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RESEARCH ARTICLE

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A *Brucella melitensis* H38ΔwbkF rough mutant protects against *Brucella ovis* in rams



Pilar M. Muñoz^{1,2*} , Raquel Conde-Álvarez³, Sara Andrés-Barranco^{1,2}, María-Jesús de Miguel^{1,2}, Amaia Zúñiga-Ripa³, Beatriz Aragón-Aranda³, Miriam Salvador-Bescós³, Estrella Martínez-Gómez^{3,4}, Maite Iriarte³, Montserrat Barberán⁵, Nieves Vizcaíno⁶, Ignacio Moriyón³ and José M. Blasco^{1,2}

Abstract

Brucella melitensis and *Brucella ovis* are gram-negative pathogens of sheep that cause severe economic losses and, although *B. ovis* is non-zoonotic, *B. melitensis* is the main cause of human brucellosis. *B. melitensis* carries a smooth (S) lipopolysaccharide (LPS) with an N-formyl-perosamine O-polysaccharide (O-PS) that is absent in the rough LPS of *B. ovis*. Their control and eradication require vaccination, but *B. melitensis* Rev 1, the only vaccine available, triggers anti-O-PS antibodies that interfere in the S-brucellae serodiagnosis. Since eradication and serological surveillance of the zoonotic species are priorities, Rev 1 is banned once *B. melitensis* is eradicated or where it never existed, hampering *B. ovis* control and eradication. To develop a *B. ovis* specific vaccine, we investigated three *Brucella* live vaccine candidates lacking N-formyl-perosamine O-PS: Bov::CAΔwadB (CO₂-independent *B. ovis* with truncated LPS core oligosaccharide); Rev1::wbdRΔwbkC (carrying N-acetylated O-PS); and H38ΔwbkF (*B. melitensis* rough mutant with intact LPS core). After confirming their attenuation and protection against *B. ovis* in mice, were tested in rams for efficacy. H38ΔwbkF yielded similar protection to Rev 1 against *B. ovis* but Bov::CAΔwadB and Rev1::wbdRΔwbkC conferred no or poor protection, respectively. All H38ΔwbkF vaccinated rams developed a protracted antibody response in ELISA and immunoprecipitation *B. ovis* diagnostic tests. In contrast, all remained negative in Rose Bengal and complement fixation tests used routinely for *B. melitensis* diagnosis, though some became positive in S-LPS ELISA owing to LPS core epitope reactivity. Thus, H38ΔwbkF is an interesting candidate for the immunoprophylaxis of *B. ovis* in *B. melitensis*-free areas.

Keywords: Sheep, brucellosis, *Brucella*, *B. ovis*, rough, vaccine

Introduction

Brucellosis is a worldwide extended disease caused by gram-negative bacteria of the genus *Brucella* that has a severe impact on animal and human health [1]. The genus includes several species among which *B. melitensis* primarily produces brucellosis in sheep and goats and is the major cause of human brucellosis [2]. *B. melitensis* cells have a surface smooth (S)-type lipopolysaccharide

(LPS) made of a lipid A-core oligosaccharide linked to an N-formylperosamine O-polysaccharide (O-PS) that carries the epitopes relevant in S *Brucella* serodiagnostic tests [3]. Sheep can also be infected by *B. ovis*, a species that displays a rough (R) LPS because it lacks a complete set of functional O-PS genes [4, 5]. Although non-zoonotic, *B. ovis* causes a serious disease manifested by genital lesions in rams and placentitis and abortions in ewes that result in important economic losses [6].

While some countries have eradicated *B. melitensis* through the combined use of vaccination and test and slaughter programs [7, 8], none has eradicated *B. ovis*. Attempts to eradicate *B. ovis* infection, sometimes

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misnamed ovine epididymitis, have often been focused exclusively on ram testing and culling, a strategy that has failed consistently because it overlooks the important epidemiological role of ewes [9–11]. Moreover, testing and culling becomes economically unfeasible when prevalence is moderate or high, making vaccination against *B. ovis* indispensable [11, 12]. Nevertheless, the only vaccine available for the immunoprophylaxis of *B. melitensis* and *B. ovis* is *B. melitensis* Rev 1, a live-attenuated S strain that elicits antibodies to the O-PS that interfere in *B. melitensis* serodiagnostic tests. Since these vaccinal antibodies encumber *B. melitensis* eradication and surveillance and this zoonotic species is a priority, Rev 1 is banned after *B. melitensis* eradication, and the same reasons make Rev 1 unsuitable for use in *B. melitensis*-free countries but yet affected by *B. ovis*. Hence, the development of *B. ovis*-specific vaccines is of great interest, as proven by the dramatic increase in *B. ovis* prevalence in European Union countries shortly after Rev 1 withdrawal [6, 13].

