# Stability of the *B. abortus* S19 vaccine strain with a eukaryotic expression plasmid encoding the G glycoprotein from the rabies virus

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## Abstract

*Brucella abortus* S19 is an intracellular vaccine strain against bovine brucellosis. Rabies is a lethal disease in cattle. Plasmids encoding the G glycoprotein from the rabies virus induce a protective immune response in different animal species. A vector called pBBR4-CMV-Ggp-SV40+, which encodes the G gene, regulated by the cytomegalovirus eukaryotic expression promoter, and which can be used to transform the *B. abortus* S19 vaccine strain, was constructed. The stability of the transformant strain was tested both *in vitro* and *in vivo*. In the *in vitro* assays, *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ was grown for 5 sequential passages, and for the *in vivo* assays, female BALB/c mice were infected. Colony-forming unit counting and plasmid identification were performed in each passage and in the spleens at 7 days post-infection. To test the plasmid stability in the strain, all parameters were determined with and without antibiotic. The bacterial concentration was lower with antibiotic than without it, but the bacterial growth was more homogeneous. The plasmid was identified in antibiotic- and non-antibiotic-treated isolated colonies under both *in vitro* and *in vivo* conditions. The plasmid construct was also transfected into BHK-21 cells, which express the G glycoprotein. The *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ strain showed stability, representing a suitable candidate vector for developing a bivalent vaccine against brucellosis and rabies. This is the first time that a *Brucella* species has been transformed with a eukaryotic expression plasmid.

*Keywords:* G glycoprotein; Rabies virus; *Brucella abortus* S19; CMV promoter.

## Introduction

Rabies and brucellosis are considered as major zoonotic diseases. These diseases affect cattle, constitute an animal health problem, and have repercussions for human health (Monath *et al.*, 2013).

Bovine brucellosis (BB) is mainly caused by the intracellular bacterium *Brucella abortus* (Díaz, 2013). There are different vaccine strains for BB prevention. *B. abortus* RB51 is a rough strain that lacks the O-chain in its lipopolysaccharide (LPS),

and for this reason, it does not induce detectable antibodies in diagnostic tests, which helps to differentiate vaccinated from infected animals (Schurig *et al.*, 2002). However, this vaccine strain's level of protection is still controversial. *B. abortus* S19 is an attenuated smooth strain due to the presence of LPS with the O-chain, which induces antibodies that are detectable in serological assays. This feature is a major problem associated with vaccination with the S19 strain; there is also a 2-3% risk of abortion. Nevertheless, the strain forms a highly immunogenic vaccine that has proven to be the most effective vaccine against BB (Nicoletti, 1990). The vaccine's attenuation capability has been associated with a deletion in the *ery* operon that is detected by PCR (Sangari *et al.*, 1994), amplifying a 361-bp fragment that distinguishes the *B. abortus* S19 strain from other *B. abortus* vaccines and field strains, in which a 1063-bp amplicon has been identified (Mukherjee *et al.*, 2005). In Mexico, 3- to 6-month-old calves are vaccinated with the S19 vaccine at a classical dose of $10^{10}$ colony-forming units per mL (CFU/mL) of reconstituted vaccine (equivalent to 5 mL), whereas heifers older than 6 months are administered a reduced dose ($3 \times 10^8$ CFU/mL).

The etiologic agent of bovine paralytic rabies (BPR) is an RNA-enveloped virus of the *Lyssavirus* genus, which is divided into at least 11 genotypes (Rupprecht and Plotkin, 2013). Genotype 1 is distributed worldwide and includes the classical rabies virus; other genotypes are limited to specific geographic regions. On the American continent, only genotype 1 exists. The main transmitter of the virus in cattle is the hematophagous bat (*Desmodus rotundus*), which inhabits tropical and subtropical areas (Lee *et al.*, 2006; Johnson *et al.*, 2014). The rabies virus contains the G glycoprotein (Ggp), an envelope protein that has been recognized as the main viral antigen because it induces protective neutralizing antibodies (Cox *et al.*, 1977; Ross *et al.*, 2008). The Ggp is the only outer protein of the virus, and it contains 524 amino acids, which form several antigenic sites. Antigenic site I is formed by both linear and conformational epitopes, antigenic site II is discontinuous, and antigenic site III is a continuous conformational antigenic epitope. Another important site is referred to as G5: this linear epitope includes antigenic site IV, described as containing only one amino acid (Kuzmina *et al.*, 2013). DNA vaccine plasmids encoding the Ggp are useful for inducing an anti-rabies immune response (Perrin *et al.*, 2000). The pGQH plasmid contains a G gene directed by the cytomegalovirus (CMV) eukaryotic expression promoter, which produces a high antibody titer, protects against rabies in mice and dogs (Tesoro *et al.*, 2006), and is also efficient as post-exposure prophylaxis in rabbits and mice (Tesoro *et al.*, 2008).

