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# The Use of Homologous Antigen in the Serological Diagnosis of Brucellosis Caused by *Brucella melitensis*

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With 8 figures and 2 tables

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## Summary

In the European Union the serological diagnosis of brucellosis caused by *Brucella melitensis* is performed using the heterologous antigen of *B. abortus* S99. The possible higher sensitivity or ability of an early detection of antibodies by a homologous antigen may prove very useful in the final phases of an eradication programme. Results obtained in sheep experimentally infected by *B. melitensis* biovar 3 were compared using *B. abortus* S99, *B. melitensis* M1, M2 and M3 antigens in the Rose Bengal plate test (RBPT), the complement fixation test (CFT) and an enzyme-linked immunosorbent assay (ELISA) test. Forty-six sheep from an officially brucellosis-free flock were experimentally infected intraconjunctivally with *B. melitensis* biovar 3. Prior to infection, all animals were tested first against *Brucella* antibodies, weekly for 2 months post-infection (PI) and then monthly for a further $7\frac{1}{2}$ months. All sera were tested against the antigens listed above using RBPT, CFT and ELISA. Using a Bayesian approach, test sensitivities were estimated and compared. Their ability for the early detection of antibodies was evaluated through a regression model based on a logit response model, using the number of days PI as the independent variable and the logit of the fraction of positive animals as the dependent variable. No significant differences were detected among the various antigens used, either in terms of sensitivity or in terms of antibody kinetics; however, the CFT was significantly less sensitive than the RBPT and ELISA and it also showed a lower rate of increase of percentage positive animals ($\beta$-coefficient of regression analysis).

## Introduction

The serological diagnosis of *Brucella melitensis* infection, in the European Union (EU), is performed using the *B. abortus* biovar 1 strains Weybridge S99 or USDA 1119-3 heterologous antigen (EC, 2000). The officially recognized tests are the complement fixation (CFT) and Rose Bengal Plate (RBPT) test (O.I.E., 2000). In Europe, the enzyme-linked immunosorbent assay (ELISA) is not used officially for the diagnosis of brucellosis in sheep and goats. In a previous study (Nannini et al., 1992), the ability of antigen S99 to detect antibodies in 30 sheep experimentally infected with *B. melitensis* biovar 2 was found to be satisfactory [100 and 97% positive to RBPT and CFT, respectively, 1 month post-infection (PI) which decreased to 97 and 83% 6 months PI]. Despite the satisfactory results using the S99 antigen, Corbel (1985) suggested that, in the final phase of an eradication programme, a homologous antigen may prove more useful as it might detect antibodies earlier and be also more sensitive than a heterologous antigen. In a study (Alton et al., 1971) using sera obtained from cattle, rabbit, goat and human beings infected with various strains of *B. abortus* and *B. melitensis*, the performances of three antigens prepared from *B. abortus*, *B. suis* and *B. melitensis*, were compared in the tube agglutination test. All sera were strongly positive, but in most instances the titre was higher when using the homologous than the heterologous antigen. A later study (Corbel, 1985), indicated that the use of the M antigen (derived from *B. melitensis* biovar 1) in the RBPT, when testing cattle infected with an M-dominant strain of *B. abortus* (biovar 5), performed better than the classical A-dominant antigen. In another study (Connell and Dubray, 1986) on the standardization of RBPT for the diagnosis of *B. melitensis* infection in sheep and goats, a higher sensitivity was observed with the *B. abortus* S99 antigen than with the *B. melitensis* antigens.

Using sheep experimentally infected with *B. melitensis* biovar 3, this study compares the performance of antigens prepared with *B. abortus* S99, *B. melitensis* M1, M2 and M3 using the RBPT, CFT and ELISA tests.

## Material and Methods

### Animals

Forty-six brucellosis-free Fabrianese and cross-bred ewes, aged between 1 and 5 years, and of which 26 were pregnant, were experimentally infected intraconjunctivally with a field strain of *B. melitensis* biovar 3. A freshly isolated field strain was inoculated into two guinea pigs and re-isolated from their spleens 21 days later in 10 ml of Farrel's medium. After 48 h of growth in medium at 37°C, the re-isolated *Brucella* was titrated. A dose of $5 \times 10^8$ colony-forming units (CFU) in 100 $\mu$l was inoculated into the conjuctiva of both eyes (50 $\mu$l each) of the experimental ewes according to the provisions of the European Pharmacopoeia (Conseil de l'Europe, 2000).

