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Original article

Expression of *Babesia bovis* rhoptry-associated protein 1 (RAP1) in *Brucella abortus* S19

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Abstract

Brucella abortus strain 19 (live vaccine) induces a strong humoral and cellular immune response and therefore, it is an attractive vector for the delivery of heterologous antigens. The objective of the present study was to express the rhoptry-associated protein (RAP1) of *Babesia bovis* in *B. abortus* S19, as a model for heterologous expression of immunostimulatory antigens from veterinary pathogens. A plasmid for the expression of recombinant proteins fused to the aminoterminal of the outer membrane lipoprotein OMP19 was created, pursuing the objective of increasing the immunogenicity of the recombinant antigen being expressed by its association to a lipid moiety. Recombinant strains of *B. abortus* S19 expressing RAP1 as a fusion protein either with the first aminoacids of β-galactosidase (S19pBB-RAP1) or *B. abortus* OMP19 (S19pBB19-RAP1) were generated. Plasmid stability and the immunogenicity of the heterologous proteins were analyzed. Mice immunized with S19pBB-RAP1 or S19pBB19-RAP1 developed specific humoral immune response to RAP1, IgG2a being the predominant antibody isotype. Furthermore, a specific cellular immune response to recombinant RAP1 was elicited in vitro by lymphocytes from mice immunized with both strains. Therefore, we concluded that *B. abortus* S19 expressing RAP1 is immunostimulatory and may provide the basis for combined heterologous vaccines for babesiosis and brucellosis.

Keywords: *Brucella abortus* S19; *Babesia bovis*; RAP1

1. Introduction

Babesia species are intra-erythrocytic, tick-transmitted apicomplexan parasites that infect a wide variety of vertebrate hosts and cause severe diseases in wild and domestic animals. Bovine babesiosis is commonly caused by *Babesia bovis* or *Babesia bigemina*. Three-quarters of the world's cattle population live in *Babesia* enzootic regions, subtropical and tropical areas, where the disease causes significant economic losses. Babesiosis is characterized by fever, hemolytic anemia and anorexia. Particularly, *B. bovis* causes the most severe cases of bovine babesiosis, and due to the sequestration of infected red blood cells in the microvasculature of the brain, neurological signs also result. The animals that survive the acute phase become persistently infected and are resistant to developing clinical disease upon reinfection with a homologous strain [1]. Among the few *B. bovis* antigens that have been characterized, the rhoptry-associated protein, RAP1, is considered a good candidate for vaccine development. RAP1 of *B. bovis* is a 60-kDa protein localized to the apical surface and within the rhoptries of merozoites [2]. RAP1 has

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T-helper-lymphocyte epitopes in the aminoterminal region and B-cell epitopes in both the amino and carboxyterminal regions. Immunization of cattle with recombinant RAP1 was shown to significantly reduce parasitemia upon subsequent infection [3]. Recently, this antigen was expressed in the BCG strain of mycobacteria and the recombinant strain elicited both humoral and cellular responses against RAP1 in mice, demonstrating the promising aspect of this antigen [4]. Nevertheless, although BCG serves as a good model to study the immunogenicity of the antigen, this strain cannot be used to vaccinate bovines because the immune response it elicits interferes with diagnostic testing.

Brucella abortus is a facultative intracellular pathogen which causes brucellosis, a major zoonosis, in several countries. Brucellosis causes abortion and infertility in cattle and a clinical manifestation known as undulant fever in humans [5]. The vaccines used for control for bovine brucellosis are attenuated *B. abortus* S19 (smooth strain) and RB51 (rough strain) [6]. Both strains induce a strong Th1 response and generate cell-mediated immunity (CMI), while S19 also induces a strong humoral response against surface antigens, particularly O antigen [7]. S19 vaccine has been widely used for more than 50 years and the immune response it elicits, both in animal models as well as in the natural host, has been extensively studied.

Expression of antigens from diverse pathogens has been evaluated in *B. abortus* RB51 rough strain, with the aim of increasing its immunogenicity [8,9]. Also, a repetitive antigen of *Trypanosoma cruzi* was expressed in the periplasm of *B. abortus* S19 aiming to tag the vaccine strain and therefore provide an additional means to differentiate vaccinated animals from infected animals [10]. Taken together, these features form a strong basis for using *B. abortus* as a vaccine carrier to induce a Th1 biased immune response against specific antigens. The mechanism of protective immunity to *B. bovis* is thought to involve both CD4-T-helper1 lymphocytes and antibody responses [11]. Thus, *B. abortus* S19 could be an attractive alternative as a live carrier of *Babesia* immunoprotective antigens.

