Vet. Res. 34 (2003) 297–305
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DOI: 10.1051/vetres:2003005

Original article

Evaluation of a modified Rose Bengal test and an indirect Enzyme-Linked Immunosorbent Assay for the diagnosis of *Brucella melitensis* infection in sheep

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(Received 9 August 2002; accepted 9 December 2002)

Abstract – A modified Rose Bengal test (mRB) and an indirect ELISA (iELISA) with Protein G as the conjugate, were evaluated for the diagnosis of *Brucella melitensis* infection in unvaccinated sheep with a known bacteriological status, and their diagnostic efficacy was compared with that of the standard Rose Bengal (RB) and Complement Fixation (CF) tests used in the current eradication campaign in EU countries. All tests showed 100% specificity when testing the sera from 212 *Brucella*-free sheep. When testing the sera from 219 *Brucella melitensis* culture-positive sheep, both the mRB and iELISA tests were more sensitive (98.6% and 96.8%, respectively) than the RB and CF tests (95.0% and 92.7%, respectively). These results were similar when testing the sera from 181 animals belonging to infected flocks but found bacteriologically negative, suggesting that the mRB or iELISA tests could advantageously replace the current RB procedure used as the screening test.

sheep / *Brucella melitensis* / serological diagnosis / modified rose bengal test / indirect ELISA

1. INTRODUCTION

Brucellosis is an important infectious disease mainly affecting cattle, sheep and goats. The disease in cattle is most commonly caused by *Brucella abortus* whilst brucellosis in sheep and goats, a disease highly prevalent in southern EU countries, is caused by *Brucella melitensis*, a very important zoonotic agent [12, 15, 25, 29].

The diagnostic method known to produce the best results in terms of specificity is the isolation of *Brucella* organisms from the suspected animal. However, this method has a limited sensitivity, is expensive and cumbersome and has the added difficulty of being unpractical to apply at a large scale in control campaigns. Accordingly, the indirect diagnosis of disease based on serological tests is of choice in the eradication programmes. The standard Rose Bengal (RB) and Complement Fixation (CFT) tests are the main serological tests used to detect antibodies against

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B. abortus and *B. melitensis* infections. Both tests have been used for several decades, proving to be successful for eradicating bovine brucellosis in some countries, and are the official tests currently used in the EU for the eradication of *B. melitensis* infection in small ruminants. Nevertheless, there is evidence that both tests are significantly less effective for the diagnosis of brucellosis in sheep and goats than in cattle [12, 15].

The EU rules consider that the best strategy for diagnosing sheep and goat brucellosis is the combined use of RB as the screening test and the CF as the confirmatory test. However, it has been reported that a relatively high proportion of sheep and goats from *B. melitensis*-infected flocks show negative results in the standard RB test but positive in the CF test, questioning the sensitivity of the former as a screening test [4]. Thus, the simultaneous use of both is recommended to obtain a maximal sensitivity [4, 12]. To improve the sensitivity of the RB test a simple modification consisting in increasing the volume of sera to be tested has been recommended [4, 5]. This RB modified test (mRB) significantly increases the sensitivity of the RB standard procedure and considerably reduces the problem of sera being RB negative but CF positive (Blasco J.M., personal communication). During recent years, different indirect Enzyme-Linked Immunosorbent Assays (iELISAs) have been developed using more or less purified S-LPS as the antigen and have been reported to be at least as sensitive and specific as the combination of both RB and CF tests for the diagnosis of brucellosis in ruminants [1, 6, 11, 17–19, 22–24, 28, 30]. In fact, an iELISA with polyclonal conjugate (anti-IgG H+L) has been reported to be effective enough for the diagnosis of the infection in sheep and goats [5, 7, 14]. However, one of the problems of this test is that the conjugate used results in a high background reactivity when testing sera from *Brucella*-free animals, reducing the specificity of the test [14]. The use of Protein G as the conjugate reduces this problem increasing ELISA specificity [9, 22], with the additional advantage of protein G being a suitable reagent in enzyme immunoassays designed for all domestic ruminant species [22].

