Evaluation of an indirect ELISA for the detection of *Brucella* antibodies in cow's milk

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Summary

An indirect ELISA was developed by the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' (IZSA&M) for the detection of *Brucella* antibodies in cow's milk. Specific monoclonal antibody was used against a bovine IgG_1 epitope and complies with European Commission requirements. The test accuracy was evaluated on milk samples from the regions of Abruzzo and Molise in Italy. The negative samples came from 1,250 officially brucellosis-free herds from the Molise region (Italy). The positive samples were taken from three herds in the Abruzzo and Molise regions where animals positive to the official tests were present and *Brucella abortus* was isolated. Test specificity was 99.8% (with a confidence interval [CI] of 99.6%-99.9%), while sensitivity was 100% (CI 91.2%-100%). The probability of detecting antibodies in positive milk samples was higher than 50% up to a dilution of 1:256 in negative milk. The probability of identifying an infected herd in the dairy cattle population under study was 88.6% (CI 73.9%-95.3%).

Keywords

Brucella abortus – Brucellosis – Cattle – Control – Enzyme-linked immunosorbent assay (ELISA) – Italy – Milk.

Introduction

The adoption of Directive 97/12/CE (3), implemented by Legislative Decree No. 196 on 22 May 1999, gave herds and territories in member states of the European Union the possibility of gaining and retaining «officially brucellosis-free» status through laboratory tests performed on bulk milk samples.

When compared to serological tests, this possibility offers significant advantages both in terms of costs and human resources.

Performing laboratory tests on bulk milk can reduce the number of tests and samples required for each individual herd, with the consequent reduction of costs. Moreover, while serum sampling in Italy has to be performed by a veterinarian, milk sampling for brucellosis diagnosis can be conducted as part of the routine milk quality control activities.

In a previous study, the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' (IZSA&M) standardised an enzyme-linked immunosorbent assay (ELISA) on cow's milk using polyclonal antibody anti-bovine IgG conjugated with peroxidase (2). However, the ELISA protocol described in the Manual of diagnostic tests and vaccines for terrestrial animals of the Office International des Épizooties (OIE) (8), requires the use of a monoclonal antibody anti-bovine IgG_1 as conjugate.

European Commission (EC) Regulation 535/2002 of 21 March 2002 amending Annex C to Council Directive 64/432/EEC and amending Decision 2000/330/EC (3), defined standardisation

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requirements for bulk milk sample tests.

During the period between 2002 and 2003, a trial was conducted in Local Health Unit No. 3 in Campobasso (Molise region) on the use of the ELISA on milk in «officially brucellosis-free» herds in order to retain their status.

An indirect ELISA was developed by the IZSA&M for the detection of *Brucella* antibodies in cow's milk («Teramo ELISA»). Monoclonal antibody was used and was standardised in accordance with the requirements stipulated in Regulation 535/2002 (3).

Furthermore, sensitivity and specificity of the Teramo ELISA were evaluated using field samples from the above-mentioned trial.

Materials and methods

Milk samples

Negative bulk milk samples were collected during bovine brucellosis prophylaxis activities performed in 2002 and 2003 in Local Health Unit No. 3 «Centro Molise». A total of 3,493 bulk milk samples from 1,250 «officially brucellosis-free» herds were collected. The samples were divided in two groups: the first group (group A) was composed of the first 1 022 samples collected, that were from as many «officially brucellosis-free» herds; the second group (group B) was composed of the remaining 2,471 samples. Positive milk samples were collected between 2001 and 2003 from three herds in Abruzzo and Molise, where *Brucella abortus* had been isolated. Three samples of bulk milk and 36 samples of milk from single infected animals were collected. Samples were stored at –20°C until tested.

