*A diagnostic protocol to identify water buffaloes (*Bubalus bubalis*) vaccinated with* Brucella abortus *strain RB51 vaccine*

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Vaccination.

Summary
The use of live vaccine strain RB51 for vaccination of domestic water buffaloes (*Bubalus bubalis*) at risk of infection with *Brucella abortus* is permitted notwithstanding the plans for the eradication and only under strict veterinary control. The antibodies induced by RB51 vaccination are not detectable using conventional diagnostic techniques; therefore, it is necessary to have a specific diagnostic tool able to discriminate vaccinated from unvaccinated animals. The combination of a complement fixation test (CFT) with specific RB51 antigen (RB51-CFT) and a brucellin skin test has been demonstrated to be a reliable diagnostic system to identify single cattle (*Bos taurus*) vaccinated with RB51. So far, no data are available in the international scientific literature regarding the use of this test association in water buffalo. For this reason the suitability of this test combination has been evaluated in a water buffalo herd. One hundred twenty-seven animals farmed in a herd of Salerno province (Campania, Southern Italy), in the context of a presumptive unauthorized use of RB51 vaccine were chosen for this study. All tested animals resulted negative to Rose Bengal test (RBT) and complement fixation test (CFT) used for the detection of specific antibodies against *Brucella* field strains. Seventy-one animals (56%) developed RB51 antigen-specific CFT (RB51-CFT) antibodies against RB51 vaccine in a first sampling, while 104 animals (82%) gave positive result to a second serum sampling conducted 11 days after the intradermal inoculation of the RB51 brucellin. One hundred and seven animals (84%) showed a positive reaction to the RB51-CFT in at least 1 sampling, while 111 animals (87%) resulted positive to the RB51 brucellin skin test. Thus, analysing the results of the 3 testing in parallel, 119 animals (94%) were positive to at least 1 of the performed tests. The results suggest that the use in parallel of the RB51 brucellin skin test with RB51-CFT may represent a reliable diagnostic system to identify water buffaloes vaccinated with RB51 vaccine.

*Un protocollo diagnostico per identificare bufali domestici (*Bubalus bubalis*) vaccinati con* Brucella abortus *ceppo RB51*

Parole chiave
Brucella abortus,
Brucellina,
Brucellosi,
Bufalo domestico *(Bubalus bubalis)*,
Fissazione del complemento,
Intradermoreazione,
Italia,
RB51,
Vaccinazione.

Riassunto
L'uso del vaccino vivo derivato dal ceppo RB51 di *Brucella abortus* in bufali domestici (*Bubalus bubalis*) a rischio di infezione può essere consentito in deroga al piano nazionale di eradicazione e solo sotto stretto controllo veterinario. Gli anticorpi vaccinali indotti da RB51 non sono rilevabili con le tecniche diagnostiche convenzionali, pertanto è necessario disporre di uno strumento diagnostico specifico in grado di discriminare gli animali vaccinati da quelli non vaccinati. La combinazione della prova di fissazione del complemento (CFT) con antigene specifico RB51 (RB51-CFT) con la prova di intradermoreazione alla brucellina ha dimostrato di essere un sistema diagnostico affidabile per identificare singoli bovini (*Bos taurus*) vaccinati con RB51. Non sono attualmente disponibili in letteratura scientifica dati sull'uso di questa associazione nel bufalo domestico. L'efficacia di questa combinazione di prove è stata, pertanto, valutata in un allevamento bufalino. Centoventisette animali allevati in un'azienda della provincia di Salerno (Campania, Italia meridionale), nel contesto di un

