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An indirect ELISA for the detection of antibody in milk from sheep experimentally infected with *Brucella melitensis* biovar 3

M. Tittarelli, A. Giovannini, F. De Massis, M. Di Ventura & V. Caporale

Summary

An indirect ELISA was evaluated for the detection of *Brucella* antibodies in milk (m-ELISA) from sheep experimentally infected with *B. melitensis* biovar 3. At the end of the second reproductive cycle (13 months post infection), the milk of 22 lactating sheep was tested using the m-ELISA. Sera from the same sheep were also tested for *Brucella* antibodies using the Rose Bengal test (RBT) and the complement fixation test (CFT). The first serum sampling after parturition showed 100% sensitivity in both the RBT and the CFT (confidence interval [CI] 94-100%), but in subsequent samplings the sensitivity of the RBT decreased to 73% (CI 55-85%). Similarly, the sensitivity of the CFT decreased two months after the first sampling, when respective sensitivities of 95% (CI 81-98%) and 81% (CI 61-93%) were recorded for the final two samplings. The sensitivity of the m-ELISA decreased initially (68% on the third sampling, CI 50-81%), but then increased to 95% (CI 81-98%) for the final sampling. When disease prevalence in a flock is below 5%, the estimated probability of not detecting an infected flock through m-ELISA bulk milk testing is over 25%. Under field conditions in Italy (average sheep flock size of 70), the probability that the infection will not be detected is over 25% when four (or less) infected milking sheep are present in the flock. The results show that the m-ELISA is not a reliable screening test for bulk milk samples when the prevalence of brucellosis in a sheep flock is low.

Keywords

Brucella melitensis, Brucellosis, Enzyme-linked immunosorbent assay, Italy, Milk, Sheep.

Introduction

An extremely efficient means for screening dairy herds for the presence of *Brucella* is by bulk milk testing. Milk from these animals can be obtained cheaply and more frequently than blood samples and is often available centrally at dairies. The indirect milk ELISA (m-ELISA) is the most sensitive and specific test for this purpose, but the milk ring test (MRT) is a suitable alternative if the m-ELISA is not available (3, 8, 10). However, the MRT is not effective for the testing of milk from sheep and goats (1, 5). The possible use of an indirect m-ELISA for the detection of *Brucella* antibodies in sheep milk has been evaluated only twice (4, 5), when the performance of the m-ELISA was tested in a naturally infected flock. The present study was designed to evaluate the m-ELISA under controlled experimental conditions. The performance of this assay was also compared with conventional serological tests for the diagnosis of sheep brucellosis, namely the Rose Bengal test (RBT) and the complement fixation test (CFT).

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The aims of the present study were as follows:

a) to evaluate the sensitivity and the specificity of the m-ELISA for the detection of *Brucella* antibodies in sheep milk in controlled experimental conditions
b) to compare these values (in the same animals) against those obtained using conventional serological tests (RBT and CFT)
c) to establish the value of the m-ELISA for screening flocks against brucellosis by bulk sheep milk testing.

Materials and methods

Animals

Forty-six brucellosis-free Fabrianese and crossbred sheep aged between 1 and 5 years were experimentally infected intraconjunctivally with a field strain of *B. melitensis* biovar 3 in the third month of their first reproductive cycle. A freshly isolated field strain was inoculated into two guinea-pigs and re-isolated from the spleen 21 days later in 10 ml Farrells medium (7). After 48 h of growth in medium at 37°C, the re-isolated *Brucella* was titrated. A dose of 5×10^8 colony-forming units (cfu) in 100 µl was inoculated into the conjunctiva of both eyes (50 µl each) of the experimental sheep in accordance with the provisions of the *European Pharmacopoeia* (6). Of the 46 sheep, 25 were pregnant at the time of inoculation; 24 aborted between 4-6 weeks post infection (pi), while one animal had a normal parturition. Three animals died between one to five months pi. The remaining sheep were naturally inseminated after oestrus synchronisation, eight months pi. Twenty-six sheep became pregnant and all had normal parturition between weeks 56 and 58 pi (second reproductive cycle). This study includes data obtained from the monitoring of 22 of these animals that were lactating at the end of the second reproductive cycle. A flowchart of the experimental design is presented in Table I. Fifty-one sheep from an officially brucellosis-free flock were used as controls.

