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Safety of *B. abortus* rough mutant strain RB51 administration in Buffalo cows

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ABSTRACT: The objective of this study was to determine if *B. abortus* rough mutant strain RB51 is eliminated in Buffalo milk. Five milk buffaloes were inoculated with the triple of the recommended calfhood dose (3.0 – 10.2 x 10^{10} cfu/ml) of *B. abortus* RB51 strain by subcutaneous route in the right axillary region. Milk samples were taken aseptically on a daily basis for the first 30 days and weekly for the second and third months. The samples were inoculated on Brucella Medium Base (BMB) (Oxoid) and Rifampin Brucellae Medium (RBM) and incubated under 10% CO_2 at 37°C for 10 days. The suspicious colonies were re-cultured in BMB and RBM. PCR analysis was also performed on milk samples.

There were no isolations of bacteria with characteristics of Brucella from any of the milk samples collected during 90 days of the study. However Brucella RB51 DNA was detected on day 2 and 3 post vaccination in one buffalo cow and on day 21 post vaccination in another buffalo cow.

It was concluded that the strain used at this dose wasn't eliminated by milk in Buffaloes inoculated during lactation, however PCR positive results underline the necessity of milk pasteurization in order to minimize food-chain exposure.

INTRODUCTION - In Italy approximately 250.000 buffaloes (Bubalus bubalis) are reared mainly for the production of milk used to produce mozzarella cheese. The Buffalo herds are distributed across 2.246 farms (ISTAT 20) mostly in Campania region. *Brucella abortus* is the aetiologic agent of brucellosis in cattle (*Bos taurus* and *Bos indicus*) and domestic water buffalo (*Bubalus bubalis*) (Corbel M.J, 1997; Fosgate et al., 2002a). Although these two species belong to the Bovidea family they are not classified in the same genus, yet conclusions from cattle research have been extrapolated to water buffalo without appropriate verification. Recently, Fosgate et al. (2002b) reported differences in sensitivities and specificities of brucellosis serological tests between cattle and water buffalo. Fosgate et al. (2003), further demonstrated that the *B. abortus* strain RB51 (RB51) commercial live vaccine, administered at the recommended calfhood dose, failed to protect water buffalo from

infection following natural exposure to *B. abortus* biovar 1. RB51 is a genetically stable, rough mutant strain of *Brucella abortus* smooth strain 2308, derived from serial passage on trypticase soy agar with varying concentrations of rifampin (Schurig et al., 1991). The RB51 vaccine has been proven safe and effective against abortion and infection in cattle caused by *B. abortus* (Olsen et al., 1999) and American bison (Olsen et al., 2003).

RB51 strain, used in Campania Region according to a special eradication programme for the control of brucellosis in buffaloes, does not interfere with routine serological surveillance for brucellosis (Stevens et al., 1994). However RB51 has been demonstrated to have/maintain tropism for the placenta, and cause placentitis, which induces abortion in pregnant cattle, bison and buffaloes (Palmer et al., 1996; Galiero et al., 2006).

Regardless of the attributes of this vaccine, there are still some aspects that are not elucidated, especially in buffaloes.

On the other hand, it is important to know if this strain is excreted in milk, since occupational and foodchain exposure exists, though this has not been documented to date. The objective of this study was to determine if the RB51 strain is eliminated in the milk of vaccinated buffaloes during lactation.

MATERIAL AND METHODS - Three *Brucella*-free and non-vaccinated unexposed adult milk buffaloes and two *Brucella*-free and RB51 vaccinated adult milk buffaloes (vaccinated as calves with the triple of the recommended calfhood dose for two times four week operative), in the first and second third of lactation, were injected subcutaneously in right axilary region with the triple of the recommended calfhood dose ($3.0 – 10.2 \times 10^{10}$ cfu/ml) of *B. abortus* RB51 strain colonies forming units (cfu)/ml Milk samples were aseptically collected from each teat daily for the first 30 days and weekly for the second and third months.

