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Simple and Rapid Field Tests for Brucellosis in Livestock

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Abstract

Four simple and rapid field tests for the serodiagnosis of brucellosis in cattle, goat, sheep and swine were developed. The performance of the assays was investigated using serum samples collected in Portugal from animals originating from herds with a defined sanitary status with respect to the presence of brucellosis. The sensitivity calculated for the bovine, caprine, ovine and swine *Brucella* lateral flow assays based on results obtained for samples collected from animals with culture confirmed brucellosis was 90%, 100%, 90% and 73%, respectively. None of the samples from animals from herds free of brucellosis reacted in the flow assays indicating a high specificity. However, as expected, some degree of reactivity was observed when testing selected serum samples that reacted non-specific in reference tests for brucellosis.

Keywords: Brucellosis; diagnostic; field test; bovine; ovine; caprine; swine; livestock

27 1. Introduction

28

29 Brucellosis is one of the most important bacterial zoonoses worldwide and in particular in
30 developing countries the disease may have important economic, veterinarian and public health
31 consequences (Godfroid et al., 2005; Smits et al., 2005; Pappas et al., 2006; Franco et al.,
32 2007). Knowledge of the spread and prevalence of the infection is essential when planning
33 control measures. Testing of livestock for brucellosis is done by culture and serology or by
34 testing milk samples (Nielsen, 2002). Tests for brucellosis approved by the World Health
35 Organization for Animal Health (OIE) require specific expertise and laboratory support. Testing
36 of livestock is cumbersome when dealing with farms located in remote areas or with animals
37 from nomadic populations and migratory farmers. Involvement of a laboratory may cause
38 delay in testing and reporting and requires complex logistics such as the identification and
39 tracing of animals and owners. In order to prevent the further transmission and spread of the
40 infection the presence of brucellosis requires the prompt instigation of control measures, and
41 hence a rapid test result is desirable.

42 We earlier developed a rapid and simple test, the *Brucella* IgM/IgG lateral flow assay
43 (LFA), for the serodiagnosis of human brucellosis (Smits et al., 2003; Irmak et al., 2004). This
44 LFA is highly sensitive and specific and may be used as a point-of-care diagnostic by
45 application on a drop of whole blood collected by finger prick. Application of the LFA does
46 neither require specific expertise, expensive equipment, electricity and refrigeration, nor
47 training, making this assay format ideal for use in resource poor countries. Here, we have
48 adapted the LFA for the serodiagnosis of brucellosis in different livestock species. Separate
49 *Brucella* LFAs were created for testing cattle, goat, sheep and swine, and the diagnostic value
50 of these assays was assessed by testing serum samples collected in Portugal from animals
51 sent for slaughtering because of evidence of brucellosis as well as from control animals free of
52 brucellosis. Results are compared with culture and those of routine serological testing in the
53 Rose Bengal test (RBT) and the complement fixation test (CFT).

54

55

56 2. Materials and Methods

57

58 The *Brucella* LFAs consist of a porous nitrocellulose detection strip flanked at one end by a
59 reagent pad and by an absorption pad at the other end. A sample application pad flanks the
60 reagent pad in turn. The composite detection strip is contained in a plastic assay device with a
61 round sample well positioned above the sample application pad and a test window positioned
62 above the detection zone. The detection zone contains two distinct lines, a test line and a
63 control line. The test line was obtained by spraying *Brucella*-specific antigen onto the
64 nitrocellulose strip. A crude *Brucella* lipopolysaccharide (LPS) preparation prepared from
65 *Brucella abortus* strain 1119-3 was used as antigen to capture specific serum antibodies
66 (Smits et al., 1999; Smits et al., 2003). LPS is used as it is the optimal antigen in indirect
67 antibody tests for brucellosis regardless of the serotype of the infecting strain and of the
68 animal species (Diaz-Aparacio et al., 1994; Nielsen et al., 1996; Alonso-Urmeneta et al.,
69 1998). The control line was obtained by spraying immunoglobulin G (IgG) antibodies from
70 cattle, goat, ovine or swine to obtain detection strips for test devices for these four animal
71 species. Test and control lines were sprayed using a BioDot Quanti 2000 BioJet. Detection
72 reagents for the LFAs for the four animal species were prepared by conjugating affinity purified
73 antibodies against cattle, goat, ovine and swine Ig (H+L) antibodies to 40nm colloidal gold
74 particles. These conjugates were sprayed and dried onto the conjugate pads of the composite
75 strips using the AirJet of the BioDot Quanti 2000 machine. The concentration and amount of
76 LPS and of the conjugate applied to the test strips was optimized using panels of defined
77 positive and negative control sera. The tests were performed by the addition of 5 μ l serum to
78 the sample pad of the assay device followed by the addition of 130 μ l sterile running fluid
79 consisting of phosphate-buffered saline, pH 7.6, containing 1.67% bovine serum albumin and
80 3% Tween 20. Test results are read after 10 min by visual inspection for staining of the test
81 and control lines in the test window of the assay device. Tests are scored negative when no
82 staining is observed at the test line and scored positive when the test line stains. The control
83 line should stain in all cases. The test line may stain at different intensities depending on the
84 titer of specific antibodies in the sample. Positive results may be subjectively rated 1+ when
85 staining is weak, 2+ when staining is moderately strong, 3+ when staining is strong, and 4+
86 when staining is very strong. Assay devices sealed in a moisture resistant and airtight foil