sospetto uso non autorizzato di vaccino RB51, sono stati selezionati per questo studio. Tutti gli animali saggiati sono risultati negativi alla prova di sieroagglutinazione rapida con antigene Rosa Bengala (RBT) e alla prova di fissazione del complemento (CFT) utilizzato per l'individuazione di anticorpi specifici contro ceppi di campo di *Brucella*. Settantuno animali (56%) hanno sviluppato anticorpi specifici contro il vaccino RB51 rilevati mediante prova di fissazione del complemento specifica (RB51-CFT) al prelievo iniziale, mentre 104 animali (82%) hanno fornito un risultato positivo alla prova RB51-CFT effettuata 11 giorni dopo la prova di inoculazione intradermica di brucellina RB51. Centosette animali (84%) hanno mostrato una reazione positiva alla RB51-CFT in almeno uno dei due prelievi, mentre 111 animali (87%) sono risultati positivi al test di intradermoreazione alla brucellina RB51. Pertanto, analizzando i risultati delle tre prove in parallelo, 119 animali (94%) sono risultati positivi ad almeno una delle prove effettuate. I risultati suggeriscono che l'utilizzo in parallelo della prova RB51-CFT e della prova di intradermoreazione con brucellina RB51 può essere una combinazione diagnostica affidabile per identificare i bufali domestici vaccinati con il vaccino RB51.

Introduction

Brucellosis is one of the most important zoonotic diseases worldwide and is responsible for heavy economic losses due to late term abortions, stillbirths, and parturition of weakly calves (Neta *et al.* 2010). The disease is also a serious public health problem wherever the infectious agent is present. In Italy, although a constant decrease of human cases has been observed in the last decade (from an incidence of 1.84 cases/100,000 inhabitants to 0.29 cases/100 000 inhabitants), brucellosis still remains one of the major zoonoses in 4 regions of Southern Italy (Campania, Apulia, Calabria, and Sicily), accounting for the 86% of all Italian cases[1]. A particular epidemiological situation is represented by the infection in domestic water buffalo (*Bubalus bubalis*), which is mostly farmed in Campania region, where the 73% (272,540 heads out of a total of 375,278 in Italy) of National stock of this species is farmed[2]. Historically, the most common strains isolated from water buffalo populations in Italy have been mainly *Brucella abortus* biovars 1, 3, 6 and *Brucella melitensis* biovar 3 (Di Giannatale *et al.* 2008). The objective of brucellosis eradication was introduced in the Italian legislation in 1994[3], when the vaccination of animals was forbidden and a test-and-slaughter strategy of seropositive animals was adopted. The main goal of the eradication plan was to achieve the officially brucellosis free (OBF) status for herds and territories. However, despite the application of eradication measures, the brucellosis epidemiological scenario in water buffalo of Campania region, and especially in Caserta province, still remains problematic (Caporale *et al.* 2010). As a consequence, European and Italian authorities decided to adopt additional measures to face the persistence of infection and to reduce the impact of the disease in both human and animal health. On 2 August 2007, and for the first time for water buffalo, the European Commission approved the use of *B. abortus* strain RB51 vaccine (RB51) under strictly controlled conditions for the immunization of animals at risk of infection with *B. abortus* in the Caserta province and in the surrounding areas with the highest incidence of brucellosis[4]. RB51 is a genetically stable, rough mutant strain primarily produced by several passages of *B. abortus* smooth strain 2308 in media supplemented with sub-inhibitory concentrations of rifampicin and penicillin (Schurig *et al.* 1991). This strain has proven safety and efficacy against abortion and infection in cattle (Cheville *et al.* 1996, Lord *et al.* 1998, Olsen 2000), although there are reports on abortions induced by RB51 vaccine in pregnant dairy cows (Yadzi *et al.* 2009).

However, preliminary studies conducted in Trinidad (Fosgate *et al.* 2003) found that the RB51 commercial vaccine, administered at the recommended calfhood dose, failed to protect water buffalo from infection following natural exposure to a wild strain of *B. abortus* biovar 1. To increase the efficacy of RB51 vaccine in buffalo, some authors proposed to immunize water buffalo using a vaccination protocol different from the one used in cattle. This

[1] http://brucellosi.izs.it/brucellosi/ accessed on 31.12.2011.

[2] Data acceded on 31.12.2011; http://statistiche.izs.it/portal accessed on 31.12.2011.

[3] Italian Ministry of Health. 1994. Decree n. 651 of 27 August 1994. Regolamento concernente il piano nazionale per la eradicazione della brucellosi negli allevamenti bovini. *Off J*, **277**, 26.09.1994.

