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Development of a dual vaccine for prevention of *Brucella abortus* infection and *Escherichia coli* O157:H7 intestinal colonization

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ABSTRACT

Zoonoses that affect human and animal health have an important economic impact. In the study now presented, a bivalent vaccine has been developed that has the potential for preventing the transmission from cattle to humans of two bacterial pathogens: *Brucella abortus* and Shiga toxin-producing *Escherichia coli* (STEC). A 66 kDa chimeric antigen, composed by EspA, Intimin, Tir, and H7 flagellin ($EITH_7$) from STEC, was constructed and expressed in *B. abortus* Δ*pgm* vaccine strain (*Bab*Δ*pgm*). Mice orally immunized with *Bab*Δ*pgm*($EITH_7$) elicited an immune response with the induction of anti-$EITH_7$ antibodies (IgA) that clears an intestinal infection of *E. coli* O157:H7 three times faster ($t = 4$ days) than mice immunized with *Bab*Δ*pgm* carrier strain ($t = 12$ days). As expected, mice immunized with *Bab*Δ*pgm*($EITH_7$) strain also elicited a protective immune response against *B. abortus* infection. A *Brucella*-based vaccine platform is described capable of eliciting a combined protective immune response against two bacterial pathogens with diverse lifestyles—the intracellular pathogen *B. abortus* and the intestinal extracellular pathogen STEC.

1. Introduction

Zoonoses have an important economic impact that have been estimated as more than a hundred billion dollars over the last twenty years [1]. Brucellosis remains the most distributed zoonosis worldwide affecting humans, livestock and wildlife. To date a few vaccines have been approved for cattle (*Brucella abortus* S19 strain and RB51 strain) [2,3]. *B. abortus* S19 strain is the most used vaccine worldwide and the "gold standard" to which any other new vaccine is compared. Shiga toxin-producing *Escherichia coli* (STEC) is another important bacterial pathogen transmitted from cattle to humans. It is the causative agent of hemorrhagic enteritis and the life-threatening hemolytic uremic syndrome (HUS) [4]. STEC is able to survive in the cattle intestine, producing no evident symptoms, and then is shed to the environment within animal feces. During slaughter, meat can get contaminated with STEC which is then transmitted to humans. Since no vaccines are available for humans, and antibiotic treatment is avoided because it favors the appearance of HUS symptoms [5,6], the reduction of bacterial fecal shedding in cattle [7] becomes critical for the controlling the spread of this zoonosis. In a recent report it was estimated that vaccination against STEC in cattle may reduce animal shedding by 50% and human cases by 85% [8].

STEC virulence relies on its Shiga toxin (Stx) and the type three secretion system (TTSS) [9,10]. After secretion, Stx binds to the host cell Gb3 receptor, becomes internalized and then inhibits protein synthesis that results in cell death [11]. However, to efficiently deliver the Stx to the host, STEC needs to attach first to the intestinal epithelial cells, a TTSS-mediated step. TTSS translocates proteins of diverse enzymatic activities that leads to actin polymerization beneath the attached bacteria, a process named pedestal formation [12]. EspA is a TTSS effector protein that polymerizes, forming a filamentous tip on the membrane that is essential for bacterial attachment and TTSS activity [13]. Tir is "injected" into the host cells by the TTSS and exposed on the host membrane functioning as a receptor for Intimin, a bacterial outer membrane protein [14]. Tir–Intimin interaction triggers a host-signaling cascade leading to the rearrangement of the intestinal epithelial cellular architecture. Immunization with STEC proteins (EspA, Tir, Intimin or flagellin) induce a humoral response that interferes with the noted STEC-host cell interaction. Based on these mechanisms, two vaccines candidates have been developed for the prevention of STEC intestinal colonization in cattle [15,16].

In this report we explore the use of the *B. abortus* Δ*pgm* strain (*Bab*Δ*pgm*) as a vaccine carrier to express a chimeric antigen

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containing the STEC peptides EspA$^{36-192}$, Intimin$^{653-935}$ (Tir-binding domain), Tir$^{258-361}$ (Intimin-binding domain) [17] and the H_7 domain of flagellin$^{352-374}$ [18]. *Bab*Δ*pgm* strain is the result of an in-frame deletion of the gene encoding the phosphoglucomutase (*pgm*) [19], an enzyme that converts glucose-6-P to glucose-1-P. This strain is not able to produce UDP-glucose, which is necessary for the synthesis of bacterial polysaccharides such as the cyclic β-1,2-glucans (CβG) [20] crucial for virulence and host inflammatory responses [21–24]. *Bab*Δ*pgm* displays a rough phenotype due to a defect in LPS synthesis, failing to place the O-antigen on the membrane and producing only a small amount of a 45 kDa intracellular precursor [20]. *Bab*Δ*pgm* has a severe attenuation of virulence and vaccination with this strain induces a significant protection against a wild type *B. abortus* infection challenge in mice [19]. In addition, recent vaccine trials in cattle have shown that *Bab*Δ*pgm* is a very promising vaccine candidate for bovines [25].