Because the main measure of control for both diseases is vaccination, alternatives to facilitate vaccine management are currently being studied. Several issues have been considered in the development of a new option:

1. Intracellular attenuated bacteria are useful as vectors to deliver DNA plasmids directly to professional antigen-presenting cells (APC), such as macrophages and dendritic cells (Dietrich *et al.*, 2001). *B. abortus* is an intracellular bacterium, and virulent strains are able to survive inside macrophages. The *B. abortus* S19 vaccine strain is destroyed by phagocytic cells (Arenas *et al.*, 2000; Pizarro-Cerdá *et al.*, 1998), but it possesses the characteristics of a bacterial vector, and thus, *B. abortus* S19 offers the possibility of delivering a plasmid to APCs after intracellular disintegration of the strain.

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2.  DNA vaccine plasmids encode an antigen controlled by a eukaryotic promoter, such as the pGQH plasmid described before. *B. abortus* can be stably transformed with prokaryotic plasmids of the pBBR1MCS family (Elzer *et al.*, 1995), but it has never been transformed with a eukaryotic expression plasmid.

Therefore, the objectives of this study were as follows: (1) to construct a plasmid encoding the Ggp from the rabies virus regulated by the CMV promoter and to use it to transform the *B. abortus* S19 vaccine strain and (2) to evaluate the *in vitro* and *in vivo* stability of the transformant strain and to assess the protein expression resulting from the plasmid construct. If these goals were achieved, the initial stage of the development of a bivalent vaccine against rabies and brucellosis would be accomplished.

## Materials and Methods
### Plasmids
The pGQH plasmid described by Tesoro *et al.* (2008) contains a G gene (1576-bp) flanked by the *Xba*I site. The gene used in the present study was obtained from the brain of a person who died from rabies transmitted by a hematophagous bat (isolate HQ01-IMSS). Plasmid pBBR1MCS-4 is a moderate-copy plasmid maintained as an extra-chromosomal element. This plasmid is a member of the pBBR1MCS plasmid family but differs based on marker selection: pBBR1MCS is *cm*[r], and pBBR1MCS-4 is *amp*[r] (Kovach *et al.*, 1995).

### Bacterial strains and culture conditions
The *B. abortus* S19 strain and *Escherichia coli* TOP-10 (F-*mcrA*Δ (mrr-hsdRMS-mcrBC) φ80*lacZ*ΔM15 ΔlacX74 *nupG recA*1 *araD*139Δ (ara-leu) 7697 *galE*15 *galK*16 *rpsL* (Str) *endA*1 λ-) were grown in tryptic soy broth (TSB) at 37°C with orbital shaking or in tryptic soy agar (TSA). Medium containing 100 μg/mL ampicillin (Amp) was used when needed.

### Plasmid construction
Plasmid pGQH was digested with the *Bam*HI and *Bgl*II restriction enzymes, with compatible site ends. The fragment containing the G gene regulated by the CMV promoter was excised and ligated into pBBR1MCS-4, which was previously digested with *Bam*HI (Fig. 1). The insert to be cloned also included a region referred to as the SV40 enhancer/promoter. This region, which contains the origin of replication for eukaryotic cells and which induces episomal plasmid replication, was incorporated into the insert to keep the plasmid present in the host.