[4] European Commission (EC). 2007. Commission Decision 2007/561/EC of 2 August 2007 approving the amendment to the programme for the eradication of bovine brucellosis in Italy for the year 2007, approved by Decision 2006/875/EC, as regards buffalo brucellosis in Caserta, Region Campania. *Off J*, **L 213**, 15.08.2007.

protocol includes the vaccination of impuberal animals between the ages of 6 and 8 months with a first dose 3 times higher than the one used for cattle, followed by a second dose administered 1 month after the first administration (Iovane *et al.* 2007). This vaccinal scheme has been proved to be safe in young animals (Iovane *et al.* 2007) and to protect against infection caused by the wild type of *B. abortus* (Caporale *et al.* 2010).

It worthwhile noticing that when RB51 is used in adult buffaloes, it could be excreted in milk (Longo *et al.* 2009), and could induce abortion in pregnant females (Galiero 2009). RB51 is devoid of the lipopolissacaride (LPS) O-side chain (Schurig *et al.* 1991) and, therefore, the vaccination with this strain would not induce the production of antibodies detectable by the conventional brucellosis serologic tests listed by European legislation (Diptee *et al.* 2007, Schurig *et al.* 2002, Stevens *et al.* 1994, Stevens *et al.* 1995). This characteristic, even if useful for the differentiation of RB51 vaccinated animals from animals infected by *B. abortus* field strains, requires the availability of diagnostic tools specifically addressed to the detection of anti-RB51 antibodies (Tittarelli *et al.* 2008). In cattle, the tests useful for this purpose are a dot blot test (Olsen *et al.* 1997) and a complement fixation test (CFT) using specific RB51 antigen (RB51-FDC) (1, 2), Tittarelli and colleagues (Tittarelli *et al.* 2009) suggested that the gamma interferon test is not suitable for the detection of cattle vaccinated with RB51 at calfhood. In addition, the combination of RB51-CFT with a RB51 brucellin skin test has proven to be particularly effective in identifying animals vaccinated with RB51 vaccine (De Massis *et al.* 2005). To date, the use of these methods has been documented mainly in cattle. The association of RB51-CFT with RB51 brucellin skin test has been implemented, in addition to a proper epidemiological investigation, by the Italian National Reference Centre for Brucellosis, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale' (IZSAM) in Teramo, to design a diagnostic protocol to be used by the National Veterinary Services when an illegal use of RB51 vaccine is suspected in cattle[5]. To these days, no data are available in international scientific literature on the use of this protocol in domestic water buffalo.

The aims of this paper are:

- to report the results of the application of a diagnostic protocol based on the combination of RB51-CFT with a RB51 brucellin skin test, when applied to domestic water buffalo (*Bubalus bubalis*) suspected of having been vaccinated with RB51;
- to evaluate the suitability of this diagnostic protocol for the identification of water buffalo vaccinated with RB51 vaccine.

Materials and methods

Herd tested

The diagnostic protocol was applied to a buffalo herd in the Salerno province (Campania Region, Italy) after the isolation of RB51 from a lymph node sampled from a serologically negative buffalo at slaughter. The herd was under the control of the veterinary services due to an outbreak of bubaline brucellosis in which *B. abortus* had been isolated. At the time of isolation of strain RB51, no vaccination against buffalo brucellosis was officially approved in Salerno province. According to national rules on brucellosis eradication, all animals which resulted positive to the classical serological test for brucellosis (RBT and CFT) had already been slaughtered and the herd was awaiting the restoring of the Officially-free status for the disease after a first negative result on a whole herd testing with RBT and CFT.

The isolated strain has been identified by PCR-RFLP (Restriction Fragment Length Polymorphism) performed by the National Reference Laboratory brucellosis in IZSAM, according to the method described in the chapter 2.4.3 of OIE Manual of diagnostic tests and vaccines for terrestrial animals.

Diagnostic protocol

According to the diagnostic protocol, all animals in the herd aged more than 12 months (127 heads) were tested to confirm the suspicion of unauthorized use of RB51, as follows:

- intradermal injection of 0.1 ml of RB51 brucellin produced in accordance with the method described by De Massis and colleagues (De Massis *et al.* 2005) for cattle, and simultaneous collection of blood samples to be tested by a CFT with specific RB51 antigen (CFT-RB51) (sampling time: t_0);
- collection of an additional blood sample, 11 days after brucellin RB51 inoculation to be tested by CFT-RB51 (t_1), as described for cattle by De Massis and colleagues (De Massis *et al.* 2005).