Reference standard

Milk from cows from *Brucella abortus*-infected herds that gave positive results to the Rose Bengal test (RBT) and complement fixation test (CFT) was used as the positive reference milk (PRM) standard. The milk from which *Brucella abortus* was isolated was stored freeze-dried at the IZSA&M. Milk from cows from «officially brucellosis-free» herds and that had given negative results to the RBT and CFT was used as the negative reference milk (NRM) standard. The milk that gave negative results in regard to *Brucella* spp. isolation was freeze-dried and stored at the IZSA&M.

To define mean values and standard deviations (SD) of optical density (OD) of reference standards, 70 calculation repetitions of the ODs of PRM and NRM were performed.

To standardise the method, the national standard serum (NSS) against *Brucella abortus* and the OIE international standard serum (OIEISS) were used.

Antigen

A smooth lipopolysaccharide antigen (s-LPS) of Brucella abortus S99 (Weybridge) was used. This was produced by the IZSA&M in accordance with the technique described by Hendry *et al.* (6). The working titre of the antigen was defined according to Alton *et al.* (1) using 1:1,000, 1:2,000, 1:3,000, and 1:4,000 s-LPS dilutions. The final working titre of the antigen was 1:2,000; this dilution gave the expected values for PRM and NRM.

Titration curve with standard sera

The level of antibodies in PRM was evaluated with reference to NSS. Twofold dilutions of NSS in bovine negative serum, extending from 1:250 to 1:4,000, were prepared. These were diluted 1:10 in NRM. To define the titration curve of the NSS, 70 repetitions of OD calculation of the undiluted NSS and 70 repetitions of OD calculation of the single dilutions were performed.

Using this method, milk ELISA positive reference values were determined, as follows:

– strong positive (100% positivity): the value of the NSS dilution in negative milk (OD corresponds to the value assessed for PRM)

– medium positive (50% positivity): the value of NSS dilution in negative milk (OD corresponds to 50% of the value assessed for PRM).

To ensure the test was standardised in line with EC Regulation 535/2002 (3), the same trial was performed using dilutions of the OIEISS (excluding the undiluted OIEISS).

ELISA performance

All procedures used NUNC-immuno™ Plate PolySorp™ Surface plates. Plates were adsorbed with 100 µl per well of s-LPS antigen diluted in carbonate-bicarbonate buffer 0.06M pH 9.6 and incubated overnight at room temperature. The following day, the plates were washed three times with phosphate buffered saline 0.01 M (PBS) + 0.05% Tween 20, pH 7.2 (PBST). Each sample was tested twice, using 100 µl of milk per well. Sixteen wells were used as controls. Eight wells contained 100 µl of buffer (PBS 0.01 M + 1% yeast extract), to assess the enzymatic reaction without the sample (blank), four wells contained 100 µl PRM (positive control), and four wells contained 100 µl NRM (negative control).

Plates were shaken for two minutes on an orbital shaker and then incubated for an hour at 37°C. Plates were then washed three times with PBST, and 100 µl of anti-IgG_1 bovine monoclonal antibody were added per well, conjugated with alkaline phosphatase, using a previously determined optimal dilution obtained by checkerboard titration. After shaking, the plates were incubated for 30 min at 37°C, then washed three times with PBST. After adding 100 µl substrate p-nitrophenylphosphate per well, plates were incubated for 45 min at room temperature, away from direct light. The OD values of wells were read at 405 nm using a microplate reader. OD values minus blank values were used to determine sample results, expressed in percent positivity (PP) of the sample in relation to PRM using the following formula:

$$PP = \frac{\text{milk sample OD mean} - \text{NRM OD mean}}{\text{PRM OD mean} - \text{NRM OD mean}} \times 100$$

The cut-off value was calculated as the mean plus three SD of PP values resulting from the analysis of 1,022 negative bulk milk samples from as many 'officially brucellosis-free' herds (group A).

Evaluation of ELISA performances

The specificity value of Teramo ELISA was further defined by testing the 2 471 additional negative bulk milk samples (group B). The sensitivity evaluation of the test was conducted by analysing 39 undiluted positive individual and bulk milk samples. Moreover, to simulate bulk milk samples belonging to herds infected at various levels of prevalence, twofold dilutions in negative milk of the 36 individual positive milk samples were analysed, commencing from dilution 1:2, up to a dilution of 1:512.

