The use of green fluorescent protein as a marker for *Brucella* vaccines

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ABSTRACT

Brucellosis is an important malady of productive and wildlife animals and a worldwide zoonosis. The use of effective vaccines and the corresponding diagnostic tests that allow differentiating infected from vaccinated animals are essential tools to control the disease. For this, a prototype of *Brucella abortus* S19 vaccine expressing green fluorescent protein (S19-GFP) was constructed. The S19-GFP was readily identified under ultraviolet light by macroscopic and microscopic examination and maintained all the biochemical characteristics of the parental S19 vaccine. S19-GFP replicated *ex vivo* and *in vivo*, and protected mice against challenge with virulent *B. abortus* to the same extent as the isogenic S19. An immunoenzymatic assay designed to measure anti-GFP antibodies allowed the discrimination between mice vaccinated with S19-GFP and those immunized with S19. Both vaccines raised antibodies against lipopolysaccharide molecule to similar levels. This experimental model constitutes a "proof of concept" for the use of *Brucella*-GFP vaccines and associated diagnostic tests to distinguish vaccinated from naturally *Brucella* infected animals.

1. Introduction

Brucellosis is a disease of terrestrial and marine mammals and an important zoonosis [1]. *Brucella abortus* and *Brucella melitensis* are the most important etiological agents of domestic ruminants. For more than 60 years, the control and eradication programs around the world have used live attenuated *B. abortus* S19 and *B. melitensis* Rev1 vaccine strains for protecting large and small domestic ruminants, respectively [1, 2, 3]. These vaccines have been used in combination with recurrent diagnosis and removal of the reactive animals [1, 2, 3, 4]. In the last decade, however, its use has been restricted based on claims that the serological and bacteriological diagnosis between infected and vaccinated animals is not straightforward [5, 6]. Indeed, both *B. abortus* S19 and *B. melitensis* Rev1 are smooth attenuated strains capable of generating antibodies against the O-polysaccharide chain of the lipopolysaccharide (LPS) molecule, which is the main bacterial antigen used in the diagnosis of brucellosis [7]. In order to bypass this difficulty, conjunctival vaccination route [2, 4,8], alternative diagnostic tests [7, 9] and mutant vaccines have been used [10, 11]. Conjunctival vaccination with *B. abortus* S19 in bovine or *B. melitensis* Rev1 in caprine and ovine, is an efficient route of immunization inducing lower and less persistent antibodies against LPS. Although these approaches minimize the diagnostic problems of differentiating infected from vaccinated cattle, they do not solve the serological interferences [12, 13].

An alternative strategy to avoid the serological interference has been the development of attenuated *B. abortus* and *B. melitensis* rough vaccines [11, 14, 15]. However, all the O-polysaccharide defective mutants that have been generated are less efficient in protecting animals against virulent infection than the smooth S19 or Rev1 vaccines [10, 16, 17]. After several field trials, the use of rough *B. abortus* RB51 vaccine against bovine brucellosis

remains controversial [10, 17, 18]. Moreover, in countries where the disease is endemic and the use of rough RB51 vaccine is compelled, brucellosis remains as an important prevalent disease [10, 18, 19, 20].

An interesting option has been the development of *B. abortus* S19 and *B. melitensis* Rev1 deficient in the antigenic periplasmic protein 26kD (bp26), and an associated ELISA for the identification of negative vaccinated reactors against this protein [21, 22, 23, 24, 25, 26, 27]. However, antibodies against bp26 are only present in a fraction of the infected animals, precluding the straightforward differentiation between vaccinated and field infected cattle [25, 28, 29].

Here, we have explored the use of green fluorescent protein (GFP) as a xenogenic positive marker for the construction of a new prototype of *B. abortus* S19 vaccine (S19-GFP) and the development of complementary diagnostic assays. We have demonstrated that the S19-GFP display very similar biological properties as the parental vaccine S19 and allowed the discrimination between mice immunized with S19-GFP and infected with non-fluorescent brucellae.

