Use of chemiluminescence for the serological diagnosis of bovine and ovine brucellosis with indirect and competitive enzyme-linked immunosorbent assays

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Summary

The official methods specified in the national bovine and ovine/caprine brucellosis eradication plan are the Rose Bengal and complement fixation tests. In the current phase of the eradication plan, it is often difficult to interpret the results obtained with the official tests. Consequently, additional tests that offer greater sensitivity and specificity are thus required. For this reason, two methods, the indirect chemiluminescence enzyme-linked immunosorbent assay (i-ELISA CL) and the competitive chemiluminescence ELISA (c-ELISA CL) that use a chemiluminescent substrate to determine anti-*Brucella* antibodies in bovine and ovine serum were validated. The methods are based on the detection of anti-*Brucella* antibodies in serum by catalysis of a chemiluminescent enzyme substrate (luminol/H_2O_2/enhancer system) by peroxidase conjugated to secondary anti IgG antibodies in i-ELISA CL and to monoclonal anti-lipopolysaccharide (LPS) antibodies in c-ELISA CL. From the results obtained, a cut-off of 60% for bovine serum and 37.5% for ovine serum, expressed as positivity rate (PR), were established Using these cut-off values, for the i-ELISA CL, 100% sensitivity and specificity was obtained for bovine serum and 100% sensitivity and 99.8% specificity for ovine serum. Cut-off values of 30% for bovine serum and 40% for ovine serum, expressed as inhibition rate, were selected for c-ELISA CL, which ensured 100% sensitivity and specificity in both cases.

Keywords

Animal, Bovine, Brucellosis, Chemiluminescence, Enzyme-linked immunosorbent assay, ELISA, Ovine, Serology.

Introduction

In the European Union, strategies to combat bovine and ovine/caprine brucellosis aim to eradicate the infection, i.e. to eliminate the disease and its aetiological agent from the area (4). This objective has been pursued the identification and slaughter of all animals with positive results to serological or bacteriological tests, the prohibition of vaccination and attaining the 'officially free' status of both farms and areas of the European Union.

The official methods specified in the national brucellosis bovine and ovine/caprine eradication plan of are Rose Bengal test (RBT) and complement fixation test (CFT) (1, 2, 3).

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In the current phase of the eradication plan, it is often difficult to interpret the results obtained with the official tests, and additional tests that offer greater sensitivity and specificity are required.

Chemiluminescence is the emission of visible or near-visible radiation (wavelengths in the range of 300-800 nm) when electrons excited by an exergonic chemical reaction return to their ground state (7). One of the most important applications of chemiluminescent methods is to be found in the development of immunological methods using chemi-luminescent tracers for direct labelling of antibodies and antigens or enzymatic tracers and chemiluminescent substrates. It is very convenient to use the enzymatic labels because it enables amplification and stabilisation of the signal, thus allowing extremely low limits of detection (10).

One of the most commonly used chemiluminescent analytical compounds is luminol (9), the structure of which consists of two adjacent rings. The aromatic ring is unaffected by the chemiluminescent reaction, while the second, a non-aromatic heterocyclic ring, is oxidised and emits photons on return to its ground state after excitation. Luminol requires hydrogen peroxide and can be catalysed by metal ions and enzymes, such as peroxidase.

Two enzyme-linked immunosorbent assays (ELISAs), the indirect (i-ELISA CL) and the competitive (c-ELISA CL) ELISAs which use a chemiluminescent substrate to determine anti-*Brucella* antibodies in bovine and ovine serum were validated in this study. The methods are based on the detection of anti-*Brucella* antibodies in serum by the catalysis of a chemiluminescent enzyme substrate (luminol/H_2O_2/enhancer system) by peroxidase conjugated to secondary anti-IgG antibodies in i-ELISA CL and to monoclonal anti-lipopolysaccharide (LPS) antibodies in c-ELISA CL.

Materials and methods

Serum panel

The tests were validated using a panel of 1 500 sera, as follows:

- 745 bovine sera from brucellosis-free farms in the regions of Abruzzo, Basilicata, Calabria, Liguria, Sardinia and Veneto
- 43 bovine sera from 12 brucellosis-infected farms in the regions of Abruzzo, Calabria and Molise from which *Brucella* spp. had been isolated
- 636 ovine sera from brucellosis-free farms in region of Sardinia
- 76 ovine sera from brucellosis-infected farms in regions of Abruzzo, Calabria and Sicily from which *Brucella* spp. had been isolated.