Brucella O-antigen has been used as specific antigen for diagnosis of brucellosis in S19-vaccinated herds, allowing the discrimination between infected and vaccinated animals [26]. Differential diagnostic properties of the O-antigen have been explained by the presence of a non-immunodominant epitope (named as tip epitope) [27] that only induces antibody production in the context of a persistent infection (*e.g.* wild type *Brucella*) but not in a transient infection (*e.g.* S19 vaccine strain). Thus, S19 revaccination is avoided because animals will seroconvert as the result of a second dose and the benefits of having a differential diagnosis test are consequently lost. Interestingly, *Bab*Δ*pgm* has been proposed for adult cattle revaccination because it reinforces protection against *Brucella* infection without boosting anti-O-antigen titres [19].

We present here the development of a *Brucella*-based vaccine platform oriented to protect against two zoonoses, providing i) a therapeutic vaccine to reduce STEC fecal shedding ii) a protective vaccine at the mucosal level for *B. abortus* and STEC, the point of entry for both bacterial pathogens.

2. Materials and methods

2.1. Bacterial strains, plasmids, culture media and growth conditions

All bacterial strains and plasmids used are listed in Table S1. *E. coli* strains were cultured at 37 °C on LB medium [28] or Sorbitol Mac Conkey agar (SMAC) (Britania, Argentina). *B. abortus* strains were cultured on tryptic soy agar (TSA) or tryptic soy broth (TSB) (Becton Dickison, USA) at 37 °C on a rotary shaker (250 rpm). When necessary, culture media were supplemented with antibiotics at the following concentrations: ampicillin 50 μg/ml, kanamycin 50 μg/ml, nalidixic acid 5 μg/ml for *Brucella* and 20 μg/ml for *E. coli*. All experiments involving *B. abortus* were conducted in a BSL3 facility.

2.2. Designing the recombinant chimeric antigen for STEC vaccine development

A synthetic gene encoding EspA, Intimin, Tir and flagellin H7 domain ($EITH_7$) from STEC EDL933 strain was synthesized by GenScript Company (USA). The peptide $(EAAAK)_4$ was intervened between individual proteins to promote their proper folding [29]. A FLAG epitope (DYKDDDDK) was fused to the C-terminal of $EITH_7$ to monitor protein expression (Fig. 1A). The β-lactamase signal sequence (*bla* SS$^{1-35}$) was fused to the N-terminal of the $EITH_7$ for secretion to periplasma, a cellular localization that improve the efficiency of antigen presentation [30].

2.3. Expression and purification of recombinant proteins

The encoding sequence of $EITH_7$ was subcloned into pGEX expression system (GE Healthcare Life Sciences) and transformed into *E. coli* BL21. GST-$EITH_7$ was purified by glutathione–Sepharose chromatography (GE Healthcare Life Sciences) and dialysed against PBS. Individual components of $EITH_7$ chimeric protein EspA, Intimin and Tir were subcloned into pET-26b(+) (Table S1). The resulting His-tagged proteins were purified by Ni-NTA-his bind Resin columns accordingly to the manufacturer's protocol. H7 flagellin from EDL933 was purified as described previously [31].

2.4. Generation of anti-$EITH_7$ antibodies

Eight-week old BALB/c mice were immunized intraperitoneally with 10 μg of purified recombinant GST-$EITH_7$ protein using aluminium hydroxide as adjuvant and boosted at 2 and 4 weeks with 5 μg of GST-$EITH_7$. A week after the last immunization, mice were bled and sera were store at −20 °C for further use.