The resulting DNA construct was transformed in *E. coli* TOP-10. The new plasmid construct was characterized by enzyme restriction: *Bam*HI linearized it, and *Xba*I released the G gene. The recombinant plasmid was called pBBR4-CMV-Ggp-SV40+ and was purified using the EndoFree Plasmid Giga Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The G gene was sequenced from the pure plasmid using the outer primers Ggp1 and Ggp2 as well as the in-
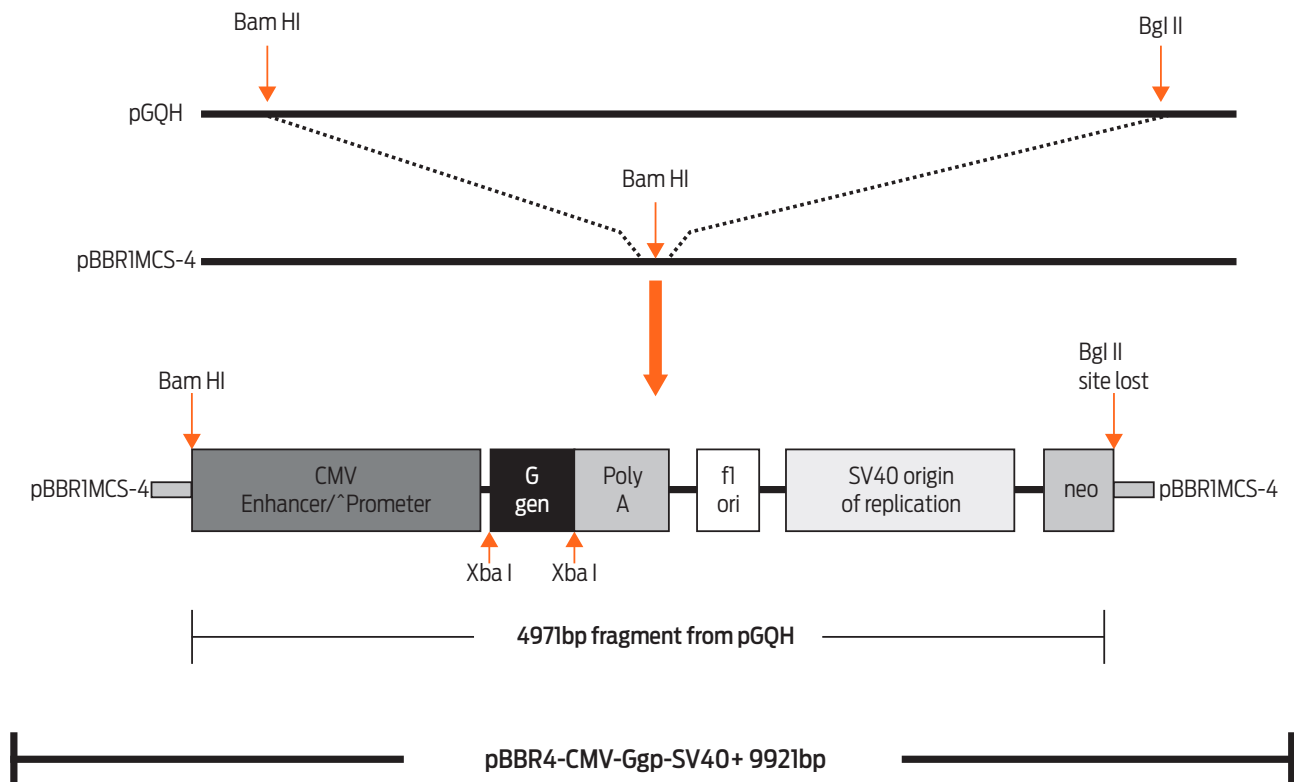
**Figure 1.** Plasmid pBBR4-CMV-Ggp-SV40+ construction diagram.

ner primers Gli1 and Gli2. For the designed inner primers, the amplified fragment, which was 736-bp in size, included most of the glycoprotein's antigenic sites.

### Transformation of *B. abortus* *S19*

Plasmid pBBR4-CMV-Ggp-SV40+ was electroporated into the *B. abortus* S19 strain by applying a 2.5-Kv pulse and using 3 μg of plasmid. Amp-resistant (Ampr) colonies were analyzed by PCR using pBBR primers (5'-GTAAAACGACGGCCAGT-3' and 5'-CGAGGTCGACGGTATCG-3') to amplify a 5100-bp fragment, corresponding to the insert ligated in pBBR1MCS-4. The amplification protocol was as follows: denaturation at 96°C for 1 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 64°C for 45 sec, extension at 72°C for 5 min, and a final incubation at 72°C for 10 min. The same colonies were analyzed using the *ery* primers described by Sangari *et al.* (1994) and Mukherjee *et al.* (2005) to identify the 361-bp amplicon of the deleted *ery* operon as an attenuation genetic marker. The following reaction controls were included: the *B. abortus* RB51 strain for the non-deleted *ery* operon and *E. coli* harboring pBBR4-CMV-Ggp-SV40+ as the negative *ery* control and the positive plasmid control, respectively. The transformant strain was called *B. abortus* S19 pBBR4-CMV-Ggp-SV40+.

### In vitro *stability of plasmid pBBR4-CMV-Ggp-SV40+*

The stability of *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ was evaluated following the method referenced by Elzer *et al.* (1995). The strain was grown in TSB-Amp

for 48 h. A total of 100 μL of the culture was transferred to 5 mL of either TSB or TSB-Amp and incubated for 48 h to obtain passage 1. The process was successively repeated until passage 5. Serial dilutions of each passage were performed to determine the CFU/mL in triplicate under 3 conditions: 1) bacteria cultivated in TSB and isolated in TSA (TSB/TSA); 2) bacteria cultivated in TSB and isolated in TSA-Amp (TSB/TSA-Amp); and 3) control culture, consisting of bacteria cultivated in TSB-Amp and isolated in TSA-Amp (TSB-Amp/TSA-Amp). Randomly sampled colonies from each condition were tested by PCR to identify the 5100-bp amplicon from pBBR4-CMV-Ggp-SV40+.

