Efficacy of *Brucella abortus* vaccine strain RB51 compared to the reference vaccine *Brucella abortus* strain 19 in water buffalo

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Summary

Approximately 250 000 water buffalo (*Bubalus bubalis*) live in the Campania region of southern Italy where the breeding of this species is very popular. Of these animals, almost 150 000 are concentrated in the Caserta province where the prevalence of *Brucella abortus* in this species represents approximately 20% at herd level. The Italian brucellosis eradication programme provides a slaughter and vaccination strategy for this province. *B. abortus* strain RB51 (RB51) has become the official vaccine for the prevention of brucellosis in cattle in several countries. The aim of this study was to evaluate the efficacy of RB51 in water buffalo compared to the *B. abortus* S19 vaccine (S19). The study was performed in accordance with a protocol described in mice. Female buffalo aged five months were inoculated. Five received a RB51 dosage on two occasions that was three times greater than that approved for use in cattle and a booster after one month, five received *B. abortus* S19 vaccine at the standard dosage and three controls received a phosphate buffer solution. Buffalo were then challenged with a virulent *B. abortus* strain 544 thirty days post vaccination. Antibodies that developed in the five animals vaccinated with RB51 were not detected by the Rose Bengal test or complement fixation test (CFT) and were also tested by CFT prepared with RB51 antigen. After culling, *B. abortus* was cultured from the spleen, retropharyngeal and supra-mammary lymph nodes. A statistical evaluation was performed to assess the immunogenicity values obtained in buffalo vaccinated with S19, compared to those obtained in buffalo vaccinated with the RB51 vaccine and in the unvaccinated control group.

Keywords

Brucella, *Brucella abortus*, Brucellosis, Buffalo, Italy, Strain 19, Vaccine, Water buffalo.

Introduction

Brucella abortus is the principal agent of brucellosis in buffalo. High prevalence is recorded in the Campania region of southern Italy where most Italian water buffalo (*Bubalus bubalis*, Linnaeus, 1758) are raised. Due to the persistence of this high prevalence of *Brucella* (strain *B. abortus* 1 and 3) infection despite years of control and eradication programmes conducted in Campania, the European and Italian authorities decided to adopt a vaccination strategy to reduce the impact of infection on both human and animal health.

The brucellosis vaccine employed in buffalo worldwide is strain 19 (9). The main drawback in the use of this vaccine is the production of antibody that is detectable by the official tests used for the diagnosis of brucellosis. An alternative vaccine (strain RB51) has been developed for use in cattle, using a rifampicin-resistant rough mutant of *B. abortus*. This

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vaccine has proved safe and effective in the field against bovine brucellosis and exhibits negligible interference with diagnostic serology (8). The use of vaccine RB51 at the same dose as that used in cattle proved ineffective in protecting water buffalo from the challenge with a wild strain (5). Nevertheless, the RB51 vaccine interferes less with diagnostic serology in buffalo (4).

Some authors have suggested the possibility of effectively immunising buffalo with the RB51 vaccine using a vaccination scheme that differs from that used in cattle (7), namely: vaccination of impuberal buffalo with a dose that is three times greater than that used in cattle and a booster after one month.

The authors evaluated the protocol suggested by Iovane *et al.* (7) and compared its efficacy to that of strain 19 administered in accordance with standard protocol (9). The effectiveness of vaccination was evaluated in relation to the colonisation by a wild strain of *B. abortus* (strain 544) of spleen and lymph nodes of buffalo and mice vaccinated with RB51 or strain 19. The trial performed in mice will be the published separately.

Materials and methods

Animals

Thirteen impuberal female water buffalo, aged between five and six months, from officially brucellosis-free herds in the province of Salerno (Campania, southern Italy) were randomly allocated to three experimental groups (Table I), as follows:

- Group R: 5 animals vaccinated with RB51
- Group S: 5 animals vaccinated with S19
- Group C: 3 control animals.

Animals were kept in the stables of the *Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise* in Teramo and, after a period of 7 days of adaptation to the new environment, the animals were clinically examined, received parasitological, biochemical and electrophoretic tests for complete blood cell counts and underwent the official serological tests for brucellosis.