We developed a vector for expressing heterologous antigens in *B. abortus* associated to OMP19, an outer membrane lipoprotein [12,13], expecting the heterologous antigen to be associated to a lipid moiety in the *Brucella* membrane. In this way we generated recombinant *B. abortus* S19 strains expressing RAP1 antigen from *B. bovis*. This study highlights the use of *B. abortus* S19 as a vehicle for the expression and presentation of *B. bovis* antigens and supports further investigation on this strategy for the development of heterologous vaccines for the control of bovine babesiosis.

2. Materials and methods

2.1. Bacterial culture conditions

B. abortus strain 19 was used from our strain collection. *Escherichia coli* DH5α and Top 10 were purchased from Invitrogen. *E. coli* was grown in either liquid or solid Luria–Bertani (LB) medium containing appropriate antibiotics at the following concentrations: ampicillin (100 μg/ml); kanamycin (50 μg/ml). *Brucella*e were grown at 37 °C in trypticase soy broth (TSB) or tryptose agar (TA) (DIFCO), supplemented with antibiotics (kanamycin, 25 μg/ml), when appropriate.

2.2. Plasmids

For the construction of pBB19, primers 5′gaattccattcttc ctgcggcttt3′ and 5′accggtgcgaaacattatcgagattacca3′ were used to amplify a region corresponding to part of *omp19*, from the genomic DNA of *B. abortus* S19 (NC007618). Amplification conditions were 95 °C, 2 min, followed by 35 cycles of 95 °C, 1 min; 58 °C, 1 min and 72 °C. The amplified gene fragment was cloned into the pGEM-T Easy vector. Subsequently, the EcoRI insert was subcloned into pBBR1MCS2 [14] to obtain pBB19. In order to construct vectors for RAP1 expression in *B. abortus*, the *rap1* gene was amplified by PCR from the genomic DNA of *B. bovis* R1A extracted with a Puregene kit (Gentra, USA) from *B. bovis* R1A (AF030062), kindly provided by Dr. Ignacio Echaide (INTA-Rafaela, Argentina) [15]. Primers 5′aagcttagaatcattagcggc3′ and 5′tctagagaggtatccggcggt3′ were used, with HindIII restriction site incorporated into the forward primer and XbaI restriction site in the reverse primer to allow the directional cloning of the full length *rap1* into PBBR1MCS2. In order to subclone *rap1* without the sequence that encodes for its signal peptide into pBB19, primers: forward 5′ctgcaggctttcgccac aatcagagagt3′ and reverse 5′ctgcagtctagagaggtatccggcggt3′ were used. PstI sites (underlined) were incorporated into both primers. Amplification was performed at 95 °C, 2 min, followed by 35 cycles of 95 °C, 1 min; 58 °C, 1 min and 72 °C, 1.5 min. The amplified gene fragments were cloned into the pGEM-T Easy vector (Promega) and then subcloned into HindIII-XbaI of pBBR1MCS2, or PstI of pBB19 to generate pBB-RAP1 or pBB19-RAP1, respectively. The recombinant plasmids were transformed into *E. coli* DH5α cells and analyzed by sequencing to check for correct framing and orientation. Subsequently, the purified plasmids were used to electroporate *B. abortus* S19.

2.3. Transformation of B. abortus S19

Recombinant plasmids were electroporated into *B. abortus* S19 according to methods previously described [16]. Transformant colonies were confirmed by colony PCR using *rap1* specific oligonucleotides. Finally, total proteins from the kanamycin-resistant *B. abortus* S19 colonies (rS19) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with monoclonal antibodies (Mab) specific for *B. bovis* RAP1 protein (Mab BABB75A4 kindly provided by Dr. Carlos Suarez, Animal Disease Research Unit, USDA Agricultural Research Service, Pullman, Washington). *B. abortus* S19 strains containing plasmids pBBR1MCS, pBB19, pBB-RAP1, pBB19-RAP1 were designated S19pBB, S19pBB19, S19pBB-RAP1, S19pBB19-RAP1, respectively.

2.4. Subcellular fractioning of S19pBB-RAP1 and S19pBB19-RAP1

Subcellular fractioning was performed as previously described [4]. The fractions were analyzed by Western blot using Mab BABB75A4.