2.5. Expression of $EITH_7$ in *Brucella*

The pTRC-$EITH_7$ plasmid (Fig. 1, Table S1) was transformed into *E. coli* S17.1 (λpir) strain. Conjugation between *E. coli* S17.1 (λpir)($EITH_7$) and *Bab*Δ*pgm* (Nal^r) were selected onto TSA Nal 5 μg/ml and Amp 50 μg/ml. The expression of $EITH_7$ in *Bab*Δ*pgm* strain was determined by Western blot analysis using anti-FLAG (1/5000) (Sigma-Aldrich USA) or anti-$EITH_7$ (1/2000) antibodies. HRP-anti-mouse-IgG antibody was used at 1/10000 (Sigma-Aldrich) and developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, USA).

2.6. Vaccination

The vaccination protocol is shown in Fig. S1. 8-week-old BALB/c mice were randomly divided into 3 groups of 6 mice each and ear-tagged for individual identification. Each group was orally vaccinated with 5×10^9 CFU of *Bab*Δ*pgm*, *Bab*Δ*pgm*($EITH_7$) or PBS as negative control at 0, 2 and 4 weeks. Prior to vaccine administration, 0.1 ml of sodium bicarbonate (10%) was administered by oral gavage to neutralize the acidic content within mouse stomach. In addition a group of three mice (naïve group) was reserved as a control of *B. abortus* 2308 challenge infection as described in Section 2.8.

2.7. Challenge of vaccinated mice with STEC O157:H7

Protection was evaluated as follows: food and water were removed from mouse cages 6 h prior to the oral infection challenge with 0.1 ml containing 1×10^9 CFU STEC O157:H7 [32]. Bacterial fecal shedding was monitored daily over 25 days. Briefly, mouse fecal pellets were collected, suspended in 500 μl of PBS, serially diluted and plated onto SMAC agar with 20 μg/ml of nalidixic acid. Culture plates were incubated at 37 °C for 24 h and CFU were determined.

2.8. Challenge of vaccinated mice with *B. abortus* 2308

One week after all the animals experimentally infected with *E. coli* O157:H7 became negative for fecal shedding (12 weeks post-immunization), mice were orally infected with 1×10^9 CFU of *B. abortus* 2308. As a control for *Brucella* infection challenge, a group of 3 mice (naïve group) was also infected with *B. abortus* 2308. Prior to the inoculation, mice were administered 0.1 ml of 10% sodium bicarbonate. At 1 week post-infection, animals were euthanized, spleens removed and homogenized in 2 ml of PBS. Tissue

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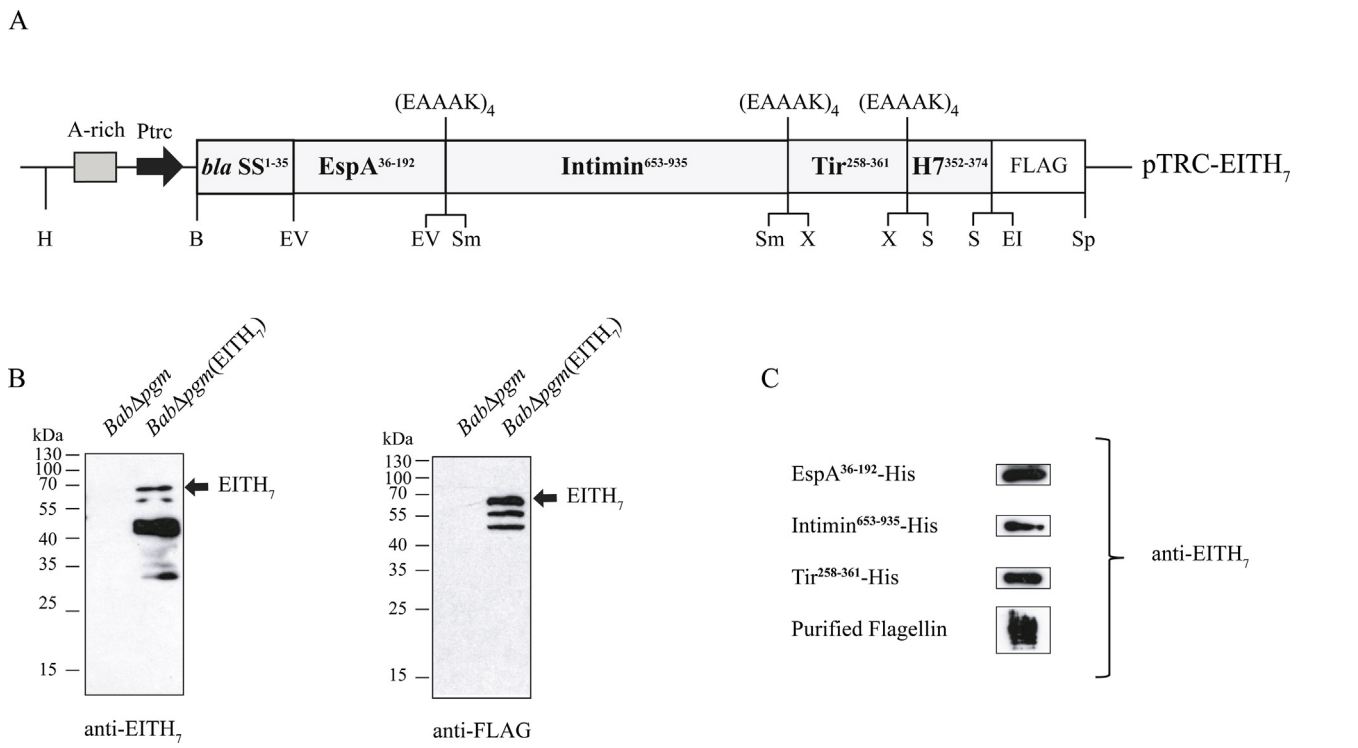