In Portugal, a complex strategy with the sequential or parallel use of the RB and CFT was established, depending on the sanitary classification of the flock (Norms for the Laboratory Analysis of Eradication Plans for Brucellosis, Agriculture Ministry, DGV, Circular No. 80/DSSA, 14/06/00). In the case of *Brucella*-Free flocks, confirmation of each positive sera in the RB test has to be done by the CF test. In *Brucella* infected flocks, however, the RB test is used directly as a confirmatory test. If herds being tested for a long time (more than 24 months) remain having RB positive animals, all sera being RB negative are also tested by the CF test. These procedures and test combinations complicate a lot the official eradication campaign and considerably increase their costs, justifying the search of tests of increased sensitivity and/or specificity. The aims of the present work were: (i) to compare the diagnostic performance of both mRB and iELISA / protein G in sheep with a known bacteriological status with that of the standard RB and CF official tests in the EU, and (ii) to determine the diagnostic performance of both tests in infected flocks having animals positive in the classical RB and/or CF test, but in which *B. melitensis* could not be isolated.

2. MATERIALS AND METHODS

2.1. Samples

The sheep sera used in this study were collected at slaughter from 400 unvaccinated animals belonging to several flocks in which *B. melitensis* infection was bacteriologically proven. Fragments from the liver, spleen, uterus (when the animals

were pregnant) and the whole submaxillary and supramammary lymph nodes were collected to assess the bacteriological status of the animals.

Samples of the intestine (ileon) were also collected from all the animals for the assessment of the eventual existence of the cross-reacting bacteria such as *Escherichia coli* O:157, *Salmonella* spp. and *Yersinia enterocolitica* O:9. The procedures and culture media used for this purpose have been described elsewhere [3, 8, 27].

Additionally, sera from 212 sheep belonging to *Brucella*-free flocks were collected as negative controls.

2.2. Bacteriological procedures

Culture and identification of *Brucella* spp. was carried out by standard procedures. Briefly, each sample was homogenised in sterile tryptose saline solution and 0.2 mL of each homogenate was smeared on each of 6 plates of the modified Farrell medium [10]. This medium was prepared with tryptose agar (Difco, Detroit, Michigan, USA) with 5% sterile equine serum and a commercial freeze-dried selective supplement (Oxoid**,** DuPont, Qualicon Inc., USA). In 1 L of the medium there was nalidixic acid 5 mg, bacitracin 25 000 IU, natamycin 50 mg, polymixin B sulphate 5 000 IU, vancomycin 20 mg and nystatin 100 000 IU. The plates were incubated for up to 10 days, at 37 °C, half of them in a normal atmosphere and the other half in a 5% CO_2 atmosphere. The presumptive identification of *Brucella melitensis* was conducted on the basis of colony morphology, Gram staining and biochemical tests (CO_2 requirement, catalase, oxidase, urease, dye sensitivity and H_2S production). Biovars were identified by agglutination with monospecific anti-A and anti-M sera [2].

According to the bacteriological results, the following groups of sera were obtained:

(i) *The infected group* composed of sera from 219 sheep in which *B. melitensis* was isolated.

(ii) *The suspected group* composed of sera from 181 sheep that were culture-negative but belonged to flocks in which *B. melitensis* was confirmed bacteriologically.

(iii) The *Brucella-free group* composed of sera from 212 sheep belonging to *Brucella*-free flocks.

2.3. Serological tests

2.3.1. Antigen Preparations

The antigens used for the Rose Bengal and Complement Fixation tests were prepared according to Hendry et al. [13], from *Brucella abortus* biovar 1, strain 99 (Weybridge, United Kingdom) and standardised according to EU requirements. For ELISA, the crude smooth Lipopolysaccharide (S-LPS) extract was used, prepared from *Brucella abortus* biovar 1, strain 99 (Weybridge, United Kingdom) by a phenol-water extraction according to Hendry et al. [13].

2.3.2. Rose Bengal (RB) test

The RB test was performed, following the procedure described by Alton et al. [2]. The plates were shaken for 4 min and any agglutination that appeared within this time was recorded as a positive reaction.

2.3.3. Modified Rose Bengal (mRB) test

This test was performed following the procedure described by Blasco et al. [4], mixing 75 μL of sera and 25 μL of the antigen. The plates were shaken for 4 min and any agglutination that appeared within this time was recorded as a positive reaction.

2.3.4. Complement Fixation (CF) test

The CFT was performed on a microplate, following the "warm" procedure described by Alton et al. [2].

The sera were considered positive if they showed at least 50% hemolysis at a 1/4 dilution (i.e. ≥ 20 ICFTU).

