the
94 three infected farms (A, B and C) that had been culled on the first tests were collected at
95 abattoir to culture the *Brucella* strain involved in the outbreak. *B. melitensis* was
96 recovered from all samples, and cultures were identified as belonging to biotype 3 by
97 agglutination with A and M-specific antisera (OIE, 2008). In addition, seven workers in
98 total (four from farm A and three from farm B) were also found to be infected by *B.*
99 *melitensis* biotype 3. No increase in the abortion rates in all four farms had been
100 reported before the outbreak was detected.
101 Among possible sources of disease, infected sheep flocks located in the vicinity
102 of farm D were considered as the most likely source. However, due to the fact that no
103 positive animals were detected during the whole outbreak in this farm (with the
104 exception of the two cows that were sero-positive only two days after arrival) this
105 possibility was ruled out in the beginning. Nevertheless, a thorough investigation
106 revealed that on the first week of May one sheep of unknown origin was kept in farm D
107 for four days, showing a lack of appropriate biosecurity measures. On May 25th one cow
108 that was about to return to farm C before calving aborted in farm D (in the pre-
109 movement yard), and was then transported to farm C without any supplementary
110 analysis following abortion. This animal was later positive in the first analysis
111 performed in farm C, although it was negative in the pre-movement *Brucella* test carried
112 out before the abortion. Most animals sharing the pre-movement yard with this animal
113 were later positive in both RB and CF tests in the first analysis performed in their

114 destination farms (A, B and C), even though they were also negative in pre-movement
115 test. Therefore, the cow aborting on the 25th of May could have been the first case;
116 animals infected in farm D would have spread the infection on their destination farms
117 once they had been transported just before parturition.

118 *Molecular epidemiology*: in order to confirm the suspected link between cattle,
119 human and sheep strains, five strains from farm A, three from farm B and one from
120 farm C were submitted to the Spanish National Reference Laboratory for Animal
121 Brucellosis for molecular characterization using multiple locus variable number tandem
122 repeats analysis (MLVA) on 15 polymorphic loci (MLVA-15) as previously described
123 (Le Fleche et al., 2006). In addition, isolates from two infected farm workers and from
124 several positive sheep flocks located in the vicinity of farm D were analyzed.
125 Comparison of the results observed in the eight conserved loci included in panel 1 by Le
126 Fleche et al. (2006) with those available in the MLVAbank (http://minisatellites.u-
127 psud.fr/MLVAnet) revealed profile 51 was present in all strains. Patterns obtained in the
128 seven highly variable loci included in panel 2 are summarized in Table 2. MLVA
129 analysis revealed four different patterns in panel 2 due to polymorphisms on loci
130 Bruce09 and Bruce16: profile I was identified in two cattle isolates from farm A, and
131 profile II was present in one sheep strain, cattle isolates from farms A (n=3), B (n=3)
132 and C (n=1) and in the two human isolates (Table 2).

133 *Outcome*: due to the large number of animals involved several options for its
134 control were discussed. Finally, strategies were applied depending on the *B. melitensis*
135 infection prevalence detected on every premise: the high numbers of reactors detected in
136 farms A and B, together with the uncertainty about the possible evolution of the
137 outbreak and sanitary and socioeconomical reasons, made stamping out the most
138 feasible control option, and all animals in both farms were culled 140 days after

139 detection of the outbreak. A different approach was adopted on farm C, which had a
140 lower seropositivity rate (Table 1): all animals were tested every 15-20 days using CFT
141 and RBT, with immediate removal of positive reactors. Besides, biosecurity measures
142 were maximized regarding handling of pregnant animals, disinfection of all material in
143 contact with positive reactors, control of movement of all animals, etc. A similar
144 strategy was established in farm D: all pregnant cows held in farm D were not moved
145 back to their farms of origin before calving to avoid entering animals in the infected
146 farms; instead, all cows calved in farm D in a separated area. Special care was taken to
147 avoid any contact of these animals with the rest of the cattle in farm D, as it could not
148 be discarded that they were infected, in order to minimize the risk of spreading the
149 infection. Handling of these high risk animals by human workers was performed with
150 extreme care. The heifers were tested eight and 30 days post-birth to maximize the
151 possibilities of detecting remaining infected animals after calving, in order to overcome
152 difficulties in the diagnosis of *Brucella* infections in primiparous heifers, especially
153 difficult to detect before calving.
154 The evolution of results of diagnostic tests confirmed the viability of a
155 conservative approach to control the outbreak in farms C and D: on farm C only eight
156 more reactors were detected on following tests (Table 1), and since then no more
157 positive reactors have been detected by any diagnostic test, confirming the eradication
158 of the disease. In farm D only two positive animals were detected and both were likely
159 infected in their origin farms, although the origin of the outbreak could have taken place
160 there; segregation of cows on the pre-movement yard would have made possible that
161 only animals in that particular group would have been affected. Nevertheless all animals
162 in this farm were also subjected to regular testing for more than one year. The