Statistical analysis

Sensitivity and specificity values were calculated using a Bayesian approach (9, 11) with a Beta (s+1, n-s+1) probability distribution, where s was the number of samples correctly identified and n the total number of samples tested. In addition, 95% confidence intervals (CI) were calculated. A least square linear regression of PPs in relation to the reciprocal of dilutions was conducted. The significance of the regression was evaluated using variance analysis.

Results

Teramo ELISA cut-off value calculation

The mean PP value of the 1,022 negative milk samples (group A) was –0.069% (SD 3.75). Based on results, a PP cut-off value of 11.2% (PP mean plus 3 SD) was defined, considering samples with

PP<11.2% as negative and those with PP≥11.2% as positive.

Titration curve of standard sera

The NSS titration curve for Teramo ELISA is given in Figure 1. The PRM had a mean OD value of 1.034 (SD 0.053), while the medium positive showed 0.517 (SD 0.026), and the NRM 0.020 (SD 0.012). OD values of PRM and medium positive reference milk were placed between NSS undiluted and a 1:2 500 dilution of NSS in negative milk, while the OD value of NRM fell below the 1:40,000 NSS dilution in negative milk (Fig. 1).

The titration curve of the OIEISS for Teramo ELISA kit is shown in Figure 2.

At the 1:10,000 OIEISS dilution in negative milk, Teramo ELISA showed a positive reaction, with a PP mean value (on the total number of repetitions) of 13.8% (SD 1.7%) (Fig. 2).

Evaluation of Teramo ELISA performance

The evaluation of Teramo ELISA sensitivity on milk samples of group B gave 2,467 correct results out of 2,471 negative milk samples tested. The test specificity value of Teramo ELISA was therefore 99.8% (CI 99.6%-99.9%). The probability distribution of specificity values of the test is shown in Figure 3.

The Teramo ELISA correctly identified all 39 positive milk samples. Consequently, the sensitivity value was 100% (CI 91.2%-100%). The probability distribution of sensitivity values of the test is shown in Figure 4.