2.3.5. Indirect ELISA (iELISA)

The iELISA was performed as described previously [12, 14] with some modifications. Briefly, standard 96-well polystyrene plates (Dynatech M129B) were coated at 4 °C overnight with S-LPS (0.4 μg/mL; 100 μL per well) suspended in 10 mM Phosphate Buffered Saline (PBS) (pH 7.2). Non-absorbed antigen was removed by four washings with PBS containing 0.05% Tween-20 (PBST). The sera were diluted to 1/200 in PBST. 100 μL of each dilution were added in duplicate and the plates were incubated for 1 h at 37 °C and were washed four times with PBST. The protein G conjugate (0.125 μg/mL, P-8170; Sigma, St. Louis, MO, USA) was added (100 μL per well) and the plates were incubated for 1 h at 37 °C and washed four times with PBST. Finally, 100 μL of the substrate solution (10 mg *o*-phenylenediamine (OPD, P-8287, Sigma, St. Louis, MO, USA)) in 25 mL of 0.05 M Phosphate-Citrate Buffer pH 5.0, with 10 μL of H_2O_2) were added and the optical density (OD) was determined at 492 nm in a Dynex MRX Microplate reader (Dinex Technologies Inc., Chantilly, VA, USA) after 15 min of incubation at room temperature in the absence of light. Two positive and two negative sera control tests were repeated in all plates.

The optimal antigen concentration and conjugate dilution were determined in a preliminary experiment by testing 20 sera from culture-positive animals and 20 sera from *Brucella*-free animals. The optimal serum dilution was determined by testing positive sera from *Brucella* culture-positive animals showing 213 ICFTU (International Complement Fixation Test Units) and negative sera from *Brucella*-free sheep, both diluted in PBST from 1/100 to 1/102 400.

2.4. Statistics

Data were compiled in a database and separated into negative or positive according to the results of the different serological tests.

For iELISA, the optimal cut-off value was determined by receiver operating characteristics (ROC) analysis (*Win Episcope*, version 2.0).

Test agreement (Kappa values) was calculated using standard procedures (*Win Episcope*, version 2.0).

3. RESULTS

3.1. Bacteriological results

A total of 219 animals out of the 400 examined were culture positive. The isolated strains corresponded to *Brucella melitensis* biovar 3 in all cases. There was no isolation of cross-reacting bacteria in any of the corresponding 400 intestinal samples examined bacteriologically.

3.2. Serological results

The distribution of the OD values of the *Brucella melitensis* culture-positive and *Brucella* free populations in the iELISA is represented in Figure 1. As it can be seen, the *B. melitensis* infected animals were not fully discriminated from the *Brucella*-free ones. However, the mean OD value ± SD for the sera from the *Brucella*-free animals was 0.060 ± 0.046 and this value was 1.675 ± 0.562 for the sera from *Brucella melitensis* culture-positive animals.

The optimal cut-off value after ROC analysis (resulting in 96.8% sensitivity and 100% specificity) was 0.250. The same OD distribution for the animals from the suspected group is also represented in Figure 1 and as it can be seen, this OD distribution was practically identical to that described for the culture positive animals.

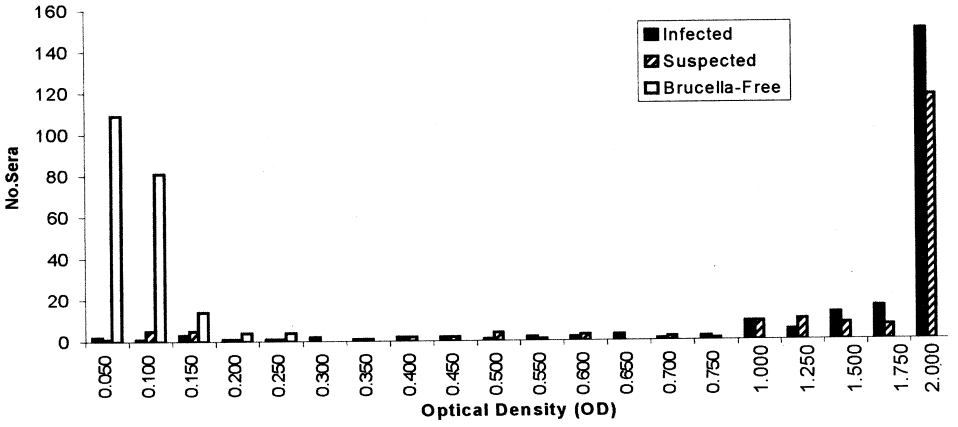

Figure 1. Distribution of iELISA OD values when testing the 219 sera from *B. melitensis*-infected animals, the 181 sera from suspected sheep and 212 sera from *Brucella*-free animals.

For comparative purposes, the sensitivity was calculated as the percentage of culture positive animals identified as positive in a given test and the specificity was defined as the percentage of *Brucella*-free animals identified as negative in the corresponding test. The best sensitivity results (Tab. I; group I) were obtained with the mRB (98.6%) and iELISA (96.8%). Both the CF and the standard RB were significantly ($P < 0.005$) less sensitive (92.7% and 95.0%, respectively) than both mRB and iELISA. All tests resulted in 100% specificity when testing the sera from the animals belonging to the *Brucella*-free flocks (Tab. I; group III animals).