Serological tests

All serum samples were tested to identify the presence of antibodies using the RBT and CFT, with antigens produced with the strain 99 *Brucella abortus* biovar 1 (Veterinary Laboratories Agency, Weybridge), as described in the *Manual of standards for diagnostic tests and vaccines* (13). The c-ELISA was conducted as described by Portanti *et al.* (8) and i-ELISA performed in accordance with the *Manual of standards for diagnostic tests and vaccines* (13).

Description of i-ELISA with chemiluminescent detection

The test was conducted using F16 Black PolySorp (NUNC™, Denmark) 96-well micro-plates adsorbed with 100 µl per well of smooth-LPS (s-LPS) antigen of *Brucella abortus* 99 (Weybridge) produced by the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del 'G. Caporale'* (IZS A&M) according to the technique described by Hendry *et al.* (6) and diluted 1:3 500 in carbonate-bicarbonate buffer 0.05 M, pH 9.6.

The plates were incubated overnight at +4°C and then washed three times with saline phosphate buffer 0.01 M + 0.05% Tween 20, pH 7.2 (phosphate buffered saline Tween: PBST) and further incubated for 2 h at +4°C with 250 µl of blocking solution (PBS 0.1 M + 1% polyvinylpyrrolidone) per well. After three

washing cycles, 100 µl/well of each sample were distributed in duplicate, diluting 1:50 in PBS. Bovine brucellosis-positive national standard serum (NSS) and ovine brucellosis-positive standard serum, both produced by IZS A&M, were used as the positive reference standards. Bovine brucellosis-NSS and ovine brucellosis-NSS from officially brucellosis-free farms, both produced by IZS A&M, were used as the negative reference standards. Both standards, examined as for a serum sample, were freeze-dried and preserved in a 1 ml-bottle.

Plates were incubated for 1 h at room temperature and then washed three times with PBST. They were then incubated for 1 hour with 100 µl/well horseradish peroxidise (HRP)-labelled anti-IgG antibody (Sigma-Aldrich Corporation, St Louis, Missouri) diluted 1:40 000 in PBS. After incubation, the plates were washed again and 100 µl/well of SuperSignal® ELISA Femto chemiluminescent substrate (Pierce, Rockford, Illinois), based on a luminol/H_2O_2/enhancer system, ware added. The signal, expressed as counts per second (CPS), was measured immediately with a Victor3™V Multilabel Counter model 1420 light meter (PerkinElmer, Fremont, California).

The positivity rate (PR) of the samples with respect to the positive reference standard was calculated as follows:
PR = (mean sample CPS/mean positive reference serum CPS) × 100.

Description of c-ELISA with chemiluminescent detection

The test was conducted using the commercial kit produced by IZS A&M (8), and F16 Black PolySorp 96-well microplates adsorbed with 100 µl per well of s-LPS antigen of *Brucella abortus* 99 diluted 1:3 500 in carbonate-bicarbonate buffer. The peroxidase-conjugate anti-*Brucella* spp. monoclonal antibody (Mab-HRP) was diluted 1:1 000 in dilution buffer.

After incubation and washing of the Mab-HRP, 100 µl/well of SuperSignal® ELISA Femto chemiluminescent substrate (Pierce) ware added and the signal, expressed as CPS, was measured immediately with a Victor3™V multilabel counter model 1420 light meter.

The inhibition rate (IR) of the sample with respect to the CPS value of the MAb-HRP control was calculated as follows:
IR = (mean sample CPS/mean MaB-HRP control CPS) × 100.

Statistical analysis

The optimum cut-off value was determined through the receiver operator characteristic (ROC) curve on the basis of the expected diagnostic sensitivity and specificity values (5, 11).

The 95% confidence intervals were calculated for the sensitivity and specificity values using the Beta probability distribution (s+1, n-s+1), where s = total number of positive samples and n = total number of samples examined.