Serological testing

All animals were tested for the presence of antibodies against *Brucella* spp. using the RBT and the CFT prior to experimental infection. They were tested periodically after infection (12). The serum samples were collected immediately after parturition and then periodically up to the end of lactation, according to the protocol given in Table II. Both tests were performed using *B. abortus* biovar 1 strain 99 as antigen (Veterinary Laboratory Agencies, Weybridge) in accordance with the methods described in *Manual of diagnostic tests and vaccines for terrestrial animals* (10). To evaluate the specificity of the test, sera from 51 brucellosis-free sheep were tested using the RBT and CFT.

Milk sampling

Milk was collected from the 22 lactating sheep following parturition at the end of the second reproductive cycle, according to the protocol given in Table II. Milk samples were collected from parturition up to the end of lactation (about two months) and then tested using the m-ELISA. To evaluate the sensitivity of the m-ELISA, samples were not diluted. However, to evaluate the effect of milk dilution, tenfold dilutions (from 1:10 to 1:5 120) were analysed in negative milk of the same individual positive milk samples. To evaluate the specificity of the m-ELISA, milk from 51 brucellosis-free sheep were also tested.

Milk-ELISA procedure

The m-ELISA was performed according to a method described previously (4), with some modifications. A smooth lipopolysaccharide (s-LPS) antigen from *B. abortus* biovar 1 strain S99 was prepared for the ELISA according to the technique described by Hendry *et al.* (9). The milk samples and controls were distributed in duplicate into medium-capacity binding microplates. The antigen was diluted to the

optimal working concentration in carbonate-bicarbonate buffer 0.05 M (pH 9.6). The diluent/washing buffer was 0.01 M phosphate-buffered saline (PBS) (pH 7.2) plus 0.05% Tween 20 (PBST). A commercial rabbit anti-sheep IgG (whole molecule) peroxidase conjugate was used. The chromogen was a 0.16 M solution of 2,2´-azino-bis- (3-ethylbenzthiazoline-6-

Table I
Outcome of first and second reproductive cycles in 46 ewes examined for brucellosis in Italy

	First reproductive cycle		Second reproductive cycle		
	0 dpi	29-42 dpi	240 dpi	392-406 dpi	394-429 dpi
Ewe No.	Status	Outcome of first parturition	Status	Outcome of second parturition	Lactating
101	Not pregnant	—	Pregnant	Parturition	Yes
102	Pregnant	Abortion	Pregnant	Parturition	Yes
103	Pregnant	Abortion	Pregnant	Parturition	Yes
104	Pregnant	Abortion	Pregnant	Parturition	Yes
105	Not pregnant	—	Dead	—	—
106	Not pregnant	—	Pregnant	Parturition	Yes
107	Pregnant	Abortion	Pregnant	Parturition	Yes
108	Not pregnant	—	Pregnant	Parturition	Yes
109	Pregnant	Abortion	Not pregnant	—	—
110	Pregnant	Abortion	Not pregnant	—	—
111	Not pregnant	—	Pregnant	Parturition	No
112	Not pregnant	—	Pregnant	Parturition	Yes
113	Pregnant	Abortion	Not pregnant	—	—
114	Pregnant	Abortion	Pregnant	Parturition	Yes
115	Pregnant	Abortion	Pregnant	Parturition	No
116	Not pregnant	—	Not pregnant	—	—
117	Pregnant	Abortion	Not pregnant	—	—
118	Not pregnant	—	Not pregnant	—	—
119	Not pregnant	—	Pregnant	Parturition	No
120	Not pregnant	—	Dead	—	—
121	Not pregnant	—	Not pregnant	—	—
122	Pregnant	Abortion	Dead	—	—
123	Pregnant	Abortion	Pregnant	Parturition	Yes
124	Not pregnant	—	Pregnant	Parturition	Yes
125	Not pregnant	—	Not pregnant	—	—
126	Not pregnant	—	Not pregnant	—	—
127	Pregnant	Abortion	Pregnant	Parturition	Yes
128	Not pregnant	—	Pregnant	Parturition	Yes
129	Not pregnant	—	Pregnant	Parturition	Yes
130	Pregnant	Abortion	Pregnant	Parturition	Yes
131	Pregnant	Abortion	Not pregnant	—	—
132	Not pregnant	—	Pregnant	Parturition	Yes
133	Pregnant	Abortion	Pregnant	Parturition	Yes
134	Pregnant	Abortion	Pregnant	Parturition	Yes
135	Not pregnant	—	Pregnant	Parturition	Yes
136	Pregnant	Parturition	Pregnant	Parturition	No
137	Pregnant	Abortion	Not Pregnant	—	—
138	Pregnant	Abortion	Not pregnant	—	—
139	Pregnant	Abortion	Pregnant	Parturition	Yes
140	Not pregnant	—	Not pregnant	—	—
141	Pregnant	Abortion	Pregnant	Parturition	Yes
142	Pregnant	Abortion	Not pregnant	—	—
143	Not pregnant	—	Pregnant	Parturition	Yes
144	Not pregnant	—	Not pregnant	—	—
145	Pregnant	Abortion	Not pregnant	—	—
146	Pregnant	Abortion	Not pregnant	—	—