## Inoculation of BALB/c mice with the strain *B. abortus S19 pBBR4-CMV-Ggp-SV40+*

Three 6- to 8-week-old female BALB/c mice were inoculated intraperitoneally (i.p.) with 0.25 mL of PBS containing $\sim 10^6$ CFU of *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ or 0.25 mL of PBS only (control group). The animals were sacrificed by cervical dislocation 7 days later. Their spleens were weighed and homogenized in 1 mL of PBS and plated on either TSA or TSA-Amp, and the CFU/spleen value was determined by serial dilution. A multiplexed PCR was applied to the spleen colonies to simultaneously identify the deleted *ery* operon and the G gene. In the case of the rabies protein, the following inner primers were used: Gli1, forward 5'-ACA-CAATCCGTACCCTGACT-3', and Gli2, reverse 5'-CCCGTTTACATGAGGATGAC-3'. Both amplicons of the G gene (736-bp) and of the *ery* operon deletion (361-bp) were obtained as follows: denaturation at 96°C for 1 min, 30 cycles of denaturation at 95°C for 30 sec, annealing at 64°C for 45 sec, extension at 72°C for 1 min, and a final incubation at 72°C for 10 min.

All experimental procedures and animal care were performed in compliance with the *Guide for the care and use of laboratory animals* (2011), NOM-062-ZOO-1999, which enforces the proper use and care of laboratory animals in Mexico, and with the approval of Benemérita Universidad Autónoma de Puebla (license BCB/CCUAL/118/2013).

## Glycoprotein expression in eukaryotic cells

BHK-21 cells were cultured in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) during the growth phase. Cells in the logarithmic growth phase were harvested, and $1 \times 10^6$ cells were transfected by electroporation with 15 μg of the pBBR4-CMV-Ggp-SV40+ plasmid. A pulse of 140 V for 25 msec was applied to a 0.2-cm cell containing 0.1 mL of cellular suspension. Protein expression was monitored at 24, 48 and 72 h post-transfection; a control of non-transfected cells was also included. A polyclonal human IgG containing 150 IU/mL anti-rabies neutralizing antibodies was employed as a primary antibody at a 1:50 dilution. The protein was revealed with a 1:100 dilution of goat anti-human IgG-FITC.

## Statistical analysis

The CFU data were log-transformed and after examining the residuals, the variances appeared homogeneous within the range of the treatment means. The data from
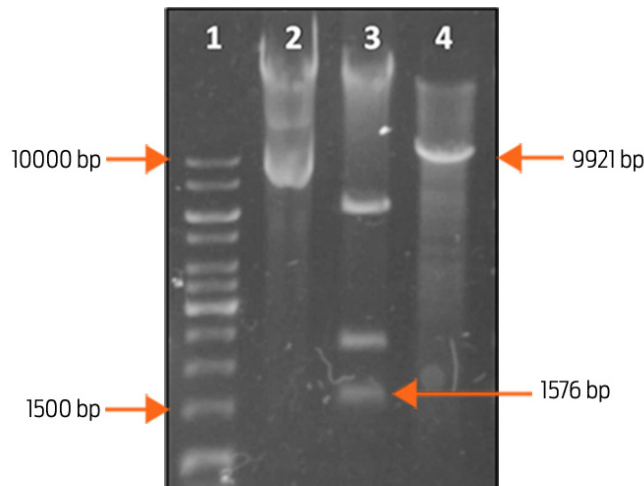
**Figure 2.** Digestion of plasmid pBBR4-CMV-Ggp-SV40+ with two restriction enzymes. Lanes: 1, 1 Kb DNA ladder; 2, purified pBBR4-CMV-Ggp-SV40+; 3 and 4, plasmid restricted with *Xba*I and *Bam*HI, respectively. The 9921-bp fragment corresponds to the expected size of the construct. The 1576-bp fragment was released from the plasmid and corresponds to the G gene.
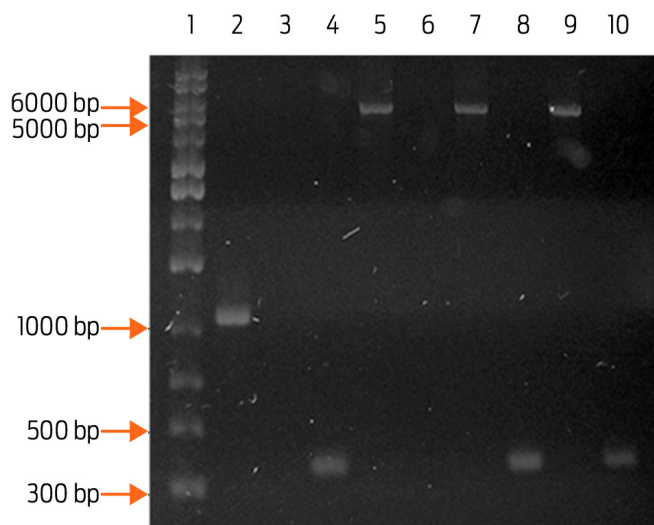