Results

Table I presents the origin of the sera examined with the chemiluminescent methods and other serological tests.

On the basis of the results obtained with i-ELISA CL, a cut-off of 60% for bovine serum (Fig. 1) and 37.5% for ovine serum (Fig. 2), expressed as positivity rate (PR), was established, determining 100% test sensitivity and specificity for bovine serum and 100% sensitivity and 99.8% specificity for ovine serum (Figs 3 and 4).

Table I
Sera used for validation

Species	Abruzzo	Basilicata	Calabria	Liguria	Molise	Sardinia	Sicily	Veneto	Total
Bovine	86	74	66	221	13	49	0	279	788
Ovine	65	0	5	0	0	636	6	0	712

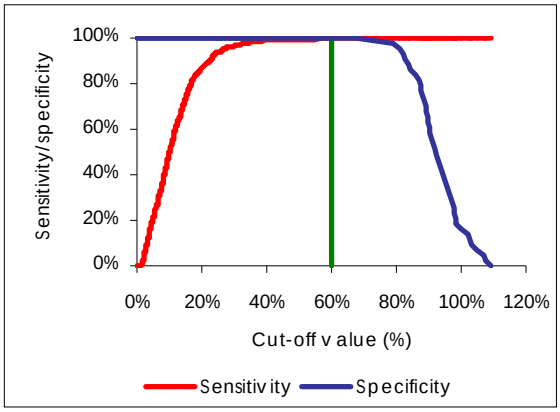

Figure 1
Cut-off of indirect enzyme-linked immunosorbent assay with chemiluminescent detectioncalculated as a function of the various sensitivity and specificity values of bovine sera

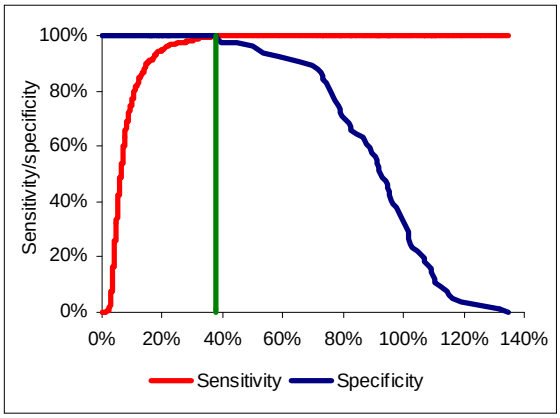

Figure 2
Cut-off of indirect enzyme-linked immunosorbent assay with chemiluminescent detection calculated as a function of the various sensitivity and specificity values of ovine sera

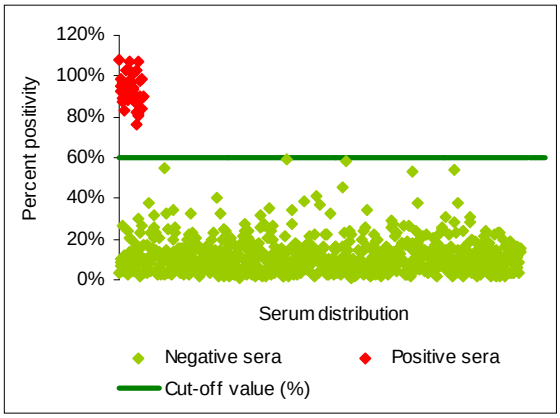

Figure 3
Positivity rate distribution for bovine sera

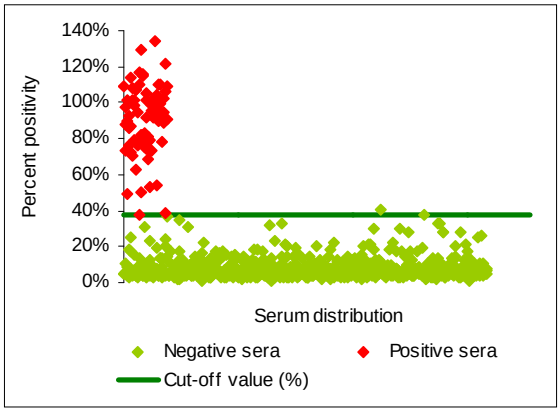

Figure 4
Positivity rate distribution for ovine sera

On the basis of the results obtained with c-ELISA CL, a cut-off of 30% for bovine serum (Fig. 5) and 40% for ovine serum (Fig. 6), expressed as IR, was established, determining 100% test sensitivity and specificity for both bovine and ovine sera (Figs 7 and 8).