Animals resulted positive to RB51-CFT (at t_0 or t_1) and/or to the brucellin intradermal reaction were considered as vaccinated with RB51. All animals were also tested with RBT and CFT both at sampling time t_0 and t_1.

[5] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale'. Centro di Referenza Nazionale per le Brucellosi. 2010. Protocollo per la gestione di un allevamento in cui si sospetti vaccinazione con vaccino RB51 per brucellosi. brucellosi.izs.it/brucellosi/common/mostra_articolo.do?id=172.

Sample collection

Blood samples were collected via coccygeal venipuncture from local veterinary services in sterile vacutainer tubes without anticoagulant. Samples were refrigerated without delay and were kept at +4°C during transport and dispatched within 24 hours to the National Reference Laboratory for Brucellosis, IZSAM.

Serological tests

All serum samples were tested for anti-*B. abortus* and anti-RB51 antibodies. Rose Bengal test (RBT) and CFT, both performed according to the methods described in chapter 2.4.3 of the OIE Manual of diagnostic tests and vaccines for terrestrial animals5, were used for anti-*B. abortus* antibody detection.

Presence of anti-RB51 antibodies was evaluated using an RB51 antigen-specific CFT (RB51-CFT), as previously described (Adone and Ciuchini 1999, Adone *et al.* 2001). A dilution of 1:4, showing 100% fixation, was considered as threshold for positivity.

Eleven days after the first blood sampling all animals were bled and tested again with the same serological tests.

Skin test

In each tested animal, 10 cm^2 of healthy clean skin on left shoulder was shaven with scissors. A tuberculin syringe was used to inject 0.1 ml of RB51 brucellin intradermally. Before injection, the skin thickness was measured and registered for each animal. The skin reaction was evaluated 72 hours after the inoculation, by measuring the difference of the skin thickness at the injection site. A spring meter (Aesculap) was used to measure the skin thickness. An increase of at least 1.5 mm was considered as a positive result.

Statistical analysis

The increase of RB51-CFT antibody titre after brucellin inoculation was evaluated using the Wilcoxon test for paired samples (Siegel and Castellan 1988).

The statistical correlation between the RB51-CFT antibody titres before and after brucellin inoculation was measured using the Spearman's rank correlation coefficient (Spearman'*r*). Calculations were performed using Xlstat® software, a Microsoft® Excel® add-in.

Results and discussion

The results of the serological tests are presented in Table I. Sera from all tested animals gave always negative results to RBT and to CFT tests for antibodies against field strains of *B. abortus*.

A total of 71 animals (56%) and 104 animals (82%) gave a positive response to serological CFT-RB51 at t_0 and t_1 time, respectively.

Overall, 107 buffalos (84%) showed a positive reaction to RB51-CFT in at least 1 sampling, whereas 111 animals (87%) gave a positive reaction to RB51 brucellin skin test (Table II).

Combining in parallel the results obtained to RB51-CFT and RB51 brucellin skin test, 119 animals (94%) resulted positive to at least 1 of the performed diagnostic tests; in particular, 99 animals (78%) were positive on both diagnostic tests, 12 animals (10%) were positive only to RB51 brucellin skin test, and 8 animals (6%) showed a positive reaction only to RB51-CFT. Eight animals (6%) were negative to both diagnostic tests (Table II). The mean and standard deviation of skin thickness values observed after 72 hours post inoculation are reported in Figure 1.

Table III presents the results of RB51-CFT before and after RB51 brucellin inoculation, in comparison with the results of the latter test. Twenty animals (16%) showed negative results to RB51-CFT on both samplings, whereas 12 of these animals reacted to RB51 brucellin skin test. Thirty-six animals (28%) with negative results to RB51-CFT at t_0 sampling developed a serological positive (anamnestic) response to RB51-CFT after RB51 brucellin inoculation.

