

Use of the complement fixation and brucellin skin tests to identify cattle vaccinated with *Brucella abortus* strain RB51

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Summary

In the European Union, RB51 vaccine can be used only under strictly controlled conditions for the immunisation of cattle at risk of infection with *Brucella abortus*. A diagnostic system is therefore necessary to distinguish vaccinated from unvaccinated animals. To avail of a rapid and accurate diagnostic tool, the authors produced, controlled and evaluated an experimental brucellin prepared from strain RB51 (RB51 brucellin). The potency of this brucellin was evaluated in guinea-pigs sensitised with *B. abortus* RB51 and compared with a commercially available brucellin. Both allergens produced similar biological activity in guinea-pigs. The RB51 brucellin skin test was performed in 10 cattle 414 days after calthood vaccination with RB51 when they were negative to the complement fixation test with RB51 antigen (RB51-CFT). The skin test revealed 60% sensitivity (with a 95% confidence interval [CI] of 30.8%-83.3%) and 100% specificity (CI 60.7%-100%). These findings limit the use of the skin test only for screening to detect RB51 vaccinated herds, not individual animals. Nevertheless, following intradermal inoculation of RB51 brucellin, a transient antibody increase to the RB51-CFT was observed from day 9 to day 20 post inoculation with RB51 brucellin. This transient antibody increase, when evaluated in parallel with the RB51 brucellin skin test results, enables detection of individual vaccinated animals (sensitivity 100%; CI 76.2%-100%).

Keywords

Brucella, Brucellin, Brucellosis, Cows, Complement fixation test, Diagnosis, Hypersensitivity, RB51.

Introduction

For the first time, the European Union approved the use of *Brucella abortus* strain RB51 vaccine (RB51) for the immunisation of cows at risk of infection with *Brucella abortus* in 2002 (European Commission Decision 2002/598/CE) (12). The competent authority of Member State is required to submit to the Commission and to the other Member States detailed information on the vaccination programme, in particular regarding the area of vaccination, age of the animals to be vaccinated and the test system in place to identify vaccinated animals. Furthermore, the competent authority must ensure that vaccinated animals are not subject to intra-Community trade, in particular by applying additional methods of marking and registration.

In healthy cattle, RB51 vaccine, 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 (22, 23). However, these antibodies can be revealed using specific tests, such as the dot-blot test (17), and the complement fixation test with RB51 antigen (RB51-CFT) (1, 2). Nevertheless, cattle infected with *B. abortus* can reveal a serological reaction

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to a conventional test, even if they have been vaccinated with RB51 (8).

Brucella infection can also be detected in cattle when recourse is made to the brucellin skin test, giving a skin delayed-type hypersensitivity (SDTH) reaction. The level of specificity ranges between 95% and 100% when performed in cattle infected with *B. abortus*, with a level of sensitivity between 70% and 75% (15).

In previous studies, the skin test conducted on cattle vaccinated with RB51 provided controversial results, either with brucellin prepared from protein extract of *B. abortus* or with the homologous RB51 brucellin (7, 8, 9). Since the biological activity of different batches of brucellin may vary, some authors highlighted the need to estimate the potency of the brucellin according to the European pharmacopoeia standard for tuberculin (acceptable if greater than 66% and equal to or lower than 150% of the reference antigen), in order to obtain statistically significant SDTH results indicative of brucellosis (6).

The aim of this study was to evaluate the ability of a homologous RB51 brucellin to identify cattle vaccinated with RB51 more than one year after calfhoo d vaccination, and to study the possibility of producing an anamnestic humoral response by this allergen.

Materials and methods

Animals and vaccination

Fifteen Friesian calves, aged between four to six months and obtained from officially brucellosis-free herds, were selected at random and divided into two groups of five and ten animals. The ten animals were vaccinated subcutaneously with RB51 in accordance with the instructions of the manufacturers (2 ml reconstituted solution, containing 10×10^9 colony forming units [CFU]). The five control animals were inoculated

subcutaneously with 2 ml sterile saline solution.

