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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
1. Introduction

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

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

2. Materials and Methods
The *Brucella* LFAs consist of a porous nitrocellulose detection strip flanked at one end by a reagent pad and by an absorption pad at the other end. A sample application pad flanks the reagent pad in turn. The composite detection strip is contained in a plastic assay device with a round sample well positioned above the sample application pad and a test window positioned above the detection zone. The detection zone contains two distinct lines, a test line and a control line. The test line was obtained by spraying *Brucella*-specific antigen onto the nitrocellulose strip. A crude *Brucella* lipopolysaccharide (LPS) preparation prepared from *Brucella abortus* strain 1119-3 was used as antigen to capture specific serum antibodies (Smits et al., 1999; Smits et al., 2003). LPS is used as it is the optimal antigen in indirect antibody tests for brucellosis regardless of the serotype of the infecting strain and of the animal species (Diaz-Aparacio et al., 1994; Nielsen et al., 1996; Alonso-Urmeneta et al., 1998). The control line was obtained by spraying immunoglobulin G (IgG) antibodies from cattle, goat, ovine or swine to obtain detection strips for test devices for these four animal species. Test and control lines were sprayed using a BioDot Quanti 2000 BioJet. Detection reagents for the LFAs for the four animal species were prepared by conjugating affinity purified antibodies against cattle, goat, ovine and swine Ig (H+L) antibodies to 40nm colloidal gold particles. These conjugates were sprayed and dried onto the conjugate pads of the composite strips using the AirJet of the BioDot Quanti 2000 machine. The concentration and amount of LPS and of the conjugate applied to the test strips was optimized using panels of defined positive and negative control sera. The tests were performed by the addition of 5 µl serum to the sample pad of the assay device followed by the addition of 130 µl sterile running fluid consisting of phosphate-buffered saline, pH 7.6, containing 1.67% bovine serum albumin and 3% Tween 20. Test results are read after 10 min by visual inspection for staining of the test and control lines in the test window of the assay device. Tests are scored negative when no staining is observed at the test line and scored positive when the test line stains. The control line should stain in all cases. The test line may stain at different intensities depending on the titer of specific antibodies in the sample. Positive results may be subjectively rated 1+ when staining is weak, 2+ when staining is moderately strong, 3+ when staining is strong, and 4+ when staining is very strong. Assay devices sealed in a moisture resistant and airtight foil
containing some desiccant may be stored at 4-27ºC without loss of activity. The stain at the
test and control lines of exposed tests is stable after drying.

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

In addition, we tested 25 bovine and 25 swine serum samples from the serum bank of the
Central Institute for Animal Health (CIDC-Lelystad), The Netherlands. These samples had been
collected in the Netherlands, a country that is free of brucellosis and were selected because of
false-positive reactivity in routine serological tests for brucellosis. All selected bovine samples
from the CIDC-Lelystad reacted in the serum agglutination test (SAT) at a titre of between 1:15 and ≥1:120, 80% reacted in the Coombs test at a titre of between 1:20 and ≥1:200, and 25% reacted in IgG ELISA at a titre of between 1:100 and 1:3,200. All selected pig samples that were taken from the serum bank of this institute reacted in the SAT at a titre of between 1:15 and 1:60, 44% reacted in the Coombs test at a titer of 1:20, and 28% reacted in the RBT.

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

1. Results

Blood culture was attempted for twelve cows and all goats, sheep and pigs originating from infected herds and yielded positive results in 92, 40, 55 and 100% of these four animal species, respectively (Table 1). All isolates from cattle were identified as B. abortus biovar 3, those of goat and sheep as B. melitensis biovar 3, and those of pigs were classified as B. suis sp. Serological testing in RBT and CFT revealed the presence of antibodies against Brucella in 73-89% of the serum samples from animals originating from infected herds. B. melitensis biovar 3 could also be isolated from the blood of 38% of the goats and 77% of the sheep from herds that had been free of brucellosis before but included reactors upon recent testing. In
addition, *B. suis* sp. was cultured from the blood of one pig with a not further specified sanitary status. The RBT and CFT tests yielded positive results in 46% of the goat and in 86% of the sheep from herds that had become infected recently. All serum samples from herds with a sanitary status free of brucellosis and from the pig herds with a sanitary status that was not specified tested negative in these classical serological tests.

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

Based on the results obtained for animals with culture confirmed brucellosis the sensitivity of the LFAs were calculated to be 90% for the bovine *Brucella* LFA, 100% for the caprine *Brucella* LFA, 90% for the ovine *Brucella* LFA and 73% for the swine *Brucella* LFA. Culture has a limited sensitivity and CFT is often used in stead of culture or to confirm culture negative
animals. The sensitivity of the CFT did not differ much from that of the LFA; only for the bovine samples a higher sensitivity (100%) could be calculated for the CFT.