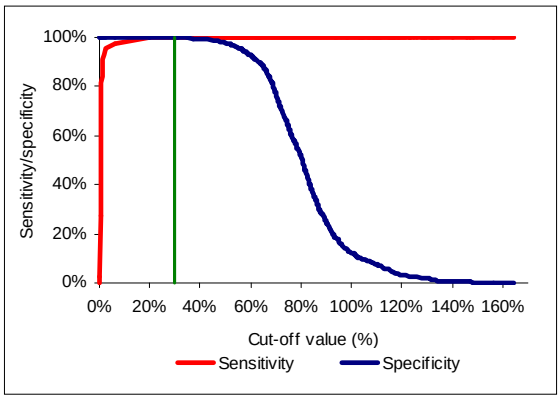

Figure 5
Cut-off of competitive enzyme-linked immunosorbent assay with chemiluminescent detection calculated as a function of the various sensitivity and specificity values of bovine sera

The results of the 745 negative bovine sera and 636 negative ovine sera examined with RBT, CFT, i-ELISA, c-ELISA, i-ELISA CL and c-ELISA CL are given in Table II.

The results of the 43 positive bovine sera and 76 positive ovine sera examined with RBT, CFT, c-ELISA, i-ELISA, i-ELISA CL e c-ELISA CL are presented in Table III.

Table IV gives the 95% confidence intervals for the sensitivity and specificity values calculated for the two chemiluminescent methods.

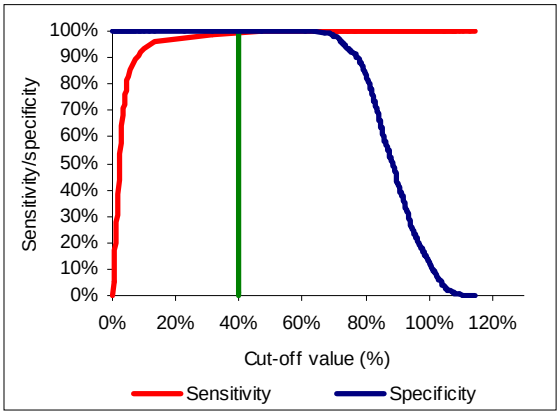

Figure 6
Cut-off of competitive enzyme-linked immunosorbent assay with chemiluminescent detection calculated as a function of the various sensitivity and specificity values of ovine sera

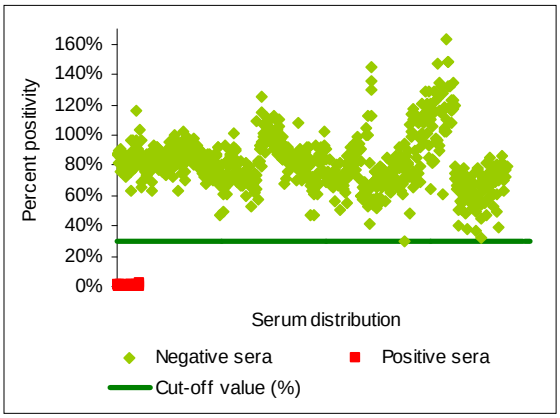

Figure 7
Inhibition rate distribution for bovine sera

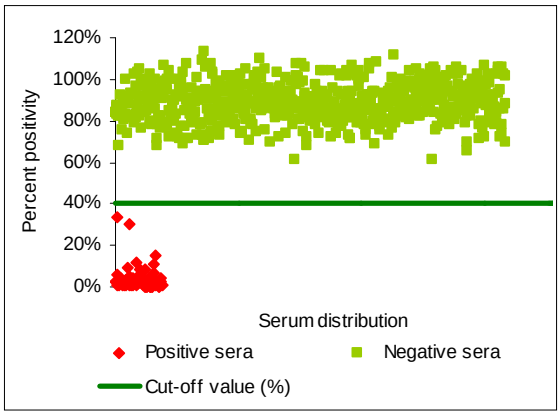

Figure 8
Inhibition rate distribution for ovine sera

The use of chemiluminescence, which offers greater detectability in comparison with more conventional spectrophotometric and spectrofluorimetric techniques (12), improved the performance of the i-ELISA and c-ELISA methods. In particular, comparing the level of specificity for standard ELISAs (indirect and competitive) and for ELISA methods that use a chemiluminescent substrate, significant differences were identified as follows:

