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

O. Portanti¹,², M. Tittarelli¹, T. Di Febo¹, M. Luciani¹, M. T. Mercante¹, A. Conte¹ and R. Lelli¹

Addressess of authors: ¹Istituto Zooprofilattico Sperimentale dell’Abruzzo e, del Molise ‘G. Caporale’. Campo Boario, 64100 Teramo, Italy; ²Corresponding author: Tel.: +39 861 332221; fax: +39 861 332251; E-mail: o.portanti@izs.it

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 4BSA) 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 ovine and 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; Erdenebatar 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 Freund's adjuvant (CFA) was inoculated by the intraperitoneal route, followed by the administration of incomplete Freund's 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 MAbs-secretion 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 MAbs 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 Kawai, 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.
ELISA Kit for the Diagnosis of Brucellosis 495

Brucella MAb-HRP (MAb 4B5A) (anti-\textit{B. melitensis}) ovine and bovine control sera were obtained from animals and the microplates were incubated for 1 h at RT. Positive \( l_{50} \) Tween 20 (PBST). About 200 \( l \) 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 \( l \) serum samples, 50 \( l \) positive bovine serum control and 50 \( l \) 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 \textit{B. melitensis} biotype 2, while negative ovine and bovine control sera were obtained from animals tested by RBT, CFT and \textit{Brucella}-culture. After incubation, microplates were washed with PBST and 50 \( l \) MAb-HRP (MAb 4B5A) (anti-\textit{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 \( l \) 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 \( l \) of sulphuric acid 0.5 N and the optical density (OD) measured at 450 nm with a microplate reader.

Competitive ELISA versus \textit{Brucella} spp. (\textit{Brucella}-Ab c-ELISA)
The 96-well ELISA microplates were coated with 100 \( l \) 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 \( l \) 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 \( l \) serum samples, 50 \( l \) positive bovine serum control and 50 \( l \) 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 \textit{B. melitensis} biotype 2, while negative ovine and bovine control sera were obtained from animals tested by RBT, CFT and \textit{Brucella}-culture. After incubation, microplates were washed with PBST and 50 \( l \) MAb-HRP (MAb 4B5A) (anti-\textit{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 \( l \) 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 \( l \) of sulphuric acid 0.5 N and the optical density (OD) measured at 450 nm with a microplate reader.

Standardization and validation of the \textit{Brucella}-Ab c-ELISA
The assay was standardized using 12 sera from infected sheep, goats and cattle to reveal the presence of \textit{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: \textit{B. melitensis} biotypes 1, 2, 3, \textit{B. melitensis} Rev. 1, \textit{B. abortus} (S99), \textit{S. enteritidis}, \textit{S. abortus ovis}, \textit{Y. enterocolitica} O:9 and \textit{E. coli}.

An intralaboratory \textit{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); \textit{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 \textit{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 \textit{Brucella}-Ab c-ELISA and the CFT and RBT was assessed using Cohen’s Kappa (Schoerner et al., 1990).

Results
Monoclonal antibodies
Ten \textit{B. melitensis} biotype 2-specific MABs cell lines were obtained. MABs cross-reacted with the other \textit{Brucella}, with the exception of \textit{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 \textit{Brucella}-Ab c-ELISA. MAB 4B5A was conjugated with peroxidase and called anti-\textit{Brucella-LPS} MAb.

\textit{Brucella}-Ab c-ELISA
\textit{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 specificity 99.9% (Table 4). The agreement index of the \textit{Brucella}-Ab c-ELISA against the CFT was significant (Table. 5, 6). As (i) no differences existed between CFT and \textit{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 \textit{Brucella}-Ab c-ELISA is shown using this latter as gold standard.

An assessment of cross-reactions in the \textit{Brucella}-Ab c-ELISA revealed cross-reactions only for serum antibodies containing \textit{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
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 2. Sheep and goat sera: results obtained using \textit{Brucella}-Ab c-ELISA, complement fixation and Rose Bengal tests

<table>
<thead>
<tr>
<th>Sera</th>
<th>\textit{Brucella}-Ab c-ELISA</th>
<th>Complement fixation test</th>
<th>Rose Bengal test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>141</td>
<td>130</td>
<td>132</td>
</tr>
<tr>
<td>Negative</td>
<td>856</td>
<td>–</td>
<td>865</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sera</th>
<th>\textit{Brucella}-Ab c-ELISA Field</th>
<th>Complement fixation test Field</th>
<th>Rose Bengal test Field</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>265</td>
<td>265</td>
<td>265</td>
</tr>
<tr>
<td>Negative</td>
<td>748</td>
<td>748</td>
<td>748</td>
</tr>
</tbody>
</table>

Table 4. \textit{Brucella}-Ab c-ELISA sensitivity and specificity for cattle, sheep and goat validation sera

<table>
<thead>
<tr>
<th>Characteristics of the test</th>
<th>Percentage</th>
<th>Confidence limit below$^*$</th>
<th>Confidence limit above$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity (cattle sera)</td>
<td>100</td>
<td>99.26</td>
<td>99.97</td>
</tr>
<tr>
<td>Specificity (cattle sera)</td>
<td>99.9</td>
<td>99.55</td>
<td>100</td>
</tr>
<tr>
<td>Sensitivity (sheep/goat sera)</td>
<td>100</td>
<td>98.65</td>
<td>99.99</td>
</tr>
<tr>
<td>Specificity (sheep/goat sera)</td>
<td>100</td>
<td>99.57</td>
<td>100</td>
</tr>
</tbody>
</table>

$^*$Minimum 95% confidence values derived from a Beta distribution.
$^1$Maximum 95% confidence values derived from a Beta distribution.

LPS, lipopoly saccharides.

$^{*}(\text{Abs X/}\text{Abs B. melitensis biotype 2}) \times 100$.

$^\dagger$MAb used to develop \textit{Brucella}-Ab c-ELISA.
Table 5. Sensitivity, specificity and measure of agreement index of c-ELISA Kit for the Diagnosis of Brucellosis

<table>
<thead>
<tr>
<th>Complement fixation test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella-Ab c-ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>271</td>
<td>0</td>
<td>271</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>856</td>
<td>856</td>
</tr>
<tr>
<td>Total</td>
<td>271</td>
<td>856</td>
<td>1127</td>
</tr>
</tbody>
</table>

Sensitivity 100%
Specificity 100%
Predicted positive values 100%
Predicted negative values 100%
Efficacy of the test 100%
Kappa index 0.96
Kappa significance < 0.05

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

<table>
<thead>
<tr>
<th>Complement fixation test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella-Ab c-ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>265</td>
<td>0</td>
<td>265</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>748</td>
<td>748</td>
</tr>
<tr>
<td>Total</td>
<td>265</td>
<td>748</td>
<td>1013</td>
</tr>
</tbody>
</table>

Sensitivity 100%
Specificity 100%
Predicted positive value 100%
Predicted negative value 100%
Efficacy of the test 100%
Kappa index 1.00
Kappa significance P < 0.05

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 goats 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.

Acknowledgements

The authors wish to thank Attilio Pini and Giorgio Filippini, for their advice in the preparation of this manuscript.

References