Vaccine

The RB51 vaccine was kindly provided by CZ Veterinaria in Pontevedra (Spain), the European distributor of the product, under license from the Colorado Serum Company in Denver (USA). Once reconstituted, the vaccine contained 5×10^9 CFU/ml of *B. abortus* strain RB51

Brucellins

Commercially available brucellin

Brucellergene OCB, the commercial equivalent of the brucellin from the Institut national de la recherche agronomique (INRA) brucellin (16), was used as a reference standard.

RB51 brucellin

The RB51 brucellin was produced as follows: the freeze-dried vaccine, once reconstituted with the diluent supplied and following the instructions of the manufacturers, was inoculated on glycerol dextrose agar plates. After 48 h incubation at $37^\circ \pm 2^\circ\text{C}$, single colonies were harvested and then inoculated onto glycerol dextrose agar slopes. After 48 h incubation at $37^\circ \pm 2^\circ\text{C}$, the growth was harvested by adding 5 ml sterile saline and checked for purity (Gram staining). It was then used to seed 60 Roux flasks containing glycerol dextrose agar. After 96 h incubation at $37^\circ \pm 2^\circ\text{C}$, the growth was harvested with sterile saline (10 ml per Roux flask). After checking for purity (Gram staining) and roughness (3), the *Brucella* cells were killed in the fermentor by raising the temperature to $70^\circ \pm 2^\circ\text{C}$ for 90 min. The cell suspension was harvested by centrifugation at $10\,000 \times g$ for 30 min at $+4^\circ \pm 2^\circ\text{C}$. The supernatant was discarded and the cells suspended in sterile distilled water (1:40 w/v). The suspension was agitated for 1 h and pH was adjusted to 9.6 with 0.5 N NaOH solution and then placed in an autoclave for 120 min under a steam stream, cooled at room temperature and then centrifuged at $10\,000 \times g$ for 30 min at $+4^\circ \pm 2^\circ\text{C}$. The supernatant was added

with 40% trichloroacetic acid (TCA) (1:10 v/v) and stored at room temperature for 24 h. The precipitate was then suspended in 1% TCA (1:10 v/v) and again stored at room temperature for 24 h. The precipitate was washed twice with a water solution of 5% sodium chloride with 0.5% of an 85% phenol solution, until the pH was set at 2.6 ± 0.1 . The supernatant was then discarded and the precipitate harvested by centrifugation at $6\,000 \times g$ for 20 min at $+4 \pm 2^\circ\text{C}$ and re-suspended in phosphate buffered saline (pH 11) to the 40% (w/v) of the initial bacterial growth. The suspension was then stirred for 1 h and diluted 1:10 (v/v) in phosphate buffered saline (pH 7.2) and the protein titre measured using the Kjeldhal method. The suspension was diluted in phosphate buffered saline (pH 7.2) with 0.01% sodium merthiolate to the protein concentration of 1 mg/ml (14).

Controls

Sterility and safety controls of RB51 brucellin were performed in accordance with the recommendations of the OIE *Manual* (16). Potency (biological activity) control was conducted according to a technique described previously (5), with the modifications listed below.

A total of 12 white guinea-pigs, each weighing 350-400 g, were used. Six guinea-pigs were sensitised by a subcutaneous injection of 1 ml of RB51 vaccine (5×10^9 CFU); the remaining six were used as controls and were inoculated subcutaneously with 1 ml sterile saline solution. Thirty days after the inoculation, the left and right flanks of the guinea-pigs were cleanly shaven and each guinea-pig was injected intradermally with 0.1 ml RB51 brucellin and 0.1 ml Brucellergene OCB, each diluted 1:20, 1:100 and 1:500. To avoid errors due to individual skin sensitivity, doses were coded and randomised for site of injection. Each guinea-pig received a total of three injections on each flank. The diameter of the erythema zone at the

injection site of each allergen dilution was measured with callipers 24 h and 48 h after the intradermal injection.

The potency of RB51 brucellin was estimated according to the European pharmacopoeia (10) standard for tuberculin (acceptable if greater than 66% and less than or equal to 150% of the potency of the reference antigen), considering the potency of standard brucellin (Brucellergene OCB) as 100%.