Fig. 1. (A) Schematic representation of pTRC-EITH$_7$ vector. The construct contains the synthetic EITH$_7$ gene encoding peptides derived from EspA, Intimin, Tir and H7 antigens under the control of the Ptrc promoter in the broad host range vector pBBR1MCS-4. An A-rich region and a β-lactamase signal sequence (bla SS$^{1-35}$) were placed upstream of the -35 promoter region. The peptide (EAAAK)$_4$ was placed between the individual peptide domains. The epitope reporter FLAG was fused in frame with the chimeric antigen. (B) Western blot analysis of the expression of the chimeric protein in *Bab*Δ*pgm* strain. Arrows indicate the expression of the full-length EITH$_7$ protein (66 kDa). In addition, a few reactive bands with lower molecular masses were detected using anti-EITH$_7$ (Left panel) or anti-FLAG (Right panel) antibodies likely derived from proteolysis. Restriction enzymes: H: *Hin*DIII; B: *Bam*HI; EV: *Eco*RV; Sm: *Sma*I; X: *Xho*I; S: *Sal*I; EI: *Eco*RI; Sp: *Spe*I. (C) Western blot analysis of individual component proteins of the chimeric EITH$_7$. Equal amounts of the His tagged proteins EspA-His, Intimin-His, Tir-His, and purified H7 flagellin from EDL933 were analyzed by SDS-PAGE and Western blot using anti-EITH$_7$ antibody.

homogenates were serially diluted and plated on TSA with nalidixic acid 5 μg/ml to determine the number of CFU per spleen.

2.9. Antibody determination in feces and sera

Purified GST-EITH$_7$ (250 ng/well) or heat-killed *Brucella* (HKB) (250 ng/well) were used to pre-coat a 96 wells-microplates for the indirect ELISA test. HKB was obtained by heating a bacterial suspension of *Bab*Δ*pgm* for 2 h at 60 °C. To determine fecal antibodies, feces were resuspended in PBS in the presence of 2 mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). The suspension was clarified by centrifugation, its supernatant diluted (1:2) and then used for an ELISA test during the same day. Sera were diluted 1/25 and used in the ELISA test. To reveal antibody presence, HRP-conjugated anti-mouse IgA (1/500) or HRP-anti-mouse-IgG (1/1000) were added, and incubated for 1 h at 25 °C. HRP activity was determined by the absorbance at 450 nm following the oxidation of 3,3′,5,5′ Tetramethylbenzidine (Sigma-Aldrich) with its substrate hydrogen peroxide.

2.10. Western blot

*Bab*Δ*pgm* whole-cell lysates were electrophoresed on 12% SDS-PAGE and transferred onto nitrocellulose membranes using a semidry transfer procedure. Serum reactivity against the whole lysates was determined by Western blot analysis using a pool of sera from the control, *Bab*Δ*pgm* or *Bab*Δ*pgm*(EITH$_7$) immunized mice (1/100). Detection was performed using HRP-anti-mouse-IgG (Sigma-Aldrich) and developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

2.11. Statistical analysis

One-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test (GraphPad Software) was used to analyze the significance ($P < 0.05$) difference in the duration of fecal shedding between groups and for results of protection assay against *B. abortus* infection. ELISA data significance was assessed by Mann–Whitney test.