87 containing some desiccant may be stored at 4-27°C without loss of activity. The stain at the
88 test and control lines of exposed tests is stable after drying.

89 Bovine, caprine, ovine and swine serum sample selected from the serum bank of the
90 Laboratório Nacional de Investigação Veterinária (LNIV) in Lisbon were used for test
91 evaluation. Samples from LNIV included in the study had been collected at different
92 slaughterhouses in Portugal and the samples from each species could be subdivided in two or
93 more of the following groups according to the sanitary status of the herd from which they
94 originated. These groups are known to be infected with brucellosis, previously free of
95 brucellosis but evidence of recent infection, free of brucellosis, and sanitary status not
96 specified. Samples were obtained from 37 cows, 48 goats, 68 sheep and 33 pigs. Eighteen
97 cows, 20 goats, 22 sheep and 10 pigs were from herds known to be infected with brucellosis,
98 13 goats and 22 sheep were from herds with a sanitary status previously free of brucellosis
99 but evidence of recent infection, and 19 cows, 15 goats and 24 sheep were from herds with a
100 sanitary status free of brucellosis. The sanitary status of the herds to which 13 pigs belonged
101 was not specified because in Portugal there is no eradication plan for brucellosis in this
102 species. The sanitary status was defined following to the classification issued by Veterinary
103 National Authority (DGV) to define regions and herds according to the presence or absence of
104 brucellosis. According to this classification a herd is considered officially free of brucellosis if,
105 for bovines, it does not include animals vaccinated for brucellosis with the exception of
106 females vaccinated more than three years ago, all animals have been free of clinical signs of
107 brucellosis for at least the last 6 months, and all animals of more than 12 months of age have
108 been subjected to one or two rounds of serological testing with negative results, and, for small
109 ruminants, it does not include any animal vaccinated for brucellosis, all animals have been
110 free of clinical signs of brucellosis for the last 12 months, and all animals of more than 6
111 months of age at the time of blood collected have been subject to with an interval of 6 months
112 two rounds of serological testing in RBT with negative results.

113 In addition, we tested 25 bovine and 25 swine serum samples from the serum bank of the
114 Central Institute for Animal Health (CIDC-Lelystad), The Netherlands. These samples had been
115 collected in the Netherlands, a country that is free of brucellosis and were selected because of
116 false-positive reactivity in routine serological tests for brucellosis. All selected bovine samples

117 from the CIDC-Lelystad reacted in the serum agglutination test (SAT) at a titre of between
118 1:15 and $\geq 1:120$, 80% reacted in the Coombs test at a titre of between 1:20 and $\geq 1:200$,
119 and 25% reacted in IgG ELISA at a titer of between 1:100 and 1:3,200. All selected pig
120 samples that were taken from the serum bank of this institute reacted in the SAT at a titre of
121 between 1:15 and 1:60, 44% reacted in the Coombs test at a titer of 1:20, and 28% reacted
122 in the RBT.