2.5. Mice immunization, obtaining of sera and splenocytes

Female BALB/c mice of 12 weeks of age were used. They were raised in the animal house of the Instituto de Biotecnología (INTA), under appropriate conditions of temperature, water and feeding. Animals were inoculated intraperitoneally (i.p.) with 1×10^6 colony forming units (CFUs) of the indicated strains or with purified recombinant RAP1 (rRAP1) (25 μg/ml in Freund's adjuvant, Sigma). Negative control mice were treated with saline alone (PBS). Mice were euthanized by cervical dislocation, bled by cardiac puncture (when requiring sera) and their spleens removed. For determining bacterial infection, spleens were weighed, homogenized in 5 ml of PBS and used for *Brucella* colony counting by serial dilution and plating on TA with or without the corresponding antibiotics. Colonies were examined after 3 days at 37 °C.

2.6. Humoral immune response

Antibody responses were monitored by ELISA and Western blot (WB). The presence of serum immunoglobulin G (IgG), IgG1, and IgG2a isotypes with specificity for RAP1 was determined by indirect ELISA as previously described [17]. Recombinant RAP1 (rRAP1) was obtained as detailed elsewhere [4] and used in a concentration of 5 μg/ml in carbonate buffer, pH 9.6, to coat the wells of polystyrene plates (100 μl/well; Nunc-Immuno plate, MaxiSorp surface). For Western blot (WB), membranes were probed with anti-RAP1 monoclonal antibody in a 1:200 dilution or mice sera in a 1:100 dilution. Alkaline phosphatase-conjugated anti-mouse immunoglobulin G (Sigma) was used at a dilution of 1:2000. For detection, BCIP/NBT Color Development (Promega) was used.

2.7. Cellular immune response

Splenocytes were obtained by a previously described method [4]. The cells were incubated at 37 °C in 5% CO_2 in the presence of different antigens indicated in the text (1 or 5 μg of recombinant RAP1, 5 μg of *B. abortus* S19 crude extract, 0.5 μg of concanavalin A, or RPMI alone). After 72 h, the supernatant was collected and IFN-γ production was determined by ELISA using 0.1 μg of capture-purified anti-mouse IFN-γ (clone R4-6A2) (BD Pharmingen, San Diego, CA) and 0.05 μg of detection-biotinylated anti-mouse IFN-γ (BD Pharmingen) per well according to the manufacturer's instructions. The assays were performed in triplicate.

2.8. Statistical analysis

Spleen weight and counts of bacteria in the spleen were analyzed, due to the dispersion observed, by the non-parametric test of Kruskal–Wallis after which a multiple comparisons test was undertaken. The data for ELISA and IFN-γ production were subjected to analysis of variance, and the means were compared using Tukey's test.

3. Results

3.1. RAP1 localizes in the membrane of B. abortus S19

Expression vectors pBBRMCS2 [14] and pBB19 [this study] were used for expression of the *B. bovis rap1* gene in *B. abortus* S19 strain. Plasmid pBBRMCS2 (pBB) is a replicative vector that allowed cytoplasmic expression of the fusion protein to the aminoterminal portion of β-galactosidase. Based on pBB we developed plasmid pBB19 which is also a replicative vector containing both the promoter and the region that encodes for the aminoterminal portion of *B. abortus* OMP19, a membrane-associated lipoprotein. In this plasmid, a foreign gene can be cloned in frame with the signal peptide-coding nucleotide sequence of *omp19* to obtain a lipidated fusion protein that can be directed to the outer membrane. RAP1 was successfully expressed in S19 both as full length fusion to the first 36 aminoacids of β-galactosidase and as a truncated fusion, without its signal peptide, to the first 21 aminoacids of OMP19. Western blot analysis using anti-rRAP1 antibodies revealed reactive bands with apparent molecular masses of 66 kDa (β-galactosidase-RAP1) and 62.5 kDa (19-RAP1) in whole extracts of recombinant S19 strains (Fig. 1). In both cases, some degradation of RAP1 was observed. As a control, recombinant purified RAP1 (rRAP), expressed and purified from *E. coli*, was used. Although the expected molecular weight of rRAP is expected to be 66 kDa, it suffers extended degradation (Fig. 1, lane 4).