The results in terms of apparent sensitivity (i.e., the percentage of suspected animals with positive results in a given test) obtained in the different tests with sera from the 181 culture negative animals belonging to infected flocks (suspected group II) are also presented in Table I. The apparent sensitivity of both mRB and iELISA were identical (92.8%) and in both cases significantly higher ($P < 0.005$) than that of both the standard RB and CF tests (87.8% and 87.3%, respectively).

Table I. Results of the serological tests when testing sera from *B. melitensis* infected (Group I), suspected (Group II), and *Brucella*-free (Group III) sheep.

Groups	No. animals	RBm		iELISA[a]		RB[a]		CFT[a]	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
I									
Infected	219	216[a]	3	212	7	208	11	203	16
		(**98.6**)	(**1.4**)	(**96.8**)	(**3.2**)	(**95.0**)	(**5.0**)	(**92.7**)	(**7.3**)
II									
Suspected	181	168	13	168	13	159	22	158	23
		(**92.8**)	(**7.2**)	(**92.8**)	(**7.2**)	(**87.8**)	(**12.2**)	(**87.3**)	(**12.7**)
III									
Brucella-free	212	0	212	0	212	0	212	0	212
			(**100**)		(**100**)		(**100**)		(**100**)

[a] Number (%). (RB: standard Rose Bengal test; mRB: modified Rose Bengal test; iELISA: indirect ELISA with S-LPS as the antigen and protein G as the conjugate; CFT: Complement Fixation test).

Table II. Agreement (Kappa values) between the several serological tests when testing sera from culture positive (n = 219) and *Brucella*-free animals (n = 212).

	CFT +	CFT –	Kappa	RBm +	RBm –	Kappa	iELISA +	iELISA –	Kappa
RB +	197	11	0.921	208	0	0.963	205	3	0.954
RB –	6	217		8	215		7	216	
RBm +	202	14	0.930						
RBm –	1	214							
iELISA +	201	11	0.940	211	1	0.972			
iELISA –	2	217		5	214				

Table III. Agreement (Kappa values) between the several serological tests when testing sera from the suspected animals (n = 181) and *Brucella*-free animals (n = 212).

	CFT +	CFT –	Kappa	RBm +	RBm –	Kappa	iELISA +	iELISA –	Kappa
RB +	147	12	0.878	159	0	0.953	154	5	0.901
RB –	11	223		9	225		14	220	
RBm +	152	16	0.885						
RBm –	6	219							
iELISA +	155	13	0.916	163	5	0.948			
iELISA –	3	222		5	220				

The Kappa values of the different tests were calculated when testing the sera from culture positive and *Brucella*-free animals (Tab. II) and from suspected and *Brucella*-free animals (Tab. III). The agreement between the tests was generally high. When comparing culture positive and *Brucella*-free animals, iELISA and mRB showed the highest agreement (0.972; Tab. II). The highest agreement (Kappa = 0.953) when comparing the suspected and *Brucella* free animals was found among RB and mRB tests (Tab. III).

4. DISCUSSION

As in other EU countries [4], in the current *B. melitensis* eradication programme conducted in Portugal, a relatively high percentage of sheep which are negative in the RB screening test but positive in the CF confirmatory test has been identified. As seen in Tables II and III, figures as high as 3% (in infected animals) and 7% (in suspected animals) resulted in RB negative but CF positive. In fact, several EU countries including Portugal are simultaneously applying both tests to increase sensitivity in the current *B. melitensis* eradication programme. This strategy can lead to a considerable lack of specificity in countries using Rev1 vaccination in replacement animals or affected by the false positive serological reactions due to cross-reacting bacteria, a problem that also seems to affect sheep and goats [12, 16]. Moreover, this test combination is very expensive, time consuming and represents a complication of importance for most EU countries applying the official *B. melitensis* eradication programme in small ruminants.

The RB plate agglutination test antigen used in the EU is standardised without reference to the cell concentration, in such a way that a positive reaction can be

achieved at a dilution of 1/47.5 with the OIEISS reference serum and a negative reaction can be obtained at a dilution of 1/55 of this same serum [26].