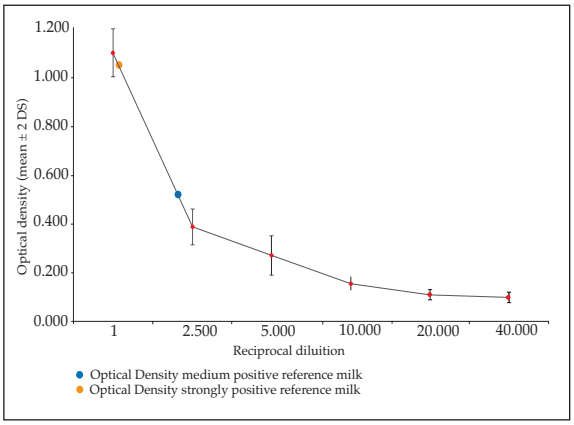

Figure 1
Titration curve of the national standard serum - Teramo ELISA

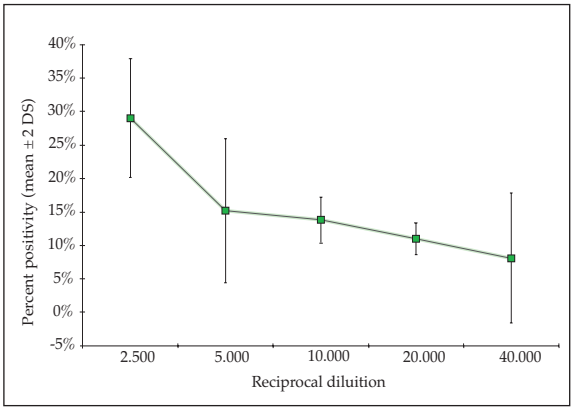

Figure 2
OIEISS titration curve - Teramo ELISA

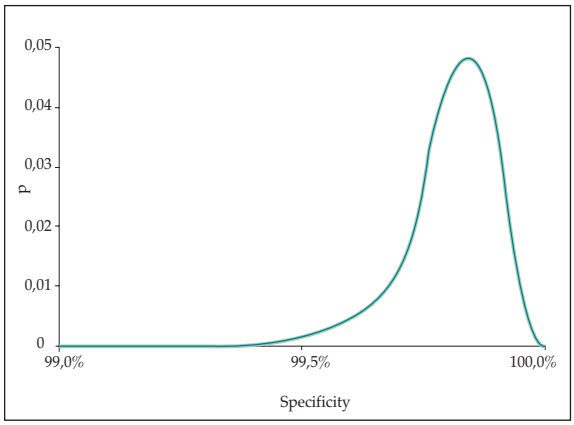

Figure 3
Specificity of Teramo ELISA on 2,471 negative milk samples

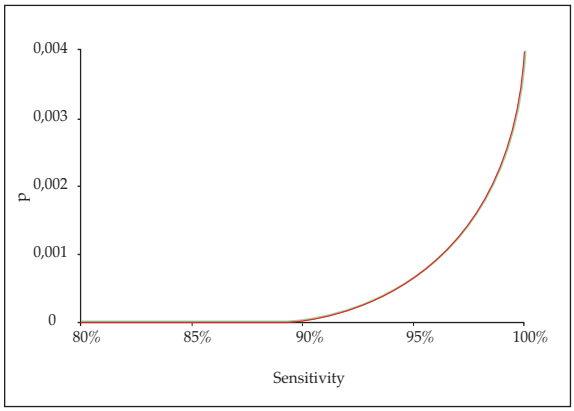

Figure 4
Sensitivity of Teramo ELISA on 39 positive milk samples

The results of dilutions in negative milk of the 36 positive milk samples from single infected animals, expressed as the percentage of samples correctly identified as positive at the given dilution, are presented in Figure 5.

Statistical analysis

The probability of obtaining a positive result by increasing the sample dilution (individual positive milk samples) followed a logarithmic model defined by the equation $y = -0.1055Ln(x) + 1.09$; with an R^2 value of 0.975032.

The F value resulted from the regression analysis of variance of such sample dilutions was significant ($p<0.01$).

Discussion and Conclusions

Teramo ELISA complies with European Union standardisation requirements (3, 4), since at a 1:10,000 dilution of OIEISS in negative milk the test gave a positive reaction (Fig. 2).

The test showed high sensitivity and specificity values comparable to other similar studies.

The ELISA kit previously produced by the IZSA&M (2) that provided an expected specificity of 99.5%, was standardised with a sensitivity value of 94.4% (CI 83.9%-97.8%).

In a study conducted on milk samples from Argentina, Canada and Chile, Nielsen *et al.* (7) developed an ELISA with sensitivity and specificity values of 96.5% (±2.5) and 99.9% (±0.05), respectively. Vanzini *et al.* (10) evaluated the method in a study conducted on dairy cows in Argentina, and observed 99.1% specificity (CI 98.9%-99.9%) and 99.6% sensitivity (CI 98.6%-99.9%).

The Teramo ELISA, when used on milk samples from infected animals and on bulk milk samples from infected herds, provides 100% sensitivity (CI 91.2%-100%).

The sensitivity of the Teramo ELISA, even if its most probable value is higher than the values found by other authors, must be considered taking into account the wide confidence interval that in any case is lower than the interval found in the previous Teramo ELISA kit (2).