Altering the antigenic structure of *S Brucella* has been the preferred strategy to develop vaccines that circumvent the interference in S serological tests. Logically, O-PS removal was the first choice and some R mutants have been investigated as *B. ovis* vaccines. The empirically developed *B. abortus* RB51 (the only R vaccine currently marketed) does not confer adequate protection against either *B. melitensis* or *B. ovis* [14, 15]. However, experiments in mice show that genetically well-defined R vaccines are superior to RB51 and could be effectual against *B. ovis* [16]. An antigen removal strategy was also followed to obtain a *B. melitensis* Rev 1 lacking protein BP26 but, although effective against *B. ovis*, this Rev 1 derivative obviously triggers anti-O-PS antibodies and the associated BP26-iELISA is not sensitive enough to be used as differential test [17]. Following a positive tagging strategy, a Rev 1 vaccine carrying the green fluorescent protein (GFP) gene and its ancillary GFP-ELISA were investigated in sheep [18]. Yet, co-inoculation of the GFP-tagged vaccine with soluble GFP plus a GFP booster in Freund's adjuvant ten weeks later are necessary to trigger GFP antibodies matching the persistence of O-PS antibodies, a protocol of no practical use. Finally, Rev1::*wbdRΔwbkC*, a construct carrying *wbdR* (an *E. coli* LPS acetyl-transferase gene) instead of *wbkC* (the *Brucella* LPS formyl-transferase gene) conferred protection against *B. ovis* in mice and displayed a N-acetyl-perosamine O-PS structure triggering antibodies that could be differentiated from those induced by *B. melitensis* in this laboratory model [19].

Since it is a naturally R species, a homologous *B. ovis* vaccine could also be an alternative to Rev 1. Classical bacterins in adjuvant are unsatisfactory as *B. ovis*

vaccines [10, 12] and, although adjuvant-encapsulated outer membrane-rich extracts are effective [20, 21], the complexity of their preparation and the need for revaccinations make these vaccines expensive and unpractical. Recently, a polymeric BLSOmp31 antigen in oil adjuvant has been proposed as a *B. ovis* vaccine but the vaccine did not prevent infection in rams [22, 23]. An alginate-encapsulated *B. ovis* mutant in a putative ABC transporter has been reported to confer perfect protection in rams [24, 25] but this claim was based on a deficient bacteriological search for the challenge strain as only minimal aliquots of diluted homogenates were cultured and some important target organs [26] were not examined. In mice, we have reported that Omp25d and Omp22 mutants, a triple Omp10-Omp31-SP41 mutant and mutants with a truncated LPS-core lateral branch are attenuated and protect against *B. ovis* [27–29]. In parallel, we have investigated *B. ovis* CO₂-dependence, a characteristic of this species that would encumber a practical use of homologous vaccines. This trait relates to mutations in carbonic anhydrase (CA) genes and can be abrogated by insertion of CA homologues of CO₂-independent brucellae without affecting virulence in mice [30, 31].

In this work, we pursued three of the above-summarized strategies to develop *B. ovis* vaccines minimizing the interference in O-PS serological tests. For the R vaccine approach, we investigated a *B. melitensis* H38 *wbkF* mutant carrying an R-LPS with intact core oligosaccharide. This choice was based on previous works that show that dysfunction of this gene in this strain, while not matching Rev 1, results in adequate attenuation and better protection against *B. melitensis* in sheep than that obtained with mutants in other LPS genes [32, 33]. In the Rev 1 background, we studied the above-described Rev1::*wbdRΔwbkC*. Finally, we also examined a *B. ovis* CO₂-independent construct deleted in *wadB*, a gene necessary for full assembly of the R-LPS core oligosaccharide lateral branch of brucellae.