Skin test

In all heifers (including all vaccinated and control animals), 10 cm² of healthy clean skin was shaven from the side of the neck with scissors 414 days after vaccination. A tuberculin syringe was used to inject intradermally 0.1 ml of RB51 brucellin (right side of the neck) and 0.1 ml of Brucellergene OCB (left side of the neck) to all heifers. The reaction was read at 24 h, 48 h and 72 h after inoculation, by measuring the difference of the skin thickness at the injection site against that on day zero. A spring meter was used to measure the skin thickness.

Serological tests

All heifers were tested for anti-*B. abortus* and anti-RB51 antibodies before vaccination, on the day of brucellin inoculation, and at days 6, 9, 13, 16, 20, 28 and 34 post brucellin inoculation (PBI). Anti-*B. abortus* antibodies were verified using the Rose Bengal test (RBT) and complement fixation test (CFT), in accordance with the methods described in the OIE *Manual* (16). Anti-RB51 antibodies were monitored with a RB51 antigen-specific CFT (RB51-CFT) (1, 2).

Statistical analysis

In guinea-pigs, mean values, confidence intervals (CI) of the mean and the variation coefficient of the diameters of the erythema zones were calculated for each allergen dilution. To check if a statistically significant difference existed in the response to allergens within each dilution, the Wilcoxon test (21) was applied, comparing the same dilutions

of the two allergens.

The potency of the RB51 brucellin was estimated according to the European pharmacopoeia recommendations for tuberculin (6, 10), and using Brucellergene OCB as the reference standard (potency considered as 100%). Sensitivity distributions of both DTH allergens were estimated using a Bayesian approach (20); the probabilities of the various possible sensitivity values were estimated using a binomial likelihood function and an uninformed *Uniform* (0,1) prior distribution. A posterior Beta distribution and the 95% CI were calculated for sensitivity and specificity (24).

Results

RB51 brucellin control

Sterility and safety controls of RB51 brucellin gave the expected results. The results of potency controls among sensitised guinea-pigs for RB51 brucellin and reference brucellin (Brucellergene OCB) are presented in Table I. Activity is expressed as the diameter (mm) of skin reaction erythemas. For both allergens, the maximum reaction was recorded at 24 h PBI, while at 48 h PBI, a decrease in intensity of response was observed. None of the allergens

caused adverse reactions in guinea-pigs. None of the control guinea-pigs revealed SDTH reactions. The Wilcoxon test did not reveal significant differences in the erythema diameters between RB51 brucellin and Brucellergene OCB at dilutions 1:20 (Wilcoxon test $z=0$; $p>0.05$), 1:100 ($z=-1.786$; $p>0.05$), 1:500 ($z=-0.412$; $p>0.05$). The biological activity of RB51 brucellin was 139% of the standard (Brucellergene OCB). This value complies with the European pharmacopoeia (10) standard for the estimation of potency of new tuberculin batches (accepted if greater than 66% and less than or equal to 150% of the reference antigen potency).

Skin test on heifers

None of the heifers showed pain, necrosis or local lymph node enlargement at the site of inoculation. In all vaccinated heifers, the maximum value of skin thickening was observed 72 h PBI. (Table II). None of the control heifers showed skin thickening at the injection site 72 h PBI, except for one heifer that showed a thickening of the skin of 0.5 mm. Seven of the ten vaccinated heifers gave a positive reaction to RB51 brucellin, while five out of ten vaccinated heifers presented a positive reaction to the reference brucellin (Brucellergene OCB)

Table I
Diameter (mm) of erythema zones in guinea-pigs sensitised with RB51 and then inoculated with two brucellins

Guinea-pig	Inoculated allergen and dilution					
	RB51 brucellin			Brucellergene OCB		
	1:500	1:100	1:20	1:500	1:100	1:20
1	0	10	16	0	8	14
2	0	11	15	0	9	15
3	0	9	11	0	10	14
4	0	10	17	0	8	10
5	0	8	12	0	8	15
6	0	10	16	0	0	13
Mean	0	9.67	14.50	0	7.17	13.50
95% upper confidence interval	0	8.58	11.95	0	3.39	11.54
95% lower confidence interval	0	10.75	17.05	0	10.95	15.46
Variation coefficient	0	0.11	0.17	0	0.50	0.14