123 Culture, and RBT and CFT for serum samples from Portugal were performed at the
124 Laboratory for Brucellosis Diagnosis of the LNIV; antigens for RBT and CFT were prepared at
125 the Laboratory for Antigen Production at LNIV. Culture and the RBT and CFT were performed
126 according to routine laboratory procedures. Blood cultures were performed using Farrell
127 medium supplemented with horse serum and antibiotics (Alton et al., 1988). The RBT was
128 performed as described in Annex C of Council Directive 64/432/EEC and Alton and colleagues
129 (1988). The CFT was performed by the warm fixation method, based on procedures described
130 by Alton and colleagues (1988). The CFT was considered positive when giving $\geq 50\%$ fixation
131 degree at a dilution of 1:4 or higher. SAT, ELISA, Coombs and RBT for samples from the
132 Netherlands were performed according to routine procedures at the CIDC-Lelystad. The
133 *Brucella* LFAs were performed at the laboratory of KIT Biomedical Research. All LFAs were
134 performed at 18-24°C.

135

136

137 **1. Results**

138

139 Blood culture was attempted for twelve cows and all goats, sheep and pigs originating
140 from infected herds and yielded positive results in 92, 40, 55 and 100% of these four animal
141 species, respectively (Table 1). All isolates from cattle were identified as *B. abortus* biovar 3,
142 those of goat and sheep as *B. melitensis* biovar 3, and those of pigs were classified as *B. suis*
143 sp. Serological testing in RBT and CFT revealed the presence of antibodies against *Brucella* in
144 73-89% of the serum samples from animals originating from infected herds. *B. melitensis*
145 biovar 3 could also be isolated from the blood of 38% of the goats and 77% of the sheep from
146 herds that had been free of brucellosis before but included reactors upon recent testing. In

147 addition, *B. suis* sp. was cultured from the blood of one pig with a not further specified
148 sanitary status. The RBT and CFT tests yielded positive results in 46% of the goat and in 86%
149 of the sheep from herds that had become infected recently. All serum samples from herds with
150 a sanitary status free of brucellosis and from the pig herds with a sanitary status that was not
151 specified tested negative in these classical serological tests.

152 The *Brucella* LFAs for the four livestock species tested positive in the majority of the
153 samples from the animals from herds known to be infected with brucellosis (Table 1). The
154 percentage of animals from infected flocks that reacted in the *Brucella* LFA ranged from 64%
155 for sheep to 90% for goat. With the exception of the caprine *Brucella* LFA the number of
156 animals from infected flocks that tested positive in the LFAs was in the same range as the
157 number of culture positive animals; the number of goats that tested positive in the caprine
158 *Brucella* LFA (90%) was notably higher than the number of culture positive goats (40%). The
159 same observation was made when testing goats from herds that had become infected
160 recently; while the pathogen could be isolated from 38% of these goats, 85% of them tested
161 positive in the caprine *Brucella* LFA. None of the animals from flocks free of brucellosis and
162 also none of the pigs with a non-specified sanitary status tested positive in the LFA. For most
163 groups the percentage of animals that reacted in the LFA was similar to the number of animals
164 that reacted in the RBT or the CFT. Again notable exceptions are the groups of infected goats
165 and goats from herds that had become infected recently which showed higher percentages of
166 animals that reacted in the LFA than in the RBT and CFT. All LFAs were easy to read and the
167 staining intensity of the test line was moderately strong (2+) to very strong (4+) for 93% of
168 the positive bovine samples, 66% of the positive goat samples, 85% of the positive ovine
169 samples and 75% of the positive swine samples. Examples of exposed bovine *Brucella* LFAs
170 performed with samples collected from animals originating from infected cattle herds and from
171 animals of herds free of brucellosis are presented in figure 1.

172 Based on the results obtained for animals with culture confirmed brucellosis the sensitivity
173 of the LFAs were calculated to be 90% for the bovine *Brucella* LFA, 100% for the caprine
174 *Brucella* LFA, 90% for the ovine *Brucella* LFA and 73% for the swine *Brucella* LFA. Culture has
175 a limited sensitivity and CFT is often used in stead of culture or to confirm culture negative

176 animals. The sensitivity of the CFT did not differ much from that of the LFA; only for the
177 bovine samples a higher sensitivity (100%) could be calculated for the CFT.