### Sampling

All animals were tested for *Brucella* antibodies prior to experimental infection and, once infected, were tested for the following $9\frac{1}{2}$ months, according to the protocol given in Table 1. A total of 648 sera were collected and tested by RBPT, CFT and ELISA, using the four antigens listed above.

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Table 1. Sampling protocol for experimentally *Brucella*-infected sheep

| Sampling no. | Date | Dpi | Sampled animals |
|---|---|---|---|
| 1 | 16/06/1999 | 0 | 46 |
| 2 | 25/06/1999 | 9 | 46 |
| 3 | 01/07/1999 | 15 | 45 |
| 4 | 08/07/1999 | 22 | 45 |
| 5 | 16/07/1999 | 30 | 45 |
| 6 | 22/07/1999 | 36 | 44 |
| 7 | 29/07/1999 | 43 | 44 |
| 8 | 05/08/1999 | 50 | 43 |
| 9 | 12/08/1999 | 57 | 44 |
| 10 | 20/10/1999 | 126 | 43 |
| 11 | 09/02/2000 | 238 | 39 |
| 12 | 27/03/2000 | 285 | 41 |

Dpi, days PI.

### Serological testing

The four antigens were prepared in the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, using *B. abortus* biovar 1 strain S99, *B. melitensis* biovar 1 strain 16/M, *B. melitensis* biovar 2 strain 63/9 and *B. melitensis* biovar 3 strain Ether, all provided by the Veterinary Laboratories Agency (Weybridge, UK). Whole cell antigens for RBPT and CFT were prepared and tests performed following Alton et al. (1988). Smooth lipopolysaccharide (LPS) ELISA antigens were extracted following Hendry et al. (1985) and the tests performed following set standards for diagnostic tests and vaccines (O.I.E., 2000).

### Statistical analyses

Sensitivity values for both tests and antigens were estimated and compared using a Bayesian approach (Sivia, 1996). Probability of the various possible sensitivity values were estimated using a binomial likelihood function and an uninformed *Uniform(0,1)* prior distribution. A group of posterior $\beta$-distributions was calculated for the entire experimental period (using all 602 sera derived from repeated sampling of the 46 animals). As the use of repeated sampling data introduces a dependency in the data set, the following two cross-sections were considered: (i) the first month after infection (i.e. the 45 sera collected 30 days PI) and (ii) the last sampling performed $9\frac{1}{2}$ months PI (i.e. 39 sera collected 285 days PI). Similar probability distributions were calculated using data from a previous study (Nannini et al., 1992) in which 30 sheep had been infected with *B. melitensis* biovar 2 and tested using S99 antigen in RBPT, CFT and ELISA; only the samples collected by Nannini et al. (1992) 35 days PI are assessed in the present analysis. The Bayesian analysis was performed using MS-Excel for Windows.

The CFT antibody titres obtained using the four antigens were compared by Friedman's non-parametric analysis of variance and the contrasts were evaluated using the Wilcoxon test (Siegel and Castellan, 1988).

The ability for early antibody detection was evaluated using a logit response model (Norušis, 1997); the number of days PI was the independent variable and the logit of the fraction of positive animals was the dependent variable. These latter two analyses were performed using SPSS for Windows (version 12.0).

## Results

All animals were negative for brucellosis before infection. All except one of the pregnant ewes aborted during the last month of pregnancy; *B. melitensis* was isolated from either the liver, the abomasum or from the brain of the aborted foetuses. Five animals died during the experiment, one as a sequel to the abortion; the remainder died of causes unrelated to brucellosis between 2 weeks and 8 months PI.

The respective results obtained using the RBPT, the CFT and the ELISA are compared in Table 2. These sensitivity values reflect two phases of infection in the animals: the first phase is one of steeply increasing antibody production (lasting approximately 1 month), and the second phase of more stable antibody production. During the first month, and using all antigens, test sensitivities showed little increase in the first 9 days PI, the values ranging between 0 and 4%, but reached 100% on the 36th day PI in both the ELISA and the RBPT and only on the 50th day in the CFT (Table 2). Following the 50th day PI, all test sensitivities stood at 100%. As a result of the antibody kinetics, different test sensitivities should be calculated for the two phases of infection: (i) the first month PI and (ii) the remaining period up to 1 year PI. The probability distributions of the estimates of sensitivity for the 30th day PI are shown in Fig. 1 and for the last day of PI in Fig. 2. All 12 samplings were also considered together; the resulting probability distributions of the estimates are shown in Fig. 3. These