2.12. Ethics statement

The protocol of this study (reference number 10/2011) was approved by the Committee on the Ethics of Animal Experiments of the Universidad Nacional de San Martín, under the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

3. Results

3.1. The chimeric antigen EITH$_7$ is expressed from the vector pTRC in *Bab*Δ*pgm*

An improved expression vector was constructed to maximize antigen expression in *Brucella*. For this, a chimeric antigen was designed that contained EspA$^{36-192}$, Intimin$^{653-935}$, Tir $^{258-361}$, and H7 flagellin$^{352-374}$ (Fig. 1A). As shown in Fig. 1A, the lacZ promoter present in the vector pBBR1MCS-4 was replaced by the stronger promoter Trc. An A-rich UP element described for the improved heterologous expression of proteins in *Ocrobactrum arthropii* [33] was added upstream to improve antigen expression in *Brucella* (Fig. 1A). As shown in Fig. 1B the strain *Bab*Δ*pgm*(EITH$_7$) efficiently

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expressed EITH$_7$ antigen. In addition to the full-length EITH$_7$ protein (66 kDa), a few extra reactive bands were detected most likely deriving from non-specific proteolysis. To analyze if all the components of the chimeric protein EITH$_7$ are individually immunogenic, recombinant proteins EspA-His, Intimin-His, Tir-His and the H$_7$ flagellin purified from EDL933 were analyzed by Western blot and revealed with anti-EITH$_7$ antibody. As shown in Fig. 1C all the individual components of EITH$_7$ were highly immunogenic.

3.2. Mice orally immunized with BabΔpgm(EITH$_7$) are protected against a challenge with Escherichia coli O157:H7

An oral immunization protocol was designed to determine the dual protection properties against both STEC and *B. abortus* infection. As shown in Fig. S1, previous to the challenge with *E. coli* O157:H7, mice were orally immunized three times with *BabΔpgm*(EITH$_7$), *BabΔpgm* or PBS. Eight weeks post-immunization mice were orally infected with *E. coli* O157:H7 and bacterial shedding in the feces was monitored to study protection. As shown in Fig. 2A, when mock-immunized mice (PBS control group) were infected with *E. coli* O157:H7, an intestinal infection was established with bacterial fecal shedding that persisted for almost three weeks. Similar results were observed for mice immunized with the *BabΔpgm* carrier strain that showed clearing of intestinal infection within eighteen days post-infection (Fig. 2B). Mice immunized with *BabΔpgm*(EITH$_7$) strain eliminated the infection significantly faster than either *BabΔpgm* or PBS immunized mice and as shown in Fig. 2C no bacterial fecal shedding was detected at day six post-infection. As shown in Fig. 3 the average time for STEC fecal shedding in *BabΔpgm*(EITH$_7$) immunized mice was reduced three-fold ($t = 4$ days) compared with *BabΔpgm* immunized ($t = 12$) or PBS ($t = 14$) control groups. These results indicate that immunization with *BabΔpgm*(EITH$_7$) elicited a protective immune response that drastically reduced the time of fecal shedding of STEC. Although it has been reported that *B. abortus* and *E. coli* O157:H7 share common antigenic properties (due to O-antigen similarities) [34], as shown in Figs. 2 and 3 the contribution of cross-protection between *B. abortus* and *E. coli* O157:H7 was not significant (Figs. 2 and 3; see PBS *vs* *BabΔpgm* group). Remarkably, protection against *E. coli* O157:H7 challenge infection was EITH$_7$-specific (Figs. 2 and 3; see *BabΔpgm vs BabΔpgm*(EITH$_7$)).

