Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

# Delta-*pgm*, a new live-attenuated vaccine against *Brucella suis*

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ARTICLE INFO

*Article history:*
Received 11 January 2016
Received in revised form 4 February 2016
Accepted 8 February 2016
Available online xxx

*Keywords:*
*Brucella suis*
Live vaccine

ABSTRACT

Brucellosis is one of the most widespread zoonosis in the world affecting many domestic and wild animals including bovines, goats, pigs and dogs. Each species of the *Brucella* genus has a particular tropism toward different mammals being the most relevant for human health *Brucella abortus*, *Brucella melitensis* and *Brucella suis* that infect bovines, goats/camelids and swine respectively. Although for *B. abortus* and *B. melitensis* there are vaccines available, there is no efficient vaccine to protect swine from *B. suis* infection so far. We describe here the construction of a novel vaccine strain that confers excellent protection against *B. suis* in a mouse model of infection. This strain is a clean deletion of the phosphoglucomutase (*pgm*) gene that codes for a protein that catalyzes the conversion of glucose-6-P to glucose-1-P, which is used as a precursor for the biosynthesis of many polysaccharides. The Delta-*pgm* strain lacks a complete lipopolysaccharide, is unable to synthesize cyclic beta glucans and is sensitive to several detergents and Polymyxin B. We show that this strain replicates in cultured cells, is completely avirulent in the mouse model of infection but protects against a challenge of the virulent strain inducing the production of pro-inflammatory cytokines. This novel strain could be an excellent candidate for the control of swine brucellosis, a disease of emerging concern in many parts of the world.



## 1. Introduction

Brucellosis, a zoonotic disease produced by species of the genus *Brucella* spp., still inflicts important economical losses worldwide due to a decrease in the livestock reproductive efficiency and is one of the most wide spread zoonosis [1]. While the relevant clinical manifestations of the disease in animals is late abortion and, with less impact, sterility due to orchitis or epididymitis in males [2], in humans it is a chronic and debilitating disease [3]. To date, prevention of the disease is achieved through detection (diagnosis) and vaccination with attenuated live vaccines. In the case of bovine brucellosis two live attenuated strains are currently used in different countries as vaccines: S19 and RB51 [4]. Strain S19 was isolated over 60 years ago by serial passaging and selection; it has a complete lipopolysaccharide (LPS, smooth strain), which complicates discrimination between vaccinated and infected animals, but it has a high residual virulence [4]. As with S19, strain RB51 was also isolated by serial passaging and selection for mutants with membrane alterations [5]. This strain is highly attenuated in mice and bovines and is rough (no assembled LPS) which allows it to be used in adult animals, as it does not induce persistent serological titers allowing a proper diagnostic differentiation between vaccinates and infected animals. Despite these advantages over S19, this strain is less efficient in inducing protection, is rifampicin resistant (an antibiotic of choice in humans) and the genetic basis of its attenuation are still not fully known [5]. For these reasons there are still efforts to obtain better vaccines, particularly for countries were extensive farming practices are employed and for which neither S19 nor RB51 have been efficient in completely eradicating the disease.

Brucellosis in swine is caused by *Brucella suis*, a species with higher zoonotic potential than *B. abortus* and, in countries like Argentina, a neglected disease [6]. As with bovines, the disease is characterized by late abortion in pregnant animals and sterility in males and, in consequence, the disease lowers the reproductive rates [7]. Additionally, and due to its high zoonotic capacity, *B. suis* is important in human infections, particularly in slaughterhouse workers where infection is acquired due to aerosolization of the bacteria during the process. For pigs no efficient vaccine exists to

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http://dx.doi.org/10.1016/j.vaccine.2016.02.025
0264-410X/

date and, in almost all countries, no vaccination takes place. Only China vaccinates with an attenuated strain (*B. suis* S2) [8] and, even though it is accepted that the vaccine induces good levels of protection in mice [9], it has not been widely used in other parts of the world. Even though the strain was isolated over 30 years ago there are very few reports that have determined its protective efficiency [10] and, additionally, the strain is smooth rendering impossible the discrimination between infected and vaccinated animals.

We have generated and characterized a rough *B. abortus* vaccine strain that is a clean deletion of the phosphoglucomutase (*pgm*) gene, which is highly attenuated in mice but induces very good levels of protection [11,12]. This mutant is unable to convert glucose-6-P to glucose-1-P and, thus, cannot synthesize a subset of sugar nucleotides essential for the biosynthesis of many polysaccharides. For this reason the mutant does not synthesize cyclic beta glucans, a virulence factor important for the intracellular cycle of the bacteria [13,14], and cannot assemble a complete LPS [12]. This last characteristic makes this strain attractive for adult vaccination as it does not induce persistent antibody titers and would allow the discrimination between vaccinated or infected animals. Our data indicate this strain is highly attenuated in bovines, induces a high level of protection against abortion and colonization and does not generate persistent antibody titers in animals that have received up to two doses of the vaccine (manuscript in preparation). Because of the very good performance of this new *B. abortus* vaccine strain in mice and bovines we decided to generate the equivalent mutant in *B. suis* to determine its virulence and protective properties. In the present manuscript we describe the construction of a *B. suis* Delta-*pgm* mutant strain, the analysis of its biochemical properties and virulence as well as its protective capacity in the mouse model of infection. Our results show that this novel strain is highly attenuated but induces a robust protective immune response indicating that it could be potential new vaccine for the control of brucellosis in swine, a neglected disease of great concern in many developing countries where no vaccination takes place.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

