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Development and Validation of a Competitive ELISA Kit for the Serological Diagnosis of Ovine, Caprine and Bovine Brucellosis

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Summary

A competitive ELISA (*Brucella*-Ab c-ELISA) was standardized and validated for the detection of Brucella antibodies in cattle, sheep and goat sera using a monoclonal antibody (MAb 4B5A) produced against *Brucella melitensis* biotype 2. The specificity and sensitivity of the assay were 100% to a 67.5% cut-off point (B/Bo%). When compared with an indirect ELISA, the *Brucella*-Ab c-ELISA did not demonstrate cross-reactions when testing positive sera for antibodies to some Enterobacteriaceae. A comparison was made between the *Brucella*-Ab c-ELISA and the complement fixation and Rose Bengal tests. Results demonstrated that the *Brucella*-Ab c-ELISA is a valuable tool for the serological diagnosis of bovine and ovine/caprine brucellosis.

Introduction

The Rose Bengal test (RBT) and complement fixation test (CFT) are commonly used for the routine serological diagnosis of ovine/caprine and bovine brucellosis (Davies, 1971; Anon, 1992, 1994; ; Anon, 1995; Corbel, 1997; Lucero et al., 1999). Although the two diagnostic methods are highly sensitive and specific, additional tests are required to clarify unspecific reactions caused by the presence of antibodies against the cross-reactive Enterobacteriaceae (Bundle et al., 1984; Nielsen and Duncan, 1990; Diaz-Aparicio et al., 1993; Gall et al., 1998; Kittelberger et al., 1998; Samartino et al., 1999; Erdenebaatar et al., 2003). This need has become more obvious in the final stages of the brucellosis eradication programmes, especially where RBT is used as a screening test for its high sensitivity and CFT is used as a confirmatory test for its high specificity.

When the prevalence of the disease diminishes, the probability of identifying a true positive case decreases because the number of cases is too small to be detected. To overcome this disadvantage, a highly sensitive and specific competitive ELISA (c-ELISA) was developed, using a monoclonal antibody that was capable of revealing antibodies against the various strains of *Brucella* spp., but no other micro-organisms that are notorious for being cross-reactive.

Material and Methods

Monoclonal antibodies

Monoclonal antibodies (MAbs) against *Brucella* spp. were produced by immunizing Balb/c mice, with *Brucella melitensis* biotype 2 that had been heat inactivated and ultrasonicated. An emulsion of bacterial proteins suspended in phosphate buffered saline (PBS) 0.01 M, pH 7.2 and complete Freunds adjuvant (CFA) was inoculated by the intraperitoneal route, followed by the administration of incomplete Freunds adjuvant (IFA) (Harlow and Lane, 1988).

The splenocytes of killed animals were used for cell fusion with the mouse cell line myeloma Sp2/O-Ag-14. Hybridomas were cultivated for 2 weeks, as described by Lelli et al. (Lelli et al., 2003). The MAb-secreting hybridomas against *B. melitensis* biotype 2 were cloned in accordance with the limiting-dilution method (Campbell, 1987; Goding, 1993; Lelli et al., 2003).

Monoclonal antibodies-secreting hybridomas were screened by indirect ELISA (i-ELISA) (Malavasi and Bargellesi-Sevesi, 1992) using microplates coated with *B. melitensis* biotype 2. Cross-reactions with other *Brucella* strains were monitored, namely: *B. melitensis* biotype 1, *B. melitensis* Rev. 1 strain, *B. abortus* biotypes 1 (S19, S99), 2, 3 and 6, *B. suis* biotype 1 and *B. ovis* (all supplied by the *Brucella* Reference Culture Collection, Weybridge, GB), *B. abortus* S99 and certain Enterobacteriaceae lipopolysaccharides (LPS) (*Salmonella enteritidis*, *Salmonella typhimurium*, *Escherichia coli* and *Yersinia enterocolitica* O:9) produced following the same procedure used for *B. abortus* LPS described in the next paragraph; in this regard, *B. ovis* cross-reactions were evaluated even if it belongs to the rough form, to include all *Brucella* species of animal concern.

The MAb isotype was determined by ELISA using the ImmunoPure® monoclonal antibody isotyping kit I (Pierce Biotechnology, Rockford, IL, USA). MAbs (isotype IgG) were purified using affinity chromatography with protein A (Hermanson et al., 1992; Ansari and Chang, 1993) and conjugated with peroxidase as described in the literature (Morgan-Capner et al., 1979).

