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# An ELISA for the evaluation of gamma interferon production in cattle vaccinated with *Brucella abortus* strain RB51

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**Summary**

The results of an enzyme-linked immunosorbent assay (ELISA) implemented for the detection of gamma interferon (γ-interferon) production in cattle vaccinated with *Brucella abortus* strain RB51 are presented. A purified protein fraction derived from RB51 (RB51 brucellin) has been used as antigenic stimulus for whole blood. The test was evaluated for 300 days in ten heifers vaccinated at calfhood with $10 \times 10^9$ colony-forming units of RB51 and in five control heifers. All animals came from officially brucellosis-free herds. Vaccinated animals started to give positive results from day 17 post vaccination (pv) until day 239 pv. All vaccinated animals gave a positive reaction at least once (with a stimulation index exceeding 2.5). Nevertheless, if sampling on day 20 pv is excluded (90% of vaccinated animals gave positive results), the sensitivity of the test varies from 20% to 70%, with a 40% average. A stimulation index over 2.5 was also recorded in three control animals. The results suggest that the γ-interferon test is not suitable for the detection of cattle vaccinated with RB51, either at the individual or at the herd level.

**Keywords**

*Brucella abortus*, Brucellosis, Eradication, ELISA, Enzyme-linked immunosorbent assay, Gamma interferon, RB51, Vaccination.

## Introduction

The European Commission (EC) Decision 2002/598/CE of 15 July 2002 (6) approved the use of *Brucella abortus* strain RB51 vaccine (RB51) for the immunisation of cows at risk of infection with *B abortus*.

The competent authority of a member state is required to submit to the EC and to the other Member States detailed information on the vaccination programme, in particular regarding the area of vaccination, the age of the animals to be vaccinated and the test system in place to identify vaccinated animals.

RB51, the rough mutant of the virulent strain *B. abortus* 2308, does not lead to the production of antibodies that can be detected using serological tests listed by European legislation (11, 14, 15). This characteristic, even if it could be useful for the distinction of the vaccinated animals from infected ones, however requires the availability of alternative tests able to identify animals vaccinated with RB51, in order to satisfy the European rules and to enable pursuance of the implementation of the vaccination programme. To date, tests useful for this purpose are a dot-blot test (10), a complement fixation test (CFT) with specific RB51 antigen (RB51-CFT) (1) and the combination of RB51-CFT with a brucellin skin test (5). A useful method to reveal the presence of a cell-mediated immune response against

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*B. abortus* can be the detection of gamma interferon (γ-interferon) production following lymphocyte stimulation with the specific antigen. The test utilises, *in vitro*, the same mechanism that can be evoked *in vivo* by the brucellin skin test. Previous studies conducted on this topic demonstrated that *Brucella* spp. is able to elicit a macrophage response through the production of γ-interferon by stimulated T lymphocytes both in mice (7, 13) and in cattle infected with *B. abortus* (17). Studies performed on blood sampled from cattle vaccinated with RB51 and then stimulated with the homologous vaccinal strain showed promising results for the specific identification of cattle treated with RB51 (2). Although the test is less practical to perform compared to serological tests, it may be useful to identify cattle vaccinated with RB51.

The aims of our study were as follows:

- to evaluate the γ-interferon production in lymphocytes from cattle vaccinated with RB51 at calfhood
- to evaluate the possibility of identifying cattle vaccinated with RB51 at calfhood through the use of an enzyme-linked immunosorbent assay (ELISA) for the detection of γ-interferon.

# Materials and methods

## RB51 vaccine

The RB51 vaccine was kindly provided by CZ Veterinaria in Pontevedra (Spain) which is the European producer and distributor of the product, under licence from the Colorado Serum Company in Denver. Once reconstituted according to the manufacturer's instructions, the vaccine contained $5 \times 10^9$ colony-forming units (cfu)/ml.

## Animals and vaccination

Fifteen Friesian calves, aged between four and six months, obtained from officially brucellosis-free herds, were selected at random and divided into two groups of five and ten animals. Prior to the experiment, animals were tested using the Rose Bengal test (RBT) and CFT, in accordance with the *Manual* of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (18). The animals were also tested with the dot-blot test and the RB51-CFT. All tests gave negative results. The ten animals were then vaccinated subcutaneously with RB51 in accordance with the instructions of the manufacturers (2 ml reconstituted solution, corresponding to $10 \times 10^9$ cfu). The five control animals were inoculated subcutaneously with 2 ml sterile saline solution. The animals were kept in isolation, on the same premises, for the entire experiment duration.