Subcellular fractioning of rS19 revealed that RAP1 was localized in association to the membrane fraction not only when expressed as a fusion protein to OMP19 (Fig. 2a) but

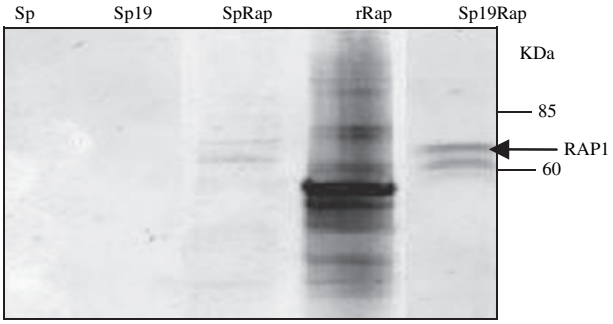

Fig. 1. Expression of RAP1 in *B. abortus* S19. Western blot of *B. abortus* S19 extracts using anti-RAP1 monoclonal antibody Mab BABB75A4 (dilution 1:200). Lanes: Sp: S19pBB, Sp19: S19pBB19, SpRAP: S19pBB-RAP1, rRAP: recombinant RAP expressed in *E. coli* and Sp19RAP: S19pBB19-RAP1. Molecular weight markers (in kDa) are shown on the right. RAP1 is indicated with an arrow. Note: rRAP (66 kDa) purified from *E. coli* presents a characteristic degradation pattern (lane 4).

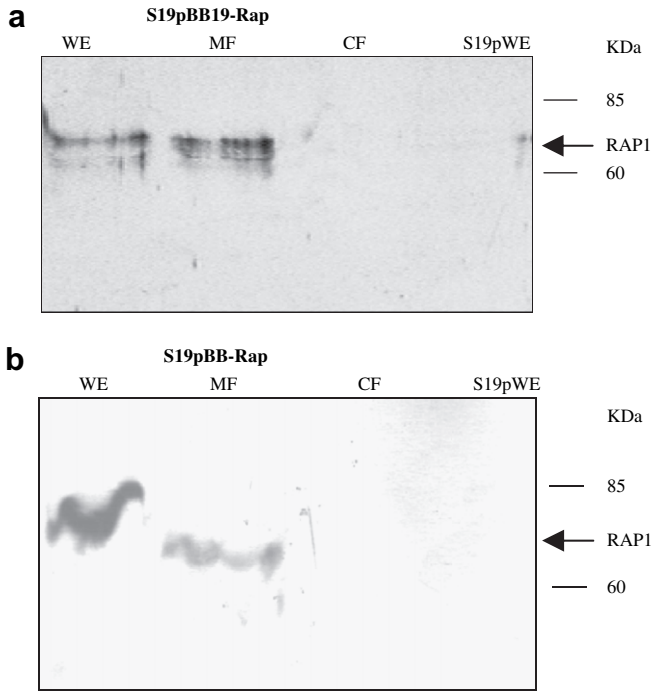

Fig. 2. Subcellular localization of rRAP1 expressed in S19. Subcellular fractions were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Western blotting was performed using Mab BAB75A4 at a dilution of 1:200. Panel (a): S19pBB19-RAP1. Panel (b): S19pBB-RAP1. References: WE: whole extract, MF: membrane fraction, ultracentrifugation pellet P150000, CF: cytoplasmic fraction, S150000 and S19pWE: S19pBB whole extract. Molecular weight markers (in kDa) are shown on the right. The expected bands are indicated with solid arrows.

also as a fusion protein to β-galactosidase (Fig. 2b), possibly due to RAP1 intrinsic properties and/or its own signal peptide. Similar results were recently reported for the expression of truncated RAP1 in *Mycobacterium bovis* [4], suggesting that RAP1 may have other signals that direct it to membrane localization. The purity of the membrane fractions was confirmed by the detection of OMP19 using anti-omp19 antisera and the absence of any bands using anti-L7/L12 antisera in additional Western blot assays (data not shown).

3.2. RAP1 does not affect infection characteristics of B. abortus *S19 in the mice model*

In order to evaluate the stability of the replicative vectors carrying *rap1* gene constructs, groups of five mice were infected with each strain and, at different time points, spleens were collected and bacteria recovered.