Table 2. Results obtained in 12 samplings with the RBPT, CFT and ELISA tests and S99, M1, M2, M3 antigens

| Dpi | Animals tested | RBPT (%) | | | | CFT (%) | | | | ELISA (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | S99 | M1 | M2 | M3 | S99 | M1 | M2 | M3 | S99 | M1 | M2 | M3 |
| 0 | 46 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 9 | 46 | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 2 | 2 | 4 | 2 |
| 15 | 45 | 58 | 71 | 73 | 40 | 29 | 14 | 11 | 14 | 49 | 44 | 42 | 44 |
| 22 | 45 | 91 | 87 | 96 | 93 | 49 | 45 | 55 | 55 | 93 | 91 | 93 | 93 |
| 30 | 45 | 100 | 96 | 100 | 93 | 82 | 64 | 77 | 77 | 98 | 100 | 98 | 100 |
| 36 | 44 | 100 | 100 | 100 | 100 | 98 | 95 | 98 | 100 | 100 | 100 | 100 | 100 |
| 43 | 44 | 100 | 100 | 100 | 100 | 95 | 95 | 98 | 95 | 100 | 100 | 100 | 100 |
| 50 | 43 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 57 | 44 | 100 | 100 | 100 | 100 | 100 | 98 | 100 | 100 | 100 | 100 | 100 | 100 |
| 126 | 43 | 100 | 100 | 100 | 100 | 100 | 98 | 100 | 100 | 100 | 100 | 100 | 100 |
| 238 | 39 | 100 | 100 | 100 | 100 | 100 | 97 | 95 | 97 | 100 | 100 | 100 | 100 |
| 285 | 41 | 100 | 100 | 100 | 100 | 100 | 95 | 100 | 95 | 100 | 100 | 100 | 100 |

Dpi, days PI.

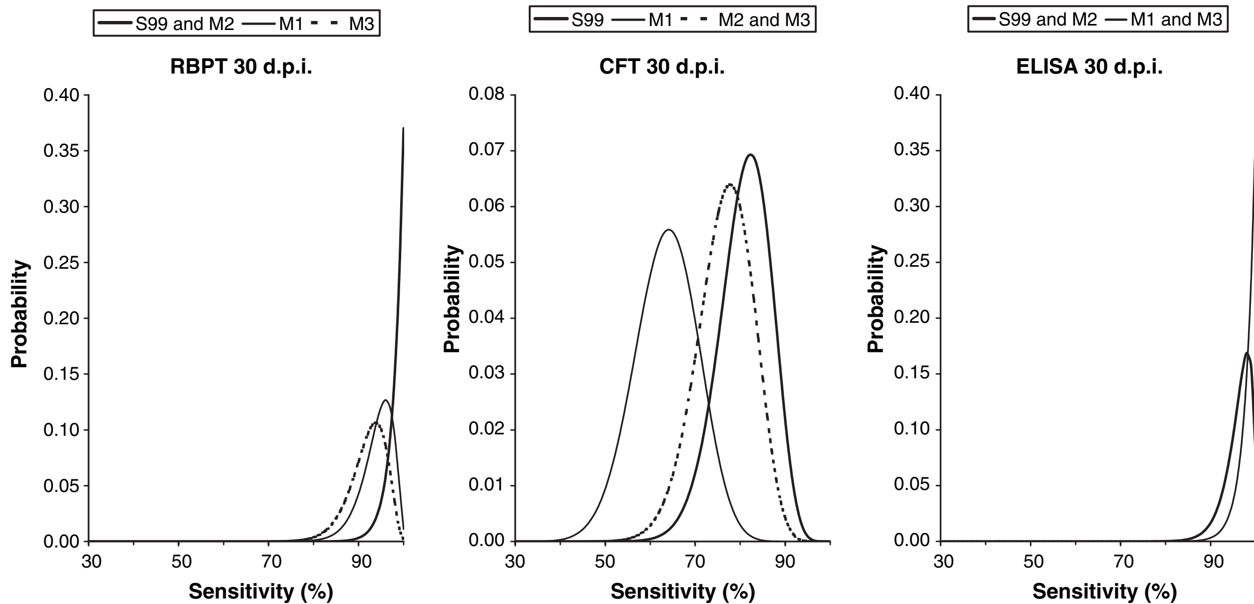


Fig. 1. Probability distributions of the estimates of sensitivity of serological tests for brucellosis 30 days PI.