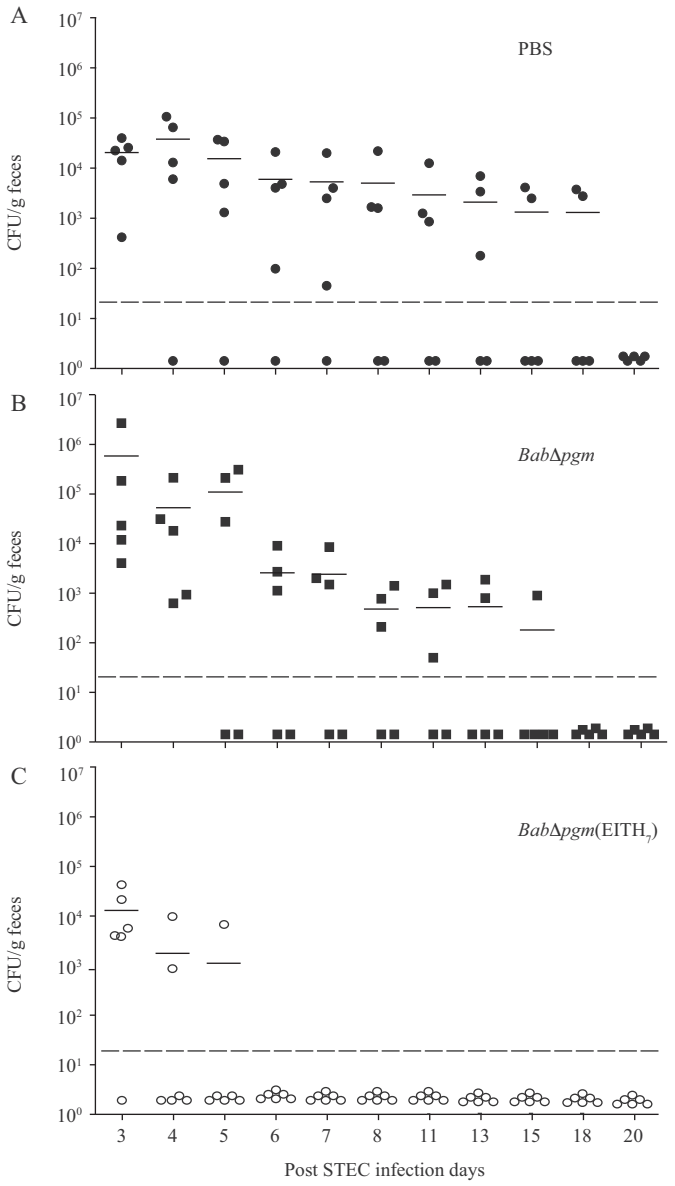

Fig. 2. Fecal shedding profiles of mice vaccinated with PBS (●) (A), *BabΔpgm* (■) (B) or *BabΔpgm*(EITH$_7$) (○) (C). Bacterial counts of STEC recovered in feces were Log$_{10}$ transformed and plotted against fecal sampling days. The mean of bacterial shedding of each group is shown as a horizontal line. Dotted lines represent shedding detectable levels (>10^2 CFU/g feces).

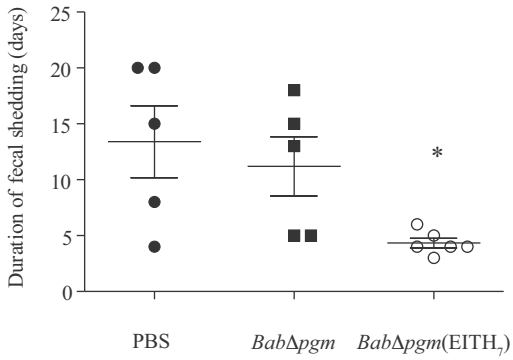

Fig. 3. Duration of fecal shedding in vaccinated mice orally challenged with STEC. The mean duration of fecal shedding in mice was calculated by averaging the total number of days in the 20-day fecal sampling period (following oral inoculation with STEC) that the mice shed detectable levels (>10^2 CFU/g feces). The mean of the duration of shedding of each group of mice is shown as a horizontal line intersected by a vertical line representing the standard error of the mean. The differences were considered significant at $P < 0.05$ (*), one-way analysis of variance (ANOVA) with Bonferroni *post-hoc* test.

3.3. Protection against STEC challenge infection correlates with the induction of anti-EITH$_7$ IgA antibodies in feces

As shown in Fig. 4A, a significant increase in anti-EITH$_7$ specific antibodies were detected in the feces of mice immunized with *BabΔpgm*(EITH$_7$) compared with mice vaccinated with the *BabΔpgm* strain. In addition, specific IgA antibodies against the vaccine carrier were also detected in the feces of mice immunized with *BabΔpgm*(EITH$_7$) compared with the PBS control group (Fig. 4B).

3.4. Oral immunization with BabΔpgm(EITH$_7$) or BabΔpgm induces a strong humoral immune response against Brucella

Although anti-EITH$_7$ antibodies were detected in the feces from mice immunized with *BabΔpgm*(EITH$_7$), no anti-EITH$_7$-IgG antibodies were detected in the sera from the same experimental group (data not shown). However, *BabΔpgm*(EITH$_7$) immunized mice (and also *BabΔpgm* immunized group) showed in their sera a strong antibody response against *Brucella* carrier (Fig. 5).