Materials and methods

Bacterial strains, conservation and growth conditions

The parental strains used for the construction of the vaccine candidate mutants are described below. Bov::CA is a *B. ovis* PA derivative carrying the active carbonic anhydrase (CAI and CAII) genes of *B. abortus* 2308 W in a stable Tn7 insert in chromosome II that is CO₂-independent and retains the virulence of the wild-type in the mouse model [30, 31]. *B. melitensis* H38 is a S virulent strain used previously in the analysis of LPS genes [32], and *B. melitensis* Rev 1 is the OIE recommended sheep brucellosis vaccine [34].

B. ovis PA (wild-type, virulent, serum and CO₂-dependent) and its kanamycin-resistant virulent

derivative (BoPA-Km^R) have been respectively used as challenge strains in sheep and mice in previous works [19, 35].

All the above bacteria and the corresponding mutants (see below) were maintained freeze-dried or in skimmed milk at -80°C in the CITA (Zaragoza, Spain) *Brucella* culture collection. To prepare the bacterial suspensions for the experiments described below, the stocks were rehydrated or thawed and seeded onto Blood Agar Base (BAB no. 2, OXOID, USA) or BAB with 5% sterile calf serum (BAB-S, for *B. ovis* PA) plates, for 3–4 days at 37°C in air or, for *B. ovis* PA, in 10% CO₂ atmospheres.

Construction and characteristics of vaccine candidates

The origin and characteristics of the primers and plasmids used in this work are presented in Additional files 1 and 2, respectively.

Bov::CAΔwadB was constructed using the suicide plasmid pJQKΔ*wadB* (pYRI-2 in [36]), which was introduced into *Bov::CA* by conjugation with *E. coli* S17 λpir [37]. The first recombination event was selected by nalidixic and kanamycin resistance and the double recombination by nalidixic and sucrose resistance, and kanamycin sensitivity. Deletion of *wadB* was confirmed by PCR using oligonucleotides *wadB*-F1 and *wadB*-R4 (Additional file 1), which amplified a fragment of 570 base pairs (bp) in the mutant and a fragment of 1011 bp in the sibling revertant strain. Oligonucleotides *wadB*-F1 and *wadB*-R5 (Additional file 1), which amplify a fragment of 471 bp only in the wild-type strain, were used to verify the deletion.

Brucella melitensis H38Δ*wbkF* (R mutant in-frame deleted in the bactoprenol priming for O-PS polymerization gene; henceforth H38Δ*wbkF*) was constructed using the same methodology and genetic tools described for the homologous *B. suis* Bs2Δ*wbkF* mutant [38]. Briefly, pJQKΔ*wbkF* was transformed into competent *E. coli* S17 λpir (Additional file 2) and, after conjugation with *B. melitensis* H38, the first recombination event was selected by nalidixic acid and kanamycin resistance, and the double recombination by nalidixic and sucrose resistance, and kanamycin sensitivity. Deletion of *wbkF* was confirmed by PCR using oligonucleotides *wbkF*-F1 and *wbkF*-R4 (Additional file 1), which amplified a fragment of 953 bp in the mutant and a fragment of 1796 bp in the sibling revertant strain. Oligonucleotides *wbkF*-F1 and *wbkF*-R6 (Additional file 1), which amplified a fragment of 680 bp only in the wild-type strain, were used to verify the deletion.

Rev1::*wbdRΔwbkC* construction and characterization has been described in a previous work [19]. Briefly, Rev1::*wbdR* was obtained inserting *wbdR* in chromosome II of *B. melitensis* Rev 1 using a Tn7 strategy [39]. Then, *wbkC* (formyltransferase gene) was deleted to obtain

Rev1::*wbdRΔwbkC*, which carries *N*-acetyl-perosamine instead of *N*-formyl-perosamine as proved by immunochemical and NMR analyses [39].

All vaccine candidates were stored at -80°C in skimmed milk and grown as described above.

Virulence and protection studies in mice

These experiments were carried out using seven-week-old female BALB/c mice (ENVIGO, Harlan) that were maintained in a BSL-3 facility (ES/31-2010-000132) with water and food ad libitum for 2 weeks before and during the experiments in accordance with the current European (directive 86/609/EEC) and Spanish (RD 53/2013) legislations.