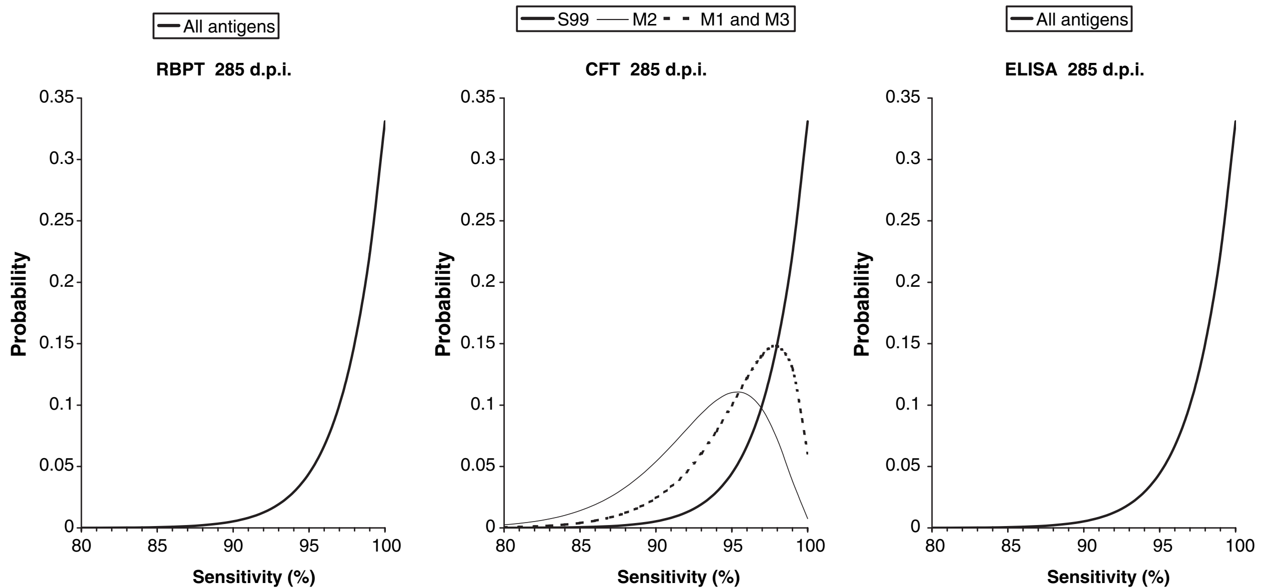


Fig. 2. Probability distributions of the estimates of sensitivity of serological tests for brucellosis 285 days PI.

indicate that the sensitivity of the RBPT ranged between 87 and 90% depending upon the antigen used (S99 = 89%, M1 = 89%, M2 = 90%, M3 = 87%), that of the CFT ranged between 81 and 84% (S99 = 84%, M1 = 81%, M2 = 82%, M3 = 82%), and that of the ELISA was a consistent 88% for all antigens.

The results of the 46 animals tested on the first sampling (day 0) can be used to estimate test specificity (Fig. 4). The probability distribution of sensitivity estimates obtained in a previous study (Nannini et al., 1992) and shown in Fig. 5, are used to compare the performance of S99 antigen in *B. melitensis* biovar 2 and in biovar 3 infections.

If each of the three tests is examined separately, and antigen performances are compared, it will be seen that all curves overlap. The overlap of areas below the 30-day PI curves (Fig. 1) is between 26% and 100% in the case of RBPT, between 18 and 100% for CFT and between 63% and 100% for ELISA. At the end of the experiment (285 days PI, Fig. 2), the overlap had increased to 100% for all antigens using both the RBPT and the ELISA, but was lower (ranging between 41% and 100%) when using the CFT. The amount of overlap in the distributions indicates the similarity of the probability of the test sensitivities.

The test performances for each of the three *B. melitensis* antigens indicate the CFT to be of lower sensitivity when compared with RBPT and ELISA using the 30-day PI data (Fig. 6). These differences decrease over increasing PI period and disappear 50 days PI when all animals tested positive in all