## Sampling protocol

Blood samples were collected via jugular venipuncture in sterile tubes containing lithium-heparin as an anticoagulant on day 0 (just before vaccination) and on days 1, 2, 6, 9, 13, 14, 17, 20, 29, 43, 58, 76, 91, 104, 119, 162, 239, 268, 300 post vaccination (pv). Samples were refrigerated without delay; they were kept at +4°C during transport and despatched to the laboratory within 6 h. Simultaneously, serum samples were collected from the same animals and tested with the RBT and CFT.

## Whole blood stimulation

All ELISA γ-interferon tests were performed no later than 6 h after bleeding. The whole blood stimulation was performed using a purified proteic fraction of RB51 (RB51 brucellin), produced by the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'* (IZS A&M) as previously described (5). Stimulation was performed in duplicate in 24-well microplates by mixing 100 µl of RB51 brucellin diluted in phosphate buffered saline (PBS) (40 µg of protein content) to 1 ml of the blood under examination. As a negative control, 100 µl of PBS were added to 1 ml of the blood being tested. Plates were incubated for 16-24 h at 37°C in a humidified atmosphere with 5% $CO_2$. After incubation, plates were centrifuged for 10 min at 800 × g and supernatant plasma was harvested.

## Gamma interferon assay

The level of γ-interferon in the plasma harvested after stimulation was evaluated using an ELISA (17). Microplates were activated with 100 µl of commercial

monoclonal antibody anti-bovine γ-interferon (Eurokit, Gorizia), diluted in carbonate-bicarbonate buffer, pH 9.6 (5 µg/ml) and incubated overnight at 37°C. Plates were then saturated with diluent buffer (PBS + bovine albumin) and incubated for 2 h at room temperature. After washing with saline solution containing 0.01% Tween 20 (200 µl/well), 100 µl of plasma previously harvested and 100 µl of PBS were distributed per well. For each plate, bovine RB51-negative reference sera, bovine RB51-positive reference sera, (prepared from the blood of a calf vaccinated with RB51), and PBS (to verify the reaction in the absence of a sample) were used as internal controls. All samples and controls were assayed in duplicate. Plates were then incubated for one hour at room temperature and, after washing, 100 µl of commercial monoclonal antibody anti-bovine γ-interferon (Eurokit Gorizia), conjugated with peroxidase and diluted in diluent buffer (5 µg/ml), were added. After one hour of incubation at room temperature, the plates were washed and 100 µl of substrate were added. The optical density (OD) values were read in a microplate reader ($\lambda$ = 650 nm). The results were expressed as stimulation index (SI), calculated as the ratio between the mean OD of tested sample and the mean of the optical densities of negative control wells as described by Weynants *et al.* (17). SI values greater than 2.5 were considered positive.

### Statistical analysis

The sensitivity and specificity of the γ-interferon test were estimated and compared using a Bayesian approach (12). Bayesian inference is an application of the Bayes theorem (3) 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 ELISA γ-interferon test results were expressed as the percentage of animals correctly identified as vaccinated (sensitivity, calculated on the vaccinated group) or non-vaccinated (specificity, calculated on the non-vaccinated group) on animals tested; the upper and lower 95% confidence intervals (CI) were calculated using a beta probability distribution (16). Calculations were performed using MS-Excel® for Windows®, version 2000.