dpi = days post infection

Table II
Sampling date, number of lactating sheep and total milk and serum samples collected

Sampling date (days post infection)	Sampling No.	No. of lactating sheep	No. of milk samples collected	No. of blood samples collected
363	1	0	0	22
394	2	19	16	22
413	3	22	22	22
415	4	22	19	22
419	5	22	22	22
422	6	22	14	NA
429	7	22	22	22

NA not available

sulphonic acid) in citrate buffer (pH 4.5). Sodium fluoride was used to stop the enzymatic reaction. The optical density (OD) values were read in a microplate reader (λ = 405 nm). Strong positive (considered as 100% positivity) and negative standards were used. The results were expressed as percent positivity (PP) of the sample tested in relation to the strong positive control using the following formula:

$$PP = \frac{\text{Mean OD of tested sample}}{\text{Mean OD of positive control}} \times 100$$

The test cut-off value was set at 10% PP.

Demographic data

Data on sheep and goat populations and data from the 2004 National Brucellosis Eradication Campaign were kindly provided by the Italian Ministry of Health.

Statistical analysis

Sensitivity and specificity values were estimated and compared using a Bayesian approach (11). Bayesian inference is an application of the Bayes theorem (2) that allows the investigator to integrate any previous knowledge (expressed as a prior probability distribution), with the likelihood of obtaining a certain result if the animal is infected or if the animal is healthy (likelihood functions), with the results obtained by the application of the tests to a given population (collected data). The likelihood functions depend on the sensitivity and specificity of the test(s) employed and on the uncertainty of their values. The final results are probability distribution of the number of infected animals correctly identified as infected (sensitivity) or of the number of healthy animals correctly identified as healthy (specificity) in the sample or in the population (posterior probability). Probabilities of the various possible sensitivity values were estimated using a binomial likelihood function and an uninformed Uniform(0,1) prior distribution. As existing knowledge on the sensitivity or specificity of tests was considered to be virtually nil, an uninformed Uniform(0,1) prior distribution was used. The Uniform (0,1) distribution states that prior to the collection of data, all true probability values are considered possible within the range defined for the number of true positives (sensitivity calculation) or true negatives (specificity calculation). The RBT and the CFT results were expressed as the percentage of positive animals tested; the upper and lower 95% confidence intervals (CI) were calculated using a beta probability distribution (13). To interpolate the data obtained from positive samples, the results were analysed by least square linear regression. After transformation into the natural logarithm, the percentile of the frequency distribution of the maximum dilution to which positivity was still detectable (dependent variable)

was regressed against the dilution (independent variable). Data of the regression were also used to estimate the probability of a flock infection not being detected through bulk milk testing in relation to the prevalence of infection within the flock. Calculations were performed using MS-Excel® for Windows®, version 2000.