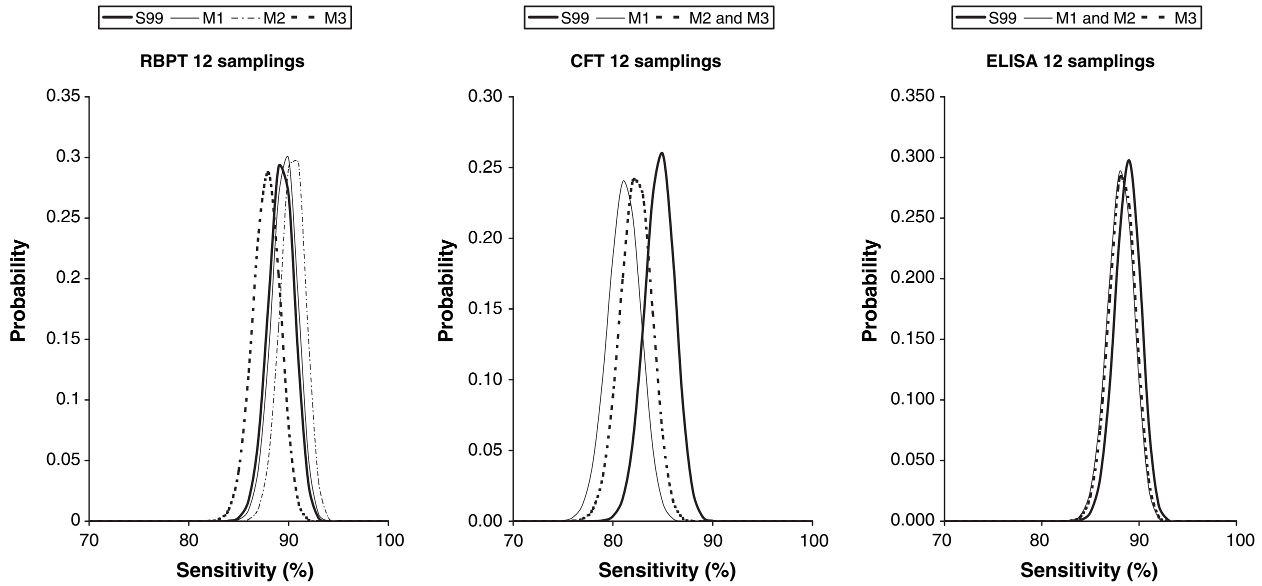


Fig. 3. Probability distributions of the estimates of sensitivity of serological tests for brucellosis in all 12 serum samples.

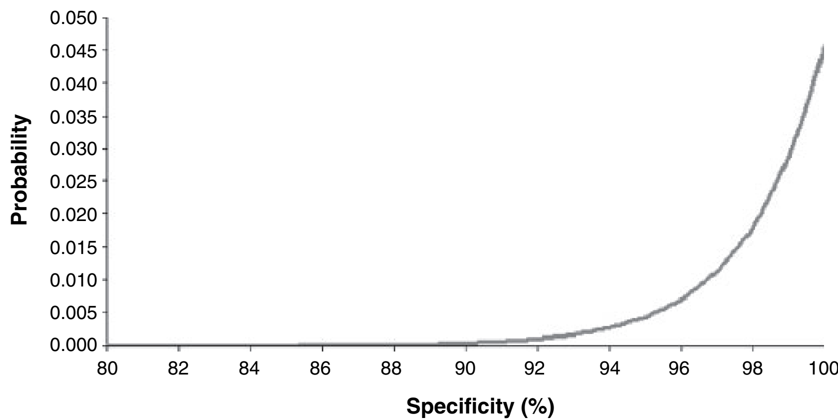


Fig. 4. Probability distribution of the specificity based on 46 uninfected sheep.

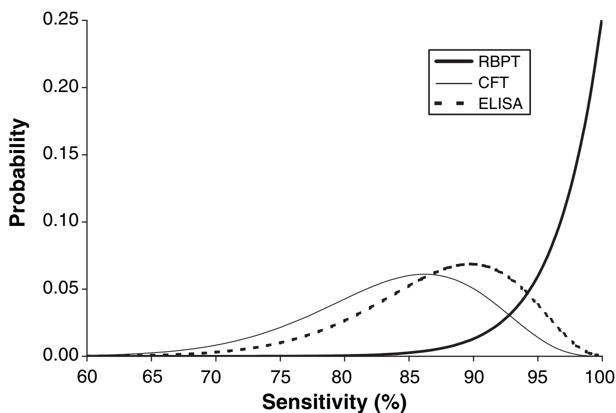


Fig. 5. Probability distribution of sensitivity estimates of serological tests for brucellosis using S99 antigen in 30 sheep experimentally infected with *Brucella melitensis* biovar 2 (Nannini et al., 1992).

tests using all antigens (Table 2). One week thereafter and subsequently, very few negative CFT results were obtained using the various M antigens (Table 2). The CFT antibody titres differed significantly amongst all four antigens both 30 days PI ($\chi^2 = 48.0$, $P < 0.001$) and 285 days PI ($\chi^2 = 64.4$, $P < 0.001$) using Friedman's non-parametric analysis of variance. In these two sample collection days, the titres were significantly higher to antigen S99 than to any of the three M antigens (Wilcoxon test, $P < 0.001$).