**Figure 3.** Identification of the *B. abortus* S19 pBBR4-CMV-Ggp-SV40+-transformed strain. Individual PCRs with pBBR and *ery* primers were applied to *B. abortus* S19 candidates. The 5100-bp fragment corresponds to the plasmid; the 361-bp fragment, to the deleted *ery* operon; and the 1063-bp fragment, to the non-deleted *ery* operon. Lanes: 1, 1 Kb DNA ladder; 2, *B. abortus* RB51 as a control for the non-deleted *ery* operon; 3, *E. coli* TOP-10 pBBR4-CMV-Ggp-SV40+ as a negative control for the *ery* operon; 4, *B. abortus* S19 with the deleted *ery* operon; 5, *E. coli* TOP-10 pBBR4-CMV-Ggp-SV40+ as a plasmid control; 6, *B. abortus* S19 as a negative plasmid control; 7 and 8, candidate 1 tested by PCR for the presence of the plasmid and the *ery* operon, respectively; 9 and 10, candidate 2 tested by PCR for the presence of the plasmid and the *ery* operon, respectively.

the *in vitro* assays were analyzed by one-way ANOVA with Dunnett's post-test, and the bacterial loads in the *in vivo* assays (CFU/spleen) were analyzed with Student's t-test using the software associated with GraphPad Prism 6 (GraphPad Software Inc. La Jolla, CA, USA). A value of $P < 0.05$ was considered significant for both tests.

## Results

### Construction of the recombinant plasmid and transformation of the *B. abortus* S19 strain with plasmid pBBR4-CMV-Ggp-SV40+

After extraction, plasmid pBBR4-CMV-Ggp-SV40+ was digested with *Bam*HI and *Xba*I to verify the size of the construct and to release the G gene, respectively. Digestion with *Xba*I released 3 fragments of 6057-, 2283-, and 1576-bp in size. The last one corresponded to the G gene (Fig. 2). Digestion with *Bam*HI produced a 9921-bp linearized plasmid, which indicated loss of the *Bgl*II site (Fig. 2). The complete sequence of the G gene was obtained, and preliminary results showed that Mexican isolate HQ01-IMSS maintained the amino acids considered to be invariable in all of the Ggp's antigenic sites. Figure 3 shows the deletion of the *ery* operon and the presence of the recombinant plasmid in the *B. abortus* S19 strain after electroporation.

### Plasmid pBBR4-CMV-Ggp-SV40+ was stable under *in vitro* conditions in the *B. abortus* S19 strain

The bacterial concentrations in each medium ($Log_{10}$ CFU/mL) were as follows:

- Control culture (TSB-Amp/TSA-Amp) = 8.54
- TSB/TSA-Amp culture = 8.44
- TSB/TSA culture = 9.13.