*B. suis* 1330 was used as the wild-type strain. *Brucella* strains were cultured in Tryptic Soy Agar (TSA; Difco/Becton-Dickinson, Sparks, MD), or in Tryptic Soy Broth (TSB) at 37 °C on a rotary shaker for 16–24 h. Work with *Brucella* was performed at the Biosafety Level 3 laboratory facility at the Universidad Nacional de San Martín. All *Escherichia coli* strains were grown in Luria–Bertani (LB) agar and broth at 37 °C. If necessary, media was supplemented with antibiotics at the indicated final concentrations: Ampicillin 100 μg/ml, Kanamycin, 50 μg/ml and Nalidixic Acid, 5 μg/ml.

### 2.2. Recombinant DNA techniques

#### 2.2.1. Construction of the B. suis Δpgm mutant strain

To construct the *B. suis Δpgm* mutant strain, the regions flanking the *pgm* gene were amplified and ligated using the recombinant PCR technique [15]. The primers used for PCR amplification were BamHI-up-F (5′-CAGGATCCGGGCCGTCTGGTCTT-3′) and pgm-up-R (5′-CCCTACTGCCCTACTCCCGTTCGTTTCAATCG-3′) to amplify a 445-bp upstream region, and pgm-down-F (5′-AGTAGGGCAGTAGGGCAGT-3′) and XbaI-down-R (5′-ATTCTAGACGCCGGATACCGGCACG-3′) to amplify a 447-bp downstream region. BamHI-up-F and XbaI-down-R were used in the overlapping PCR. The resulting fragment was digested with BamHI and XbaI, and ligated to the pK18mobSacB plasmid digested with the same enzymes. The plasmid *pK18mobSacB/Δpgm* was introduced in the *B. suis* wild type strain by biparental mating using the *Escherichia coli* S17λ*pir* strain. Double recombination events ($Km^s$ $Sac^r$) were selected, and the gene knockout was confirmed by colony genomic PCR.

#### 2.2.2. Construction of B. suis Δpgm(pgm) complemented strain

The plasmid named pBBE30 [12] expressing the *pgm*, was introduced in the *B. suis Δpgm* strain by biparental mating. This plasmid contains a 4.3 kb pairs of the genome of *B. abortus* 2308 that contains the *pgm* gene which has 100% identity at the DNA level with the *B. suis* 1330 genomic region.

### 2.3. Intracellular replication assays

A standard antibiotic protection assay was performed in HeLa cells and murine macrophage-like J774 A.1 cells [16]. Cells were seeded in 24-wells plates in suitable culture medium at $10^5$ cells per ml and incubated overnight at 37 °C. *Brucella* strains were grown in TSB with the appropriate antibiotics for 24 h and diluted in culture medium prior to infection. The suspension was added at the indicated multiplicity of infection (100:1 for J774 A.1 cells; 500:1 for HeLa cells) and centrifuged at 300 g for 10 min. After 1 h of incubation at 37 °C, cells were washed and fresh medium containing 100 μg/ml of streptomycin and 50 μg/ml of gentamicin was added. At different time post-infection, cells were washed and lysed with 0.1% Triton-100X. The intracellular CFU were determined by direct plating on TSB agar plates.

### 2.4. Mouse infections

Mice infections were performed as previously described [12]. Groups of five 8 to 9-week-old female BALB/c mice were intraperitoneally inoculated with $10^5$ CFUs of *B. suis* 1330 wild type, *Δpgm* or complemented strains in PBS. At 3 weeks post-infection, spleens from infected mice were removed and homogenized in 2 ml of PBS. Serial dilutions from individualized spleens were plated on TSA with the appropriate antibiotics and the number of CFU per spleen determined.

All mice were bred in accordance with institutional animal guidelines under specific pathogen-free conditions in the local animal facility (BSL-3, Institute for Research in Biotechnology) of the University of San Martín. Mouse studies were approved by the local regulatory agencies (CICUAE-UNSAM).

### 2.5. Protection experiments

Protection experiments were carried out as described previously [11]. Groups of 10 mice were vaccinated intraperitoneally with $10^7$ CFUs of the *B. suis Δpgm* strain or with PBS (non-vaccinated control). Eight weeks post-vaccination, both groups were intraperitoneally challenged with $5 \times 10^5$ CFUs of virulent *B. suis* 1330. Two weeks post-challenge, all mice were sacrificed and the numbers of CFUs recovered from spleens were determined by direct plating as described above.