The antibody kinetics during the first 2 months, which is expressed as the percentage of animals positive at each sampling point, are shown in Fig. 7. The dots in the figure represent the observed percentages, while the solid lines represent the expected percentages based on the logit response analysis. If the four antigens are compared, no statistically significant differences occur. However, the antibody response appears earlier in the RBPT and in the ELISA than in the CFT. The difference in the observed antibody responses is statistically significant except for the RBPT–CFT (using antigen M3) and the CFT–ELISA (using antigen M2) (Fig. 8).

## Discussion

Under the experimental conditions described in this paper, homologous antigens did not give a more sensitive result than

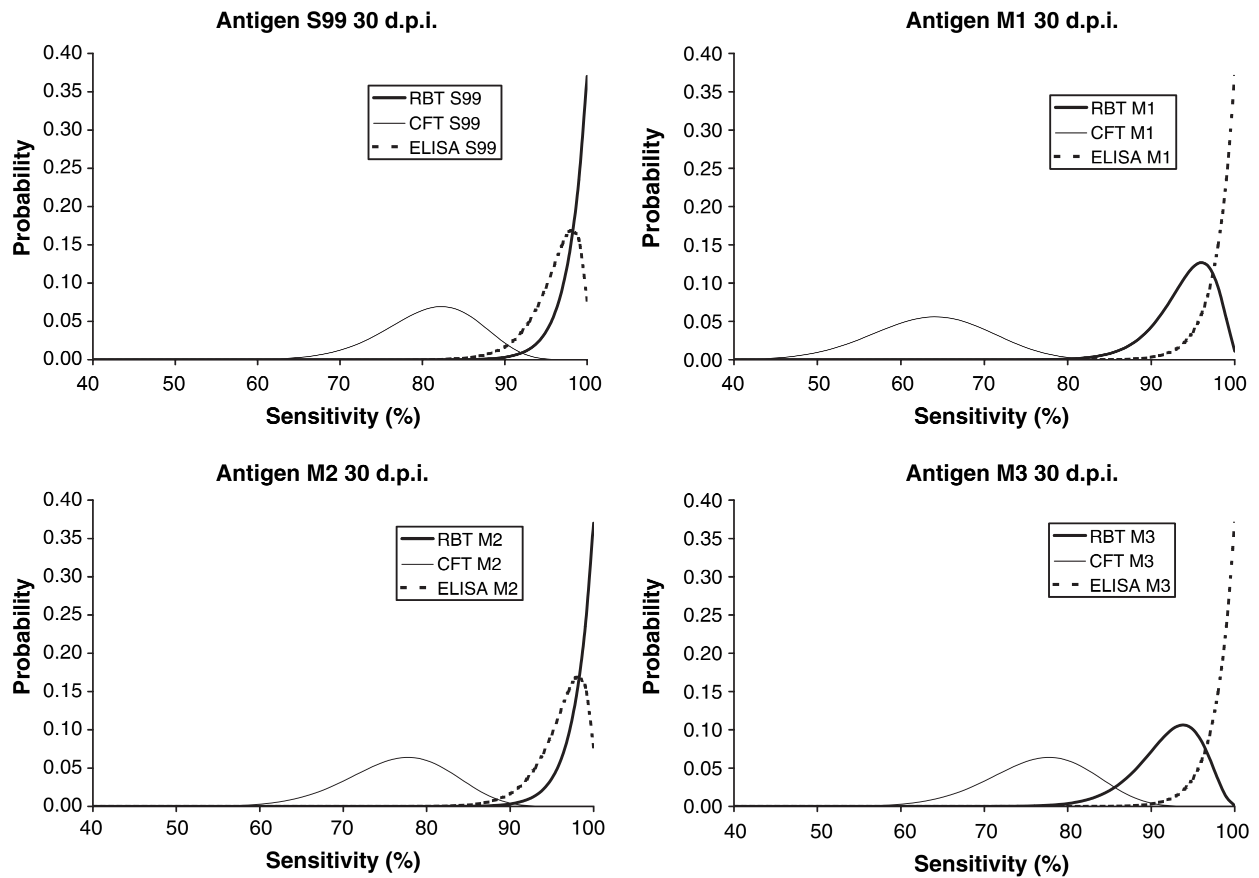


Fig. 6. Comparative performance of RBPT, CFT and ELISA 30 days PI.