- 95% confidence intervals c-ELISA vs c-ELISA CL, respectively 97.38-99.17% vs 99.6-100%
- i-ELISA vs i-ELISA CL, respectively 96.05-98.35% vs 99.6-100%.

Table II
Results of negative sera validation

Species	SAR		CFT		c-ELISA		i-ELISA		i-ELISA CL		c-ELISA CL		Total
	–	+	–	+	–	+	–	+	–	+	–	+	
Bovine	744	1	736	9	734	11	726	19	745	0	745	0	745
Ovine	636	0	632	4	629	7	634	2	635	1	636	0	636

SAR rapid serum agglutination test
CFT complement fixation text
c-ELISA competitive enzyme-linked immunosorbent assay
i-ELISA indirect enzyme-linked immunosorbent assay
c-ELISA CL competitive chemiluminescence enzyme-linked immunosorbent assay
i-ELISA CL indirect enzyme-linked immunosorbent assay with chemiluminescent detection
– negative
+ positive

Table III
Results of positive sera validation

Species	SAR		CFT		c-ELISA		i-ELISA		i-ELISA CL		c-ELISA CL		Total
	–	+	–	+	–	+	–	+	–	+	–	+	
Bovine	1	42	0	43	1	42	0	43	0	43	0	43	43
Ovine	0	76	1	75	0	76	0	76	0	76	0	76	76

SAR rapid serum agglutination test
CFT complement fixation text
c-ELISA competitive enzyme-linked immunosorbent assay
i-ELISA indirect enzyme-linked immunosorbent assay
c-ELISA CL competitive enzyme-linked immunosorbent assay with chemiluminescent detection
i-ELISA CL indirect enzyme-linked immunosorbent assay with chemiluminescent detection
– negative
+ positive

Table IV
Sensitivity and specificity of chemiluminescent methods

Test	Species	Property	Percentage	Lower confidence limit	Upper confidence limit
i-ELISA CL	Bovine	Sensitivity	100.0	93.42	100.00
		Specificity	100.0	99.60	100.00
	Ovine	Sensitivity	100.0	96.18	100.00
		Specificity	99.8	99.13	99.96
c-ELISA CL	Bovine	Sensitivity	100.0	93.42	100.00
		Specificity	100.0	99.60	100.00
	Ovine	Sensitivity	100.0	96.18	100.00
		Specificity	100.0	99.53	100.00

c-ELISA CL competitive enzyme-linked immunosorbent assay with chemiluminescent detection
i-ELISA CL indirect enzyme-linked immunosorbent assay with chemiluminescent detection

In enzymatic labels, chemiluminescent substrates enable amplification and stabilisation of the signal, thereby permitting extremely low detection limits (10).

Two indirect and competitive ELISA methods that use a chemiluminescent substrate to determine anti-*Brucella* antibodies in bovine and ovine sera were validated in this study.

The ROC curve provided the optimum cut-off values, enabling positive samples to be distinguished unequivocally from negative samples, thus improving the performance of the methods.

The sensitivity of the chemiluminescent tests that was determined on the basis of test results of sera that originated from brucellosis-infected farms in which *Brucella* spp. had been isolated, was found to be 100% for both bovine and ovine sera in both the i-ELISA CL and c-ELISA CL. Similarly, specificity, determined by examining sera that originated from officially brucellosis-free farms, was found to be 100% for bovine sera and 99.8% for ovine sera in the indirect method and 100% in the competitive method for both bovine and ovine sera.

Chemiluminescent detection techniques, which do not require incubation prior to measurement, reduce overall analysis time, while the transfer of methods to larger microplates (e.g. 384-well) could boost analytical productivity, making the procedures suitable for high throughput screening (HTS) applications.

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