2. Materials and methods

2.1. Bacterial strains, inocula and growth conditions

The reference *B. abortus* S19 and 2308 strains were originally obtained from the culture collection of the Centro de Investigación y Tecnología Agroalimentaria of Aragón, Spain. *B. abortus* 2308 expressing red fluorescent protein (2308-RFP) from *Discosoma coral* was kindly provided by Dr. Jean-Jacques Letesson (Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium). Handling of strains, growth

conditions, and typing of vaccine *B. abortus* S19 and virulent *B. abortus* 2308 were performed as described elsewhere [1, 3, 30]. Bacterial stability, inoculi, cellular and mice assays were performed as previously described in detail [28].

2.2. Construction of fluorescent B. abortus strains

B. abortus S19 and 2308 strains expressing GFP were built as previously reported [31], with some modifications. Briefly, plasmid pBBR-2-*gfp* derived from pBBR1MCS-2 containing a kanamycin resistance (Km^R) cassette and under the control of *lac* promoter [32], provided Diego Comerci (Instituto de Investigaciones Biotecnológicas, UNSAM, Argentina), ~~–~~was introduced in competent *B. abortus* cells by electroporation in a BTX630 (Genetronics, Inc) apparatus. Successfully transfected, brucellae were selected by Km^R in plates of agar supplemented with 50 mg/L of kanamycin. For testing *in vitro* stability of the plasmid insertion, three consecutive subcultures were performed and bacterial counts were determined in agar and agar supplemented with kanamycin. The fluorescent S19-GFP stocks were kept at -80 °C in 50% glycerol, and ~~after~~ the stability of phenotypic~~,~~ and molecular characteristics ~~and stability,~~ were confirmed in defreeze bacteria. ~~*B. abortus* 2308 expressing red fluorescent protein (2308-RFP) from *Discosoma coral* was provided by Dr. Jean-Jacques Letesson (Unité de Recherche en Biologie Moléculaire, Facultés Universitaires Notre-Dame de la Paix, Namur, Belgium).~~

2.3. Cell infections

For intracellular multiplication assays, HeLa cells (ATCC CCL-2) and murine RAW 264.7 macrophages (ATCC TIB-71) were infected with *B. abortus* strains at multiplicity of infection of 500 and 50 bacterial colony forming units (CFU), respectively, following previous

protocols [31, 33]. Adhesion and internalization of *B. abortus* strains in HeLa cells was determining by differential extracellular/intracellular immunofluorescence as described elsewhere [33, 34]. Bacterial colonies or dispersed *Brucella* cells were checked for fluorescence under the Chemi Doc XRS apparatus with adequate filter recommended for GFP (Bio Rad) or UV microscopy (Olympus BH-2), respectively.

2.4. Mice assays

Swiss CD1 female 4-6 week-old mice were from the Animal Facility Unit of the University of Costa Rica. Mice were handled, bled and sacrificed according to international recommendations (http://www.felasa.eu/recommendations.htm) and local guidelines of the "Comité Institucional para el Cuidado y Uso de los Animales of the Universidad de Costa Rica", in agreement with the corresponding law "Ley de Bienestar de los Animales N° 7451" of Costa Rica.

Residual virulence and protection assays in the mouse models were carried out following standard protocols [3, 30, 35] with slight modifications. Briefly, for virulence studies, groups of 25 mice were intraperitoneally inoculated with $1x10^5$ CFU/mouse of *B. abortus* strains, and spleen counts determined at different days after infection. For protection studies, three groups of six mice each were injected with 0.1 mL of PBS for controls, or immunized subcutaneously with $1x10^5$ CFU/mouse of S19 or S19-GFP, respectively. Then, all mice were challenged sixty days later with $5x10^4$ CFU/mouse of the virulent *B. abortus* 2308 by the intraperitoneal route. Two weeks after challenge, the number of *B. abortus* 2308 in the spleens of vaccinated mice was determined. In both assays, the data was transformed to logarithms and the mean and standard deviation of CFU/gram of spleen was estimated, followed by statistical analysis.