Preparation of the *Brucella abortus* lipopolysaccharide

Smooth lipopolysaccharide (S-LPS) used for the coating of ELISA microplates was prepared by a phenolic extraction of *B. abortus* biotype 1 S99 (Nakane and Kawaoi, 1974; Hendry et al., 1985). *Brucella abortus* biotype 1 S99 was cultivated in brain heart infusion agar (BHIA). The bacterial mixture recovered was centrifuged at 4000 ***g*** for 20 min and the pellet re-suspended in sterile deionized water. The pellet was added to 85% phenol, pre-heated at 66–70°C and incubated for 20 min. The phenol extract was recovered after centrifugation

at 20 000 ***g*** for 20 min at 4°C. After diluting 1 : 10 with deionized water, the phenol extract was dialysed in a physiological NaCl-solution. The S-LPS was precipitated with three volumes of methanol/sodium acetate and incubated overnight at −20°C. The S-LPS pellet was re-suspended in sterile deionized water and stored at −80°C.

Competitive ELISA versus *Brucella* spp. (*Brucella*-Ab c-ELISA)

The 96-well ELISA microplates were coated with 100 μl S-LPS per well at the dilution of 1 : 3500 in carbonate/bicarbonate buffer (50 mM pH 9.6), incubated overnight at room temperature (RT) and washed three times with PBS containing 0.05% Tween 20 (PBST). About 200 μl blocking solution of PBST containing 1% yeast extract was added to the microplates that were then incubated for 1 h at RT. After the PBST washing, 50 μl serum samples, 50 μl positive bovine serum control and 50 μl negative bovine serum control were added to the wells and the microplates were incubated for 1 h at RT. Positive ovine and bovine control sera were obtained from animals experimentally infected with *B. melitensis* biotype 2, while negative ovine and bovine control sera were obtained from animals tested by RBT, CFT and *Brucella*-culture. After incubation, microplates were washed with PBST and 50 μl MAb-HRP (MAb 4B5A) (anti-*Brucella*-LPS), diluted 1 : 45 000 in PBS, were dispensed into the wells. The microplates were incubated again for 1 h at RT. After further PBST washings, 100 μl of chromogen substrate were dispensed into each well (3.3′, 5.5′-tetramethylbenzidine liquid substrate system for ELISA) and the plates incubated at RT for 30 min. The colorimetric reaction was blocked with 50 μl of sulphuric acid 0.5 N and the optical density (OD) measured at 450 nm with a microplate reader.

Standardization and validation of the *Brucella*-Ab c-ELISA

The assay was standardized using 12 sera from infected sheep, goats and cattle to reveal the presence of *Brucella* antibodies and an equal number of negative sera from animals from free flocks and herds. Positive sera from animals infected experimentally with the following microorganisms were tested: *B. melitensis* biotypes 1, 2, 3, *B. melitensis* Rev. 1, *B. abortus* (S99), *S. enteritidis*, *S. abortus ovis*, *Y. enterocolitica* O:9 and *E. coli*.

An intralaboratory *Brucella*-Ab c-ELISA validation was then performed by testing 1127 sheep and goat sera and 1013 cattle sera. The sera were screened using CFT and RBT in parallel (Hendry et al., 1985; Alton et al., 1988; Anon, 1992, 1994, 1995); *Brucella*-culture was then used in series with both CFT and RBT as a gold standard. Following the screening, 856 sheep and goat samples from brucellosis-free flocks/herds tested negative whilst 271 tested positive (of these, 141 were from infected herds and 130 from animals experimentally infected with *B. melitensis* biotype 2); moreover, 265 cattle samples tested positive (all coming from infected herds) and 748 tested negative (all coming from brucellosis-free herds).

Validation of statistical tests

Based on established values of diagnostic sensitivity and specificity, the optimal cut-off value (0,675) was determined using receiver operative curves (ROC) (Siegel and Castellan, 1988; Gardner and Greiner, 2000), with the OD values of the analysed sera normalized with the OD MAb-HRP control value (B/Bo). The cut-off value obtained was then used to calculate the positive/negative threshold for each microplate by multiplying the mean OD value of the MAb-HRP control by the ROC cut-off value.

Serum samples were classified as positive if their OD value was below the positive/negative threshold, and as negative if the OD value was greater or equal to the positive/negative threshold. Internal quality control was performed on each test by comparing the mean OD values of the positive and negative control sera with the positive/negative threshold. The agreement between the *Brucella*-Ab c-ELISA and the CFT and RBT was assessed using Cohen's Kappa (Schoerner et al., 1990).