Results

Serological testing

All animals were RBT- and CFT-negative before experimental infection. The RBT and the CFT results, expressed as the overall number of positive samples divided by the number tested per sampling day, are shown in Table III. The most probable test sensitivity values are reported in brackets. The serological tests performed on the first serum sampling after parturition had a sensitivity of 100% for both the RBT and the CFT (CI 88-100%). The sensitivity of the RBT decreased to 73% (CI 55-85%) in subsequent samplings. The sensitivity of the CFT also decreased about two months later, with sensitivity at 95% (CI 81-98%) and 81% (CI 61-93%) recorded for the last two samplings, respectively. The probability distributions of the RBT and CFT sensitivity estimates, on single-day samplings are reported in Figures 1 and 2, respectively. All control animals were negative to the RBT and the CFT.

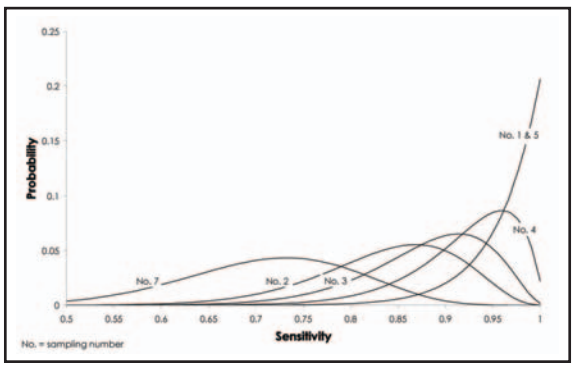

Figure 1
Probabily distributions of the sensitivity estimates of the Rose Bengal test on each single-day sampling

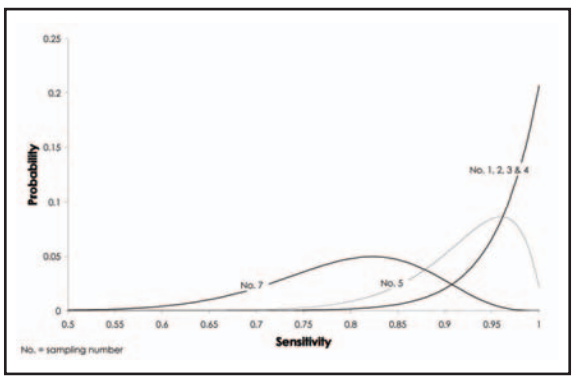

Figure 2
Probability distributions of the sensitivity estimates of the complement fixation on each single-day sampling

Table III
Results obtained using Rose Bengal test, complement fixation test and m-ELISA

Sampling date (days post infection)	Sampling No.	No. of positive samples collected RBT (% Se)	CFT (% Se)	m-ELISA (% Se)
363	1	22/22 (100%)	22/22 (100%)	NA
394	2	19/22 (86%)	22/22 (100%)	13/16 (81%)
413	3	20/22 (91%)	22/22 (100%)	15/22 68%)
415	4	21/22 (95%)	22/22 (100%)	14/19 (74%)
419	5	22/22 (100%)	21/22 (95%)	20/22 (91%)
422	6	NA	NA	12/14 (86%)
429	7	16/22 (73%)	18/22 (82%)	21/22 (95%)

Se sensitivity
NA not available

Milk testing

Milk samples from the 51 brucellosis-free sheep gave negative results to the m-ELISA.

The results of the m-ELISA on experimentally infected ewes, expressed as the overall number of positive samples tested per sampling day, are shown in Table III. The sensitivity of the m-ELISA initially decreased from 81% (CI 57-93%) on the second sampling to 68% (CI 50-81%) on the third sampling, but then increased to 91% (CI 72-97%) on the fifth and to 95% (CI 81-98%) on final sampling. The probability distribution of the estimates of the m-ELISA sensitivity for each sampling is shown in Figure 3. The probability distribution of the estimates of the specificity of the three tests (RBT, CFT and m-ELISA), calculated on samples from brucellosis-free sheep, is shown in Figure 4. The three tests demonstrated 100% specificity. The cumulative frequency distribution of the maximum dilution to which positivity was still detectable is shown in Figure 5. The frequencies of the milk antibody titres were effectively described by the logarithmic model $y=11.423\text{Ln}(x)+1.892$ ($F=316.2$, $p<0.01$) which had an adjusted determination coefficient of $R^2=0.978274$. Results of the regression analysis are shown in Figure 6. The probability that flock infection would not be detected by bulk milk testing was estimated by

