

Full Length Research Paper

# The immunological response of RB51 vaccinated buffalo calves using brucella periplasmic proteins as ELISA antigen

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Immune status of RB51 vaccinated buffaloes was evaluated using tube agglutination test (TAT) and ELISA, using both periplasmic protein antigen (PPA) and lipopolysaccharide antigen (LPS). For this purpose, three groups of buffalo calves were used. The first one received S19 vaccine subcutaneously; the second was vaccinated once subcutaneously with RB51 vaccine. The third (control) group was injected similarly with sterile saline. Concerning the S19 vaccinated group, significant TAT titers were seen 1 week post vaccination (WPV) till the maximum at the 2<sup>nd</sup> WPV. After that it was gradually decreased till the 7 WPV, then sharply before it completely disappeared at the 13 WPV. On the other hand, the LPS-ELISA showed an antibody titer as early as one WPV reached its peak at 2 WPV and persisted steadily till the 6<sup>th</sup> WPV and decreased slowly when it reached minimal level at the 16 WPV till the end of the experiment. While in RB51 vaccinated buffalo calves using the PPA-ELISA, the antibody titer began and reach the maximum as early as the first WPV, still steady till 2 WPV, fluctuating till the 6<sup>th</sup> WPV, then dropped sharply when it disappeared at 11WPV till the end of the experiment.

**Key words:** Brucella, buffaloes, ELISA, lipopolysaccharide (LPS), periplasmic proteins, RB51, S19, tube agglutination test (TAT).

## INTRODUCTION

Bovine brucellosis is one of the most important zoonotic diseases worldwide, especially in developing countries and is responsible for serious economic losses due to late term abortion, stillborn and weakly calves (Neta et al., 2010). The occurrence of the disease in humans is largely dependent on the animal reservoir (Adone et al., 2005). Brucella control programs mainly depend on vaccination, mostly with attenuated *Brucella abortus* strain 19 which provides good levels of protection against

*B. abortus* and prevents premature abortions in cattle (Nicoletti, 1990) and buffalo worldwide (OIE, 2009). However, this vaccine has the drawback of inducing O-polysaccharide-specific antibodies that interfere with the discrimination between vaccinated and infected animals during serological screening (Salmakov et al., 2010). In addition, they retain pathogenicity and sometimes cause abortion in vaccinated animals (Diptee et al., 2006) and cause the disease in humans.

To overcome the problem of serological interference, RB51, a mutant vaccinal rough strain that is devoid of the LPS O-side chain was developed (Poester et al., 2006). Consequently, cattle vaccinated with RB51 do not sero-convert on conventional brucellosis serologic tests (Robles et al., 2009). Therefore, this vaccine is more appropriate than *B. abortus* S19 for control and eradication programs that rely on serologic testing and removal of positive animals.

Generally, RB51 prevents abortion and infection in

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**Abbreviations:** TAT, Tube agglutination test; WPV, week post vaccination; BAPAT, buffered acidified plate agglutination test; RBPT, Rose Bengal plate test; LPS, lipopolysaccharide; PPA, periplasmic proteins antigen; pv, post vaccination.

cattle under experimental (Olsen, 2000) and field conditions (Lord et al., 1998); although, there are few reports about abortion induced by RB51 vaccine (Yazdi et al., 2009) and shedding of the micro-organism in buffaloes milk (Ingo et al., 2009). Most studies in cattle reported that RB51 provides protection against moderate challenge (Martins et al., 2009), but less effective than S19 against severe challenges (Moriyon et al., 2004). Moreover, elk (*Cervus elaphus*) vaccinated with RB51 are not protected from infection and abortion (Kreeger et al., 2002). Fosgate et al. (2003), further demonstrated that the RB51 vaccine, administered at the recommended calfhood dose, failed to protect water buffalo from infection following natural exposure to *B. abortus* biovar 1. To our knowledge, official use of RB51 in domestic water buffalo has not been reported. Therefore, the aim of this study was to provide a simple and effective method for the identification of RB51 vaccinated buffalo and overcome illegal vaccination beside the evaluation of the RB51 vaccine efficacy in the vaccination campaigns.