163 management of the pregnant cows in this farm avoided the spread of the disease,
164 therefore saving the heifers that were later used to restock farms A and B.
165 This *B. melitensis* outbreak affected seven workers and four cattle farms,
166 involving more than 2000 animals. The absence of evident clinical signs together with a
167 possible lack of detectable immune response to routine bovine brucellosis diagnostic
168 test in infected primiparous heifers before abortion or calving make this kind of
169 outbreaks an emerging problem difficult to handle. However, it was identified and
170 controlled minimizing the number of animals that had to be culled thanks to several key
171 actions:
172 - Immediate control measures adopted when the first cases were reported
173 allowed, through a complete epidemiological analysis, the detection of the most likely
174 source of infection, an infected ovine flock near farm D.
175 - Culture and molecular characterization of the etiological agent from all host
176 species involved confirmed the possible origin of the outbreak.
177 - Measures adopted on the different farms were based on the information
178 available, avoiding the use of alternative measures as vaccination. An exhaustive
179 diagnostic strategy was successfully implemented in farms C and D, removing all
180 infected animals in approximately two months.
181 The application of a highly discriminative characterization technique found two
182 profiles in the *B. melitensis* isolates from cattle, with subsequent repetition of the
183 analysis confirming this result. Difference between the two patters were focused on
184 locus Bruce09, which had been described as the most polymorphic loci among MLVA-
185 15 targets in different panels of *B. melitensis* strains (Garcia-Yoldi et al., 2007; Tiller et
186 al., 2009). The two most likely explanations are a possible mutation in the outbreak
187 strain leading to a different profile (with only one more TR repetition) or the

188 involvement of two different strains in the outbreak, although the second profile could
189 not be identified on other sheep strains from the same area, and therefore would be of
190 unknown origin. Limited number of sheep strains typed in this study cannot rule out this
191 possibility. However, although loci included in the MLVA-15 have demonstrated their
192 usefulness for epidemiological purposes, occasional one-step increase or decrease
193 changes in one or more loci have been reported before after *in vivo* and *in vitro* passages
194 of *Brucella* strains (Whatmore et al., 2006; Kang et al., 2009). Therefore, the difference
195 found in highly polymorphic locus Bruce09 cannot exclude a possible common origin
196 of all cattle strains.

197 The present reports highlights the importance of maintaining strict biosecurity
198 measures and regular testing of cattle herds, and routinely report cases of abortions for
199 investigation in areas free of bovine brucellosis but where *B. melitensis* is present in
200 small ruminants, as in some cases clinical presentation of the disease in cattle can go
201 unnoticed.

202 **Conflict of interest statement**

203 None.

204 **References**

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254 characterization of Variable-Number Tandem-Repeat Markers for typing of

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256

257 Table 1. Results of serological analysis in farms A, B, C and D using Rose Bengal and
258 Complement Fixation techniques.

Farm	Day [a]	Analyzed samples	Positive samples [b] (%)	RB+ [c]	CF+ [d]
A	0	579	3 (0.5)	3	3
	19	574	53 (9.2)	48	50
	53	656	28 (4.3)	25	25
	97	387	64 (16.5)	60	15
	127	272	24 (8.8)	23	12
B	34	596	131 (22)	123	106
	60	509	79 (15.6)	72	54
	96	344	121 (35.2)	120	38
	133	294	53 (18)	50	37
C	39	642	23 (3.6)	18	20
	55	528	6 (1.1)	3	5
	67	636	0 (0)	0	0
	90	515	0 (0)	0	0
	105	506	2 (0.4)	2	2
	125	493	0 (0)	0	0
	147	628	0 (0)	0	0
D	26	916	2	2	2
	43	913	0 (0)	0	0
	54	67*	0 (0)	0	0
	70	917	0 (0)	0	0
	80	8*	0 (0)	0	0

86	29*	0 (0)	0	0
100	59*	0 (0)	0	0
121	12*	0 (0)	0	0
131	945	0 (0)	0	0
139	29*	0 (0)	0	0
148	17*	0 (0)	0	0

259 [a] All dates at referred to the day of first detection of the outbreak (14th September)

260 [b] Number of positive samples to the Rose Bengale and/or Complement Fixation tests.

261 [c] Number of positive samples to the Rose Bengale Test.

262 [d] Number of positive samples to the Complement Fixation test.

263 * Includes tests performed on heifers 8 and 30 days post-calving.

264 Table 2. Number of repetitions in tandem detected by multiple locus variable number
265 tandem repeats analysis (MLVA) in highly discriminatory loci described by Le Fleche
266 et. al (2006) in *Brucella melitensis* isolates from sheep, cattle and human samples.

Number of isolates	Host	Farm of origin	Bruce 18	Bruce 21	Bruce 04	Bruce 07	Bruce 09*	Bruce 16*	Bruce 30	Profile
2	Cattle	A	7	8	7	6	9	10	3	I
3	Cattle	A	7	8	7	6	8	10	3	II
3	Cattle	B	7	8	7	6	8	10	3	II
1	Cattle	C	7	8	7	6	8	10	3	II
2	Human	-	7	8	7	6	8	10	3	II
1	Sheep	-	7	8	7	6	8	10	3	II
4	Sheep	-	7	8	7	6	7	10	3	III
1	Sheep	-	7	8	7	6	7	11	3	IV

267 *Loci were polymorphisms among isolates were found.
268

269 Figure 1. Flow diagram of movements between dairy farms (A, B and C) and
270 reproduction farm D. Blue arrows indicate movement of heifers to farm D for being
271 served. Yellow arrows indicate movement of these animals back to their farms of origin
272 approximately one week before calving. D’ represents the pre-movement yard were
273 animals were segregated within 30 days before moving back to farms A-C.