### 2.6. LPS analysis

To analyze the nature of the LPS, whole-cell extracts from *B. suis* 1330 wild type parental, *Δpgm* or complemented strains were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [11] and analyzed by immunoblotting using anti-O-polysaccharide monoclonal antibody M84 [17] and anti-mouse secondary antibody conjugated to horse-radish-peroxidase. All antibodies were diluted in

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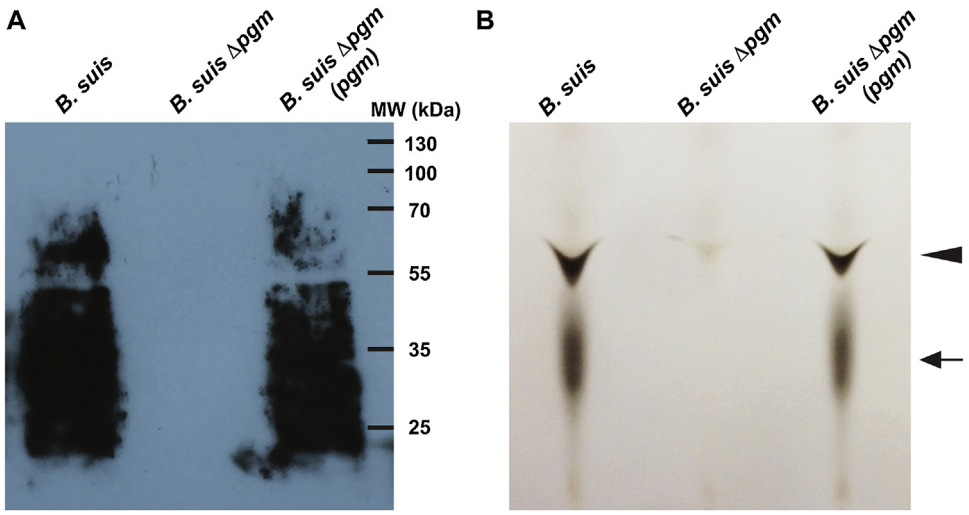


**Fig. 1.** Phosphoglucomutase (*pgm*) is necessary for the synthesis of a complete LPS and cyclic beta glucans in *B. suis*. A. Western-blot analysis of total cell extracts of the *B. suis* wild type, Δ*pgm* and complemented strains developed with an anti O-antigen monoclonal antibody (M84). B. Thin layer chromatography (TLC) of ethanol extracted cyclic beta glucans from *B. suis* wild type, Δ*pgm* and complemented strains developed by baking the plates after a treatment with 5% sulfuric acid in ethanol. Complete arrow, neutral glucans; arrowhead, charged glucans.

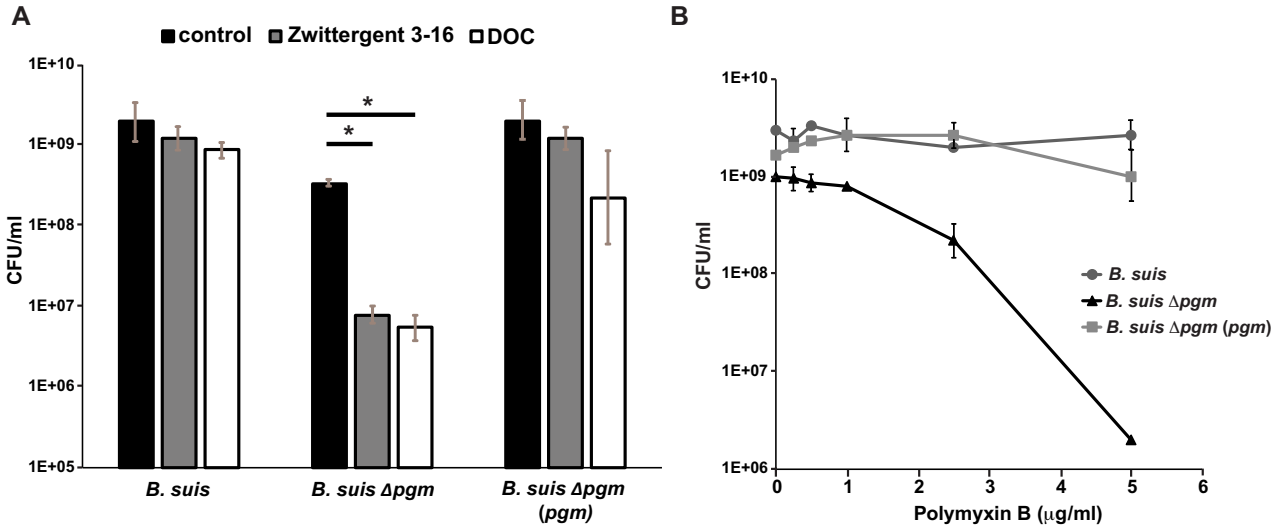


**Fig. 2.** *B. suis* Δ*pgm* is more sensitive to Zwittergent, Deoxycholate and Polymyxin B. A. *B. suis* wild type, Δ*pgm* and complemented strains were grown in TSB agar plates with or without the addition of Zwittergent 3-16 (12.5%) or Deoxycholate (DOC, 0.1%) and incubated until visible colonies were observed. The number of CFU per ml of the original culture was determined by direct counting. $^{*}P < 0.05$, *T*-test. B. *B. suis* wild type, Δ*pgm* and complemented strains were grown in TSB agar plates with increasing concentrations of Polymyxin B and incubated until visible colonies were observed. The number of CFU per ml of the original culture was determined by direct counting. $P < 0.05$, one-way ANOVA comparing the wild type versus Δ*pgm* inhibition curves.

Tris-buffered saline (TBS) – 5% non-fat milk – 0.1% Tween solution. Detection was performed by chemiluminiscence.