A statistically significant increase of the number of samples positive to RB51-CFT has been observed at t_1 (Wilcoxon T = 1618.000; 1-tailed p = 0.000013), especially evident at dilutions between 1:4 and 1:16 (Figure 2).

Table I. *Results of serological tests (number of positive on tested) carried out on 127 water buffaloes (*Bubalus bubalis*) on samples collected on the day of RB51 brucellin inoculation (t_0) and 11 days after (t_1).*

Sampling time	RBT*	CFT*	RB51-CFT*
Day 0 (t_0)	0/127	0/127	71/127
Day 11 (t_1)	0/127	0/127	104/127

RBT = Rose Bengal test; CFT = complement fixation test; RB51-CFT = RB51 complement fixation test; *number of positive animals on total tested; (t_0) = day of 1st sampling; (t_1) = day of 2nd sampling.

Table II. *Overall results of the association in parallel of Complement Fixation test using the RB51 antigen (RB51-CFT) conducted at t_0 and t_1 time and the RB51 brucellin skin test conducted at t_0 time.*

		RB51 Brucellin		Total
		Positive	Negative	
RB51-CFT	Positive	99	8	107
	Negative	12	8	20
	Total	111	16	127

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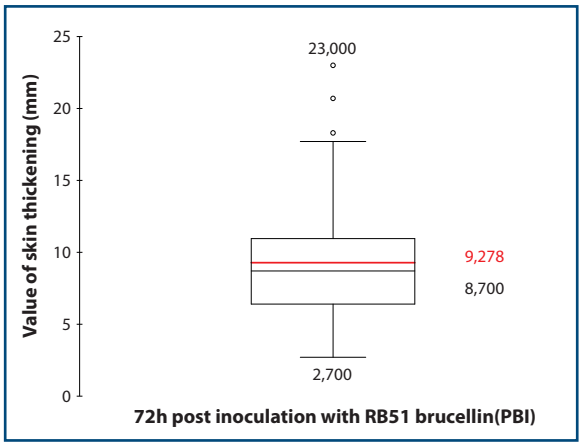

Figure 1. *Mean, maximum value, minimum value, 25th percentile, and 75th percentile of RB51 brucellin skin test results in 127 animals, tested with RB51 brucellin skin test at t_0 time.*

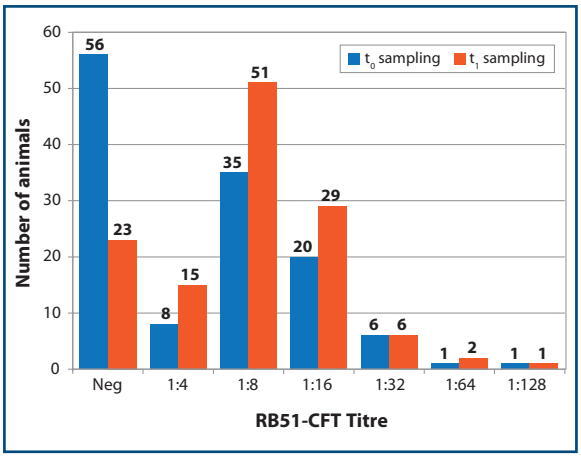

Figure 2. *RB51-Complement fixation test (CFT) antibody response prior (t_0) and after (t_1) intradermal inoculation of RB51 brucellin.*

Table III. *Comparison of the results of RB51-Complement fixation test (CFT) conducted at t_0 and t_1 time and the RB51 brucellin with skin test results conducted at t_0 time.*

		RB51-CFT				
		NN	NP	PN	PP	Total
RB51-Brucellin	N	8	0	1	7	16
	P	12	36	2	61	111
	Total	20	36	3	68	127

N = negative to RB51 brucellin skin test; P = positive to RB51 brucellin skin test; NN = negative to RB51-CFT at both samplings; NP = negative to RB51-CFT at t_0 sampling, positive at t_1 sampling; PN = positive to RB51-CFT at t_0 sampling, negative at t_1 sampling; PP = positive to RB51-CFT at both samplings.

In addition, the RB51-CFT titres before and after the inoculation of brucellin were significantly correlated (Spearman $r = 0.545$, $p < 0.0001$).