## MATERIALS AND METHODS

*B. abortus* RB51 vaccine (Professional Biological Company, 4950 York Street, Denver, CO) and *B. abortus* S19 were kindly provided by Serum and Vaccine Research Institute, Abbasia, Cairo, Egypt. Viable counts were performed at the time of inoculation.

30 Egyptian female buffalo calves, 6 months old were obtained from a "brucellosis-free" farm during 2007 to 2008. Animals had received routine preventive medicine including vaccinations and dewormings and appeared to be in good general health when examined. No animal had a history of acute or chronic illnesses. All animals were tested negative for brucellosis on buffered acidified plate agglutination test (BAPAT) and Rose Bengal plate test (RBPT) as described earlier (Alton et al., 1988). These buffalo calves were divided into three groups each, of ten animals: Group 1 received 1 to 2 x 10<sup>10</sup> CFU of S19 vaccines subcutaneously as described earlier (Uzal et al., 2000). Group 2 was vaccinated once with (1.0 to 3.4) x 10<sup>10</sup> CFU of RB51 vaccine subcutaneously in the left prescapular region according to manufacturer's recommendations for calfhood vaccination of cattle (Stevens et al., 1997). Animals in the non-vaccinated control group (Group 3) were injected similarly with 2 ml sterile saline solution. Blood was obtained by jugular venipuncture before and at zero week post vaccination (WPV) and weekly till 20 WPV. Sera were separated and stored at 4°C till used. All stages were conducted with consideration of their welfare and all procedures with animals were carried out in accordance with appropriate humane methods.

All the work was done in the National Research Center (NRC), Animal Reproduction Research Institute (ARRI) and the Central Laboratory for Evaluation of Veterinary Biologics (CLEVB).

### Serological testing and antigens

All animals were tested for anti-*B. abortus* and anti-RB51 antibodies before vaccination, on the day of vaccination (day zero) and then periodically until 20 weeks post vaccination (WPV). Anti-*B. abortus* antibodies were verified using both tube agglutination test (TAT) according to Alton et al. (1988) and ELISA using *B. abortus* M-99 crude lipopolysaccharide (LPS-ELISA) according to Plackett et al. using the *B. abortus* M-99 periplasmic proteins (PPA-ELISA)

(1976). While Anti-RB51 antibodies were monitored with ELISA prepared according to Yifan et al. (1993).

### Extraction of *B. abortus* periplasmic protein antigen (PPA)

*B. abortus* M-99 was cultured on the growth medium for 3 to 4 days at 37°C in CO<sub>2</sub> incubator. After the growth of the organism, they were harvested, and then washed 3 times using 0.15 M sodium chloride by centrifugation at 3000 rpm/15 min, pellet was re-suspended using the same buffer, 0.15 M sodium chloride and then autoclaved at 120°C/20 min (hot saline extract method). The suspension of the autoclaved organism was centrifuged at 12,000 rpm for 30 min, then the supernatant was separated and ammonium sulphate was added. The precipitated proteins were collected by centrifugation at 3000 rpm/15 min, then the pellets were resuspended in 0.01 M phosphate buffer saline (PBS). Dialysis occurred against PBS in dialysis bag overnight at 4°C; aspiration of the solution occurred and was lifted at -20°C till used.

### ELISA assay

ELISA assay on serum samples was performed as mentioned earlier (Bassiri et al., 1993) using both periplasmic and LPS antigens and the optical density was read at 490 nm.