Table II
Mean and standard deviation of skin thickness in heifers (n=10) vaccinated at calthood with RB51 and inoculated with brucellin 414 days post vaccination

Skin measurement results	Hours post inoculation with brucellin							
	(a) (b)		24h		48h		72h	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Mean skin thickness (mm)	7,65	7,85	8,6	7,85	8,7	7,95	8,9	8,5
Standard deviation	0,74	0,78	0,97	0,78	1,55	0,96	1,71	0,97

(a) RB51 brucellin
(b) Brucellergene OCB

(Table III). Skin test sensitivity and specificity values with RB51 brucellin and Brucellergene OCB, with respect to the skin thickening at the injection site (threshold), are given in Table IV.

Serological tests on heifers

All heifers gave negative results to RBT, CFT, and RB51-CFT prior to vaccination (administered 414 days before the skin test). After vaccination, the heifers developed a serological response to RB51-CFT, and again gave negative results prior to the inoculation of brucellin.

After brucellin inoculation, all heifers gave negative results to RBT and CFT. Considering as reaction threshold was a dilution of 1:4 showing 100% haemolysis, all vaccinated heifers exhibited positive reactions to the RB51-CFT (Fig. 1) for at least one sampling; all control heifers presented negative results to the RB51-CFT (Fig. 2). The antibody response in vaccinated heifers reached the maximum value at day 13 PBI, and then progressively decreased (Fig. 1). The percent distribution of animals tested and identified correctly as vaccinated (Fig. 3) shows that between days 9 and 20 PBI, the sensitivity of RB51-CFT is equal to or higher than 90% (CI 58.7%-97.7%), with a maximum value of 100% (CI 76.2%-100%) at days 9 and 16 PBI. The two heifers that gave negative results to the RB51-CFT at days 13 and 20 PBI, respectively, gave positive reactions to the RB51 brucellin skin test.

Discussion

In previous studies, the skin test on cattle vaccinated with RB51 gave controversial results, either with brucellin prepared from protein extract of *B. abortus* or with the homologous RB51 brucellin (7, 8, 9). Since various batches of brucellin may have differing biological activity, some authors suggested the need to estimate the potency of the brucellin used (6) according to the standard of the European pharmacopoeia (10) for tuberculin (acceptable if greater than 66% and equal to or less than 150% of the reference antigen potency), to obtain statistically significant SDTH results indicating

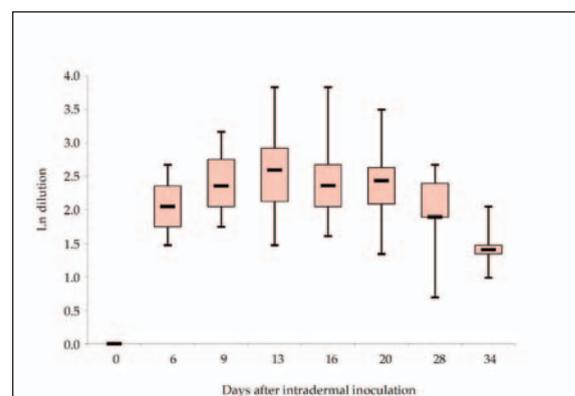


Figure 1
RB51-CFT antibody response after intradermal inoculation of RB51 brucellin and Brucellergene OCB in heifers (n=10), 414 days after vaccination with RB51

the presence of brucellosis. The RB51 brucellin used in this study complies with the requirements of the European pharmacopoeia (10) and shows a biological activity of 139% of the brucellin considered as reference antigen (Brucellergene OCB).