Comentario [MJG1]: OJO: si no se ponen los pesos de los bazos sería más correcto poner CFU/spleen. Si prefieres dejar las CFU/gramo, corregir la leyenda de las Figs. 2ª y 2B... pensé que esto estaba corregido antes de enviarlo.

2.5. Immunochemical assays

Recombinant GFP was obtained by affinity chromatography as a glutathione-S-transferase (GST-GFP) fusion protein from soluble fraction of *E. coli* XL1-Blue harboring plasmid pGEX-GFP expression system, and the purity of the fusion protein was determined by SDS-PAGE [36].

Western blotting for estimating the amount of GFP and Omp19 produced by *B. abortus*-GFP and 2308-RFP constructs was performed as described elsewhere [36]. For this, m~~M~~onospecific antibodies against GST-GFP were produced by repeated immunizations of mice or sheep as described elsewhere [36]~~.~~ And r~~R~~eactivity of the obtained antibodies against GFP was tested by agar immunodiffusion test [37]. ~~Western blotting for estimated the amounts of GFP produced by *B. abortus* GFP constructs was performed as described elsewhere [36].~~ Monospecific rabbit antibodies ~~Antibody~~ against *B. abortus* Omp19 was kindly provided by Dr. Axel Cloeckaert (INRA, UR1282, Infectiologie Animale et Santé Publique, IASP, Nouzilly F-37380, France).

Indirect enzyme linked immunosorbant assays (ELISA) for the detection of mouse anti-GFP antibodies (ELISA-GFP) was performed on 96 well plates coated with 100 µL/well of a 10 µg/mL GFP-GST solution prepared in 0.1 M PBS containing 0.01% Tween 20, following standard protocols [38]. Indirect ELISA for the detection of murine anti-*Brucella* LPS antibodies (ELISA-LPS) was performed as described before [39]. In both ELISAs rabbit anti-mouse IgG (H+L) horse radish-peroxidase conjugates (Sigma) were used as detecting reagent, ABTS as substrate, and readings were performed at 405 nm. ~~-~~The immune response against LPS and GFP was evaluated in sera of S19-GFP (n=25), 2308-GFP (n=25) or S19 (n=25) ~~vaccinated~~ inoculated mice, using as negative reference sera of PBS injected control animals (n=5) and bled at different times after infection.

2.6. Statistical analysis

In all cases, comparisons of means were performed by one way ANOVA's test, followed by the Fisher's Protected Least Significant Differences (PLSD) test [30, 35].

3. Results

3.1. B. abortus S19-GFP keeps the biological properties of S19 vaccine strain

B. abortus S19-GFP maintained the growth properties, phenotypic and bacteriological characteristics of the isogenic parental S19 strain, such as smoothness, erythritol and penicillin sensitivity and the distinctive deletion in the *ery* operon detected by the AMOS-Ery PCR test [1, 30]. *B. abortus*- 2308-GFP kept its virulent properties as reported elsewhere [31]. Bacterial colonies displayed fluorescence in agar plates grown in the presence or absence of kanamycin and were readily distinguishable from control non-fluorescent *Brucella*, mainly when grown for four or more days (Fig. 1A). Regardless of the presence or absence of kanamycin in TSA plates, all the S19-GFP CFU from mouse spleens counted displayed fluorescence (Table 1). When examined under the microscope, close to 100 % of the counted bacteria isolated from mice were fluorescent (Table 1). Those bacteria that did not display fluorescence were presumably dead, because all individual colonies plated only generated fluorescent CFU. These properties, which remained constant over time, were in agreement with previous observations, demonstrating that plasmids are very stable in *Brucella* cells [40], probably due to the absence of mechanisms to eliminate them since *B. abortus* does not naturally harbor plasmids [1].