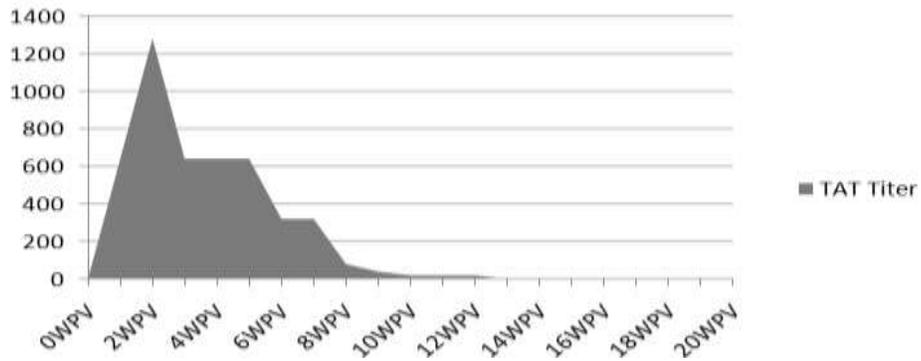
## RESULTS

Sera from all buffaloes calves (RB51-vaccinated and control) gave negative results to TAT and ELISA using *Brucella* crude LPS prior to vaccination, on day zero and during the entire study. All animals were also negative to PPA-ELISA prior to vaccination and on day zero, while after vaccination, vaccinated animals developed a serological response to RB51.

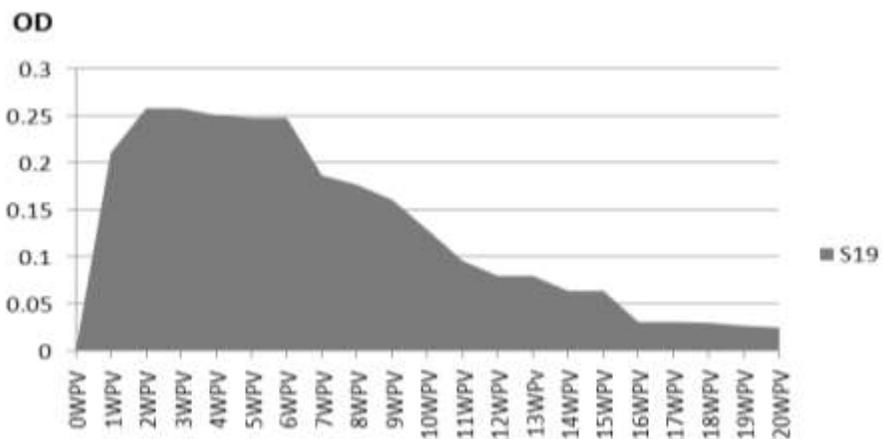
Significant TAT titers in S19 vaccinated buffaloes (Figure 1) were seen as early as 1 WPV, while the highest TAT titer was observed at the 2WPV, then gradually declined till 7 WPV, and sharply when all animals became sero-negative at 13WPV.

Concerning LPS-ELISA in S19 vaccinated buffaloes (Figure 2), antibody titer began as early as 1 WPV, reached its peak at 2 WPV and persisted steadily till the 6<sup>th</sup> WPV, then decreased slowly till it reached minimal level at the 16 WPV till the end of the experiment. By using periplasmic protein antigen (PPA) (Figure 3), the average PPA-ELISA titer in buffalo calves vaccinated with RB51 reached the maximum as early as 1 WPV, still steady till 2 WPV and fluctuated till the 6 WPV, then dropped sharply when it disappeared at the 11 WPV up to the end of the experiment. Control animals (Group 3) maintained their negative serological status during the complete experimental period.

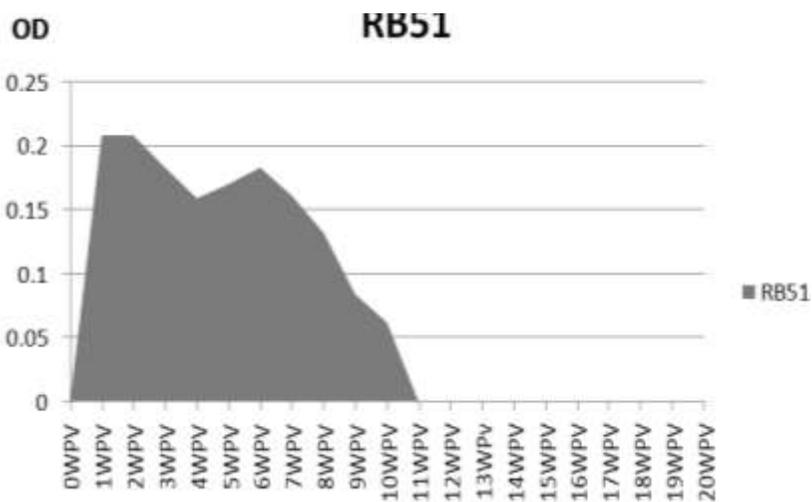
The RB51 vaccination did not interfere with the conventional diagnostic serology tests like TAT and ELISA using the LPS as antigen. The PPA-ELISA was able to detect antibodies produced by the RB51 vaccination in buffaloes.