## Results

All RBT and CFT tests gave negative results. The results of the γ-interferon assay performed on vaccinated animals, expressed as the percentage of animals providing a positive result to the test and related CI, are shown in Fig. 1. In vaccinated animals, and using RB51 brucellin diluted at 1:2.5 as stimulation antigen (40 µg of protein), the test began to give positive results from day 17 pv (40% of vaccinated animals gave positive results to the test, CI 16.7%-69.2%) reaching a peak of 90% sensitivity (CI 58.7%-97.7%) at day 20 pv. After a sharp decrease in the following sampling (10%; CI 2.3%-41.3%), the sensitivity of the test fluctuated between 20% and 70%, with an average of 40% until day 239 pv, a period in which at least one vaccinated animal reacted positive to the test. The last two samplings, at days 268 and 300 pv, provided negative results for all vaccinated animals. During the entire

experiment, all vaccinated animals gave a positive response to the test at least once. Results, expressed as a percentage of control animals (not vaccinated) that gave positive results to the test, are shown in Fig. 2. Control animals showed positive results to the test on sampling performed on days 20, 76, 91, 104, 119 and 239 pv, with a peak of three animals positive out of five (specificity 40%, CI 11.8%-77.7%) at day 76 pv. Three control animals out of five had a positive reaction to the test at least once. The sensitivity of the γ-interferon test at herd level, in relation to the animal and the number of vaccinated animals in the herd, is shown in Figure 3. Figure 4 shows the probability that the γ-interferon test would give at least one false positive result at the herd level, in relation to the specificity of the test at the animal level and to the number of the animals in the herd.

## Discussion

Inoculation of strain RB51 does not lead to the production of antibodies that can be detected using serological tests listed by European legislation (11, 14, 15). The availability of diagnostic tests able to detect animals

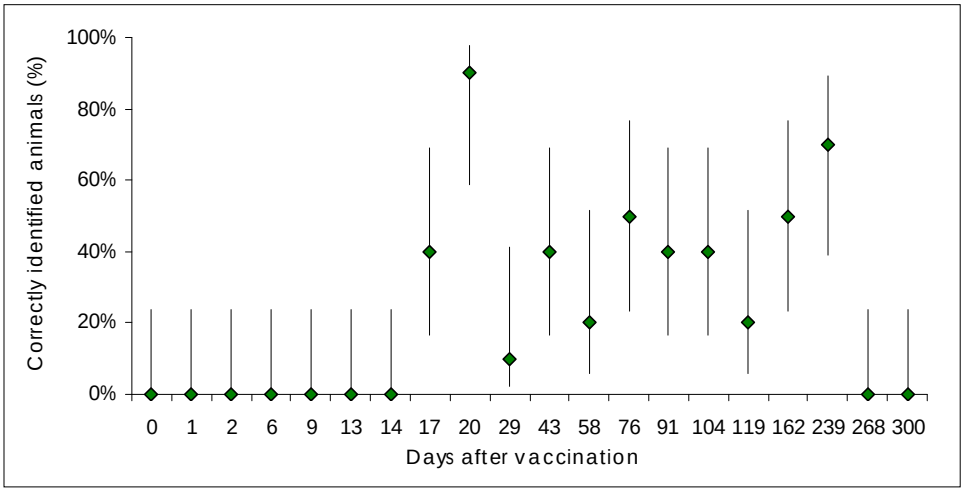


Figure 1
Percentage of vaccinated animals correctly identified by the γ-interferon test and 95% confidence intervals

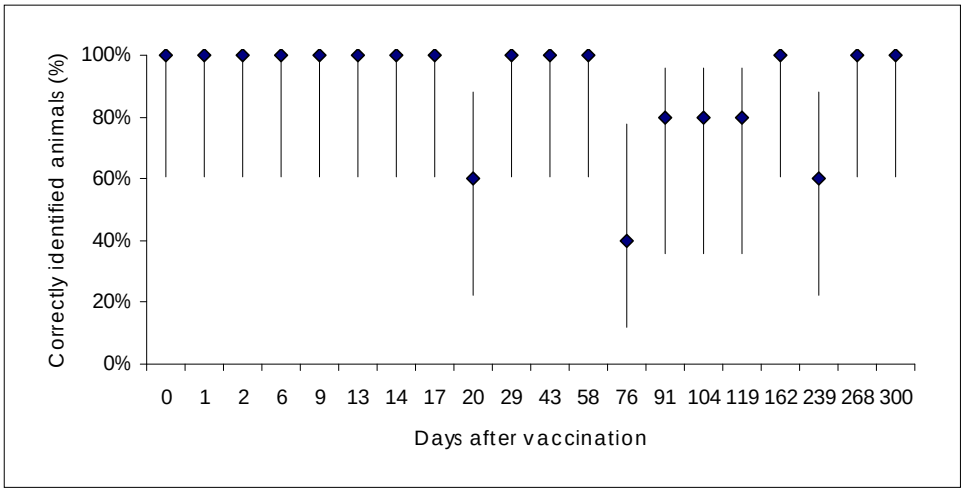


Figure 2
Percentage of non-vaccinated animals correctly identified by the γ-interferon test and 95% confidence intervals