Comparison of S19-GFP with the respective isogenic S19 demonstrated no significant differences in terms of binding to and internalization into HeLa cells, thus maintaining the reported interaction of S19 with host cells (Fig. 1B). Similarly, S19-GFP replicated to the same extent as its parental strain in HeLa cells and in macrophages (Fig. 1C). All CFU recovered from S19-GFP infected macrophages or HeLa cells were fluorescent, demonstrating the stability of the construct.

B. abortus S19 follows distinctive replication kinetics in mice, and induces significant levels of protection after challenge with virulent strains [41]. The replication profile of S19-GFP shows a characteristic peak at 14 days of infection paralleling the replication profile of the isogenic S19 reference strain (Fig. 2A). In addition, S19-GFP vaccinated mice showed a similar level of protection against challenge with virulent *B. abortus* 2308 than S19 (Fig. 2-B). In cases in which few colonies of S19-GFP were present in challenged animals, they were readily resolved from the *B. abortus* 2308 by fluorescence, without the need of a selective bacteriological agar media.

3.2. B. abortus S19-GFP induces antibodies against LPS and GFP

The rational for using a S19-GFP vaccine relies partly on its potential for inducing anti-GFP antibodies in vaccinated animals. This would allow the development of serological tests that could differentiate vaccinated from naturally *Brucella* infected animals. To test this, an ELISA-GFP for detecting antibodies against GFP in S19-GFP vaccinated animals was developed and tested. Mouse positive control serum against purified GFP demonstrated a single immunoprecipitation band (Fig. 3A) and no reaction against *B. abortus* antigens, including LPS (not shown). This positive control immune serum displayed a proportional ELISA-GFP reaction after dilution, indicating a good correlation between the binding of antibodies to the GFP antigen and the enzymatic reaction (Fig. 3A). All the mice vaccinated with S19-GFP or infected with 2308-GFP produced significant levels ($p<0.001$) of antibodies against GFP, already detectable at three weeks after inoculation and persistent up to the end of the experiment at 12 weeks after infection (Fig. 3B). All mice injected with S19-GFP showed significantly higher antibody titers ($p<-0.001$) against GFP during the 12 weeks of the assay than mice infected with *B. abortus* 2308-GFP (Fig. 3B). The differences in antibody

production between mice vaccinated with S19-GFP and those infected with 2308-GFP~~,~~ were not due to different expression of GFP between both strains, as demonstrated by immunodetection of this protein in bacterial lysates (Fig. ~~3D~~3C). Moreover, no ~~cross reaction~~cross-reaction against the coral RFP present in 2308-RFP lysates was observed with either ~~goat~~ sheep anti-GFP (Fig. ~~3D~~3C) or mice anti-GFP (not shown), demonstrating the specificity of the reaction. ~~–~~Similarly, none of the mice vaccinated or infected with non-fluorescent isogenic parental *B. abortus* S19 or 2308 strains developed cross-reacting antibodies against GFP. Although S19-GFP vaccinated mice showed variable levels of antibodies against LPS during the first weeks of infection as compared to animals vaccinated with the parental S19 strain, eventually antibodies leveled up at later times (Fig. 3D).

4. Discussion

Several attempts to construct *Brucella* vaccines exhibiting "negative" molecular markers, such as the absence of periplasmic bp26 or O-polysaccharide chain of the LPS, have been reported [10, 11, 42, 43]. Although valuable, these approaches have disadvantages. For instance, the value of vaccine candidates devoid of Omps [44] is hampered by the fact that an important proportion of naturally infected individuals do not produce antibodies against this negative cell envelope marker [25, 28, 29]. Similarly, animals vaccinated with rough ~~–~~*B. abortus* RB51 spontaneous mutant or rough *B. melitensis* punctual mutants, in addition to produce antibodies against many *Brucella* protein antigens, also generate antibodies against LPS core epitopes and in cases, to residual quantities of O chain determinants present in some of these rough bacterium, including RB51 [11, 17, 45]. These phenomena may be exacerbated after revaccination; a common practice in many low-income countries, mainly, when

concomitant infections with field *Brucella* strains are present [17, 18, 46]. In addition, it has been argued that the level of protection of rough mutants is considerable lower than that conferred by smooth attenuated vaccines [10, 11, 18, 20]. *Brucella* vaccines injected by the subcutaneous route have been shown to produce abortions and they can be isolated from tissues or aborted fetuses [13, 47, 48], hampering the expedite distinction between field *Brucella* and vaccine strains. These events complicate the direct and differential bacteriological and serological diagnosis of vaccinated and naturally infected cattle and the further use of vaccines.