The bacterial concentration was higher ($P < 0.05$) in the TSB/TSA culture than under the other two culture conditions (Fig. 4a). Whereas no differences in the bacterial concentration were observed among passages in the TSB-Amp/TSA-Amp culture ($P > 0.05$), variability was observed in both the TSB/TSA and the TSB/TSA-Amp cultures (Fig. 4b). In particular, in the
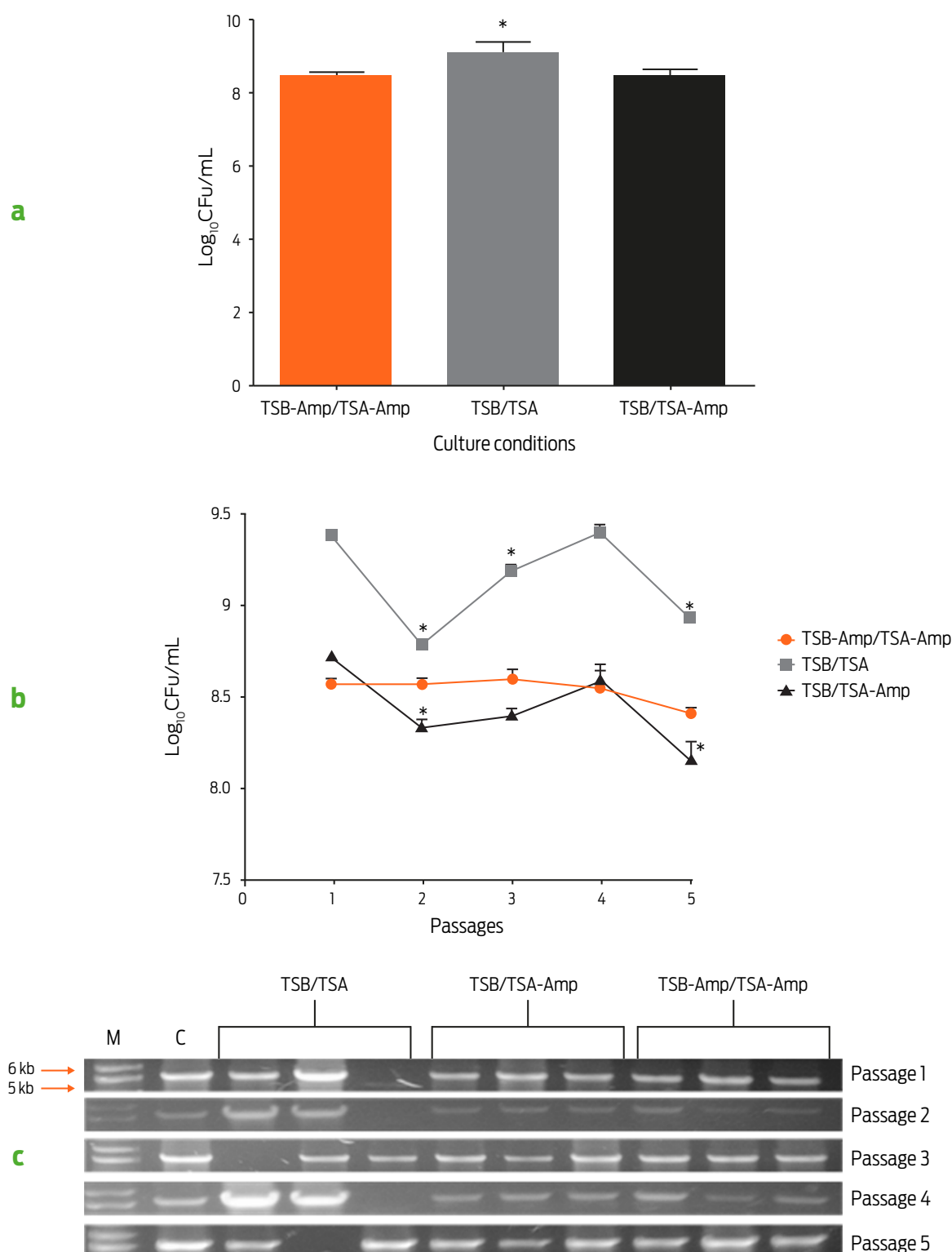
**Figure 4.** *In vitro* stability of plasmid pBBR4-CMV-Ggp-SV40+ under different conditions. (**a**) Mean concentration of *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ after 5 passages in culture with or without antibiotic; * significant difference ($P<0.05$) relative to TSB-Amp/TSA-Amp. (**b**) Bacterial concentration in each passage under different conditions; * significant difference ($P<0.05$) relative to passage 1. (**c**) Identification of the pBBR4-CMV-Ggp-SV40+ plasmid in *B. abortus* S19 colonies; M, molecular weight marker: 1 Kb DNA ladder; C, control: *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ strain; TSB, tryptic soy broth; TSA, tryptic soy agar; Amp: ampicillin (100 µg/mL).

**Table 1.** Spleen weight (wt) and bacterial concentrations of the *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ strain isolated from experimentally infected BALB/c mice.

| | Inoculation with | |
|---|---|---|
| | **PBS** | **B. abortus S19 pBBR4-CMV-Ggp-SV40+** |
| Spleen wt (g) | 0.120 ± 0.009 | 0.100 ± 0.014 |
| $Log_{10}$ CFU/spleen | | |
| TSA-Amp | | 2.8 ± 1.2 |
| TSA | | 4.0 ± 1.1 |

Mice were inoculated i.p. with 0.25 ml of PBS containing ~$10^6$ CFU of the strain or 0.25 mL of PBS only (control group). The results are the mean ± standard deviation for three mice.

TSB/TSA culture, the bacterial concentration showed a statistically significant difference ($P<0.05$) in passages 2, 3 and 5 relative to passage one, and in the TSB/TSA-Amp culture, a statistically significant difference ($P<0.05$) was found in the bacterial concentration in passages 2 and 5 relative to passage one. In addition, plasmid pBBR4-CMV-Ggp-SV40+ was identified in all TSA-Amp colonies and in different TSA colonies (Fig. 4c).

### Plasmid stability in the B. abortus *S19 pBBR4-CMV-Ggp-SV40+ strain was maintained during BALB/c mouse infection*

Table 1 shows the results from the spleen homogenates. No statistically significant difference was found between the bacterial concentrations in antibiotic- and non-antibiotic-complemented media. All analyzed colonies from both media were positive for the deleted *ery* operon. All analyzed colonies isolated in the presence of the antibiotic were positive for the G gene, and only one colony isolated in the absence of the antibiotic was positive for the G gene (Fig. 5). The plasmid was extracted from all G gene-positive colonies (data not shown).

### Plasmid pBBR4-CMV-Ggp-SV40+ induced the expression of the Ggp from the rabies virus in eukaryotic cells

After BHK-21 transfection, the Ggp was detected at 24, 48 and 72 h. The glycoprotein was not detected in the control cells. Figure 6 shows protein expression at 48 h (expression at 24 and 72 h is not shown). This result indicates that the viral protein was properly expressed in eukaryotic cells with the pBBR4-CMV-Ggp-SV40+ plasmid.