The brucellosis vaccine which is employed in buffalo worldwide is *B. abortus* strain 19. However, the use of this vaccine leads to the production of antibodies that are detectable by the official tests used for the diagnosis of brucellosis in the context of national control and eradication plans (Caporale *et al.* 2010). *Brucella abortus* strain RB51 vaccine has been developed for use in cattle and could be preferred to *B. abortus* strain 19 vaccine for its negligible interference with diagnostic serology (Diptee *et al.* 2007, Schurig *et al.* 2002, Stevens *et al.* 1994, Stevens *et al.* 1995). Nonetheless, when a strict test and slaughter policy is applied in a country or zone and the application of vaccines is, therefore, not allowed, the illegal use of vaccines is of particular concern. Indeed, the fraudulent use of a live vaccine could also be a serious public health problem. In particular, RB51 could infect humans and it is highly resistant to rifampicin, one of the antibiotics of choice for the treatment of human brucellosis. It is also worth stressing that the diagnosis of the infection produced by RB51 requires special tests not available in most hospitals (Tittarelli *et al.* 2008). In addition, field trials indicated that RB51 could be excreted in milk of buffalo vaccinated as adults (Longo *et al.* 2009) and that it could induce abortion if consumed by pregnant women (Galiero 2009). Therefore, the availability of a reliable diagnostic tool to identify animals vaccinated with RB51 is necessary.

Considering that the possibility of using of the RB51–CFT to identify cattle vaccinated at calfhood is limited in terms of time, and considering the low sensitivity of the RB51 skin test when used alone, De Massis and colleagues (De Massis *et al.* 2005) suggested the use of RB51 skin test and RB51-CFT in parallel to correctly identify all vaccinated animals. The association of these tests has been used by the Italian National Reference Centre for Brucellosis (IZSAM) along with a proper epidemiological investigation, to design an official diagnostic protocol to be applied when an illegal use of RB51 vaccine is suspected in cattle.

Actually, this type of findings has not been previously reported for water buffalo, but only for vaccinated cattle. However, the results of this field study suggest the suitability of the diagnostic protocol also when applied in water buffalo. Following RB51 brucellin inoculation in the study animals, the serological test RB51-CFT is able to reveal a significant greater number of positive animals, discovering those vaccinated heads not reacting to the serological tests at t_0. The increase of sensitivity of this diagnostic protocol, with respect to the use of the single test alone, is due to the specific and anamnestic humoral response elicited by the use of RB51 brucellin, which represents the evidence of previous vaccine administration.

Results from this field study show that 71 animals (56%) and 104 animals (82%) developed specific

antibodies against RB51 vaccine, which were revealed with the RB51 antigen-specific CFT (RB51-CFT), before and after intradermal inoculation of the RB51 brucellin, respectively. The classical tests (RBT and CFT) performed at t_0 and t_1 (*i.e.* before and after brucellin inoculation) resulted negative, therefore suggesting that animals with latent infection were no longer present in the herd under study and the reactions against brucellin were caused by vaccination.

One hundred and seven animals (84%) showed a positive reaction to the RB51-CFT in at least 1 sampling, while 111 animals (87%) resulted positive to the RB51 brucellin skin test. Thus, analysing the results of the 2 testing in parallel, 119 animals (94%) were positive to at least 1 of the performed tests. The application of a single test alone would have revealed a lesser proportion of vaccinated animals. In particular, 36 animals (28%) of the total number of animals identified as positive (Table III) developed a serological response to RB51-CFT after RB51 brucellin inoculation and all of them were also positive to the RB51 brucellin skin test (Table III).

Furthermore, many of the inoculated animals showed an increase of volume of prescapular lymph nodes located near the site of inoculation. The value of skin thickening observed (Figure 1) revealed the presence of a strong cell-mediated immune response against *B. abortus* RB51 in accordance with a previous study (Iovane *et al.* 2007).

The results of the present study suggests that the use of a diagnostic protocol based on the parallel application of a RB51 skin test and a RB51-CFT assays could be a reliable diagnostic system able to identify water buffalo vaccinated with RB51.

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