**Figure 1.** TAT serologic responses in buffaloes vaccinated with *B. abortus* S19 vaccine. TAT investigated for 20 weeks post vaccination; serum was obtained before (time 0) and at 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 WPV after buffaloes were vaccinated with S19.



**Figure 2.** LPS-ELISA serologic responses (as O.D.) in buffaloes vaccinated with *B. abortus* S19 vaccine.



**Figure 3.** PPA-ELISA serologic responses (as O.D.) in buffaloes vaccinated with *B. abortus* RB51 vaccine.

## DISCUSSION

Brucellosis is a disease of socio-economic and public health importance and has significant impact on the international trade of animals and animal products (OIE, 2004) as it causes abortions and reduced fertility in cattle and water buffalo (Mohan, 1968).

The water buffalo is potentially the most important tropical bovine species in areas where rivers and swamps abound (Shaffie, 1985), yet there is no reported effective vaccine against brucellosis in this valuable livestock species (Fosgate et al., 2003).

Recent reports (Fosgate et al., 2002) showed differences in sensitivities and specificities of brucellosis serological tests between cattle and buffalo. Furthermore, Fosgate et al. (2003) showed that the *B. abortus* RB51 administered at the recommended calfhood dose failed to protect water buffalo from infection following natural exposure to *B. abortus* biovar 1. Moreover, Kreeger et al. (2002) demonstrated that elk (*C. elaphus*) vaccinated with RB51 were not protected from infection and abortion. The poor cell-mediated immune response may be the reason for this vaccine inefficacy as reported by Olsen (2000).

As a new approach to elevate the efficiency of RB51 vaccine in buffalo, some authors have suggested the possibility of using a vaccination scheme that differs from that used in cattle (Iovan et al., 2007; Caporale et al., 2010), such as vaccination of impuberal buffalo with a triple dose than that used in cattle and a booster dose after one month. This proved safe in young animals but caused abortions in vaccinated pregnant female buffalo (Galiero, 2009). Others suggested that RB51 vaccine alone does not eliminate the problem of brucellosis in endemic zones and it is necessary to apply strict bio-security measurements, such as removing seropositive animals (Herrera-LÓpez et al., 2010).

Recently, with the gradual use of RB51 vaccine, it became necessary to develop a method capable of identifying animals vaccinated with RB51 vaccine (Robles et al., 2003). This includes a dot-blot assay using killed irradiated RB51 bacteria as an antigen (Olsen et al., 1997), iELISAs using a 5% optical density heat-killed whole RB51 organisms as an antigen (Edmonds et al., 1999), and a crude rough LPS preparation from RB51 (Uzal et al., 2000), Dot-Blot ELISA (Fosgate et al., 2003; Diptee et al., 2007), an immunoblot analysis using sonicated cell lysates from RB51 (Edmonds et al., 1999), a complement fixation test, using RB51 cultured cells in calcium-magnesium-veronal buffer (Diptee et al., 2007; Galiero, 2009; Caporale et al., 2010) and an agar gel immunodiffusion test using hot saline extract antigen from *Brucella ovis* (Robles et al., 2009). Unfortunately all these methods were unpractical and time consuming.

According to results in Figure 1, significant TAT titers in S19 vaccinated buffalo were seen as early as 1 WPV, while the highest TAT titer was observed at 2 WPV, it

declined gradually till 7 WPV, then sharply when all animals became sero-negative at 13 WPV.