Sensitivity and specificity of skin tests depend on the criteria chosen for interpretation of results. Several authors (4, 18, 19) did not agree on the value of skin thickening to be considered as a threshold. Other authors stated that, in field conditions and in herds infected with *B. abortus*, all visible and/or palpable reactions should be considered positive for the skin test (18). Others proposed a skin test threshold of 2 mm, similar to that prescribed for the tuberculosis skin test (4).

However, other authors (19), although in agreement with the statement that the true positive and negative reactions can be rapidly classified by simple observation of the injection site and by palpation, stated that, for some doubtful cases

and in order to quantify the reaction, the thickening of the skin should always be measured. These authors observed that the brucellin allergic reaction was two or three times less intense than the tuberculin PPD allergic reaction, and suggested that to be able to consider the animal as infected,

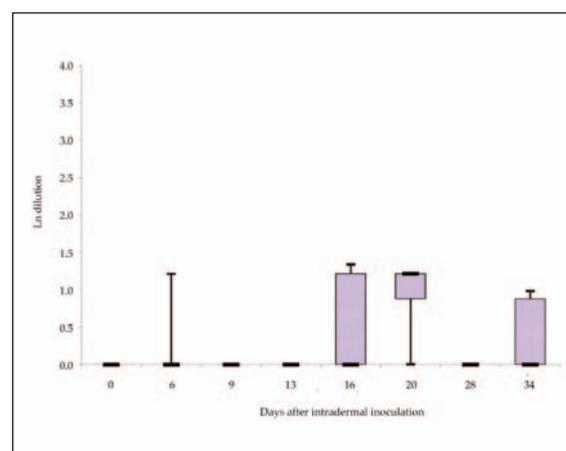


Figure 2
RB51-CFT antibody response after intradermal inoculation of RB51 brucellin and Brucellergene OCB in control heifers (n=5)

Table III

SDTH reaction (mm skin thickness) in heifers inoculated with RB51 brucellin and Brucellergene OCB 72 h after inoculation

Heifers vaccinated with RB51 and controls	Heifer No.	Initial skin thickness (mm)		Skin thickness 72 h (mm)		Difference in skin thickness (mm)	
		RB51 Brucellin	Brucellergene OCB	RB51 Brucellin	Brucellergene OCB	RB51 Brucellin	Brucellergene OCB
Vaccinated	1	7	7	7	7	0	0
	2	8	8	8	8	0	0
	3	7	8.5	8	8.5	1.0	0
	4	7	7	9	8.5	2.0	1.5
	5	9	9	13	10	4.0	1.0
	6	9	9	11	9	2.0	0
	7	7.5	8	8	8.5	0.5	0.5
	8	7	7	9	8.5	2.0	1.5
	9	7.5	8	8.5	10	1.0	2.0
	10	7.5	7	7.5	7	0	0
Unvaccinated	11	8.5	8.5	8.5	8.5	0	0
	12	8	8.5	8	8.5	0	0
	13	5.5	5.5	5.5	5.5	0	0
	14	9	9	9	9	0	0
	15	7.5	7	8	7	0.5	0

Table IV
Sensitivity, specificity and 95% confidence intervals of brucellins in relation to skin thickening at injection site (threshold)

Threshold	RB51 Brucellin		Allergen	
	Sensitivity	Specificity	Brucellergene OCB Sensitivity	Brucellergene OCB Specificity
>0 mm	70% (39-89.1%)	80% (35.9-95.7%)	50% (23.4-76.6%)	100% (60.7-100%)
≥0.5 mm	70% (39-89.1%)	80% (35.9-95.7%)	50% (23.4-76.6%)	100% (60.7-100%)
≥1 mm	60% (30.8-83.3%)	100% (60.7-100%)	40% (16.7-69.2%)	100% (60.7-100%)
≥1.5 mm	40% (16.7-69.2%)	100% (60.7-100%)	30% (10.9-61%)	100% (60.7-100%)
≥2 mm	40% (16.7-69.2%)	100% (60.7-100%)	10% (2.3-41.3%)	100% (60.7-100%)

a threshold in skin thickening of 1.1 mm for the brucellin skin test 72 h p.b.i. was necessary (19). In this study, the skin reaction (with RB51 brucellin only) in a heifer that had not been vaccinated with RB51, suggests the use of a skin thickening threshold at the injection site greater than 0.5 mm. However, to be conservative, using as the threshold ≥ 1 mm the skin test shows sensitivity at 60% (CI 30.8%-83.3%) and specificity at 100% (CI 60.7%-100%) if performed with the homologous RB51 brucellin, and sensitivity of 40% (CI 16.7%-69.2%), with specificity at 100% (CI 60.7%-100%) if performed

with Brucellergene OCB (Table IV).