### 2.7. Thin layer chromatography (TLC) analysis of Beta-Glucans

To determine the synthesis of cyclic beta glucans, cells from 3 ml of stationary phase cultures of *B. suis* 1330 wild type parental, Δ*pgm* or complemented strains were harvested by centrifugation. Glucans were extracted from cell pellets with ethanol (70% ethanol, 1 h at 37 °C). Ethanolic extracts were centrifuged, and supernatants were dried in a Speed-Vac centrifuge. Extracted glucans were dissolved in 70% ethanol and submitted to TLC on silica gel-60 plates (Merck) with 1-butanol/ethanol/water (5:5:4 vol/vol) as described previously [14]. Sugars were detected baking the plates for 10 min at 125 °C after dipping them in 5% sulfuric acid in ethanol.

### 2.8. Polymyxin B, Zwittergent 3-16 and Deoxycholate sensitivity assays

To analyze the inhibition of growth by Polymyxin B (PmB), overnight cultures of wild type, mutant and complemented strains were serially diluted and plated on TSB agar plates with increasing concentrations of PmB (0, 0.25, 0.5, 1, 2.5 and 5 μg/ml). Plates were incubated at 37 °C for 48–72 h and the number of CFU per milliliter was determined by direct counting.

To analyze the inhibition of growth by detergents, sodium Deoxycholate (DOC) and Zwittergent (ZW) 3-16 were used. Overnight cultures of wild type, mutant and complemented strains were serially diluted and plated on TSB agar plates with 0.1 mg/ml DOC or 12.5 μg/ml ZW. Plates were incubated at 37 °C for 48–72 h and the number of CFU per milliliter was determined by direct counting.

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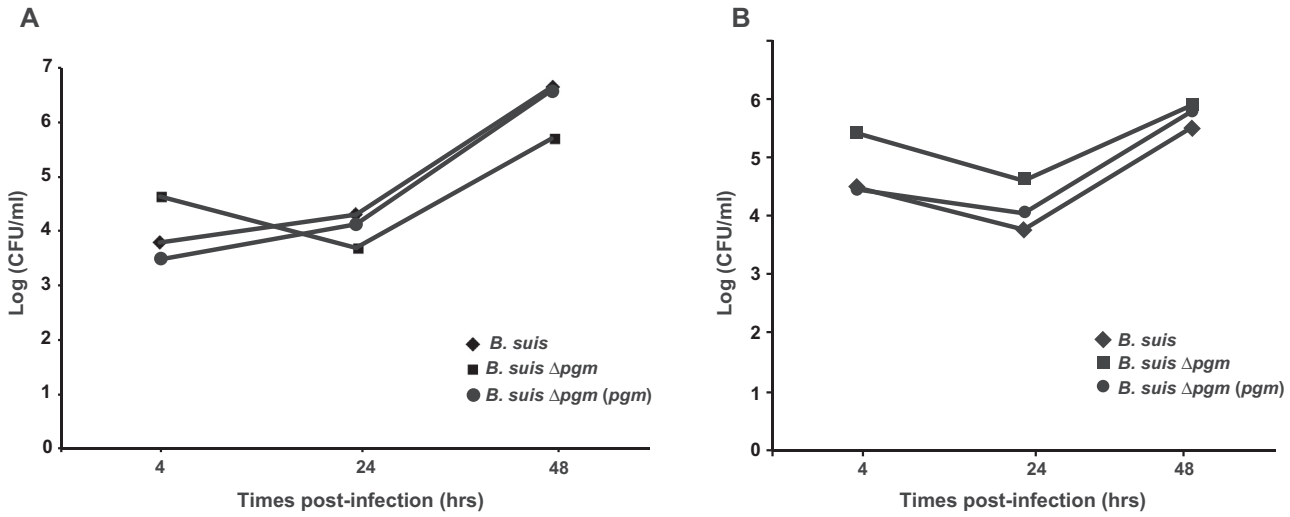


**Fig. 3.** *B. suis* Δ*pgm* is able to multiply in HeLa and J774 A.1 cells. A. Antibiotic protection assay to determine intracellular replication of *B. suis* wild type, Δ*pgm* and complemented strains in HeLa cells. B. Antibiotic protection assay to determine intracellular replication of *B. suis* wild type, Δ*pgm* and complemented strains in J774 A.1 cells.

### 2.9. Cytokine determinations

Eight weeks post-infection spleens from mice inoculated with strain *B. suis* Δ*pgm* or not were obtained under aseptic conditions. Single-cell suspensions were prepared from the spleens by disaggregation in cell culture medium (RPMI 1640 – 10% fetal bovine serum; Invitrogen), and erythrocytes were lysed with Tris-buffered ammonium chloride solution (Sigma). Splenocytes at $5 \times 10^5$ cells per well were cultured in 96-well flat-bottom plates in the presence of *B. suis* heat-inactivated whole cells in an amount equivalent to $10^6$ CFUs.

Cytokine levels were determined using commercial ELISA kits for IFN-γ, IL-10 and TNF-α determination as indicated by the manufacturer (eBioscience, Inc.).