In comparison with the S19 vaccination in buffalo, Jamal et al. (2003) reported that using TAT, the antibodies declining rate was slow from day 14 to 49 post-vaccination, after that a rapid decrease was seen till the 91<sup>th</sup> day post vaccination and negligible after that.

Concerning LPS-ELISA in S19 vaccinated buffaloes, it showed an antibody titer (Figure 2) as early as 1 WPV, reached its peak at 2 WPV and persisted steadily till the 6<sup>th</sup> WPV, and then decreased slowly till it reached minimal level at the 16 WPV till the end of the experiment, while using PPA, the average PPA-ELISA titer in buffalo calves vaccinated with RB51 shown in Figure 3 began and reached the maximum as early as 1 WPV, still steady till 2 WPV and fluctuated till the 6 WPV, and then dropped sharply till it disappeared at the 11 WPV up to the end of the experiment.

This was significantly different from the results of RB51 vaccination in cattle calves as reported by Tittarelli et al. (2008) which showed an immune response that began with an increase at day 6 post-vaccination, the antibody level remained constant for two months, then progressively decreased. All vaccinated animals remained negative from day 162 post vaccination (pv) to the end of the study (day 300 pv).

This means that the antibody titer persisted for significantly shorter period than that of S19, and since antibodies developed against RB51 may have limited abilities to opsonize smooth *Brucella* strains, therefore, the failure of RB51 vaccination to induce comparable protection to S19 vaccination may be due to antibodies induced by RB51 which failed to bind and opsonize smooth strains of *Brucella* (Fosgate et al., 2003; Olsen et al., 2006). So, in brucellosis endemic zones, vaccination with RB51 alone is not enough to control disease. It is necessary to eliminate all positive animals at the time of vaccination and all new positive animals after that for long periods of time (Herrera-LÓpez et al., 2010).

The results of this study confirm the possibility of using PPA-ELISA to identify RB51 vaccinated buffalo and moreover, monitor antibody responses to RB51 vaccination up to 11 WPV. Besides, negative results to TAT and LPS-ELISA confirm the impossibility to detect specific RB51 antibodies with the conventional serological tests which is in agreement with that of others (Diptee et al., 2007).

Obviously, more research is needed in order to evaluate the PPA-ELISA in field buffaloes and also to determine the correct dose to be used in adult and young water buffaloes in terms of safety, immunogenicity and efficacy.

## REFERENCES

- Adone R, Ciuchini F, Marianelli C, Tarantino M, Pistoia C, Marcon G, Petrucci P, Francia M, Riccardi G, Pasquali P (2005). Protective