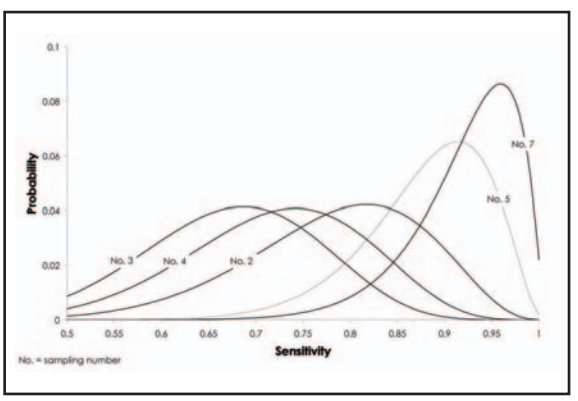

Figure 3
Probability distributions of the sensitivity estimates of the m-ELISA on each single-day sampling

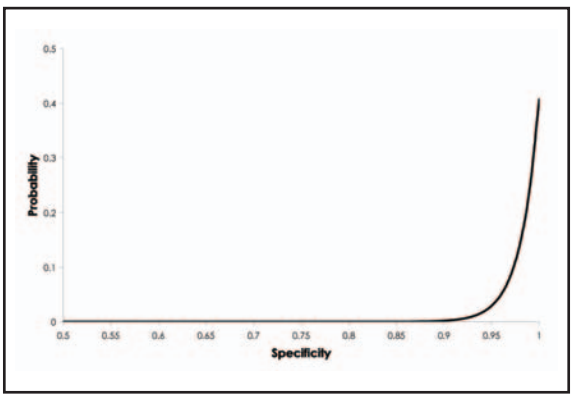

Figure 4
Probability distribution of the specificity estimates of the Rose Bengal test, complement fixation test and m-ELISA

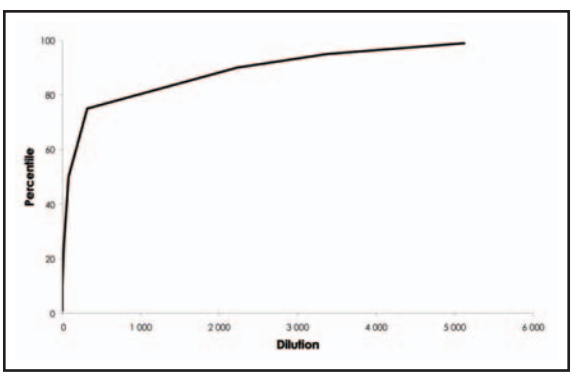

Figure 5
Maximum dilution of milk samples in which test positivity was still detectable

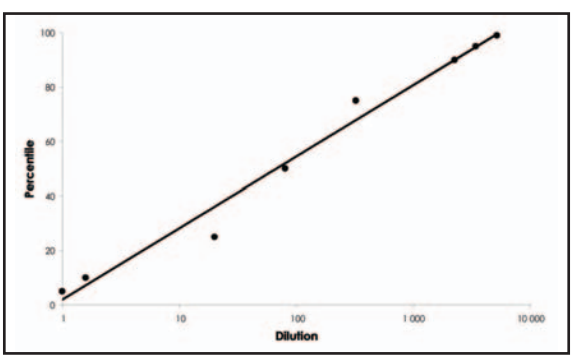

Figure 6
Results of the regression analysis on various milk dilutions

means of regression data, considering flocks at various levels of prevalence. Results are shown in Figure 7. The probability of not detecting infection in a flock is always greater than 25% when the prevalence of infection within the flock is lower than 5%. All control animals gave negative results against the m-ELISA.