Table I
Experimental animal groups

Animal No.	Group	Description
1	R	RB51 vaccination at triple dose and re-vaccination at time t_1, 30 days after the first vaccination
2		
3		
4		
5		
6	C	Control animals, inoculated with a placebo at time t_1
7		
8		
9	S	S19 vaccination at time t_1
10		
11		
12		
13		

Vaccines and vaccination

Group R

Five animals were vaccinated subcutaneously (time t_0) using a triple dose (6 ml, containing 3-10.2*10^{10} colony-forming units [cfu]) of RB51 vaccine produced by CZ-Veterinaria (Porriño) (a single dose contains 1-3 × 10^{10} cfu). These animals were re-vaccinated at time t_1, 30 days after the first vaccination, using the same triple dose.

Group S

Five animals were vaccinated subcutaneously at time t_1 (concomitant with the second inoculation of RB51 in group R) using a standard dose (5 ml containing 6-12*10^{10} cfu) of S19 vaccine produced by CZ-Veterinaria.

Group C

Three additional animals, kept as unvaccinated controls, were inoculated subcutaneously with 2 ml sterile phosphate buffer solution (PBS) at time t_1, concomitantly with the second inoculation of RB51 in the first group of animals.

The vaccine titres were validated by plate count.

Challenge

Thirty-three days (time t_2) after the last vaccination, i.e. 69 days after t_0, all 13 experimental animals were inoculated conjunctivally with 100 µl (50 µl in each eye) of

a suspension containing 10^7 cfu of *B. abortus* strain 544 (concentration of 3.1×10^8 cfu/ml) provided by the *Agence française de sécurité sanitaire des aliments* (AFSSA) (French Food Safety Agency) in Maisons-Alfort. The suspension of *B. abortus* strain 544 was prepared by reconstituting a lyophilised strain. We then measured the suspension prepared in buffer (PBS) with an optical density (OD 600) of 0.400±0.005 using a biophotometer (Eppendorf, Milan), equivalent to approximately 10^8 cfu/ml. The bacterial count of the inoculation was measured by titration on tripticase soy agar (Biolife, Milan).

Tests

All animals in the three groups were subjected to weekly serological testing for brucellosis up to 9 weeks after the first RB51 vaccination (33 days after the S19 vaccination) and twice a week after challenge until the end of the trial (t_3, 31 days post infection and 100 days after t_0). The serological tests used were the official tests for brucellosis, i.e. the Rose Bengal test (RBT) and complement fixation test (CFT), according to Italian rules, using smooth antigen (3, 9) and the CFT specific for RB51 (using rough antigen) (1, 2).

The body temperature of all animals was recorded daily after challenge.

At time t_3 (100 days after t_0) 31 days post inoculation, all animals were euthanised and the spleen, liver, kidney, lung, udder, urinary bladder, supra-mammary, retropharyngeal, mandibular, mesenteric, iliac and pulmonary lymph nodes were collected. All samples were submitted for *Brucella* isolation both in aerobic conditions and in air supplemented with 5%-10% (v/v) CO_2, according to the *Manual of diagnostic tests and vaccines for terrestrial animals* of the World Organisation for Animal Health (*Office International des Épizooties*: OIE) (9). Plate counts of spleen, supra-mammary and retropharyngeal lymph nodes were taken, using tenfold dilutions up to 1:1 000.

Certain colonies were verified from each positive sample, using identification procedures, such as *Abortus Melitensis Ovis Suis*-polymerase chain reaction (AMOS-PCR), restriction fragment length polymorphism (FRLP), growth in the presence of basic fuchsin and thionin at final concentrations of 20 μg/ml, production of H_2S and agglutination with A and M sera (9).

Statistical analysis

The different organs that gave positive results for each buffalo in the three experimental groups were compared using the Kruskall-Wallis non-parametric analysis of variance and the differences between groups were evaluated using the Mann-Whitney U test with Bonferroni confidence intervals. As three groups were compared, the Bonferroni *p* value corresponding to an overall alpha value of 0.05 was 0.0167.

Results

Clinical parameters of the animals at the beginning of the experiment were within the normal range, while parasitological and official serological tests for brucellosis were all negative.

Results of serological tests performed after the vaccination of the experimental animals are shown in Table II.

Four animals in Group R developed specific antibodies against the RB51 vaccine strain four days after revaccination, which disappeared after less than three weeks.

All five animals vaccinated with S19 developed antibodies less than two weeks after vaccination; these were specific for the smooth strains of *Brucella* and were detectable by both RBT and CFT. This positive serology persisted until the date of challenge, 33 days post vaccination.

The control animals remained serologically negative until two weeks post challenge.

No febrile temperature was recorded in any group post challenge.

Results of serological tests performed after the challenge of the experimental animals are given in Table III.