- Properties of Rifampin-Resistant Rough Mutants of *B. melitensis*. Infect. Immun., 7: 4198-4204.
- Alton GG, Jones LM, Angus RD, Verger JM (1988). Serologic methods, In Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, p. 63-134.
- Bassiri M, Ahmad S, Giavendonni L, Jones L, Saliki JT, Mebus C, Yilma T (1993). Immunological response of mice and cattle to baculovirus expressed F&H proteins of Rinderpest virus, lack of protection in the neutralizing antibody. J. Virol., 67: 1255-1261.
- Caporale V, Bonfini B, Di Giannatale E, Di Provvio A, Forcella S, Giovannini A, Tittarelli M, Scacchia, M (2010). Efficacy of *B. abortus* vaccine strain RB51 compared to the reference vaccine *B. abortus* strain 19 in water buffalo. Vet. Ital., 46: 13-19.
- Diptee MD, Adesiyun AA, Asgarali Z, Campbell M, Adone R (2006). Serologic responses, biosafety and clearance of four dosages of *B. abortus* strain RB51 in 6–10 months old water buffalo (*Bubalus bubalis*). Vet. Immun. Immunopath., 109: 43–55.
- Diptee MD, Asgarali Z, Campbell M, Fosgate G, Adesiyun AA (2007). Post-exposure serological and bacteriological responses of water buffalo (*Bubalus bubalis*) to *B. abortus* biovar 1 following vaccination with *B. abortus* strain RB51. Rev. Sci. Tech. Off. Inter. Epizoot., 26: 669-678.
- Edmonds MD, Schurig GG, Samartino LE, Hoyt PG, Walker JV, Hagius SD, Elzer PH (1999). Biosafety of *B. abortus* strain RB51 for vaccination of mature bulls and pregnant heifers. Am. J. Vet. Res., 60: 722– 725.
- Fosgate GT, Adesiyun AA, Hird DW, Johnson WO, Hietala SK, Schurig GG, Ryan J (2002). Bayesian comparison of brucellosis serologic tests without a gold standard in cattle and water buffalo (*Bubalus bubalis*) of Trinidad. Am. J. Vet. Res., 63: 1598–1605.
- Fosgate GT, Adesiyun AA, Hird DW, Johnson WO, Hietala SK, Schurig GG, Ryan J, Diptee MD (2003). Evaluation of brucellosis RB51 vaccine for domestic water buffalo (*Bubalus bubalis*) in Trinidad. Prev. Vet. Med., 15: 211-225.
- Galiero G (2009). *Brucella abortus* RB51 vaccine safety of the buffalo in the Mediterranean. Large Anim. Rev., 15: 19-22.
- Herrera-López E, Suárez-Güemesb F, Hernández-Andradea L, Córdoba-López D, Díaz-Aparicio E (2010). Epidemiological study of brucellosis in cattle, immunized with *B. abortus* RB51 vaccine in endemic zones. Vaccine, 28 (5): F59-F63.
- Iovane G, Martucciello A, Astarita S, Galiero G, Pasquali P, Adone R, Ciuchini F, Pagnini U, Guarino A, Fusco G (2007). *Brucella abortus* RB51 vaccine - First results on the safety and immunogenic activities in the Mediterranean buffalo. Prog. Vet., 1: 19-21.
- Jamal SM, Afzal M, Ahmed S (2003). The immune response of guinea pigs and buffalo calves to the locally prepared *Brucella abortus* strain 19 vaccine. Rev. Sci. Tech. Off. Int. Epiz., 22: 893-897.
- Kreeger TJ, Cook WE, Edwards WH, Elzer PH, Olsen SC (2002). *B. abortus* strain RB51 vaccination in elk. II. Failure of high dosage to prevent abortion. J. Wild. Dis., 38: 27–31.
- Longo M, Mallardo K, Montagnaro S, De Martino L, Gallo S, Fusco G, Galiero G, Guarino A, Pagnini U, Iovane G (2009). Shedding of *B. abortus* rough mutant strain RB51 in milk of water buffalo (*Bubalus bubalis*). Prev. Vet. Med., 90: 113–118.
- Lord VR, Schurig WH, Cherwonogrodzky JW, Marcano MJ, Melendez GE (1998). Field study of vaccination of cattle with *B. abortus* strains RB51 and 19 under high and low prevalence. Am. J. Vet. Res., 59: 1016–1020.
- Martins H, Garin-Bastuji B, Lima F, Flor L, Pina Fonseca A, Boinas F (2009). Eradication of bovine brucellosis in the Azores, Portugal - Outcome of a 5-year programme (2002–2007) based on test-and-slaughter and RB51 vaccination. Prev. Vet. Med., 90: 80-89.
- Mohan RN (1968). Diseases and parasites of buffaloes. Vet. Bull., 38: 647–659.