Data could suggest a higher sensitivity of the skin test performed with RB51 brucellin. However, considering the wide overlapping of confidence intervals, the difference in reactivity to the two allergens is not significant. In any case, the higher reactivity recorded among heifers inoculated with RB51 brucellin complies with the higher biological activity of this allergen in guinea-pigs (139%) in respect to Brucellergene OCB.

In animals vaccinated with RB51 vaccine, RB51 brucellin presents results similar to Brucellergene OCB when used to identify animals and/or herds infected with *B. abortus* (11, 13, 15).

The relatively low sensitivity of the test reduces the probability of identifying single animals vaccinated with RB51, but allows the use of the skin test as a method for screening to identify herds in which the vaccine has been administered. Considering that a herd vaccination is normally performed on all (or almost all) eligible animals, if all eligible animals are tested, the sensitivity at herd level would considerably increase. Considering the sensitivity estimated in this study, the presence of at least four vaccinated animals in a tested group would lead to 95% probability of having at least one positive skin test result with RB51

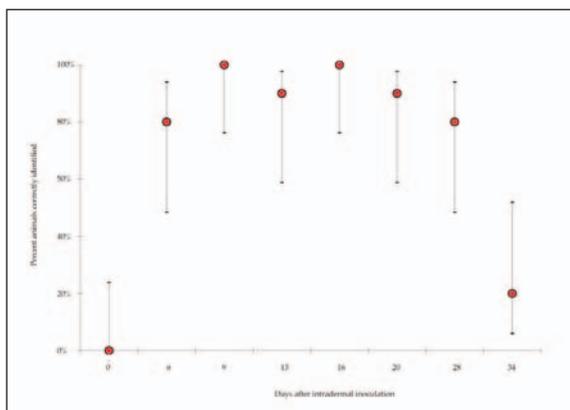


Figure 3
Distribution (%) of heifers correctly identified as vaccinated after intradermal inoculation of RB51 brucellin and Brucellergene OCB

brucellin (sensitivity 60%, CI 30.8%-83.3%). With Brucellergene OCB, the same probability could be obtained with at least six vaccinated animals (sensitivity 40%, CI 16.7%-69.2%).

The results of this study suggest the possibility of compensation of the low skin test sensitivity by testing the animals with RB51-CFT after the intradermal inoculation with RB51 brucellin. In line with the results of this study 414 days after the RB51 vaccination and considering a threshold dilution of 1:4 showing 100% haemolysis for RB51-CFT, on days 9 and 16 PBI all vaccinated heifers gave positive results to the RB51-CFT test (sensitivity 100%, CI 76.2%-100%), and between days 9 and 20 PBI eight of ten vaccinated heifers gave positive results (sensitivity 80% CI 48.2%-94.0%). All control heifers tested with RB51-CFT gave negative results PBI (specificity 100%, CI 60.7%-100%). The two vaccinated heifers negative to the RB51-CFT at days 13 and 20 PBI were positive to the RB51 skin test, thus suggesting that the use of the skin test and RB51-CFT in parallel correctly identifies all vaccinated animals between days 9 and 20 PBI. In conclusion, the skin test can be used for herd screening to identify herds vaccinated with RB51. For this purpose, both homologous and heterologous brucellins can be used, taking into consideration the different sensitivities of these two allergens. The skin test performed with the homologous brucellin 414 days after calthood vaccination with RB51, produces an anamnestic humoral response. Thus the association between the RB51 skin test and RB51-CFT could represent a reliable diagnostic system to identify single animals vaccinated with RB51

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