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

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

2. Discussion

The isolation and identification of *Brucella* bacteria offers a definite diagnosis of brucellosis. Based on the results for animals with culture confirmed brucellosis the sensitivity of the bovine *Brucella* LFA was calculated to be 90%, that of the caprine LFA 100%, that of the ovine LFA 77%, and that of the swine LFA 73%. No reactivity in the *Brucella* LFAs was observed for samples from cows, goat and sheep from herds from Portugal known to be free of brucellosis indicating a high (100%) specificity. The sensitivity of the CFT which is accepted as a confirmatory test for bovine brucellosis was somewhat higher than that calculated for the
bovine LFA. The validity of the CFT for testing other animal species for brucellosis is less well
established and it is of interest to note that the sensitivity calculated for the caprine, ovine and
swine LFAs were identical to that of the CFT. However, compared with the CFT a clearly higher
proportion of goats from infected herds and from herds that had become infected recently
tested positive in the LFA. The number of goats from these two groups that had a positive
result in the LFA was also higher than the number of animals from which the *Brucella*
pathogen could be isolated. It is well known that current tests for brucellosis in small
ruminants are not optimal and the use of a combination of tests for instance RBT and CFT is
recommended (Lilenbaum et al., 2007; Solorio-Rivera et al., 2007). Our results indicate that
the LFA is a simple, rapid and highly sensitive and specific alternative for the detection of
brucellosis in livestock, and that in particular the use of the LFA for caprine brucellosis may
help to improve the testing for brucellosis in this species. Laboratory testing for brucellosis in
swine is least well developed and also for this animal species the use of different tests is
recommended to increase the detection rate. Our results indicate that the LFA for swine
brucellosis performs similar to the RBT and CFT. Notably, the serum samples of three culture
positive pigs did not reacted in the LFA and of these only one tested positive in the CFT. It will
be worthwhile to investigate whether repeated testing of pigs could improve sensitivity.

The LFA has several practical advantages that allows testing on the spot and that may
make it the method of choice when testing animals in remote areas or when testing animals
from nomadic and other migratory populations. Practical advantages include that the use of
the LFA does neither requires specific training, expertise, electricity nor expensive equipment,
that assay devices may be stored without the need for refrigeration and that test results are
obtained almost instantaneously and by visual inspection with the unaided eye. Furthermore,
the components of the LFA are well-standardized which for instance is not the case with the
antigen used in the RBT that requires careful titration (Diaz-Aparazio et al., 1994; Blasco et
al., 1994). By using the LFA as a field test identification and tracing of animals and their
owners is much less problematic and intervening measures to control the disease could be
started without delay with less risk of further transmission and spread of the infection.
Importantly, all LFA test results were easy to read with the majority of the positive tests
reading 2+ or stronger and the test result may be shown to the owner of the animal while the
implications of the result is explained.

The antigen employed in the Brucella LFAs is a LPS extract prepared from B. abortus. LPS
is a dominant antigen in all smooth Brucella strains that are of economic importance in
livestock. The Brucella LPS is well-characterized and is formed by an O-chain consisting of an
N-formyl-persosamine homopolymer and a oligosaccharide core which is linked via a
diaminoglucose backbone to long acyl groups (Bundle at al., 1987; Caroff et al., 1984b;
Lapaque et al., 2005). The structure of the LPS in Brucella is conserved and the LPS of smooth
B. abortus, B. melitensis and B suis strains all share common epitopes. Upon infection the
antibody response initially consists of IgM response which is almost immediately followed by
the production of IgG1 antibodies and at a later stage by small amounts of IgG2 and IgA
antibodies (Corbel, 1972; Beh, 1974; Allan et al., 1976; Levieux, 1978; Nielsen et al., 1984).  
Because most cross-reacting antibodies resulting from exposure to other microorganisms such
as Yersinia enterocolitica serotype O:9 that have a structural very similar LPS are IgM, the
most important isotype for serological testing is IgG1 (Caroff et al, 1984a; Corbel, 1985).  
Similar to other serological assays for brucellosis the Brucella LFAs are based on the detection
of IgG antibodies against smooth LPS antigen (Allan et al., 1976; Lamb et al., 1979; Nielsen et
al., 1984; Butler et al., 1986). Other Brucella species that do not contain significant amounts
of smooth LPS such as B. ovis and B. canis that are rough may require the use of a different
antigen (Blasco, 1990; Carmichael and Shin, 1996). Testing of serum samples from cattle and
pigs from The Netherlands, which were selected because of known non-specific serological
reactivity with Brucella antigens in reference tests for brucellosis showed some weak reactivity
in part of these samples. This reactivity was most likely due to immunological responses to
Yersinia enterocolitica O:9 exposure. Therefore, the specificity of the LFAs may be influenced
depending on the prevalence in the population of pathogens like Yersinia enterocolitica O:9.
Cross-reactivity due to exposure to these pathogens is a known limitation of serological testing
for brucellosis (Diaz-Aparicio et al., 1993; Hilbink et al., 1995; Weynants et al., 1996). Other
factors that could influence specificity are background antibody levels due to earlier exposure
or vaccination.
In summary, we have developed a set of simple and rapid field tests for the serodiagnosis of brucellosis in livestock. The use of these bovine, caprine, ovine and swine *Brucella* immunochromatographic lateral flow assays may have important advantages when testing in remote areas where access to laboratory facilities is problematic and when testing animals from nomadic and other migratory farmers.