Results

Monoclonal antibodies

Ten *B. melitensis* biotype 2-specific MAbs cell lines were obtained. MAbs cross-reacted with the other *Brucella*, with the exception of *B. ovis*. In Table 1 the MAbs cross-reactions using the indirect ELISA are shown; the MAbs were considered cross-reactive when the value was greater than 10%. Six did not cross-react with the Enterobacteriaceae tested with the i-ELISA. From these six MAbs, MAb 4B5A, isotype IgG2a and kappa-light chain, was selected as it is the one which best discriminates between positive and negative sera in *Brucella*-Ab c-ELISA. MAb 4B5A was conjugated with peroxidase and called anti-*Brucella*-LPS MAb.

Brucella-Ab c-ELISA

Brucella-Ab c-ELISA results, compared with CFT and RBT results on tested sera are shown in Table. 2 and 3.

The cut-off value, based on results obtained from sheep, goat and cattle sera, was optimized using the ROC analysis to the value of 0.675 (B/Bo% = 67.5) against established levels of sensitivity and specificity (Figs 1 and 2). Sensitivity and specificity were 100% for the sheep and goat sera, while for cattle sera, sensitivity was calculated to be 100% and specifity 99.9% (Table 4). The agreement index of the *Brucella*-Ab c-ELISA against the CFT was significant (Table. 5, 6). As (i) no differences existed between CFT and *Brucella*-Ab c-ELISA and (ii) the only differences between CFT and RBT concerned sheep and goat sera, in Table 7 the agreement index between RBT and *Brucella*-Ab c-ELISA is shown using this latter as gold standard.

An assessment of cross-reactions in the *Brucella*-Ab c-ELISA revealed cross-reactions only for serum antibodies containing *Brucella* spp. and not for anti-LPS-antibodies in Enterobacteriaceae-positive sera. To the contrary, in the i-ELISA, using microplates coated with S-LPS, cross-reactions were observed with some positive sera for Enterobacteriaceae (Table 8). The repeatability and reproducibility of the assay expressed as the coefficient of variation was 6.8% and 8.1%, respectively.

Discussion and Conclusions

The use of the ELISA in laboratory diagnosis of brucellosis provides many advantages over serological tests (RBT and

Table 1. Cross-reactions of monoclonal antibodies (MAbs) using the indirect ELISA

	MAbs*									
Bacterial strain	9C2A	9C2D	4B5A†	4B5B	4B5C	4B5E	4B5F	4B2	3C3B	8B2C
Brucella melitensis biotype 2	100	100	100	100	100	100	100	100	100	100
B. melitensis biotype 1	106	106	89.0	79	106	100	96	70	111	31
B. melitensis biotype 1 Rev .1	100	105	88.9	61.0	90.0	96.0	84.0	51.0	105	61.0
Brucella abortus biotype S19	103	101	104	102	101	97.0	100	71.0	105	98.0
B. abortus biotype S99	104	104	98.0	71.0	91.0	83.0	93.0	74.0	123	15.0
LPSB. abortus biotype S99	107	119	99.8	84.0	100	100	90.0	82.0	170	39.0
B. abortus biotype 2	104	110	100	102	103	102	100	74.0	123	96.0
B. abortus biotype 3	98.0	106	102	106	97.0	101	101	56.0	102	110
B. abortus biotype 6	106	102	87.0	100	99.0	96.0	96.0	93.0	157	5.0
Brucella suis biotype 1	85.0	90.4	92.0	100	87.9	83.5	94.8	87.0	75.9	37.5
Brucella ovis	2.5	1.8	1.0	1.0	5.4	3.6	1.1	2.2	0.5	5.0
Salmonella, enteritidis	102	107	0.0	1.0	1.7	3.0	1.0	35.0	120	1.0
S. typhimurium	94.0	101	1.0	1.0	3.9	5.0	2.0	24.0	78.0	1.0
Escherichia coli	106	105	0.0	1.0	1.1	1.0	0.5	50.0	136	1.0
*Yersinia enterocolitica*O:9	107	110	0.0	0.5	1.4	1.0	0.5	53.0	145	1.0

LPS, lipopoly saccharides.
*(Abs X/Abs *B. melitensis* biotype 2) × 100.
†MAb used to develop *Brucella*-Ab c-ELISA.