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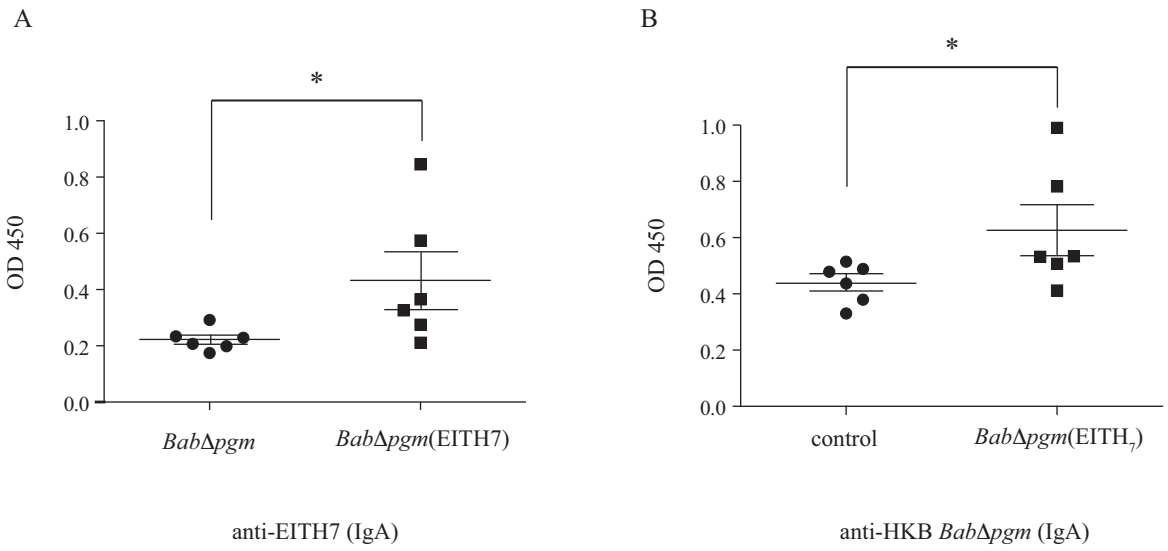

Fig. 4. (A) ELISA determination of fecal anti-$EITH_7$-IgA antibodies in mice orally immunized with *Bab Δpgm* (●) or *Bab Δpgm*($EITH_7$) (■). (B) ELISA determination of fecal anti-HKB-IgA antibodies in mice orally immunized with *Bab Δpgm*($EITH_7$) (■) compared with PBS control group (●). Fecal samples were collected at 11 weeks post-vaccination. * $P < 0.05$, Mann–Whitney test.

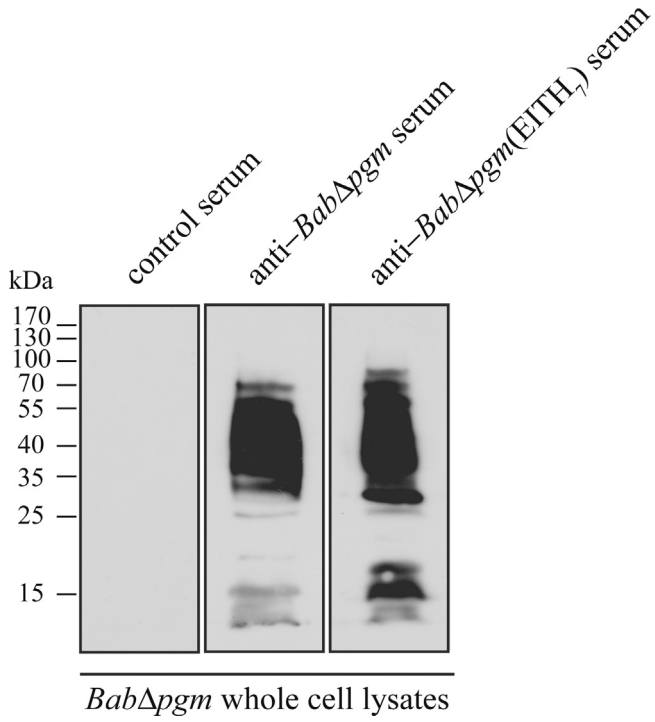

Fig. 5. Study of the induction of humoral response in sera from mice immunized with *Bab Δpgm* or *Bab Δpgm*($EITH_7$) by Western Blot analysis as described in Section 2.