Accordingly, all the mice injected with *Brucella* strains expressing GFP throughout the course of this investigation, generated statistically significant levels of specific antibodies against GFP, which were easily detected by the indirect ELISA-GFP developed here. Taking into account that GFP displays a particular structure not related to mammalian proteins or mammal commensal microorganisms [49], it is unlikely that cross-reactions arise, maintaining low background levels. Furthermore, antibodies against GFP raised in sheep and mice do not cross react with related fluorescent proteins such as the coral RFP, which shares critical amino acid motifs and stable three-dimensional beta-can barrel structure with GFP. Although we have observed that the GFP is highly immunogenic in mice and in a restricted number of ovine tested, others have shown that the form in which this fluorescent protein is presented to the immunized animals is relevant for antibody production [50,51]. For instance, while rinderpest virus vaccine expressing membrane-anchored GFP induces good level of antibodies against GFP in cattle, that vaccine designed to produce GFP inside infected cells does not [50, 51]. In this regard it is worth noting that vaccinated mice with S19-GFP consistently generated higher levels of antibodies than the 2308-GFP infected animals, despite of the fact that both strains expressed similar quantities of GFP (Fig. 3). Interestingly, *B. abortus* S19 vaccinated cattle

consistently produce lower levels of antibodies against the LPS antigen than infected animals [2, 7, 9], an event that seems to be reversed in the case of anti-GFP antibodies, at least in the murine model used here. Therefore, the manner in which brucellosis infection proceeds seems to be a relevant factor for the production of antibodies against GFP and LPS.

The S19-GFP vaccine in addition to induce antibodies against the GFP marker antigen, it possesses other advantages that eventually could be extrapolated to alternative GFP anti-*Brucella* vaccines, such as Rev1. First, the S19-GFP is easily distinguished from other *Brucella* strains by its intrinsic fluorescence, either macroscopically or microscopically, in pure cultures or animal tissues and the presence of the *gfp* gene in vaccine strains could be detected by a specific PCR. Second, since S19 and Rev1 have been tested extensively over sixty years, and have been shown to be successful vaccines for the control and eradication of ruminant brucellosis [2, 4], the need of large and costly trials is precluded. Third, the risk and cost of production should not differ from that of S19 or Rev1 reference vaccines. Fourth, the genetics, biochemical and biological properties of these two *Brucella* vaccine strains have been extensively studied [1, 11, 52]. Fifth, conventional tests developed to distinguish infected from S19 or Rev1 vaccinated animals will remain functional. This is important because some of these tests are able to distinguish abortions and bacterial shedding due to exacerbated infections with the vaccine strain [47]. And last but not least, it is likely that these vaccines are eagerly accepted by farmers and agriculture authorities, due to the already recognized immunogenic and protective properties of its parent S19 or Rev1 reference strains.

The S19-GFP vaccine studied here is a prototype, containing a non-integrative plasmid that expresses GFP constitutively and owns an antibiotic resistant cassette. In addition it was tested in mice, widely used in experimental brucellosis, but which do not correspond to the natural hosts. In conclusion, our approach constitutes a “proof of concept” demonstrating that

brucellae expressing GFP can successfully deliver this protein as an immunogen after infection. The stability, biological behavior and the immunogenic properties of the S19-GFP, makes realistic to design efficient *Brucella* fluorescent vaccines with a single *gfp* gene encoded in the chromosome, which then could be used in domestic ruminants and may be in wild life hosts. Moreover, the S19-GFP tested here provides a standard for comparing the performance of chromosomal GFP-expressing *Brucella* vaccine candidates in the mouse model, a fact that gives value to this vaccine prototype. ~~–~~The prediction that the high immunogenic properties of the GFP protein would remain in ~~domestic cattle~~ natural hosts of *Brucella* spp, and that a combination of simple serological tests shall give the appropriate specificity and sensibility to unambiguously differentiate *Brucella* infected from *Brucella*-GFP vaccinated animals, is currently being tested in ruminants.