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a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis.


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

<table>
<thead>
<tr>
<th>Species and sanitary status (Number)</th>
<th>Culture</th>
<th>RBT</th>
<th>CFT</th>
<th>LFA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bovine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (<em>N</em>=18)</td>
<td>11 (92)*</td>
<td>16 (89)</td>
<td>15 (83)</td>
<td>16 (89)</td>
</tr>
<tr>
<td>Free (<em>N</em>=19)</td>
<td>NP⁵</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Caprine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (<em>N</em>=20)</td>
<td>8 (40)</td>
<td>16 (80)</td>
<td>15 (75)</td>
<td>18 (90)</td>
</tr>
<tr>
<td>Previously free (<em>N</em>=13)</td>
<td>5 (38)</td>
<td>6 (46)</td>
<td>6 (46)</td>
<td>11 (85)</td>
</tr>
<tr>
<td>Free (<em>N</em>=15)</td>
<td>NP</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Ovine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (<em>N</em>=22)</td>
<td>12 (55)</td>
<td>16 (73)</td>
<td>18 (82)</td>
<td>14 (64)</td>
</tr>
<tr>
<td>Previously free (<em>N</em>=22)</td>
<td>17 (77)</td>
<td>19 (86)</td>
<td>19 (86)</td>
<td>19 (86)</td>
</tr>
<tr>
<td>Free (<em>N</em>=24)</td>
<td>NP</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>Swine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected (<em>N</em>=10)</td>
<td>10 (100)</td>
<td>8 (80)</td>
<td>8 (80)</td>
<td>8 (80)</td>
</tr>
<tr>
<td>Free (<em>N</em>=10)</td>
<td>NP</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Not specified (<em>N</em>=13)</td>
<td>1 (13)†</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*NP, not performed

*Culture was performed for eight animals
Table 2. Test results of the *Brucella* lateral flow assay and conventional tests for brucellosis stratified according to result of RBT

<table>
<thead>
<tr>
<th>Species and RBT test result (Number)</th>
<th>Culture</th>
<th>CFT</th>
<th>Culture + CFT</th>
<th>LFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine RBT positive (N=16)</td>
<td>11 (100)*</td>
<td>15 (94)</td>
<td>15 (94)</td>
<td>15 (94)</td>
</tr>
<tr>
<td>Bovine RBT negative (N=21)</td>
<td>0 (0)†</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Caprine RBT positive (N=22)</td>
<td>13 (81)§</td>
<td>21 (95)</td>
<td>21 (95)</td>
<td>22 (100)</td>
</tr>
<tr>
<td>Caprine RBT negative (N=26)</td>
<td>0 (0)∫</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>7 (27)</td>
</tr>
<tr>
<td>Ovine RBT positive (N=35)</td>
<td>26 (74)</td>
<td>31 (89)</td>
<td>32 (91)</td>
<td>31 (89)</td>
</tr>
<tr>
<td>Ovine RBT negative (N=33)</td>
<td>3 (33)¶</td>
<td>1 (3)</td>
<td>3 (9)</td>
<td>2 (6)</td>
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<tr>
<td>Swine RBT positive (N=8)</td>
<td>8 (100)</td>
<td>7 (88)</td>
<td>8 (100)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Swine RBT negative (N=25)</td>
<td>3 (30)¶</td>
<td>1 (4)</td>
<td>3 (12)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Culture was performed for 11 animals
§Culture was performed for one animal
†Culture was performed for 16 animals
∫Culture was performed for 9 animals
¶Culture was performed for 10 animals
Figure 1. **Bovine *Brucella* lateral flow assay**

Assays run with serum samples from cows from herds infected with Brucella (upper panel) and from herds free of brucellosis (lower panel). Results of the reference test and the lateral flow assay are presented below each assay. RBT, Rose Bengal test; CFT, Complement fixation test; LFA, lateral flow assay. np, not performed.
### Figure 1

<table>
<thead>
<tr>
<th>Test Type</th>
<th>1st Result</th>
<th>2nd Result</th>
<th>3rd Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>pos</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>RBT</td>
<td>pos</td>
<td>pos</td>
<td>pos</td>
</tr>
<tr>
<td>CFT</td>
<td>&gt;1:106</td>
<td>&gt;1:106</td>
<td>&gt;1:106</td>
</tr>
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