178 RBT is often used as a first screening assay. The *Brucella* LFAs confirmed 89 to 100% of
179 the RBT positive samples from the different animal species (Table 2). This result was fairly
180 similar to those obtained for culture and CFT. Notably, one bovine sample, seven goat sample
181 and two RBT ovine samples that were RBT negative reacted in the LFA. The RBT negative
182 samples from goats and sheep that reacted in the LFA were collected from animals from herds
183 that had been recently found to be infected with *Brucella*. Some of these RBT negative ovine
184 samples that reacted in the LFA were culture and or CFT positive. *Brucella* was also isolated
185 from three pigs with a negative RBT and the serum sample from one of these animals reacted
186 in the CFT. However, none of the RBT negative swine samples reacted in the LFA.

187 The absence of reactivity in the *Brucella* LFAs for all samples from animals from herds free
188 of brucellosis indicates a high specificity of close to 100%. However, as indicated by the test
189 results for the bovine and swine sera from The Netherlands that were selected for testing
190 because of known false-positive reactivity in standard serological tests some degree of cross-
191 reactivity in the LFA may be expected; 56% of these cross-reactive bovine sera showed weak
192 (1+) or moderately weak (2+) staining in the LFA, and of the selected cross-reactive swine
193 sera 24% showed weak (1+) staining in the LFA. The other cross-reactive serum samples
194 tested negative in the LFAs.

195

196

197 **2. Discussion**

198

199 The isolation and identification of *Brucella* bacteria offers a definite diagnosis of brucellosis.
200 Based on the results for animals with culture confirmed brucellosis the sensitivity of the bovine
201 *Brucella* LFA was calculated to be 90%, that of the caprine LFA 100%, that of the ovine LFA
202 77%, and that of the swine LFA 73%. No reactivity in the *Brucella* LFAs was observed for
203 samples from cows, goat and sheep from herds from Portugal known to be free of brucellosis
204 indicating a high (100%) specificity. The sensitivity of the CFT which is accepted as a
205 confirmatory test for bovine brucellosis was somewhat higher than that calculated for the

206 bovine LFA. The validity of the CFT for testing other animal species for brucellosis is less well
207 established and it is of interest to note that the sensitivity calculated for the caprine, ovine and
208 swine LFAs were identical to that of the CFT. However, compared with the CFT a clearly higher
209 proportion of goats from infected herds and from herds that had become infected recently
210 tested positive in the LFA. The number of goats from these two groups that had a positive
211 result in the LFA was also higher than the number of animals from which the *Brucella*
212 pathogen could be isolated. It is well known that current tests for brucellosis in small
213 ruminants are not optimal and the use of a combination of tests for instance RBT and CFT is
214 recommended (Lilenbaum et al., 2007; Solorio-Rivera et al., 2007). Our results indicate that
215 the LFA is a simple, rapid and highly sensitive and specific alternative for the detection of
216 brucellosis in livestock, and that in particular the use of the LFA for caprine brucellosis may
217 help to improve the testing for brucellosis in this species. Laboratory testing for brucellosis in
218 swine is least well developed and also for this animal species the use of different tests is
219 recommended to increase the detection rate. Our results indicate that the LFA for swine
220 brucellosis performs similar to the RBT and CFT. Notably, the serum samples of three culture
221 positive pigs did not react in the LFA and of these only one tested positive in the CFT. It will
222 be worthwhile to investigate whether repeated testing of pigs could improve sensitivity.

223 The LFA has several practical advantages that allows testing on the spot and that may
224 make it the method of choice when testing animals in remote areas or when testing animals
225 from nomadic and other migratory populations. Practical advantages include that the use of
226 the LFA does neither requires specific training, expertise, electricity nor expensive equipment,
227 that assay devices may be stored without the need for refrigeration and that test results are
228 obtained almost instantaneously and by visual inspection with the unaided eye. Furthermore,
229 the components of the LFA are well-standardized which for instance is not the case with the
230 antigen used in the RBT that requires careful titration (Diaz-Aparazio et al., 1994; Blasco et
231 al., 1994). By using the LFA as a field test identification and tracing of animals and their
232 owners is much less problematic and intervening measures to control the disease could be
233 started without delay with less risk of further transmission and spread of the infection.
234 Importantly, all LFA test results were easy to read with the majority of the positive tests

235 reading 2+ or stronger and the test result may be shown to the owner of the animal while the
236 implications of the result is explained.