### 2.10. Glyco-iELISA assays

To detect the anti O-antigen antibody response, an indirect ELISA using a recombinant *Yersinia enterocolitica* O: 9-polysaccharide-protein conjugate (OAg-AcrA) was performed as previously described [18]. Briefly, microtiter plates (Corning® #3590) were coated with 0.125 μg/well of OAg-AcrA overnight at 4 °C and blocked for 1 h with 1% of non-fat milk in PBS – 0.1% Tween20 (blocking solution) at 37 °C. Serum samples from 21 days post-infected mice were diluted 1:25 in blocking solution, and incubated 1 h at room temperature. Samples were evaluated in triplicates. Positive (anti-carrier (AcrA) serum) and negative-control (non infected serum) samples were included in each plate. Immunoglobulins were detected with an anti-mouse HRP-secondary antibody in a colorimetric reaction and read at 450 nm using a plate reader (DTX 880 Multimode Detector, Beckman Coulter, Inc.).

## 3. Results

### 3.1. The B. suis Δpgm strain is unable to synthesize cyclic beta glucans, is rough and has an altered outer membrane

As indicated above we have identified and characterized the phosphoglucomutase (*pgm*) gene in *B. abortus* as an important virulence factor implicated in the synthesis of several key virulence molecules such as LPS and cyclic beta glucans [11,12]. To determine if the homologous gene plays a similar role in *B. suis*, we deleted the gene in the 1330 strain and determined several biochemical and virulence properties of the resulting mutant. As can be seen in Fig. 1, the *B. suis* Δ*pgm* strain is unable to synthesize a complete LPS as well as cyclic beta glucans as evidenced by western-blot with a monoclonal anti O-antigen antibody performed on whole cells (panel A), and thin layer chromatography (TLC) of ethanol extracted glucans (panel B). Both phenotypes were complemented by the expression of the *pgm* gene from a plasmid. In the case of the LPS analysis it is worth noticing that if we loaded a higher amount of bacteria of the *B. suis* Δ*pgm* strain and over-exposed the western-blot we were able to observe a 45 kDa signal consistent with a cytoplasmic O-antigen as we have previously informed with the *B. abortus* strain [11]. Due to the altered LPS structure of the mutant we decided to analyze if this strain has affected its membrane properties by determining sensitivity to a series of detergents and Polymyxin B (PmB). To test this, the wild type, mutant and complemented strains were grown in TSB plates in the presence of either 12.5% of Zwittergent 3-16 or 0.1% Deoxycholate (DOC) and growth was assessed determining the colony forming units (CFU). Fig. 2A shows that the Δ*pgm* mutant was significantly more sensitive to both detergents as it had at least 100 times less bacteria than the parental and complemented strains. To determine resistance to PmB we compared the growth of the wild type, mutant and complemented strains in increasing concentrations of this antibiotic as indicated in Section 2. As shown in Fig. 2B the Δ*pgm* strain showed a marked reduction in the resistance to PmB. Altogether these results indicate that the Δ*pgm* mutant strain has affected several key components such as the LPS and the cyclic beta glucans and that it has altered the integrity of its outer membrane as evidenced by its sensitivity to several stressful conditions, these characteristics that might render the strain less virulent than the parental wild type.

### 3.2. The pgm gene is central for the virulence process and does not induce anti O-antigen antibodies

The biochemical and membrane properties of the *B. suis* Δ*pgm* mutant indicated that the *pgm* gene could be important during the virulence process of the bacterium. To test this we initially evaluated the intracellular replication capacity of the mutant in comparison to the wild type and complemented strains in HeLa and J774 A.1 cells by an antibiotic protection assay (see

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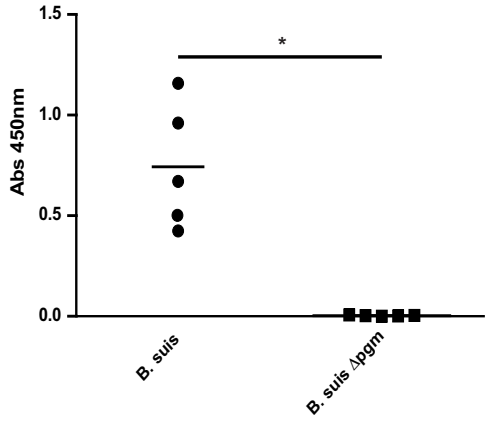


**Fig. 4.** *B. suis* Δ*pgm* does not induce an anti O-antigen antibody response. Glyco-iELISA to determine the anti O-antigen antibody response of *B. suis* or *B. suis* Δ*pgm* inoculated mice at six weeks post-infection. *$P < 0.001$, *T*-test.

Section 2). Fig. 3 shows that the mutant strain invaded the cells more efficiently (evidenced at 4 h post-infection) consistent with the fact the mutant is rough, and was able to replicate in both cell types. To further determine the role of *pgm* in the pathogenesis of the bacterium we analyzed virulence of the mutant strain in comparison with the wild type parental and complemented strains in the mouse model of infection. For this, groups of 5 female Balb/c mice were intraperitoneally infected with $10^5$ CFU of each strain and the bacterial loads in the spleens determined at 21 days post-infection. The results showed that while we recovered almost $10^6$ CFU per spleen in the wild type and complemented strains with the mutant we were unable to isolate viable bacteria indicating that infection was completely cleared and that the strain is highly attenuated in the mouse model of infection.