Vector stability was assessed by comparing the number of colony forming units (CFUs) recovered from mice spleen on solid media, supplemented or not supplemented with the selective antibiotic at 3 and 4 weeks post-infection (Fig. 3a). Although CFUs were lower in the presence of the antibiotic for the three strains, these differences were not statistically significant, indicating plasmid stability. Nevertheless it is noteworthy that we have found that pBB-RAP1 presented higher plasmid loss than pBB19-RAP, as indicated by fewer CFU counts of S19pBB-RAP1 on the solid medium supplemented with the corresponding antibiotic (TAK) than without the antibiotic (TA). Whether this could be an indication that pBB19-RAP may be a more stable candidate under different assay conditions needs to be further investigated. When these strains were grown in liquid culture, no difference was observed (data not shown).

There were also no statistically significant differences in the CFUs of S19pBB, S19pBB-RAP1 or S19pBB19-RAP1 recovered from mice spleen when the three groups were compared to each other, indicating that the three strains retain equal colonization characteristics. Accordingly, no differences in the spleen weight of infected mice were observed in any group at each time point (Fig. 3b), suggesting that the expression of RAP1 in S19 did not alter the infection characteristics of S19 in the mouse model of infection.

Expression of RAP1 following passage in vivo was confirmed by PAGE followed by Western blot of whole protein extracts of rS19 colonies recovered from the spleen of immunized mice at all time points (data not shown).

3.3. B. abortus *19 strains expressing RAP1 induce a specific cellular and humoral immune response to RAP1 in BALB/c mice*

The next question was whether the heterologous protein expressed by the transformed *B. abortus* was able to generate an immune response in the course of an experimental infection in the mouse model. Groups of five mice were intraperitoneally infected with S19pBB, S19pBB-RAP1 and S19pBB19-RAP1. As control, another group of mice was injected with saline solution (PBS). In order to study specific cellular immune response stimulation, IFN-γ production was measured. Splenocytes were collected from animals at 3 weeks post-inoculation and were stimulated for 72 h with purified RAP1 pretreated with polymyxyn B, to reduce potential LPS contamination (Fig. 4). Splenocytes from mice infected with S19pBB-RAP1 and S19pBB19-RAP1 produced significantly higher levels of IFN-γ compared to splenocytes from mice infected with S19pBB when stimulated with 5 μg of rRAP1 ($P < 0.05$ and $P < 0.01$, respectively). Although there is an apparent higher production of IFN-γ by splenocytes stimulated with rRAP1 in the S19pBB19-RAP1 group compared to the S19pBB-RAP1, this difference is not statistically significant. With lower concentrations of rRAP1 as antigen (1 μg), only splenocytes from mice infected with S19pBB19-RAP1 showed a statistically significant difference compared to the control ($P < 0.01$). Similar results were obtained in another independent experiment (data not shown). The level of IFN-γ produced by ConA or heat-killed S19 stimulated splenocytes was high in all cases. No rRAP1 specific cellular immune responses were detected in the animals inoculated with PBS (data not shown).

Humoral immune response was examined by Western blot (Fig. 5) and indirect ELISA (Fig. 6). Sera from mice inoculated either with S19pBB-RAP1 or with S19pBB19-RAP1 recognized RAP1 when either rRAP1 (Fig. 5a, b) or *B. bovis* extract (Fig. 5c) was used as an antigen (extensive degradation was observed for rRAP1). Neither S19 nor PBS immunized mice produced a detectable level of antibodies, at least by