237 The antigen employed in the *Brucella* LFAs is a LPS extract prepared from *B. abortus*. LPS
238 is a dominant antigen in all smooth *Brucella* strains that are of economic importance in
239 livestock. The *Brucella* LPS is well-characterized and is formed by an O-chain consisting of an
240 *N*-formyl-persosamine homopolymer and a oligosaccharide core which is linked via a
241 diaminoglucose backbone to long acyl groups (Bundle et al., 1987; Caroff et al., 1984b;
242 Lapaque et al., 2005). The structure of the LPS in *Brucella* is conserved and the LPS of smooth
243 *B. abortus*, *B. melitensis* and *B. suis* strains all share common epitopes. Upon infection the
244 antibody response initially consists of IgM response which is almost immediately followed by
245 the production of IgG1 antibodies and at a later stage by small amounts of IgG2 and IgA
246 antibodies (Corbel, 1972; Beh, 1974; Allan et al., 1976; Levieux, 1978; Nielsen et al., 1984).
247 Because most cross-reacting antibodies resulting from exposure to other microorganisms such
248 as *Yersinia enterocolitica* serotype O:9 that have a structural very similar LPS are IgM, the
249 most important isotype for serological testing is IgG1 (Caroff et al., 1984a; Corbel, 1985).
250 Similar to other serological assays for brucellosis the *Brucella* LFAs are based on the detection
251 of IgG antibodies against smooth LPS antigen (Allan et al., 1976; Lamb et al., 1979; Nielsen et
252 al., 1984; Butler et al., 1986). Other *Brucella* species that do not contain significant amounts
253 of smooth LPS such as *B. ovis* and *B. canis* that are rough may require the use of a different
254 antigen (Blasco, 1990; Carmichael and Shin, 1996). Testing of serum samples from cattle and
255 pigs from The Netherlands, which were selected because of known non-specific serological
256 reactivity with *Brucella* antigens in reference tests for brucellosis showed some weak reactivity
257 in part of these samples. This reactivity was most likely due to immunological responses to
258 *Yersinia enterocolitica* O:9 exposure. Therefore, the specificity of the LFAs may be influenced
259 depending on the prevalence in the population of pathogens like *Yersinia enterocolitica* O:9.
260 Cross-reactivity due to exposure to these pathogens is a known limitation of serological testing
261 for brucellosis (Diaz-Aparicio et al., 1993; Hilbink et al., 1995; Weynants et al., 1996). Other
262 factors that could influence specificity are background antibody levels due to earlier exposure
263 or vaccination.

264 In summary, we have developed a set of simple and rapid field tests for the serodiagnosis
265 of brucellosis in livestock. The use of these bovine, caprine, ovine and swine *Brucella*
266 immunochromatographic lateral flow assays may have important advantages when testing in
267 remote areas where access to laboratory facilities is problematic and when testing animals
268 from nomadic and other migratory farmers.

269

270

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274

275

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277

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- 367

368 Table 1. **Test results of the *Brucella* lateral flow assay and of conventional tests for**
 369 **brucellosis for serum samples collected from different animal species send to a**
 370 **slaughterhouse because of suspicion of brucellosis.**