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Table 1. Proportion of fluorescent *B. abortus* S19 colonies ~~and bacteria~~ isolated from spleens of vaccinated mice.

		Number of S19-GFP CFU counted in agar plates	
Mouse	CFU/spleen[a]	alone / supplemented with 50 mg/L kanamycin	under UV light
A	5.5×10^6	100/100	96 ± 3
B	5.7×10^6	100/100	98 ± 2
C	5.5×10^6	100/100	99 ± 2
D	5.3×10^6	100/100	97 ± 4

[a] Total number of CFU isolated in spleens of mice, at 14 days after intraperitoneal infection with 1×10^5 CFU/mouse.

[b] Fluorescent bacteria from ~~five~~ 100 colonies were counted under the ultraviolet light in a Chemi-Doc apparatus (BioRad), while non-fluorescent bacteria were counted in the same field by phase contrast microscopy.

Comentario [MJG3]: or by UV microscopy?

Figure legends

Fig 1. Biological characteristics of the *B. abortus* S19-GFP strain. Fluorescent S19-GFP and non-fluorescent S19 colonies stripes illuminated with UV (A). Number of intracellular and extracellular *B. abortus* S19-GFP bacteria and their corresponding parental strain in HeLa cells, at one hour after infection (B). Replication of *B. abortus* S19-GFP and their corresponding parental strain (control) in HeLa cells and Raw 264.7 murine macrophages (Mø) after 48 h of infection (C). Experiments were repeated at least three times.

Fig. 2. *B. abortus* S19-GFP and S19 replication and protection assays in mice. Twenty-five mice were ~~infected~~ inoculated intraperitoneally with 10^5 CFU of *B. abortus* S19-GFP or the parental S19 reference strain, and groups of five mice killed at the indicated times for determining the mean number of CFU in the spleens (A). Groups of six mice were subcutaneously vaccinated with 10^5 CFU of S19-GFP or S19. An additional group of six unvaccinated mice (inoculated with 0.1 mL of PBS) was used as control. After sixty days, mice were intraperitoneally challenged with 5 x 10^4 CFU of the virulent *B. abortus* 2308. After two weeks, all mice were killed and mean (n=6) CFU of virulent 2308 counted in the spleens, after logarithmic transformation (B). Experiments were repeated twice.

Comentario [MJG4]: Ojo! entre esto y los ejes de abscisas de las figures

Fig. 3. Antibody immune response against GFP and *Brucella* LPS in S19-GFP immunized mice. Monospecific mouse anti-GFP was diluted and tested by ELISA using rabbit anti-mouse IgG (H+L) horse radish peroxidase conjugate (A). The insert in "A" shows the immunodiffusion reaction of $\log_2$ serial dilutions of monospecific serum against purified 10 µg/30 µ~~l~~ L of GFP. Each point in "A" represents the average of three replicas. Antibody

response of 1/200 diluted murine serum in PBS against GFP-GST tested by ELISA in mice inoculated intraperitoneally with 10^5 CFU of *B. abortus* S19-GFP or 2308-GFP (B). Western blot of *B. abortus* S19-GFP, 2308-GFP and 2308- ~~red fluorescent protein (~~RFP~~)~~ against sheep anti-GFP and rabbit anti-~~–~~*B. abortus* Omp19 antibodies (C). ~~Murine a~~Antibody response against *B. abortus* LPS detected by ELISA in mice inoculated intraperitoneally with 10^5 CFU of S19-GFP or S19 (D). Each point in "B" and "D" represents the average of five mice.