vaccinated with RB51 is essential for the control of the efficacy and efficiency of the vaccination campaign The detection of cell-mediated immune response induced by the vaccine could be useful for the identification of cattle vaccinated with RB51. In particular, a method to reveal the presence of a cell-mediated response against *B. abortus* is the detection of γ-interferon following lymphocyte stimulation with the specific antigen.

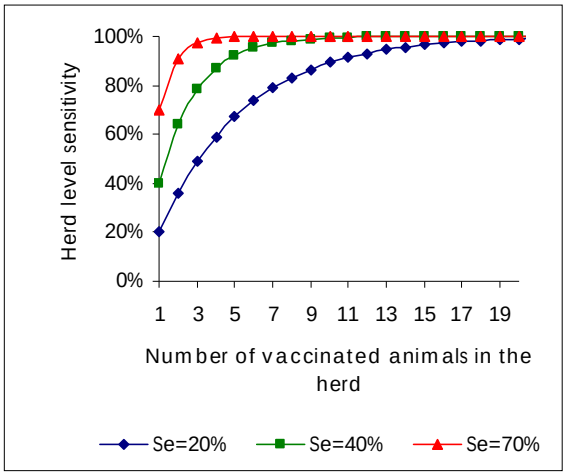


Figure 3
Sensitivity of the γ-interferon test at herd level, in relation to the animal level test sensitivity and the number of vaccinated animals in the herd

*Brucella* spp. is a facultative intracellular parasite that is able to grow and survive within host phagocytes, and it is known that cell-mediated immunity is the main host body defence mechanism. It is also known that γ-interferon plays a prominent role in cell-mediated immunity against *Brucella* spp. (9). For these reasons, high and durable γ-interferon production would be expected in vaccinated animals, as it is expected for the vaccination coverage.

Nevertheless, the higher response in terms of vaccinated animals providing positive results has only been recorded at day 20 pv; responses to subsequent sampling generally remained at low values and the test, after day 239 pv, did not provide any additional positive results (Fig. 1).

The results suggest that the test is not reliable, except on day 20 pv, for the identification of single RB51 vaccinated cattle. Previous authors reported similar results in cattle vaccinated with strain 19 (4). In particular, by using a lymphocyte stimulation test, the author reported that cattle vaccination with a single dose of *B. abortus* strain 19 induced very little of T cell memory, whereas repeated vaccination produced prolonged immunological memory. Given that in the present study the animals were vaccinated with a single dose and given that the γ-interferon test is based on the detection of cell-mediated immunity, it appears reasonable to affirm that in case of single dose vaccination with RB51 the *in vitro* T lymphocyte response is low and limited over time.

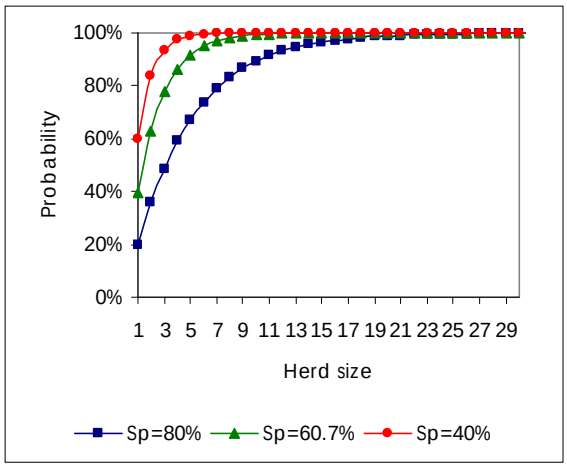


Figure 4
Probability that the γ-interferon test would give at least one false-positive result at the herd level, in relation to the specificity of the test at the animal level and to the number of animals in the herd