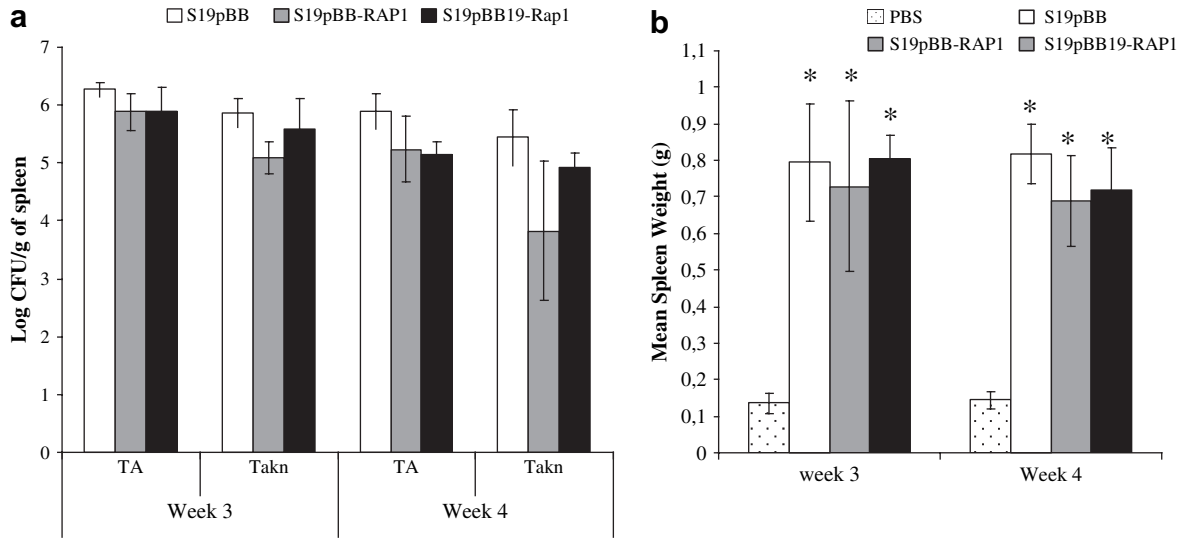

Fig. 3. Persistence of bacteria in spleen and spleen weights in inoculated mice. Numbers of bacteria in the spleen (a) and spleen weight (b) were measured at 3 and 4 weeks after vaccination. TA: tryptose agar, TaKn: tryptose agar supplemented with 25 μg/ml of kanamycin. Results are expressed as the means of five mice ± standard deviation (sd). In (b), groups with one asterisk were significantly different from the saline group ($P < 0.001$).

Western blot analysis, against either source of RAP1. The subclass of antibodies that was being generated was determined by indirect ELISA, and showed a bias towards IgG2a (Fig. 6), suggesting the development of Th1 biased immune response. Altogether, these data confirm the induction of a specific humoral and cellular immune response against RAP1, using *B. abortus* S19 as a live carrier.

4. Discussion

The most convenient and practical means to control bovine babesiosis is vaccination. The potential use of live recombinant bacteria as a platform for vaccine development is highly attractive. This strategy has been successful in inducing a robust immune response to antigens from different parasites [18,19]. In this work, we designed two different expression vectors carrying *B. bovis* antigen RAP1. We transformed strain S19 of *B. abortus* with these vectors and the RAP1 antigen was successfully expressed in both cases.

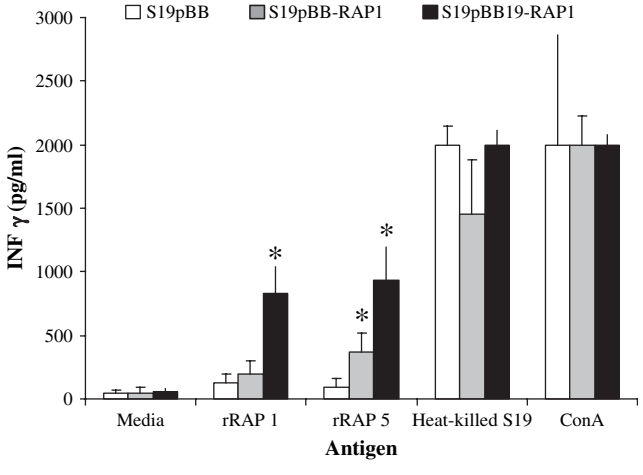

Fig. 4. Mice splenocytes IFN-γ production at 3 weeks post-infection. Bars represent groups of mice inoculated with: S19pBB (white bars), S19pBB-RAP1 (gray bars), S19pBB19-RAP1 (black bars). Antigens used: rRAP1 and rRAP5 stand for rRAP1 at a concentration of 1 and 5 μg/ml, respectively; Con A: concanavalin A; media: culture media; heat-killed S19: inactivated whole cell extract of *B. abortus* S19. Results are expressed as (pg/ml) and represent the means of five mice ± standard deviation (sd).

Regarding subcellular localization, RAP1 was localized associated to the membrane fraction, independently of the vector used. Taking into account that RAP1 is associated with the parasite membrane [20], it is possible that recombinant RAP1 molecule shows an intrinsic affinity for the cellular membrane. Having found RAP1 in the membrane fraction, even as a fusion to β-galactosidase without export signal, might constitute an evidence of protein native conformation, meaning that the intracellular expression driven by both vectors would allow proper folding of this protein. This aspect becomes more relevant since it has been recently demonstrated that immunization with recombinant RAP1 protein using adjuvants that induced strong IgG and Th1 responses in cattle was not sufficient to provide protection against challenge with a virulent strain of *B. bovis* [21]. The authors hypothesized that even though IgG responses were restimulated by challenge, structural differences in recombinant and native proteins may result in antibody responses against recombinant RAP1 incapable of neutralizing parasite infectivity. Whether RAP1 expressed in S19 presents adequate protein conformation remains to be further analyzed.