Bacteriological methods. Isolation from milk samples. For each daily sampling from each buffalo 10 ml of milk from each quarter was pooled, and the resulting 40 ml was centrifuged at 3500*g* for 15 min. The creamy layer and deposit from each sample was collected and spread onto *Brucella* Medium Base (Oxoid) with added Brucella Selective Supplement (BSSA, Oxoid, Hampshire, UK) and rifampin brucellae medium (RBM)(Hornsby et al., 2000) under 10% CO_2 at 37°C for 10 days. After 48 h, the plates were examined for growth and then daily for 10 days. Suspect *Brucella* colonies were inoculated into blood agar, MacConkey agar and RBM for secondary isolation of suspect RB51 colonies. Colonies producing haemolysis on blood agar, lactose fermentation on MacConkey and which failed to grow in the presence of rifampin were eliminated from further consideration as *Brucella* suspects. The remaining suspect colonies were subjected to Gram stain and biochemical tests. Colonies that were Gram-negative coccobacilli and positive for catalase, urease and oxidase production as determined by standard methods were deemed RB51 suspects. These were stored at -80°C in microbanks (oxoid) and later underwent to PCR for the confirmation of isolates as RB51 strain. As a control of the vaccines strains growth on BSSA and RBM media, the vaccine was inoculated in sterile buffaloes milk, 10-fold dilutions were performed (10^{-1}–10^{-9}), 20 µl of each dilution were inoculated on BSSA and RBM media; this procedure was done four times.

DNA extraction from milk samples and PCR conditions. DNA was extracted from whole milk in according to Sreevatsan et al., (2000). Preparation of PCR mixtures was con-

ducted in a separate room to avoid unintentional contamination with Brucella DNA samples. The PCR reaction was performed in a total volume of 50 µl containing 22 pmol each of primer (see Table 1), 2,5 mM each of the four deoxynucleoside trphosphate, 5 U/µl TaKaRa Taq™ polymerase, 5 µl of 10X PCR buffer (TaKaRa Taq™). After a denaturation step at 94°C for 10 min, samples were cycled 35 times in a DNA thermal cycler (Perkin-Elmer, Gene Amp PCR) as follows: 1 min and 15 s at 95°C, 2 min at 55.5°C, 2 min at 72°C and one final extension at 72°C for 5 min. Thirty microlitre samples of PCR reactions were analysed on 2% agarose gel in TBE 1 X buffer (100 mM Tris±HCl, 90 mM boric acid, 1 mM sodium ethylenediaminetetraacetic acid, ethidium bromide solution 10 mg/ml). After electrophoresis at 125 V for 120 min, gels were visualized under ultraviolet light.

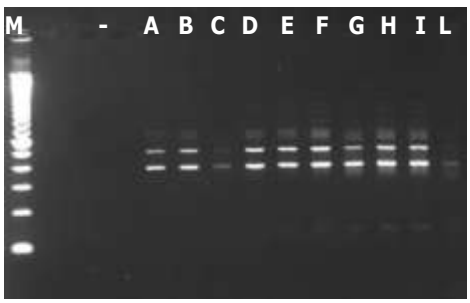
Table 1. Sequences of oligonucleotide primers used for PCR assay

Primer Sequence	(5'- 3')
412 B. abortus-specific	GAC GAA CGG AAT TTT TCC AAT CCC
416 IS711-specific	TGC CGA TCA CTT AAG GGC CTT CAT
423 RB51/2308-specific	CCC CGG AAG ATA TGC TTC GAT CC

RESULTS AND CONCLUSIONS - No bacterial colonies resembling *Brucella* were identified from any of the milk samples collected during 90 days of the study. However, Brucella RB51 DNA was detected on day 2 and 3 post vaccination in one re-vaccinated buffalo cow and on day 21 post vaccination in the other one re-vaccinated buffalo cow. The vaccine strain was able to grow on BSSA and RBM media up to dilution 1×10^{-8}. The detection limit of PCR under our experimental conditions (NET buffer, SDS at 80°C, digestion with RNase and proteinase K at 50°C, and organic extraction) was 1 to 10 *Brucella* CFU/ml of milk. (Fig 1) The original concentration of the vaccine was to 1×10^{9} cfu/ml. It was demonstrated that the BSSA media used in this experiment was suitable for the growth both vaccine strains. The identification of media components that inhibit fungal and bacterial contaminants without inhibiting RB51 has been identified, optimized and evaluated (Hornsby et al.,

Figure 1.

Detection limit of PCR: M= ladder 100 bp
-= negative A= 1 ucf/ml B= 10 ucf/ml
C= 10^2 ucf/ml D=10^3 ufc/ml E= 10^4 ufc/ml
F=10^5 ufc/ml G=10^6 ufc/ml H=10^7 ufc/ml
I=10^8 ufc/ml L=10^9 ufc/ml.



2000). Brucella RB51 DNA was detected only in re-vaccinated buffalo cows on day 2, 3 and 21 post vaccination. We can conclude that even thought the strain used at this dose wasn't eliminated by milk in Buffaloes inoculated during lactation, PCR positive results underline the necessity of milk pasteurization in order to minimize eventual food-chain exposure.

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