The fluctuations in test sensitivity (Fig. 1) limit the possibility of detecting single animals vaccinated with RB51, however, they do not preclude the use of the test as a screening method to identify the herds in which RB51 or any intact *Brucella* vaccine has been administered. Considering that herd vaccination is normally performed on all (or almost all) eligible animals, if all eligible animals are tested, the sensitivity at the herd level would greatly increase. Even considering a scenario of 20% test sensitivity (on days 58 and 119 pv), the presence of at least 13 vaccinated animals would lead to 95% probability of having at least one positive result with the γ-interferon

test (Fig. 3). Furthermore, this possibility is limited in time during the early period post vaccination up to day 17 and further, after day 20 until day 239 pv. In contrast, it has been demonstrated that after this period, namely on day 414 pv, the same animals gave a positive reaction to the skin test performed with RB51 brucellin or commercial brucellin as antigen, with a sensitivity of 60% (CI 30.8-83.3%) and 40% (16.7-69.2%), respectively (5). Therefore, the *in vivo* assay appears to have greater possibilities to identify animals vaccinated with RB51 than the *in vitro* assay (at least in temporal terms), also considering that the skin test elicits an anamnestic humoral response that can be revealed with the RB51-CFT in 100% of vaccinated animals between days 9 and 16 after brucellin inoculation (5).

Control animals gave positive results on different occasions (days 20, 76, 91, 104, 119 and 239 pv), with a peak of three animals positive out of five on day 76 pv (specificity 40%, CI 11.8%-77.7%) (Fig. 2). Animals were from officially brucellosis-free herds and they negative to the RBT and CFT performed with strain 99 as antigen (18) and, therefore, it would seem unrealistic to affirm that the positivity observed could be related to a *B. abortus* infection. On the other hand, the animals remained negative to the RBT and CFT throughout the experiment. The positive results obtained by the test in non-vaccinated animals can be explained by the presence of non-specific stimulatory components in the antigen used for the whole blood stimulation. Similar results have been observed by other authors (8) who suggested the presence of common proteins between *Brucella* genus and other Gram-negative bacteria, making them able to lead the T lymphocytes to non-specific γ-interferon production. On this basis, the authors concluded that the γ-interferon was not suitable for bovine brucellosis diagnosis in terms of specificity (8).

In addition, the low specificity of the test at the animal level has a negative influence on the specificity at the herd level (Fig. 4). Considering that the average size of the herds to be controlled within the framework of the Italian national brucellosis control programme in the Italian provinces that are not officially free from bovine brucellosis (i.e. those in which fraudulent use of RB51 could be expected) is 22 head (Italian Ministry of Health, personal communication) and considering a 80% test sensitivity at the animal level, the use of the γ-interferon as a screening test would lead to a false-positive result at herd level in 99.3% of cases (Fig. 4).

Moreover, if the lower confidence limit of the best specificity value given by the test during the experiment (100%, CI 60.7%-100%) is considered, the test would have the 100% probability of giving a false-positive result from herd sizes of 25 animals or more. Therefore, this would justify the use of γ-interferon as a screening test at herd level only if the test was followed by a high specificity confirmatory test, such as the RB51-CFT for instance (1).

Nevertheless, previous studies conducted on exactly the same subjects (5) demonstrated that the skin test (performed with RB51 brucellin or commercial brucellin) gives a specificity result of 100% (CI 60.7%-100%) on animals vaccinated with RB51. Therefore, with respect to the *in vitro* test, the delayed hypersensitivity test *in vivo* appears to give a higher guarantee for the correct identification of herds in which RB51 has not been used, to the advantage in efficacy and efficiency of the possible control procedures.

## Conclusions

If the results on day 20 pv are excluded, the γ-interferon test is not reliable in terms of sensitivity for the identification of cattle vaccinated at calfhood with a single dose of RB51, due to the poor immunological memory that the T lymphocytes show *in vitro* against the specific RB51 antigen.

The γ-interferon test is also unreliable, in terms of specificity, for the identification of animals or herds in which RB51 has not been used, due to the existence of non-specific γ-interferon production by T lymphocytes stimulated *in vitro*.

It is concluded that the γ-interferon does not give adequate guarantees for use in the

identification of cattle or cattle herds vaccinated with RB51 at calfhood.

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