Table 2. Sheep and goat sera: results obtained using *Brucella*-Ab c-ELISA, complement fixation and Rose Bengal tests

	Brucella-Ab c-ELISA		Complement fixation test		Rose Bengal test	
Sera	Field	Experimental infection	Field	Experimental infection	Field	Experimental infection
Positive	141	130	141	130	132	130
Negative	856	–	856	–	865	–

Table 3. Cattle sera: results obtained using *Brucella*-Ab c-ELISA, complement fixation and Rose Bengal tests

Sera	*Brucella*-Ab c-ELISA, Field	Complement fixation test, Field	Rose Bengal test, Field
Positive	265	265	265
Negative	748	748	748

Table 4. *Brucella*-Ab c-ELISA sensitivity and specificity for cattle, sheep and goat validation sera

Characteristics of the test	Percentage	Confidence limit below*	Confidence limit above†
Sensitivity (cattle sera)	100	99.26	99.97
Specificity (cattle sera)	99.9	99.55	100
Sensitivity (sheep/goat sera)	100	98.65	99.99
Specificity (sheep/goat sera)	100	99.57	100

*Minimum 95% confidence values derived from a *Beta* distribution.
†Maximum 95% confidence values derived from a *Beta* distribution.
$Beta(\alpha_1, \alpha_2) = \frac{x^{\alpha_1-1}(1-x)^{\alpha_2-1}}{\int_0^1 t^{\alpha_1-1}(1-t)^{\alpha_2-1}dt}$
where,
α_1 = corrected results + 1
α_2 = tested samples − corrected results + 1

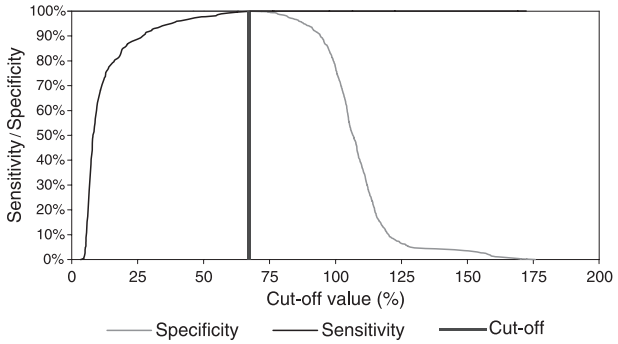

Fig. 1. Sheep and goat sera: monoclonal antibody cut-off value calculated according to sensitivity and specificity values.

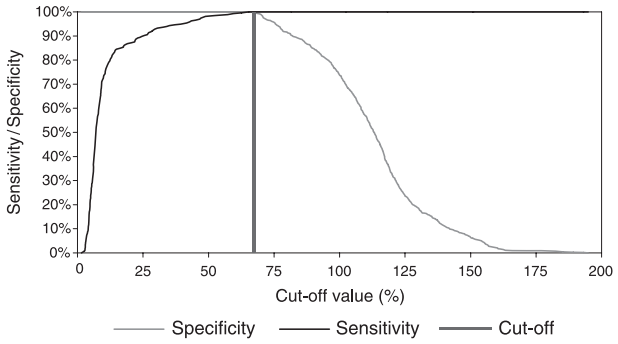

Fig. 2. Cattle sera: monoclonal antibody cut-off value calculated according to sensitivity and specificity values.

CFT) that until today have been used internationally in diagnostic laboratories. The ELISA not only simplifies laboratory procedures and facilitates the interpretation of results, but presents other advantages, as follows:

1 ELISA provides high sensitivity and specificity;
2 the assay is simple to perform and can be standardized with ease.

Table 5. Sensitivity, specificity and measure of agreement index of *Brucella*-Ab c-ELISA (sheep/goat sera) in comparison to the complement fixation test

	Complement fixation test		
	Positive	Negative	Total
Brucella-Ab c-ELISA			
Positive	271	0	271
Negative	0	856	856
Total	271	856	1127
Sensitivity	100%		
Specificity	100%		
Predicted positive values	100%		
Predicted negative values	100%		
Efficacy of the test	100%		
Kappa index	1.00		
Kappa significance	$P < 0.05$		

Table 6. Sensitivity, specificity and measure of agreement index for the *Brucella*-Ab c-ELISA (cattle sera) and complement fixation test

	Complement fixation test		
	Positive	Negative	Total
Brucella-Ab c-ELISA			
Positive	265	0	265
Negative	0	748	748
Total	265	748	1013
Sensitivity	100%		
Specificity	100%		
Predictive positive value	100%		
Predictive negative value	100%		
Efficacy of the test	100%		
Kappa index	1.00		
Kappa significance	$P < 0.05$		