All five animals in group R showed a booster reaction of their R-strain-specific antibodies detectable 9 days post infection, which nonetheless was transient, disappearing three

Table II
Serological results post vaccination

Date (days post vaccination)	Group R (vaccinated RB51)			Group S (vaccinated S19)			Group C (controls)		
	RBT*	CFT*	CFT RB51*	RBT*	CFT*	CFT RB51*	RBT*	CFT*	CFT RB51*
02/04 (0) [t_0]	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3
09/04 (7)	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3
16/04 (14)	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3
23/04 (21)	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3
30/04 (28)	0/5	0/5	0/5	0/5	0/5	0/5	0/3	0/3	0/3
02/05 [t_1]	–	–	–	–	–	–	–	–	–
06/05 (35)	0/5	0/5	4/5	0/5	0/5	0/5	0/3	0/3	0/3
13/05 (42)	0/5	0/5	4/5	5/5	5/5	0/5	0/3	0/3	0/3
20/05 (49)	0/5	0/5	0/5	5/5	5/5	0/5	0/3	0/3	0/3
27/05 (56)	0/5	0/5	0/5	5/5	5/5	0/5	0/3	0/3	0/3
03/06 (63)	0/5	0/5	0/5	5/5	5/5	0/5	0/3	0/3	0/3

RBT Rose Bengal test
CFT complement fixation text
* positive/total
[t_0] date of 1st RB51 vaccination
[t_1] date of RB51 re-vaccination and S19 vaccination

Table III
Serological results post infection

Date (days post infection)	Group R (vaccinated RB51)			Group S (vaccinated S19)			Group C (controls)		
	RBT*	CFT*	CFT RB51*	RBT*	CFT*	CFT RB51*	RBT*	CFT*	CFT RB51*
09/06 (0) [t_2]	0/5	0/5	0/5	5/5	5/5	0/5	0/3	0/3	0/3
12/06 (3)	0/5	0/5	0/5	5/5	5/5	0/5	0/3	0/3	0/3
16/06 (7)	0/5	0/5	0/5	5/5	5/5	0/5	0/3	0/3	0/3
18/06 (9)	0/5	0/5	5/5	5/5	5/5	2/5	0/3	0/3	0/3
23/06 (14)	5/5	0/5	5/5	5/5	5/5	2/5	3/3	0/3	0/3
26/06 (17)	5/5	0/5	5/5	5/5	5/5	2/5	3/3	0/3	0/3
30/06 (21)	3/5	0/5	0/5	5/5	5/5	0/5	3/3	1/3	0/3
03/07 (24)	3/5	0/5	0/5	5/5	5/5	0/5	3/3	1/3	0/3
07/07 (28)	3/5	0/5	0/5	5/5	5/5	0/5	3/3	3/3	0/3
10/07 (31) [t_3]	3/5	0/5	0/5	5/5	5/5	0/5	3/3	3/3	0/3

RBT Rose Bengal test
CFT complement fixation text
* positive/total
[t_2] date of challenge
[t_3] end of the experiment, euthanasia of animals

weeks post infection. The serological reaction to the challenge strain, detectable using RBT, appeared two weeks post infection in all five animals of the group and persisted in three of these until end of the trial. No antibody response was detectable using S-type antigen CFT (Table II).

All animals in group S (vaccinated using S19) remained serologically positive post challenge and remained so until the end of the experiment. Two of these animals also developed antibody detectable by the R-type antigen CFT from 9 to 17 days post infection.

All three control animals mounted an antibody reaction detectable by RBT 14 days post infection, whilst the development of CFT antibody response was slower, involving a single animal 21 days post infection and the remaining two buffalo only from the day 28 post infection. All remained positive until the end of the trial.

Bacteriological results obtained following the euthanasia of the experimental animals are summarised in Table IV.

All spleen, retropharyngeal and supra-mammary lymph nodes of the control animals were positive, whilst all organs of animals in group S were negative. In group R animals, one retropharyngeal lymph node sample was positive and the typed strain was *B. abortus* biovar 1 (544). The overall difference was statistically significant (Kruskall-Wallis $\chi^2 = 9.877$, two-tailed $p = 0.014$).

A comparison between groups revealed the following:

- the difference between groups R and S was not statistically significant (Mann-Whitney U test, two-tailed $p = 0.317$)
- the difference between groups C and S was statistically significant (Mann-Whitney U test, two-tailed $p = 0.01$ and the Bonferroni critical value corresponding to $\alpha = 0.05$ was 0.0167)
- the difference between groups C and R was statistically significant (Mann-Whitney U test, two-tailed $p = 0.016$ and the Bonferroni critical value corresponding to $\alpha = 0.05$ was 0.0167).