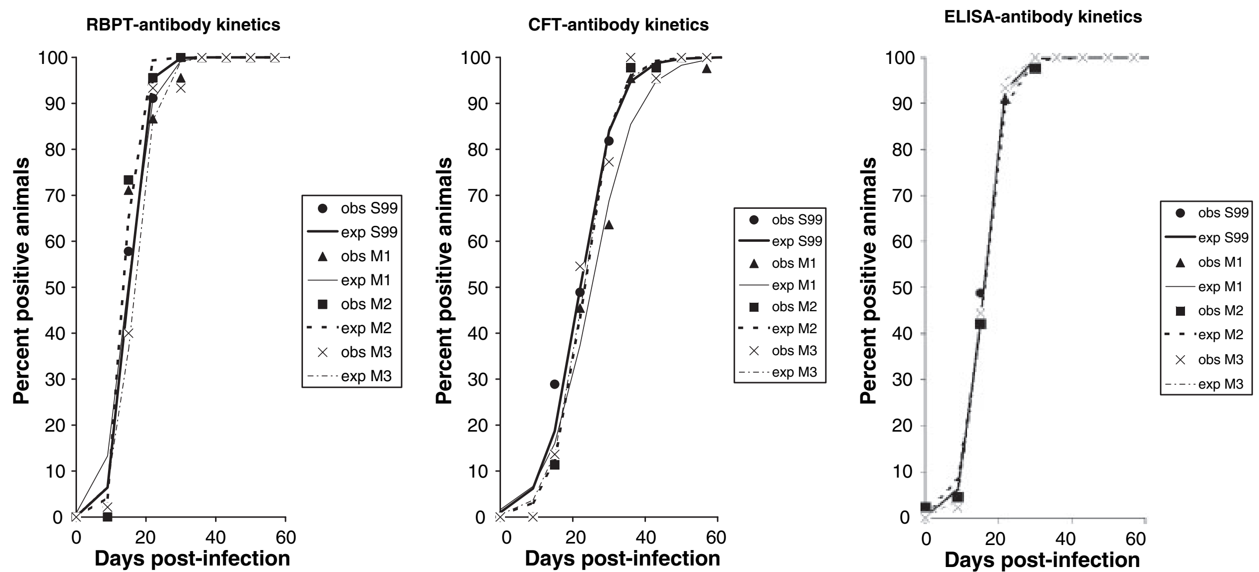


Fig. 7. Antibody kinetics during the first 2 months after experimental infection with *Brucella melitensis* biovar 3.

that obtained using the standard antigen (S99) for detecting *B. melitensis* infections in sheep. Furthermore, they do not appear capable of detecting infections earlier, thus contradicting the findings of earlier authors (Alton et al., 1988; Corbel, 1985; Connell and Dubray, 1986).

The current findings using the RBPT and the ELISA cannot be compared directly against those obtained by Alton et al. (1988) as the tube agglutination test used for antigen comparison in that study is quantitative, whereas the RBPT and the ELISA are both qualitative. The only possible comparison

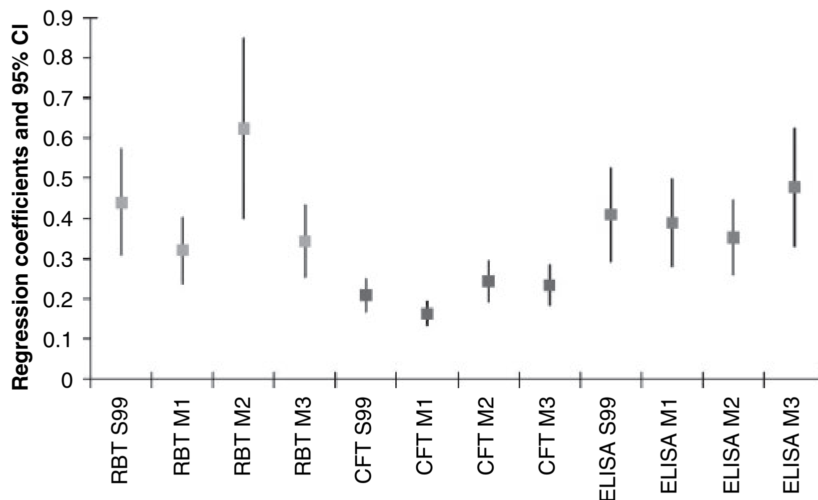


Fig. 8. Regression of the logit of the fraction of animals positive to *Brucella* antibodies and the number of days PI.

that can be made is with our CFT results. All the sera tested by Alton et al. (1988) gave positive results against all antigens, and agrees with the similar sensitivity values obtained by us. However, Alton et al. (1988) had a titre generally higher using the homologous antigen; this finding contrasts against the significantly higher CFT titres obtained by us using the heterologous antigen S99 (when compared with the homologous M antigens).