To test if inoculation of the *B. suis* Δ*pgm* induces anti O-antigen antibodies we intraperitoneally infected groups of 5 Balb/c with either the wild type or the mutant strain and, at 21 days post-infection, determined the antibody response against the O-antigen in serum. To do this, we performed an ELISA with a novel recombinant antigen that we have recently developed for the diagnosis of human and bovine brucellosis [18–20] and proved to have an excellent sensitivity and specificity. Briefly, the purified recombinant glycoconjugate was immobilized in high binding plates and an indirect ELISA was performed with a 1:25 dilution of the sera (see Section 2 for details). As can be observed in Fig. 4 the *B. suis* Δ*pgm* strain was unable to induce detectable anti O-antigen antibodies while the wild type strain induced a robust response.

Altogether the results presented so far demonstrate that the *B. suis* Δ*pgm* strain lacks key virulence factors such as LPS and cyclic beta glucans making it highly attenuated in the mouse model of infection and does not induce antibody titers against the recombinant glycoconjugate developed by our group suggesting that, if protective, it could be excellent vaccine candidate to use in combination with our novel diagnostic tool.

### 3.3. *The* B. suis Δpgm *strain is protective in mice*

The fact that the Δ*pgm* mutant strain showed a complete attenuation in mice but was able to replicate in cells, suggested that maybe this strain could be a good vaccine candidate. To evaluate this we initially measured the cytokine response of vaccinated mice in comparison with a non-vaccinated control group inoculating or not groups of eight mice intraperitoneally with *B. suis* Δ*pgm* and, at eight weeks post-inoculation, extracted the spleens, prepared total splenocytes and treated them for 72 hrs with heat-inactivated *B. suis*. Culture supernatants were collected and the levels of IFN-γ, TNF-α and IL-10 determined by ELISA (see Section 2). Fig. 5, panels A, B and C show that the secretion of the three cytokines analyzed were significantly induced compared to the non-vaccinated animals indicating that the strain triggered a robust immune response in the mice. More specifically, the high levels of IFN-γ and TNF-α strongly suggested that the *B. suis* Δ*pgm* strain induced a pro-inflammatory memory immune response consistent with strains that have shown protective properties. To test if this is also the case we performed a vaccination-challenge assay in mice as we have previously described for the *B. abortus* Δ*pgm* strain (Section 2). Groups of 10 mice were vaccinated or not with $10^7$ UFC of the *B. suis* Δ*pgm* strain and, at 8 weeks post-vaccination, challenged with $5 \times 10^5$ CFU of the virulent *B. suis* 1330 strain. Two weeks post-challenge the bacterial load in spleens was determined by direct counting of viable CFU. As can be observed in Fig. 5D, the Δ*pgm* strain induced very good levels of protection as all vaccinated animals showed a dramatic decrease in the spleen colonization (between 2,5 and 3,5 logarithms less bacteria) compared to the non-vaccinated group. These results demonstrate that *B. suis* Δ*pgm* induces excellent levels of protection in the mouse model of infection and suggest that this could be a promising candidate to perform vaccination-challenge trials in swine.

## 4. Discussion

Control and, eventually, eradication of infectious diseases is at present extremely difficult without the implementation of campaign programs that include vaccination. In the particular cases of intracellular pathogens this is even more important as in many cases antibiotics are not effective against them and control of the infectious process is achieved through a robust cellular immune response. Vaccines can be generally divided in live attenuated, inactivated whole organisms or subunits, depending on the type of infectious agent and type of immune response necessary for protection. *Brucella* is an intracellular pathogen that requires a strong T helper type 1 (Th1) immune response to elicit protection [21]. To date only live attenuated strains have been efficiently used as vaccines for the control of Brucellosis in several animals, suggesting that for this particular zoonosis a different approach might not be suitable. The obvious advantages of live attenuated vaccines, is that they harbor the complete antigenic repertoire of the virulent strain, which can promote a protective humoral and cellular immune response, but are unable to trigger the normal pathology of the disease.

Swine production has become, in the past twenty years, in one of the fastest growing livestock activities in the world due to its breeding and nutritional advantages but has raised important animal and human health concerns particularly due to the concept of the One Health approach (the swine flu is one well known example) [22]. Porcine brucellosis is an important zoonosis of animal and human health concern worldwide, particularly due to the highly zoonotic potential of *B. suis* and its elevated virulence in humans. Currently there is only one vaccine for the control of brucellosis in pigs, the S2 strain, which is only used in China and is delivered through the drinking water of the animals [8], which raises serious biosafety issues particularly due to the fact that is it a live strain. Additionally its protective efficiently has been subjected to controversy at least for some animal models [10]. For all these reasons there is still a need for the development of new *B. suis* vaccines.