Table 7. Sensitivity, specificity and measure of agreement index for the Rose Bengal Test (sheep and goat sera from flocks) compared to the *Brucella*-Ab c-ELISA

		Brucella-Ab c-ELISA		
		Positive	Negative	Total
Rose Bengal test	Positive	137	0	137
	Negative	9	852	852
	Total	146	852	1127
Sensitivity		93.8%		
Specificity		100%		
Predictive positive value		100%		
Predictive negative value		98.9%		
Efficacy of the test		99.1%		
Kappa index		0.96		
Kappa significance		$P < 0.05$		

Table 8. Cross reaction between the *Brucella*-Ab c-ELISA and indirect ELISA

Positive sera	*Brucella*-Ab c-ELISA (B/Bo%)*	Indirect ELISA (Abs)†
Brucella melitensis biotype 1	11.2	2224
B. melitensis biotype 2	0.2	2073
B. melitensis biotype 3	12.5	2111
B. melitensis biotype l(Rev. 1)	18.3	1987
Brucella abortus S 99	0.7	2001
Salmonella enteritidis	94.5	1725
S. abortus ovis	100.0	1490
Yersinia enterocolitica O:9	100.0	1682
Escherichia coli	100.0	1724

*Cut-off value: 67.5 %.
†Cut-off value: 0.600.

The MAb 4B5A used in this study is directed against a specific epitope of the S-LPS and recognizes all the biovariants of *Brucella* investigated, with the exception of *B. ovis* which belongs to the rough form. The same MAb does not react with the LPS of *Y. enterocolitica* O:9 or with the LPSs of other Enterobacteriaceae that share the same antigenic determinant. In this way, the *Brucella*-Ab c-ELISA, developed with MAb 4B5A, can be a valid diagnostic tool to avoid false-positive serological reactions and to reveal infections caused by *Brucella* spp. It is therefore more reliable when used to apply veterinary restriction measures following positive results in a herd.

The sensitivity of the *Brucella*-Ab c-ELISA was determined on the basis of results obtained by analysing positive sera to the CFT and has proved to be 100% reliable in the case of cattle, sheep and goat sera. In the same way, the specificity, determined from negative sera to the CFT, resulted in 100% for sheep and goat sera and 99.9% for cattle sera. An evaluation of the potential of the *Brucella*-Ab c-ELISA to solve the problem of false-positive serological reactions was made by analysing sera from sheep that had been infected experimentally with certain Enterobacteriaceae among which was *Y. enterocolitica* O:9. None of these sera gave positive results with the test, implying greater specificity than the CFT, which is known to cross-react with some of them (Bundle et al., 1984; Nielsen and Duncan, 1990; Diaz-Aparicio et al., 1993; Gall et al., 1998; Kittelberger et al., 1998; Samartino et al., 1999; Erdenebaatar et al., 2003). Regarding the RBT applied to sheep and goat sera when used as a screening test in flocks, the *Brucella*-Ab c-ELISA demonstrated greater sensitivity, while no difference was found when evaluating sera from sheep and goats that had been infected experimentally. In the literature it is well documented that RBT has greater sensitivity than CFT; in our study, CFT showed greater sensitivity than RBT when used as screening test. This result needs further investigations even if not important for the results of the present study, as the real status of the animals (and therefore of the sera) was known.

Sera from vaccinated animals were not included as the aim of the study was to develop a new assay with high throughput and very high characteristics in terms of specificity and sensitivity when compared with CFT; to understand if the assay is able to discriminate between vaccinated and infected animals further investigations are needed.

Using ROC analysis, an optimal cut-off value of 67.5% was determined, making a clear distinction between positive and negative sera and confirming the sensitivity and specificity results in comparison to the CFT. The level of agreement with

the CFT was calculated and is shown in Table. 3 and 4. The Kappa statistic of 1 indicates perfect agreement between the tests and the efficacy of the *Brucella*-Ab c-ELISA which was 100%.

In conclusion, the performance of *Brucella*-Ab c-ELISA is similar to other serological tests, such as the CFT, as the assay detects the same infected animals. Unlike CFT, *Brucella*-Ab c-ELISA discriminates false-positive results for anti-LPS-antibodies in Enterobacteriaceae-positive sera. Therefore, the *Brucella*-Ab c-ELISA can be usefully employed in the diagnosis of brucellosis for the three animal species tested and offers the advantage of simplicity and rapidity, in addition to providing method standardization.

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