371

372 No. positive samples (% positive)

373

| 374 Species and sanitary status (Number) | Culture | RBT | CFT | LFA |
|--|---------------------|---------|---------|---------|
| 376 Bovine | | | | |
| 377 Infected (N=18) | 11 (92)* | 16 (89) | 15 (83) | 16 (89) |
| 378 Free (N=19) | NP [§] | 0 (0) | 0 (0) | 0 (0) |
| 379 | | | | |
| 380 Caprine | | | | |
| 381 Infected (N=20) | 8 (40) | 16 (80) | 15 (75) | 18 (90) |
| 382 Previously free (N=13) | 5 (38) | 6 (46) | 6 (46) | 11 (85) |
| 383 Free (N=15) | NP | 0 (0) | 0 (0) | 0 (0) |
| 384 | | | | |
| 385 Ovine | | | | |
| 386 Infected (N=22) | 12 (55) | 16 (73) | 18 (82) | 14 (64) |
| 387 Previously free (N=22) | 17 (77) | 19 (86) | 19 (86) | 19 (86) |
| 388 Free (N=24) | NP | 0 (0) | 0 (0) | 0 (0) |
| 389 | | | | |
| 390 Swine | | | | |
| 391 Infected (N=10) | 10 (100) | 8 (80) | 8 (80) | 8 (80) |
| 392 Free (N=10) | NP | 0 (0) | 0 (0) | 0 (0) |
| 393 Not specified (N=13) | 1 (13) [†] | 0 (0) | 0 (0) | 0 (0) |

394

395

396 *

397 [§]NP, not performed

398 [†]Culture was performed for eight animals

399 Table 2. **Test results of the *Brucella* lateral flow assay and conventional tests for**
 400 **brucellosis stratified according to result of RBT**

| | | No. positive samples (%) | | | |
|--------------------------------------|-----------------------|--------------------------|---------------|----------|--|
| Species and RBT test result (Number) | Culture | CFT | Culture + CFT | LFA | |
| Bovine | | | | | |
| RBT positive (N=16) | 11 (100) [*] | 15 (94) | 15 (94) | 15 (94) | |
| RBT negative (N=21) | 0 (0) [§] | 0 (0) | 0 (0) | 1 (5) | |
| Caprine | | | | | |
| RBT positive (N=22) | 13 (81) [†] | 21 (95) | 21 (95) | 22 (100) | |
| RBT negative (N=26) | 0 (0) [‡] | 0 (0) | 0 (0) | 7 (27) | |
| Ovine | | | | | |
| RBT positive (N=35) | 26 (74) | 31 (89) | 32 (91) | 31 (89) | |
| RBT negative (N=33) | 3 (33) [‡] | 1 (3) | 3 (9) | 2 (6) | |
| Swine | | | | | |
| RBT positive (N=8) | 8 (100) | 7 (88) | 8 (100) | 8 (100) | |
| RBT negative (N=25) | 3 (30) [‡] | 1 (4) | 3 (12) | 0 (0) | |

423 *Culture was performed for 11 animals

424 [§]Culture was performed for one animal

425 [†]Culture was performed for 16 animals

426 [‡]Culture was performed for 11 animals

427 [‡]Culture was performed for 9 animals

428 [‡]Culture was performed for 10 animals

429

430 Legend to the figure

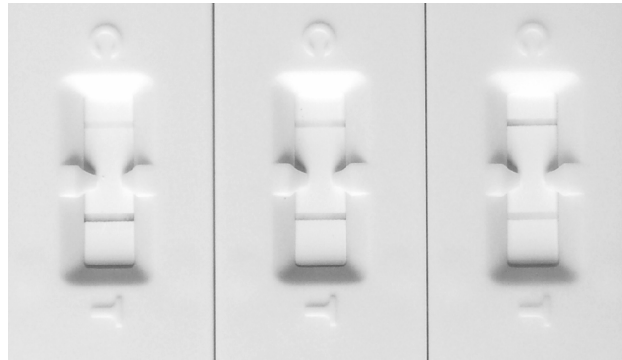
431

432 Figure 1. **Bovine *Brucella* lateral flow assay**

433 Assays run with serum samples from cows from herds infected with *Brucella* (upper panel) and
434 from herds free of brucellosis (lower panel). Results of the reference test and the lateral flow
435 assay are presented below each assay. RBT, Rose Bengal test; CFT, Complement fixation test;
436 LFA, lateral flow assay. np, not performed.

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Figure 1.



| | | | |
|---------|--------|--------|--------|
| Culture | pos | pos | pos |
| RBT | pos | pos | pos |
| CFT | >1:106 | >1:106 | >1:106 |
| LFA | 3+ | 3+ | 2+ |



| | | | |
|---------|------|------|------|
| Culture | np | np | np |
| RBT | neg | neg | neg |
| CFT | ≤1:4 | ≤1:4 | ≤1:4 |
| LFA | neg | neg | neg |