- Moriyon I, Grillo MJ, Monreal D, Gonzalez D, Marin C, Lopez-Goni I, Mainar-Jaime RC, Moreno E, Blasco JM (2004). Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet. Res., 35: 1–38.
- Neta AVC, Mol JPS, Xavier MN, Paixão TA, Lage AP, Santos RL (2010). Pathogenesis of bovine brucellosis. Vet. J., 184: 146–155.
- Nicoletti P (1990). Vaccination, p. 284-299. In Nielsen K, Duncan JR (ed.), Animal brucellosis. CRC Press, Boca Raton, Fla.
- Office International Des Epizooties, OIE (2004). International Animal Health Code 2000; 2.3.1. Available at: <http://www.oie.int/>.
- Office International Des Epizooties, OIE (2009). Bovine brucellosis, Chapter 2.4.3. In Manual of diagnostic tests and vaccines for terrestrial animals. OIE, Paris, 1-35 ([www.oie.int/eng/normes/mmanual/2008/pdf/2.04.03\\_bovine\\_bruce.pdf](http://www.oie.int/eng/normes/mmanual/2008/pdf/2.04.03_bovine_bruce.pdf)).
- Olsen SC (2000). Immune responses and efficacy after administration of a commercial *B. abortus* strain vaccine to cattle. Vet. Therap., 1: 183–191.
- Olsen SC, Fach SJ, Palmer MV, Rsacco E, Stoffregen WC, Waters WR, Rsacco E, Stoffregen WC, Waters WR (2006). Immune Responses of Elk to Initial and Booster Vaccinations with *Brucella abortus* Strain RB51 or 19. Clin. and Vacc. Immun., 10: 1098–1103.
- Olsen SC, Stevens MG, Chevillie NF, Schurig G (1997). Experimental use of a dot-blot assay to measure serologic responses of cattle vaccinated with *Brucella abortus* strain RB51. J. Vet. Diag. Invest., 9: 363-367.
- Plackett P, Cottew GS, Best SJ (1976). An indirect haemolysis test (IHLT) for bovine brucellosis. Aust. Vet. J., 52: 136-140.
- Poester FP, Goncalves VSP, Paixão TA, Santos RL, Olsen SC, Schurig GG, Lage AP, Santos RL, Olsen SC, Schurig GG, Lage AP (2006). Efficacy of strain RB51 vaccine in heifers against experimental brucellosis. Vaccine, 19: 5327-34.
- Robles CA, Abalos P, Cabrera R, Petray S, Ibarra L (2003). Humoral and cellular immune response to *B. abortus* SRB51 and S19 in Hereford heifers in Patagonia Region, Argentina. In: Brucellosis 2003 Int. Res. Conf. and 56th Bruc. Res. Conf., Pamplona, Spain, September, 15th–17th.
- Robles CA, Nielsen K, Gall D, Willems P (2009). Evaluation of three different antigens in an iELISA for the detection of antibodies against *B. abortus* RB51 in vaccinated heifers. Vet. Immunol. and Immunopath., 127: 153-155.
- Salmakov KM, Fomin AM, Plotnikova EM, Safina GM, Galimov GM, Salmakov AV, Ivanov AV, Panin AN, Sklyarov OD, Shumilov KV, Klimanov AI (2010). Comparative study of the immunobiological properties of live brucellosis vaccines. Vaccine, (28): F35-F40.
- Shaffie MM (1985). Physiological responses and adaptation of water buffalo. In: Yousef MK (Ed.), Stress Physiology in Livestock, Ungulates, vol. 2. CRC Press, Florida, USA.
- Stevens MG, Olsen SC, Palmer MV, Chevillie NF (1997). *B. abortus* strain RB51: a new brucellosis vaccine for cattle. Comparative Control Education. Pract. Vet., 19: 766–774.
- Tittarelli M, Bonfini B, De Massis F, Giovannini A, Di Ventura M, Nannini D, Caporale V (2008). *Brucella abortus* Strain RB51 Vaccine: Immune Response after Calhford Vaccination and Field Investigation in Italian Cattle Population. Clin. Dev. Immunol. Article ID 584624, doi:10.1155/2008/584624.
- Uzal FA, Samartino L, Schurig GG (2000). Effect of vaccination with *B. abortus* strain RB51 on heifers and pregnant cattle. Vet. Res. Commun., 24: 143–51.
- Yazdi HS, Kafi M, Haghkhah M, Tamadon A, Behroozikhah AM, Ghane M (2009). Abortions in pregnant dairy cows after vaccination with *B. abortus* strain RB51. Vet. Rec., 7(165): 570-1.
- Yifan Z, Yang J, Cheers C (1993). Cytokine response of T- cell subsets from *B. abortus* infected mice to soluble Brucella proteins. Infect. Immunol., 61: 2841-2847.