Stability of recombinant bacteria within the vaccinated host is a prerequisite for any potential live recombinant vaccine. There were no statistically significant differences between the CFUs of rS19 recovered from the spleen of mice on solid medium either with or without the selective antibiotic. However, we found that pBB-RAP, encoding RAP1 as a fusion to the aminoterminal of β-galactosidase, was lost more rapidly than pBB (base plasmid) or pBB19-RAP (plasmid encoding

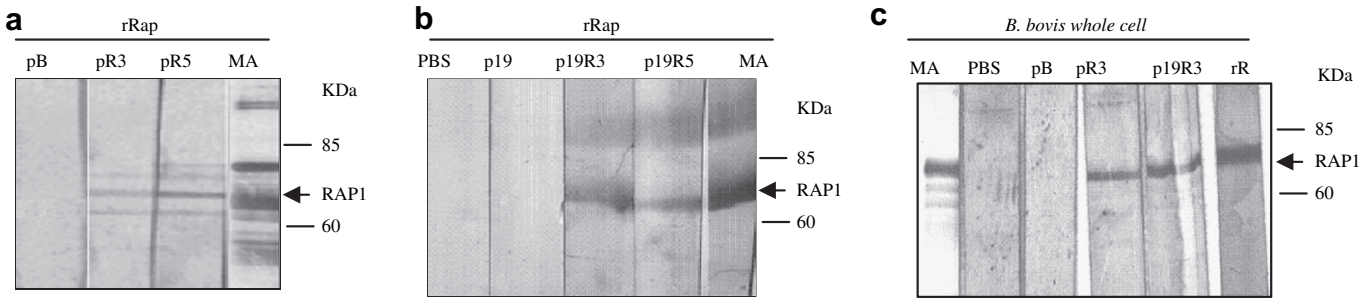

Fig. 5. Mice humoral immune response against rRAP1. Panel (a): detection of rRAP with sera from mice immunized with S19pBB-RAP1. Panel (b): detection of rRAP with sera from mice immunized with S19pBB19-RAP1. Panel (c): detection of RAP1 from *B. bovis* whole cell extract. References: pB: sera from mice immunized with S19pBB, PBS: sera from mice immunized with saline solution, pR3 and pR5: sera from mice immunized with S19pBB-RAP1, weeks 3 and 5 post-inoculation respectively, p19R3 and p19R5: sera from mice immunized with S19pBB19-RAP1, weeks 3 and 5 post-inoculation respectively, MA: anti-RAP1 monoclonal antibody MoBAB75A4 (antigen detection control), rR: sera from mice immunized with rRAP purified from *E. coli*. Molecular weight markers (in kDa) are shown on the right. The expected band is indicated with an arrow.

RAP1 as fusion to the aminoterminal of OMP19), suggesting that the fusion to OMP19 may result in a more stable plasmid. Importantly, rS19 colonies recovered from the intracellular environments were found to have maintained their ability to express RAP1 in all cases. We are now developing integrative plasmids expressing the fusion OMP19-RAP1 to avoid potential loss of recombinant vector in future in vivo assays.

The development of alternative vaccines for the control of bovine infections with *B. bovis* has been hampered by the absence of a convenient experimental animal model capable of reproducing *Babesia* infection in cattle. However, we decided to test our constructions in BALB/c mice as a first stage in rS19 based vaccine evaluation. In order to evaluate the ability of the recombinant bacteria to express the heterologous antigen and the immunogenicity of the heterologous protein expressed in *Brucellae*, immunization of BALB/c mice with S19pBB-RAP1 or S19pBB19-RAP1 was performed. Western blot analysis of sera demonstrated that both rS19 constructs were able to induce specific humoral immune responses towards rRAP1. In addition to inducing anti-RAP1 antibodies, both constructs expressing RAP1 were also able to stimulate mice splenocytes secretion of IFN-γ, when stimulated with 5 μg of recombinant protein. With a lower concentration of the antigen (1 μg), only splenocytes of mice inoculated with S19pBB19-RAP1 were able to stimulate the secretion of IFN-γ ($P < 0.01$). This may be due to the higher stability of RAP1 antigen when expressed as a fusion to OMP19, or to association to a lipid moiety, as OMP19 is a lipoprotein [12]. This issue still remains to be clarified.