Corbel (1985) found that the M antigen (derived from *B. melitensis* biovar 1) used in the RBPT was more sensitive when testing cattle infected with an M-dominant strain of *B. abortus* (biovar 5). He claimed a significant result ($P < 0.001$) when he applied chi-square analysis in the RBPT performed manually on 1703 field sera. Actually, the difference amounted to 43 discordant sera (21 of which were positive to the A antigen and negative to the M antigen, and 22 the reverse). Corbel's remaining 1660 sera were either consistently negative (1581 sera) or positive (79 sera) to both antigens. A McNemar chi-squared test performed by us on his results did not revealed any statistically significant difference ($\chi^2 = 0$; $P = 1$).

The results obtained in this study also differ from the findings of Connell and Dubray (1986) who, in an attempt to standardize the RBPT for the diagnosis of *B. melitensis* infection in sheep and goats, observed a higher sensitivity when using the *B. abortus* S99 antigen than when using the *B. melitensis* antigen. The study, based on the repeated testing of the same 31 sera from infected flocks (15 repetitions for each serum and made by five independent laboratories) indicates that the S99 antigen performed best with 82.7% of positive results; however, the data were not statistically analysed and only tables summarizing the data were presented, thus impairing a statistical comparison with our results.

Results similar to ours, and so indicating similar sensitivities for homologous and heterologous antigens, were obtained by Alonso-Urmeneta et al. (1998). A possible explanation for the similar sensitivity values with homologous and heterologous antigens may be found in the excess amounts of both A and M antigens used in relation to the amounts actually required for a serological reaction. This excess could induce an antibody response to all antigens involved, with the possible dominance of antibodies against common epitopes (Alonso-Urmeneta et al., 1998).

As regards the sensitivity of the S99-RBPT ≥1 month PI, our results agree with those reported in a previous study (Nannini et al., 1992) as no relevant differences could be detected in the respective probability distributions in the two studies. The wider range in distributions observed in the earlier study (Nannini et al., 1992) is because of the lower number of sera examined (30 versus 46). However, the sensitivity of the S99-CFT antigen was lower in this study (Nannini et al., 1992), but the biological significance of this finding is uncertain because of a one animal being found negative 1 month PI and then becoming positive upon subsequent samplings.

Although an experimental infection might be considered a simplification of the real world, this long-term study, with a high frequency of samplings in the initial phases of the infection cycle, allows us to make some inferences on possible in-field infection scenarios.

With experimental infection the principal simplification is that all animals are infected simultaneously. Estimates of test sensitivity are, therefore, influenced heavily by the time intervals between infection and sampling. Sensitivity is usually calculated at the point where the antibody curve reaches a plateau and is expressed usually by sensitivity distributions as shown in Fig. 2. This approach is correct in terms of evaluating the performance of the test under optimal conditions, but may be misleading under field conditions. In the Mediterranean region where the transhumant breeding of sheep is extensive, a certain degree of pregnancy synchronization is practiced with lambing usually occurring before the animals move to their summer pastures. Most of the serological testing in these instances is performed either before the animals depart for their summer pastures (i.e. a few months after the lambing season) or when the flocks return to their winter residences. However, and especially in southern Europe, tests may be performed twice, once in each period. Under such conditions, and during the spring testing period, a number of animals will be in the initial stages of infection and, therefore, probability distributions of sensitivity similar to those in Fig. 1 can be expected. However, during the autumn testing, when several months would have elapsed since the peak of brucellosis dissemination (i.e. during the lambing season), the probability distributions of sensitivity will be more similar to those shown in Fig. 2. Further studies are required to evaluate

the sensitivity of the tests when a longer period has elapsed following experimental infection. For this reason, the present study will be continued into the second year PI, and following a second pregnancy.

Under EU regulations set out in Annex A of Directive 91/68/CEE (EC, 1991) sheep and goat flocks that have been declared officially brucellosis-free, maintain their status even when only 25% of the adult females have been tested. If this testing protocol is used on three flocks of 100 sheep each with respective infection rates of 2, 3 and 4%, the respective probabilities of no infected sheep being detected will be 25, 12 and 6%. The present study indicates that such probability levels will be raised further by the sub-optimal performances of tests used in recently infected animals. It would be reasonable, therefore, for the regulation to be amended so that all animals in transhumant flocks be tested as they often share a common summer pasturage, thus increasing the risk of brucellosis transmission.

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