### Discussion

Vaccine plasmids consist of a gene of interest controlled by a strong eukaryotic expression promoter; the mRNA transcripts are stabilized by a polyadenylation sequence, and resistance genes for bacterial selection are included (Gurunathan *et*
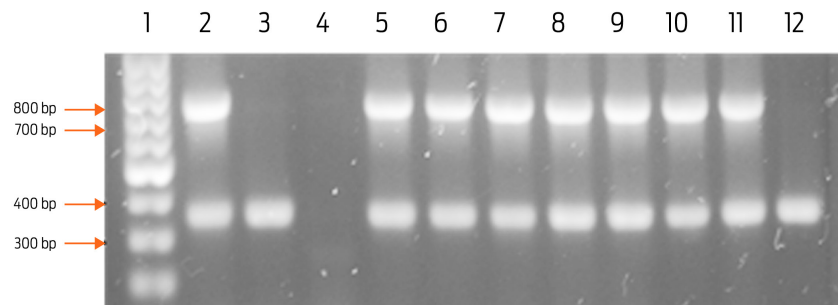
**Figure 5.** *In vivo* stability of plasmid pBBR4-CMV-Ggp-SV40+. Female BALB/c mice were infected with *B. abortus* S19 pBBR4-CMV-Ggp-SV40+. Seven days later, spleen homogenates were plated, and a multiplexed PCR was applied to isolated colonies to simultaneously detect the deleted *ery* operon (361-bp) and the G gene (736-bp). Lanes: 1, 100 bp DNA ladder; 2, *B. abortus* S19 pBBR4-CMV-Ggp-SV40+; 3, *B. abortus* S19; 4, *E. coli*; 5-10, TSA-Amp colonies; 11 and 12, TSA colonies.

*al.,* 2000). We have constructed plasmid pBBR4-CMV-Ggp-SV40+, which encodes the most immunogenic protein from the rabies virus, or the Ggp, which is ligated to the CMV promoter to strengthen gene expression in eukaryotic cells. A polyadenylation sequence and the SV40 origin of replication were also included to maintain plasmid replication in the host. The plasmid construct is based on pBBR1MCS-4, a plasmid used to transform *Brucella* species (Kovach *et al.*, 1995). The G gene sequence (isolate HQ01-IMSS) is 1575-bp in size and codes for a 524 amino-acid protein containing the sequences for antigenic sites.

Employment of intracellular attenuated bacteria improves the delivery of vaccine plasmids to a host (Dietrich *et al.*, 2001). The *B. abortus* S19 vaccine strain is both intracellular and attenuated, and it was transformed with the plasmid construct with the purpose of generating *B. abortus* S19 pBBR4-CMV-Ggp-SV40+, which maintained the marker of attenuation, or the *ery* operon deletion. The *B. abortus* S19 strain has already been transformed with plasmids encoding heterologous
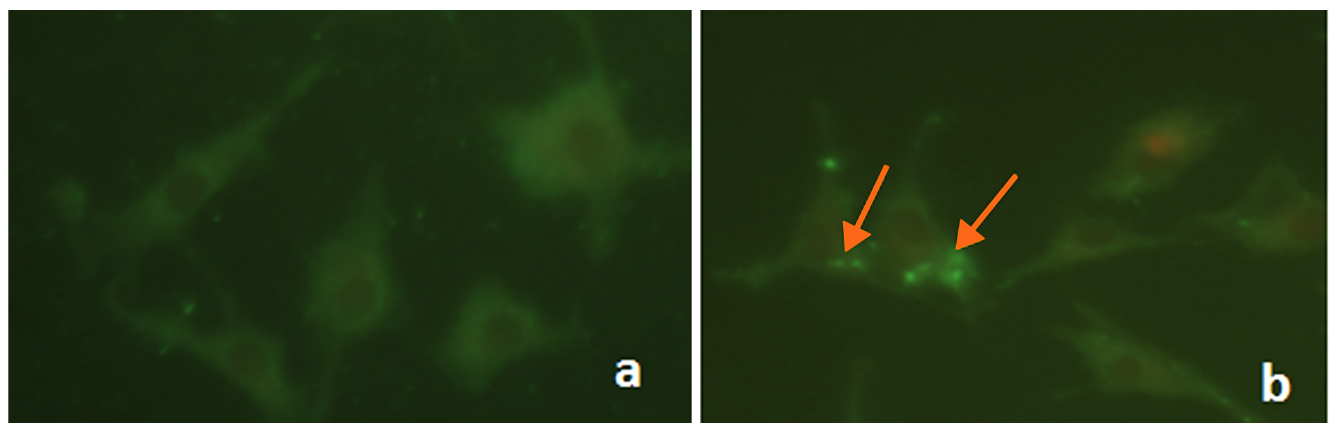


**Figure 6.** Expression of the rabies virus Ggp from plasmid pBBR4-CMV-Ggp-SV40+ in eukaryotic cells. BHK-21 cells were transfected with 15 μg of the plasmid construct. The protein was detected at 24, 48 and 72 h with polyclonal anti-rabies human IgG. **a** and **b**, non-transfected BHK-21 cells and transfected BHK-21 cells, respectively, both at 48 h (24 and 72 h not shown). The arrows indicate the Ggp.

proteins (Comerci *et al.*, 1998; Sabio y García *et al.,* 2008; Sabio y García *et al.,* 2010), but in all cases, protein expression depended on a prokaryotic promoter. This is the first report of transformation of *B. abortus* involving a eukaryotic expression promoter.