3.5. Mice orally immunized with Bab Δpgm($EITH_7$) or Bab Δpgm are protected against a challenge with B. abortus 2308

As shown in Fig. S1, one week after all the animals experimentally infected with *E. coli* O157:H7 became negative for fecal shedding (12 weeks post-immunization), mice were orally infected with *B. abortus* 2308 and, a week later, the number of bacteria in the spleen was determined to estimate protection. As shown in Table 1, all mice from the naïve group (neither vaccinated with *Bab Δpgm* nor infected with *E. coli* O157:H7) and the control group (no *Bab Δpgm* vaccinated but recovered from *E. coli* O157:H7 challenge infection) were equally infected with *B. abortus* 2308.

Table 1
Protection against *B. abortus* infection in BALB/c of mice vaccinated with PBS, *Bab Δpgm* or *Bab Δpgm(EITH7)*.

Mouse	Log CFU one week postinfection with *B. abortus* 2308			
	Naïve	Control	*Bab Δpgm*	*Bab Δpgm* ($EITH_7$)
1	3.3	4.8	0	4.6
2	4.3	3.2	4.3	0
3	3.4	2.6	0	0
4	–	4.3	0	0
5	–	3.4	0	0
6	–	3.3	0	0
X **± SD**	3.67 ± 0.58	3.6 ± 0.8	0.71 ± 1.76 *	0.76 ± 1.88*
Proportion of mice infected with *B. abortus* 2308	3/3	6/6	1/6	1/6

Three groups of 6 mice were inoculated orally 3 times with 5×10^9 colony forming units (CFU) per mouse, or PBS as a control. Twelve weeks post-vaccination, each group was orally challenged with 1×10^9 colony forming units of virulent *B. abortus* 2308 per mouse. One week later, the number of CFU in the spleens was determined.
* $P < 0.05$, One way analysis of variance (ANOVA) with Bonferroni *post-hoc* test.

In contrast, mice from the *Bab Δpgm* or *Bab Δpgm*($EITH_7$) immunized groups were equally protected against the *Brucella* challenge infection. Interestingly, the previous STEC challenge does not enhance protection against *B. abortus* infection in the spite of its O-antigen similarities.

4. Discussion

In this report we present a new approach for controlling simultaneously the transmission of two important bacterial pathogens from cattle to humans. The *Bab Δpgm* strain was tested for its induction in a mouse model of an anti-*Brucella* immune response and as a recombinant carrier vaccine for STEC antigens.

Paradoxically, although most microbial pathogens enter by the mucosal route (intestinal, respiratory, genitourinary or conjunctiva), only a few vaccines have been designed to induce protection at this level. Oral administration of vaccines promotes a mucosal immune response not only inhibiting the invasion of the host but also reducing the shedding of the pathogen with zoonotic potential to the environment.

Even though diverse sources of STEC infection have been identified, cattle intestinal colonization remains the most important

reservoir of this bacterium for human infection. For this reason, reduction of bacterial shedding in cattle feces is crucial to control the impact of this zoonosis. To date, two vaccine candidates have been developed to control STEC intestinal colonization in cattle. In both cases, purified recombinant antigens, injected intramuscular, have been combined with adjuvants compounds that provided additional inflammatory signals to improve antigen presentation. Although these vaccines have shown to be effective in reducing STEC fecal shedding in cattle, the use of recombinant protein technology plus the requirement of adjuvants for their formulation have increased production costs that limit their universal utilization, especially in developing countries. To overcome these limitations, the use of a live *Brucella* vaccine strain as a heterologous carrier for antigen delivery is an attractive option because (i) it will actively deliver antigens to the immune system tissues (ii) it will naturally engage the innate immune system, mediated by LPS or proteins, without the use of adjuvants (iii) since *Brucella* vaccination is mandatory in several countries, there is the benefit of using *Brucella* vaccines with regulatory approval as vehicles to carry additional antigens to protect against other zoonotic pathogens as well. In the present report, and as a proof of concept, it was possible to induce a protective immune response against two very different pathogens: *Brucella*, that has an intracellular niche, and STEC, that is an intestinal extracellular pathogen. The presented positive evidence for "Killing two birds with one stone" suggests that this vaccine platform has a broad potential application against a variety of pathogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.03.033.

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