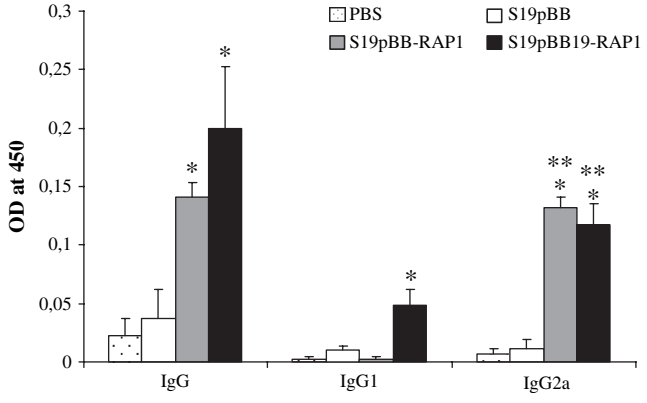

Fig. 6. ELISA detection of rRAP specific antibodies in mice serum and antibody subtype. Sera were collected from the mice of each group at 3 weeks post-inoculation (dilution 1:100). Each bar corresponds to the mean of absorbance at 450 nm of the color developed ($n = 5$ mice); error bars indicate standard deviations. Groups: PBS (dotted), *B. abortus* S19pBB (white), *B. abortus* S19pBB-RAP (gray) or *B. abortus* S19pBB-19RAP (black). In all cases, the sera were tested against rRAP1. Groups with one asterisk were significantly different from the saline and S19pBB group ($P < 0.05$). Groups with two asterisks were significantly different according to the subtype of IgG: IgG2a significantly higher than IgG1 ($P < 0.01$).

The presence of IgG2a, but not IgG1, antibodies specific for RAP1 and *B. abortus* S19 antigens in the serum of inoculated mice suggests a preferential development of a Th1 biased immune response. However, it has been documented that immunization of calves with RAP1 and adjuvants that elicit strong Th1 cell and IgG responses was insufficient to protect calves against virulent *B. bovis* challenge [21]. On the other hand, it has been suggested that innate immune response may explain the age-related cattle resistance to *B. bovis* that prevents the pathological consequences of infection [22]. When pathogen-molecular patterns bind to mammalian Toll-like receptors (TLRs), they stimulate an innate immune response so that an adaptive immune response can arise. *B. abortus* LPS and its interaction with TLR4 are a well studied example of this kind of immune response activation [23,24]. It is therefore interesting to point out that both rS19 strains were able to colonize the murine host and to express recombinant RAP1 in vivo upon immunization. Whether this active colonization would lead to a difference respect of RAP1 subunit immunization in immune response or protection in calves, needs to be further investigated.

B. abortus S19 elicits both strong cellular and humoral immune responses, the LPS in its surface being the main antigen responsible for antibody secretion [25]. This antibody response against LPS makes it not suitable for re-vaccination of adult bovines as the titers of induced antibodies interfere with the serology tests used for the detection of infected cattle. In the case of babesiosis, it may be necessary to vaccinate or

re-vaccinate adult cattle. Therefore we have also expressed RAP1 in *B. abortus* rough strain RB51, which may be used for adult cattle vaccination [26]. Preliminary experiments suggest similar results for this strain, but with a significant decrease in humoral antigenicity with respect to S19 recombinant strains. Mice inoculated with RB51 carrying pBB-RAP1 developed specific cellular immune response to RAP1, indicated by the secretion of IFN-γ by RAP1-stimulated splenocytes (unpublished data). Experiments comparing the immune response in mice towards RAP1 expressed in S19 and RB51 are currently being undertaken in our laboratory.

In summary, this report described a strategy for inducing an immune response against *B. bovis*, based on the expression of RAP1 in *B. abortus* S19. Both, humoral and cellular immune responsiveness specific to the recombinant antigen were demonstrated. These results indicate that *B. abortus* is an adequate vector to express and present antigens of *B. bovis*. More likely, to build a protective immune response against *Babesia*, multiple antigens or immunogenic epitopes of several proteins may be required [27]. Expressing such antigens in *Brucella* spp. seems therefore highly attractive and may provide the basis for future research in heterologous vaccines for bovine brucellosis and babesiosis.

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