The probability of infection after challenge depending on the vaccine used is summarised in Figure 1.

Discussion

A proper choice of the sample size for vaccination trials depends on a pre-defined threshold at which differences in effectiveness have to be detected. Such a threshold, in turn,

Table IV
Bacteriological results expressed in terms of colony-forming units/gram

Animal No.	Group	Spleen	Retropharyngeal lymph nodes	Supra-mammary lymph nodes	Other organs
1	R	N	N	N	N
2		N	N	N	N
3		N	250 cfu/g*	N	N
4		N	N	N	N
5		N	N	N	N
6	C	180 cfu/g	75 cfu/g	50 cfu/g	N
7		200 cfu/g	1 100 cfu/g	<10 cfu/g	N
8		50 cfu/g	300 cfu/g	2 200 cfu/g	N
9	S	N	N	N	N
10		N	N	N	N
11		N	N	N	N
12		N	N	N	N
13		N	N	N	N

N no *Brucella* cultured

* the biotype isolated was *Brucella abortus* biovar 1 (544)

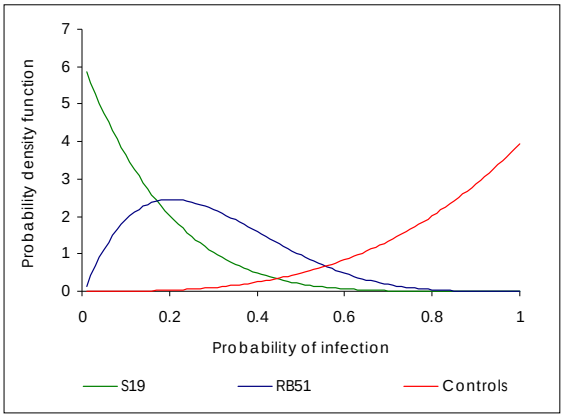

Figure 1
Probability of infection after challenge in vaccinated animals

depends on the prior knowledge of the possible difference between the vaccines being compared and on economic factors.

Since the RB51 strain has never been challenged in buffalo after a triple dose administration of vaccine and since the standard dose proved ineffective in previous trials (5), all prior knowledge indicated the possibility that a significant difference existed in the effectiveness of the two vaccine strains. As a large difference was detected statistically using relatively small sample sizes, the authors decided to refer to the samples given in the OIE *Manual* (9) for the comparison of vaccine strains in mice. This said, one has to consider the challenge strain, the dose and chosen time-scale for the evaluation of the protection by a given vaccine.

The results obtained in control animals and in those vaccinated using strain 19 were according to expectations. Therefore, animals vaccinated with RB51 can be compared to the other two groups.

Vaccine RB51, when administered at a triple dose compared to that used in cattle, and followed by a booster one month after the primary inoculation, appears to be capable of offering protection against infection due to wild type *Brucella* organisms. Even if we consider the limitations due to the low number of animals inoculated, the serological results obtained after challenge in group R animals appear to confirm that the inoculation of the agent did not induce infection since no animal developed anti-smooth complement-fixing antibodies and the CFT RB51-reacting antibodies showed a transient reaction (two of five animals became negative after two weeks from the development of a positive reaction). In the single buffalo vaccinated with RB51 where the challenge was successful, isolation was possible only from one target organ and complement-fixing antibodies were not produced at detectable levels. This appears to indicate the development of at least partial protection.

The sample size used in the experiment (five animals per group) is within the range of other trials performed with similar objectives and exactly the same sample size established by the OIE for the evaluation of vaccines in mice. The differences observed between the experimental groups after the administration of a triple dose of RB51 vaccine were very small in comparison to the results obtained in previous papers after the administration of a standard dose of RB51 vaccine. Therefore, given the sample size used, the power of the statistical tests employed was too low to detect the differences observed as statistically significant.

In order to have a rate of 90% (i.e. 90% probability of detecting a difference as statistically significant) and a confidence level of 95%, given all the other characteristics and data observed in our experiment, the sample size required would be at least 44 animals for a two-tailed statistical test or 36 animals in the case of a unilateral test. The authors intend to repeat the experiment in the future using this corrected sample size.

The safety of the vaccination protocol used has already been evaluated and was the subject of a previous publication (6). The protocol proved safe in young animals (7) whilst its application in adults has caused abortions in vaccinated pregnant female buffalo (6).

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