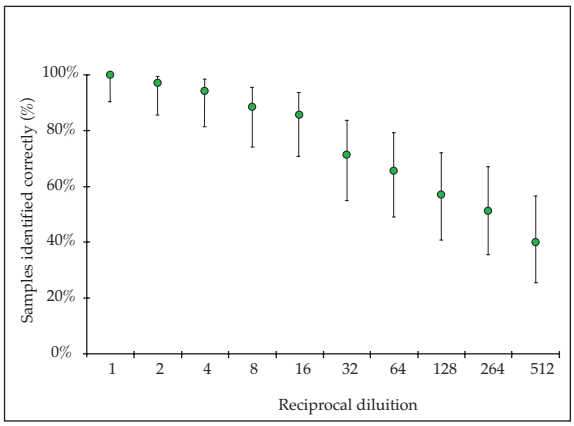

Figure 5
Results of dilutions of 36 individual positive milk samples and 95% confidence intervals

The values reported are the results of testing milk samples from single animals, while, in the field, one of the major advantages of using ELISA is that it can be used with bulk milk samples. Thus, studies conducted to define the effects of dilution on test performances are extremely important.

Nielsen *et al.* (7) has researched this issue, using twofold dilutions in negative milk of five positive milk samples from infected animals and demonstrated the possibility of identifying as positive three samples out of five at a dilution of 1:100 (sensitivity 60%; CI 22.3%-88.2%).

The ELISA kit previously produced by IZSA&M (2) in a similar study conducted on eight samples from infected animals, was able to identify as positive all samples tested up to a dilution of 1:200 (sensitivity 100%; CI 66.4%-100%) (Fig. 6).

The Teramo ELISA results give a sensitivity value of 57.1% (CI 40.8%-72.1%) at a dilution of 1:128 (Fig. 5 and 6). The probability of identifying the infection in a single animal by bulk milk testing is higher than 50% up to a dilution of 1:256

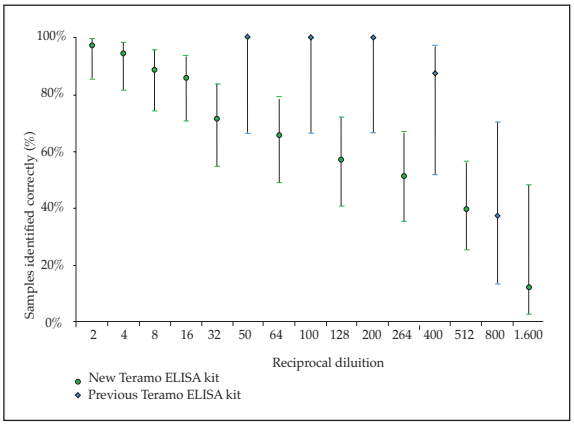

Figure 6
Comparison of sensitivity between the previous and new Teramo ELISA kit and 95% confidence intervals

(probability 51.4%; CI 35.5%-67.1%).

Even if the ELISA kit previously produced by IZSA&M (2) appears to perform better, the two kits are not significantly different, because of the overlapping of their respective CI (Fig. 6). The same applies to data obtained by Nielsen *et al.* (7).

The average national dairy herd size, during the control activities conducted in 2002, was 24.6 animals per herd, with a minimum of 8.4 per herd in the Liguria region and a maximum of 61.4 per herd in the Lombardy region. In the Abruzzo and Molise regions, there were 9.2 and 10.7 animals per herd, respectively (Italian Ministry of Health, personal communication).

Given the average national herd size, the probability of identifying as positive a single infected animal in a herd using the ELISA on milk varies from 85.7% (CI 70.2%-93.6%) to 71.4% (CI 54.8%-83.7%). In the case of the higher average herd size in Lombardy, the probability is 65.7% (CI 49.0%-79.2%), while in Abruzzo and Molise the probability is 88.6% (CI 73.9%-95.3%).

However, the overall sensitivity of the Teramo ELISA can be increased by repeating bulk milk sampling over time. The increase of sensitivity value, in this case, can be estimated by a simulation model similar to that described by Giovannini *et al.* (5). Nevertheless, since the Teramo ELISA performances do not differ significantly from those of the previous IZSA&M ELISA (2), on which the parameters of the model were based, relevant differences after changing the parameters of the model according to Teramo ELISA performances are not expected.

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