To evaluate the stability of the plasmid construct that encodes the G gene, *in vitro* and *in vivo* assays were performed. The strategy of culturing bacteria by successive passaging in media with or without a selection factor was previously established when evaluating the stability of plasmid pBBR1MCS in different *Brucella* species (Elzer *et al.,* 1995). The *in vitro* stability assays in the current study showed a significantly lower concentration of *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ in the antibiotic-complemented medium than in the medium without antibiotic, although the bacterial growth in the antibiotic-complemented cultures was homogeneous. The plasmid that we constructed is 9921-bp in size, so it might be too heavy to allow transformed bacteria to replicate. However, the variability observed in the growth of the transformant strain as well as the identification of the plasmid when these bacteria were cultured in medium without antibiotic may indicate that the plasmid confers the ability to resist adverse circumstances, although the plasmid is not essential for viability because *Brucella* does not naturally contain it (Crasta *et al.*, 2008). Therefore, without selective pressure, a spontaneous loss of plasmid could occur in part of the population.

The results of the *in vivo* assays of the concentration of transformed bacteria recovered from the spleen homogenates were similar to those reported elsewhere (Comerci *et al.,* 1998, Sabio y García *et al.*, 2008). The fact that the multiplexed PCR used was able to identify the plasmid and the *ery* operon deletion in colonies with or without antibiotic may indicate that this is a useful diagnostic tool to differentiate between the *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ strain and field strains and to help to differentiate infected animals from animals vaccinated with the *B. abortus* S19 strain (Pacheco *et al.*, 2012). These results also demonstrate the stability of plasmid pBBR4-CMV-Ggp-SV40+ in the transformed *B. abortus* S19 vaccine strain in the absence of selective pressure.

Plasmid transfection in BHK-21 cells showed that the plasmid construct expressed the Ggp from the rabies virus, evidencing the functionality of pBBR4-CMV-Ggp-SV40+ in a eukaryotic model. This result is encouraging because we can now hypothesize that *B. abortus* S19 pBBR4-CMV-Ggp-SV40+ could deliver the plasmid to macrophages after destruction of the bacteria, and simultaneous stimulation of the anti-*Brucella* response inherent to this strain and of the immune response against the viral protein could occur.

Further studies are required to produce a bivalent vaccine, and we know that current vaccine strategies for both brucellosis and rabies are working properly. However, the idea of exploiting the intracellular nature of *B. abortus* by using the S19 strain to act as a vector that delivers a plasmid encoding the main antigen of the rabies virus directly to APCs is in itself worth pursuing. Cell culture infection assays using a macrophage line to show Ggp expression from plasmid delivered by the bacterium as well as immunization studies in mouse models will be necessary to pursue this idea, and even to perform a field study in cattle although there may be regulatory hurdles. A major benefit of a bivalent vaccine would be an increase in the number of animals immunized in official vaccination campaigns because farmers would be able to have their animals vaccinated against two diseases in one

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roundup. Another benefit would be a lower production cost for the vaccine because the inert media required to produce bacterial vaccines are inexpensive compared to animal tissue cultures and infrastructure necessary to produce a viral vaccine. We have shown that *Brucella* can accept and maintain a eukaryotic expression plasmid that encodes a viral protein; this knowledge could be used to develop vaccines for other diseases, such as viral bovine diarrhea or infectious bovine rhinotracheitis, in which the main antigens are glycoproteins (Brodersen, 2014; Suman *et al.*, 2013).

## Conclusions

The *B. abortus* S19 strain was successfully transformed with plasmid pBBR4-CMV-Ggp-SV40+ to induce expression of the Ggp from the rabies virus and showed *in vivo* and *in vitro* stability. In addition, evidence indicated that the plasmid constructed induces expression of the Ggp in eukaryotic cells.

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## Author contributions

Nidia G. Pazos Salazar: conceived the study, performed all assays, and wrote the manuscript.

José A. Aguilar Setién: conceived the study.

Juan C. Benítez Serrano and José L. Calderón Chamorro: helped with the construction of the plasmid.

Rigoberto Hernández-Castro and Efrén Díaz Aparicio: helped with all assays with *B. abortus* S19.

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Original Research

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