Discussion

The sensitivity of the m-ELISA initially decreased from 81% (CI 57-93%) on the second sampling to 68% (CI 50-81%) on the third sampling, but then increased to 91% (CI 72-97%) on the fifth and to 95% (CI 81-98%) on final sampling (Table III; Fig. 3). The final value is higher than previously described (4, 5). Nevertheless, due to the overlap of respective CIs, the sensitivity values obtained for the m-ELISA in this experimental study do not significantly differ from the values reported in a previous study (4) in which the same m-ELISA had been evaluated on milk from naturally infected sheep.

The specificity of the m-ELISA was 100% (CI 94-100%; Fig. 4); other authors (4, 5) obtained similar results (specificity=100%; CI 97-100%).

The serological tests performed on the first serum sampling after parturition (Table III), had a sensitivity of 100% for both the RBT and the CFT (CI 88-100%), which is the same as reported previously in the first part of this study (12). Nevertheless, the sensitivity of the RBT decreased to 73% (CI 55-85%) in subsequent samplings (Table III; Fig. 1). The sensitivity of the CFT also decreased about two months later (Table III; Fig. 2), with a sensitivity of 95% (CI 81-98%) and 81% (CI 61-93%) recorded for the last two samplings, respectively. This post-partum decline in serological test sensitivity has been described previously (4, 12). In any event, the results of this study confirm the lower sensitivity of the m-ELISA with respect to the CFT, as previously reported under natural conditions (4).

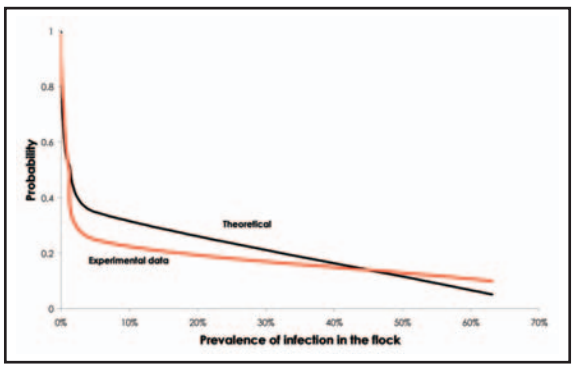

Figure 7
Probability of not detecting flock infection using the m-ELISA bulk test

The m-ELISA demonstrated a 100% ability to correctly classify non-infected animals, in full agreement with both the RBT and the CFT (Fig. 4), suggesting that all tests had similar specificity, as reported previously (4).

In regard to the possible use of the m-ELISA for screening sheep flocks for brucellosis by testing bulk milk samples, a regression analysis (Fig. 6) applied to milk dilutions (Fig. 5) showed that when the disease prevalence in a flock is below 5%, the estimated probability of not detecting an infected flock through m-ELISA bulk milk testing is always higher than 25% (Fig. 7). In 2004, the number of infected animals in controlled flocks was below 5% in almost all regions of Italy (Fig. 8). Therefore, in field conditions and considering that the average sheep population in a flock in Italy is 70, the probability that infection is not detected exceeds 25% if four infected milking sheep (or less) are present in the flock.

In conclusion, it would seem that the m-ELISA is not reliable for screening bulk milk samples from sheep when the prevalence of brucellosis at flock level is low.

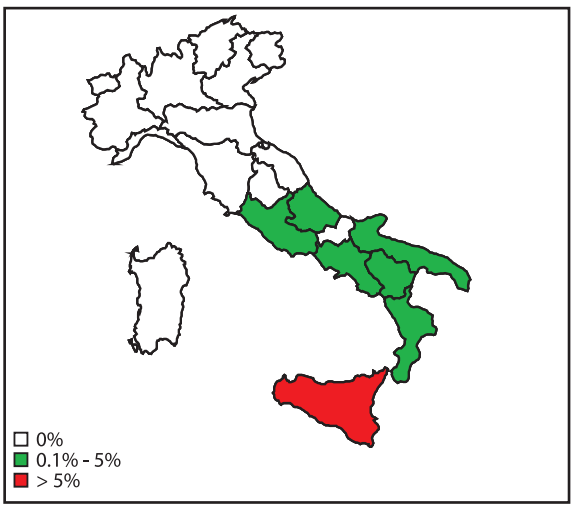

Figure 8
Percentage of brucellosis-infected sheep and goats compared to controlled animals, Italy 2004

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