In fact, it has been clearly shown that this antigen standardisation procedure significantly limits the sensitivity of the RB test in sheep and goats and explains the existence of many animals that are negative in the RB test but positive in the CF test, particularly in moderate to low prevalence conditions [4]. Suitable alternatives could be based on increasing the sensitivity limits against the OIEISS without affecting the specificity or, alternatively, modifying the antigen standardisation procedure using a wide sera panel of *B. melitensis* culture-positive and *Brucella*-free animals rather than a single reference serum [4]. Moreover, the current standard RB test procedure using equal volumes of serum and antigen is considered to be suitable (despite no rigorous existing evidence) for the diagnosis of *B. abortus* infection in cattle, but is not sensitive enough for *B. melitensis* diagnosis in sheep [4]. In fact, these authors described a modified RB test procedure (mRB), using three volumes of sera (75 μL) and one (25 μL) of the RB antigen, that significantly increased the sensitivity of the European RB antigens without affecting their specificity. In agreement with this finding, in the particular epidemiological conditions of Portugal, this simple RB test modification significantly increased the sensitivity of the RB test without affecting the specificity (Tab. I). Moreover, this modification significantly reduced the number of negative sera in the RB test but positive in the CF test (Tabs. II and III). All tests evaluated showed 100% specificity when testing sera from *Brucella* free animals and, in general, a high sensitivity for detecting culture-positive animals. The mRB and iELISA were the most sensitive tests and gave the higher proportion of agreement (Tab. II). The CF was the less sensitive test (92.7%) followed by the standard RB (95%). These results are in agreement with those previously reported by others [4, 12]; and seriously question the value of the current diagnostic strategy applied for eradicating *B. melitensis* in sheep and goats in the EU countries. Considering the higher sensitivity of the mRB with respect to the RB and that the problem of RB negative but CF positive sera was almost totally abrogated by the use of mRB (only one of 219 sera from culture positive sheep was mRB negative but CF positive, Tab. II), this simple RB test modification could help to reduce costs and complications in the current eradication campaigns conducted in the EU countries. However, like the standard RB, the mRB test has some degree of subjectivity, is slow and cumbersome to perform and not easily automated. Thus, the search for alternative screening tests is recommended with the iELISA being one of the best candidates. The sensitivity obtained (96.8%) with our iELISA was somewhat lower than that reported by others [1, 23]. Moreover, and in contrast with other experiments resulting in a better discrimination between the culture positive and *Brucella*-free populations [9, 12, 21], our sera from culture-positive animals were not clearly discriminated from those of *Brucella*-free sheep (Fig. 1). Small differences in the test reagents and standardisation conditions could account for these small discrepancies. The similar sensitivity results obtained with the mRB and iELISA suggest that either of these tests could be used in replacement of the standard RB test.

For culture negative animals belonging to infected flocks (suspected group II), the results were somewhat different to those obtained with culture positive animals (Tabs. I, II and III). Again, the mRB and iELISA showed the same sensitivity and were more sensitive than both standard tests in these suspected sera. The existence of serologically positive but culture negative sheep is a relatively frequent finding in infected flocks. In the case of Rev 1 vaccinated animals, this positive serological response in the absence of infection could be due to a secondary immunological response as a consequence of antigenic

contacts with field *B. melitensis* strains. However, vaccination was not performed in any of the flocks selected in our study. The existence of false positive serological reactions, a phenomenon of increasing importance in small ruminants [12], could also be another possibility. However, no relevant cross-reacting bacteria were isolated, even in the cultured intestinal samples of the 400 slaughtered sheep. Accordingly, a low level of sensitivity of the bacteriological procedures used could be the most probable reason to explain the existence of these 181 serologically positive but culture negative animals belonging to infected flocks. For a proper sensitivity for the isolation of *B. melitensis* from sheep, many organs and lymph nodes have to be sampled [20] and in our study only a few were sampled. Accordingly, most of these 181 suspected animals could have had true infections that were not properly detected. In fact, the serological results in these suspected animals were highly correlated with those obtained in culture positive sheep (Tabs. II and III; Fig. 1).

In conclusion, the mRB test could be a simple and economic alternative to advantageously replace the standard RB test procedure for the diagnosis of *B. melitensis* infection in the current epidemiological conditions of Portugal and many other EU countries.

In countries in which no vaccination programmes are being used, the CF test could be replaced by either the mRB or iELISA to increase diagnostic sensitivity.

ACKNOWLEDGEMENTS

The authors wish to thank Teresa Albuquerque and Patricia Temudo, from the Bacteriological Department of LNIV, Portugal.

This work was supported by a grant from the Instituto Nacional de Investigação Agrária (INIA) -PAMAF 3002. Ana Cristina Ferreira and Regina Cardoso were supported by a research-training grant from this same Institute.

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