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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 sent 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

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 µl serum to  
 78 the sample pad of the assay device followed by the addition of 130 µl 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

containing some desiccant may be stored at 4-27°C 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 include 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  $\geq 1:120$ , 80% reacted in the Coombs test at a titre of between 1:20 and  $\geq 1:200$ , and 25% reacted in IgG ELISA at a titer 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  $\geq 50\%$  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 18-24°C.

## 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 known 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-pesosamine homopolymer and a oligosaccharide core which is linked via a diaminoglucose backbone to long acyl groups (Bundle et 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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Table 1. **Test results of the *Brucella* lateral flow assay and of conventional tests for brucellosis for serum samples collected from different animal species send to a slaughterhouse because of suspicion of brucellosis.**

		No. positive samples (% positive)			
Species and sanitary status (Number)	Culture	RBT	CFT	LFA	
Bovine					
Infected (N=18)	11 (92)*	16 (89)	15 (83)	16 (89)	
Free (N=19)	NP <sup>\$</sup>	0 (0)	0 (0)	0 (0)	
Caprine					
Infected (N=20)	8 (40)	16 (80)	15 (75)	18 (90)	
Previously free (N=13)	5 (38)	6 (46)	6 (46)	11 (85)	
Free (N=15)	NP	0 (0)	0 (0)	0 (0)	
Ovine					
Infected (N=22)	12 (55)	16 (73)	18 (82)	14 (64)	
Previously free (N=22)	17 (77)	19 (86)	19 (86)	19 (86)	
Free (N=24)	NP	0 (0)	0 (0)	0 (0)	
Swine					
Infected (N=10)	10 (100)	8 (80)	8 (80)	8 (80)	
Free (N=10)	NP	0 (0)	0 (0)	0 (0)	
Not specified (N=13)	1 (13) <sup>†</sup>	0 (0)	0 (0)	0 (0)	

\*

<sup>\$</sup>NP, not performed

<sup>†</sup>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**

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

\*Culture was performed for 11 animals

<sup>§</sup>Culture was performed for one animal

<sup>†</sup>Culture was performed for 16 animals

<sup>‡</sup>Culture was performed for 11 animals

<sup>‡</sup>Culture was performed for 9 animals

<sup>‡</sup>Culture was performed for 10 animals

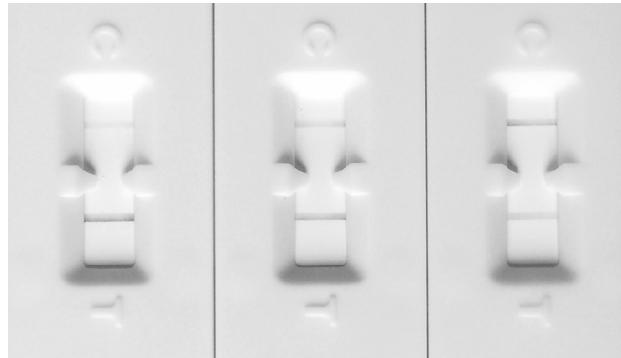
430 Legend to the figure

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.

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	$\leq 1:4$	$\leq 1:4$	$\leq 1:4$
LFA	neg	neg	neg