In the present manuscript we report the inactivation of the phosphoglucomutase (*pgm*) gene in *B. suis* that results in a strain that lacks two key virulence factors such as cyclic beta glucans and a complete LPS which turns the strain sensitive to several detergents and to Polymyxin B. Interestingly, and as we have previously reported with the *Brucella abortus* Δ*pgm* strain [12], the

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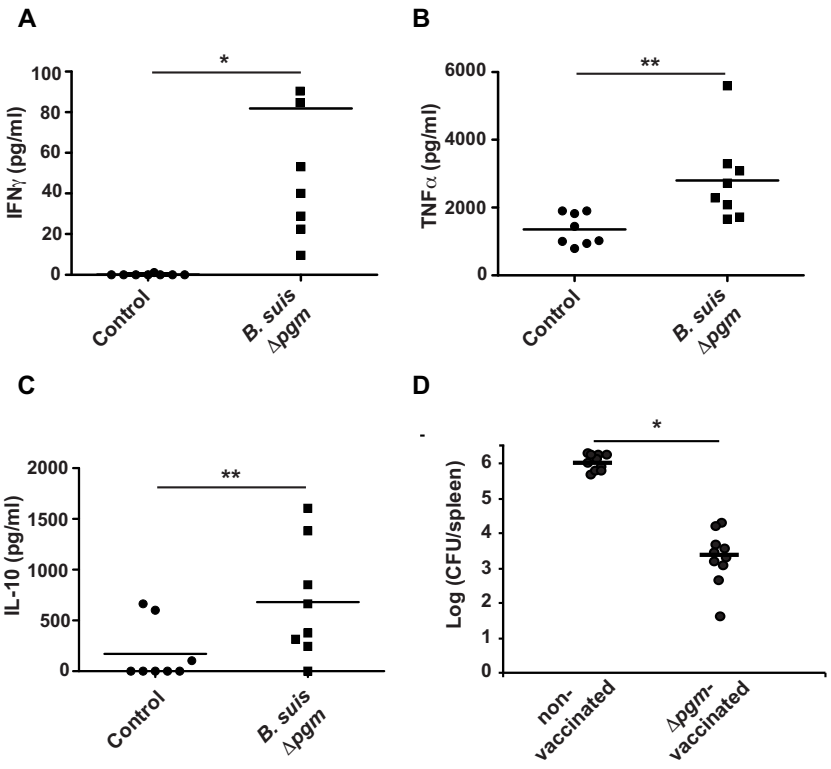


**Fig. 5.** *B. suis* Δ*pgm* induces a pro-inflammatory memory immune response and is protective in mice. A, B and C. Secretion levels of IFN-γ (A), TNF-α (B) and IL-10 (C) of total splenocytes extracted from mice intraperitoneally inoculated or not with $10^7$ CFU of *B. suis* Δ*pgm* strain and stimulated with heat-inactivated *B. suis*. Cytokine determination was performed as described in Section 2. $^*P < 0.01$, $^{**}P < 0.05$, *T*-test. D. Spleen bacterial load of groups of ten mice vaccinated or not with $10^7$ CFU of *B. suis* Δ*pgm* and, at eight weeks post-vaccination, challenged intraperitoneally with $5 \times 10^5$ CFU of wild type *B. suis*. Two weeks post-challenge the bacterial load in the spleens was determined by homogenization and direct plating as described in Section 2. $^*P < 0.00001$, *T*-test.

*B. suis* mutant was able to infect and multiply in HeLa and J774 A.1 cells, although with different kinetics compared to the wild type parental strain. Despite this, the mutant showed a dramatic decrease in its virulence capacity in the mouse model of infection, as we were unable to isolate viable bacteria from the spleens of infected mice 21 days post-infection while the wild type parental strain had close to $10^6$ CFUs. Additionally, the Δ*pgm* strain was unable to induce detectable levels of anti O-antigen antibodies determined with a novel iELISA recently developed by our group for this purpose, which could be an advantage in swine vaccination campaigns particularly to be able to discriminate vaccinated from infected animals. For all these reasons we determined the capacity of the Δ*pgm* strain to induce a cellular immune response measured as the ability to induce the production of two pro-inflammatory cytokines such as IFN-γ and TNF-α as well as its protective properties in vaccination-challenge trials in mice. It is to note that we also detected significant levels of the anti-inflammatory cytokine IL-10 that, in the context of the recent report indicating that an increase in its levels promotes *Brucella* persistent [23], might seem contradictory. We believe that the IL-10 secretion we observed upon re-stimulation is the consequence of a counterbalanced response to the pro-inflammatory cytokines triggered by the vaccine although at this stage a detailed analysis should be performed in order to understand this behavior. Nevertheless, our results clearly showed that the *B. suis* Δ*pgm* strain was able to trigger a robust cellular immune response that induced a significant level of protection against the virulent *B. suis* strain (between two and three logarithms) suggesting that it could be a potential new vaccine for the control of brucellosis in swine.

We have recently completed two vaccination-challenge trials on pregnant cattle to determine the protective properties of the *B. abortus* Δ*pgm* strain, which showed that the strain induced very good levels of protection against abortion, heifer colonization and excretion in milk (manuscript in preparation). These results, together with our new diagnostic test that can distinguish Δ*pgm* vaccinated versus infected animals [18,20], is a promising novel DIVA (Differentiate Vaccinated from Infected Animals) strategy to implement in areas were annual re-vaccination could be required to diminish the incidence of the disease. The fact that the *B. suis* Δ*pgm* strain induced very good levels of protection in the mouse model of infection but was unable to generate anti O-antigen antibodies, suggests that this strategy could be broaden to brucellosis in other species. Safety, as well as vaccination-challenge studies in pigs, should be initiated to determine if Δ*pgm* is an efficient vaccine in swine.

## Acknowledgments

We thank Diego Rey Serantes for help with the iELISA assays and members of the Ugalde laboratory for useful discussions.

This work was supported by Grants PICT-PRH08-160 and PICT-2014-1028 to J.E.U. and PICT-PRH08-230 to C.C. J.M.S., C.C. and J.E.U. are members of the National Research Council of Argentina (CONICET). M.D.G. is a fellow of the National Research Council of Argentina (CONICET).

## References

[1] Welburn SC, Beange I, Ducrotoy MJ, Okello AL. The neglected zoonoses – the case for integrated control and advocacy. Clin Microbiol Infect: Off Publ Eur Soc Clin Microbiol Infect Dis 2015;21:433–43.
[2] Corbel MJ. Brucellosis: an overview. Emerg Infect Dis 1997;3:213–21.

[3] Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. N Engl J Med 2005;352:2325–36.
[4] Schurig GG, Sriranganathan N, Corbel MJ. Brucellosis vaccines: past, present and future. Vet Microbiol 2002;90:479–96.
[5] Moriyon I, Grillo MJ, Monreal D, Gonzalez D, Marin C, Lopez-Goni I, et al. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet Res 2004;35:1–38.
[6] Samartino LE. Brucellosis in Argentina. Vet Microbiol 2002;90:71–80.
[7] Olsen SC, Palmer MV. Advancement of knowledge of *Brucella* over the past 50 years. Vet Pathol 2014;51:1076–89.
[8] Deqiu S, Donglou X, Jiming Y. Epidemiology and control of brucellosis in China. Vet Microbiol 2002;90:165–82.
[9] Bosseray N, Plommet M. *Brucella suis* S2, *Brucella melitensis* Rev. 1 and *Brucella abortus* S19 living vaccines: residual virulence and immunity induced against three *Brucella* species challenge strains in mice. Vaccine 1990;8:462–8.
[10] Blasco JM, Marin C, Jimenez de Bagues MP, Barberan M. Efficacy of *Brucella suis* strain 2 vaccine against *Brucella ovis* in rams. Vaccine 1993;11:1291–4.
[11] Ugalde JE, Comerci DJ, Leguizamon MS, Ugalde RA. Evaluation of *Brucella abortus* phosphoglucomutase (*pgm*) mutant as a new live rough-phenotype vaccine. Infect Immun 2003;71:6264–9.
[12] Ugalde JE, Czibener C, Feldman MF, Ugalde RA. Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect Immun 2000;68:5716–23.
[13] Briones G, Inon de Iannino N, Roset M, Vigliocco A, Paulo PS, Ugalde RA. *Brucella abortus* cyclic beta-1,2-glucan mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells. Infect Immun 2001;69:4528–35.
[14] Inon de Iannino N, Briones G, Tolmasky M, Ugalde RA. Molecular cloning and characterization of cgs, the *Brucella abortus* cyclic beta(1-2) glucan synthetase gene: genetic complementation of *Rhizobium meliloti* ndvB and *Agrobacterium tumefaciens* chvB mutants. J Bacteriol 1998;180:4392–400.
[15] Czibener C, Ugalde JE. Identification of a unique gene cluster of *Brucella* spp. that mediates adhesion to host cells. Microbes Infect 2012;14:79–85.
[16] Sieira R, Comerci DJ, Sanchez DO, Ugalde RA. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol 2000;182:4849–55.
[17] Nielsen KH, Kelly L, Gall D, Nicoletti P, Kelly W. Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. Vet Immunol Immunopathol 1995;46:285–91.
[18] Ciocchini AE, Serantes DA, Melli LJ, Guidolin LS, Iwashkiw JA, Elena S, et al. A bacterial engineered glycoprotein as a novel antigen for diagnosis of bovine brucellosis. Vet Microbiol 2014;172:455–65.
[19] Ciocchini AE, Rey Serantes DA, Melli LJ, Iwashkiw JA, Deodato B, Wallach J, et al. Development and validation of a novel diagnostic test for human brucellosis using a glyco-engineered antigen coupled to magnetic beads. PLoS Negl Trop Dis 2013;7:e2048.
[20] Iwashkiw JA, Fentabil MA, Faridmoayer A, Mills DC, Peppler M, Czibener C, et al. Exploiting the *Campylobacter jejuni* protein glycosylation system for glycoengineering vaccines and diagnostic tools directed against brucellosis. Microb Cell Fact 2012;11:13.
[21] de Figueiredo P, Ficht TA, Rice-Ficht A, Rossetti CA, Adams LG. Pathogenesis and immunobiology of brucellosis: review of Brucella-host interactions. Am J Pathol 2015;185:1505–17.
[22] Wielinga PR, Schlundt J. Food safety: at the center of a One Health approach for combating zoonoses. Curr Top Microbiol Immunol 2013;366:3–17.
[23] Xavier MN, Winter MG, Spees AM, Nguyen K, Atluri VL, Silva TM, et al. CD4+ T cell-derived IL-10 promotes *Brucella abortus* persistence via modulation of macrophage function. PLoS Pathog 2013;9:e1003454.

Please cite this article in press as: Czibener C, et al. Delta-*pgm*, a new live-attenuated vaccine against *Brucella suis*. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.02.025