To obtain inocula, fresh cultures (see “Bacterial strains, conservation and growth conditions”) were harvested in buffered saline solution (BSS; 0.015 M NaCl, 7 mM KH₂PO₄, 10 mM K₂HPO₄; pH 6.85) and suspensions adjusted spectrophotometrically (600 nm) to the appropriate dose (see below). Purity, absence of S-R dissociation (when appropriate) and exact doses (triplicate measurements) were assessed retrospectively following standard protocols [40].

The virulence and protection assessment in mice of Rev1::*wbdRΔwbkC* and Rev 1 parental strain have been published previously [19]. For virulence assessment of H38Δ*wbkF* and *Bov::CAΔwadB* mutants (this work), groups of 5 mice were inoculated intraperitoneally with the doses established as optimal (Figure 1) in preliminary experiments (not shown). The three vaccine candidates (*Bov::CAΔwadB*, Rev1::*wbdRΔwbkC* and H38Δ*wbkF*) were inoculated at 10⁸ CFU/mouse, a dose considered optimal for immunization with R mutants in previous experiments in mice [30, 32]; BoPA and *Bov::CA* parental strains were inoculated at 5×10^6 CFU/mouse, as established previously [31]. Also, the lowest dose of *B. melitensis* H38 (10⁴ CFU/mouse) inducing a suitable spleen colonization was determined in preliminary experiments (not shown). Mice were euthanized at two different time points, selected for each mutant according previous works [19, 31, 32]: the first point corresponds to the expected “peak of multiplication” (week 1 and week 3 for *B. melitensis* and *B. ovis* strains, respectively) and the second one, to a later stage of infection (week 5 or 8). The residual virulence was determined by plating spleen homogenates on the appropriate agar medium (see above) and calculating the mean log₁₀ CFU/spleen ± SD [41].

To evaluate the protective efficacy, groups of 5 mice were vaccinated subcutaneously with 10⁸ CFU of each vaccine candidate [19, 32]. Controls were similar groups vaccinated with 10⁵ CFU of Rev 1 or inoculated with BSS as a placebo vaccine. All mice were challenged with

5×10^6 CFU of BoPA-Km^R (see above) administered intraperitoneally 4 weeks after vaccination. The mean \log_{10} CFU/spleen \pm SD of the challenge strain in each group was determined 2 weeks later by plating spleen homogenates on BAB-S supplemented with kanamycin (50 μ g/mL) and incubation for 3–5 days in 10% CO₂. Results were compared statistically (one-way ANOVA and Fisher's Protected Least Significant Differences – PLSD- post-hoc tests) with those of unvaccinated (BSS) and Rev 1 vaccinated controls.

Ram vaccination and challenge

The experiment in rams was performed in compliance with the current European (86/609/EEC) and Spanish (RD 53/2013) legislations on the use and protection of experimental animals.

Sixty-nine 4-month-old brucellosis-free Rasa Aragonesa rams of similar weight and body condition were randomly allotted and maintained in five separate pens throughout the experiment. To prepare the inocula, suspensions of bacteria (grown and harvested in BSS as described above) were adjusted spectrophotometrically (600 nm) to the appropriate concentration (10^9 CFU/mL for Rev 1, 10^{10} CFU/mL for the mutant candidates and 10^{11} CFU/mL for *B. ovis* PA challenge strain). Purity, absence of S-R dissociation (when appropriate) and exact doses (triplicate measurements) were assessed retrospectively [40]. In the same day, three groups of 14 rams each were immunized subcutaneously in the left elbow with 4 mL of each vaccine candidate. As controls, a fourth group ($n = 14$) was vaccinated with 2 mL of Rev 1 and a fifth group ($n = 13$) was kept unvaccinated. Retrospective assessment showed that each ram received 9.6×10^9 CFU of Bov::CA Δ wadB, 2.5×10^{10} CFU of Rev1::wbdR Δ wbkC, 2.5×10^{10} CFU of H38 Δ wbkF and 1.6×10^9 CFU of Rev 1. In the next two weeks, all rams were inspected for rectal temperature and local reactions at the inoculation site every two days.

Eight months after vaccination (when Rev 1 and the vaccine candidates had been cleared [33]), rams were challenged with 2.5×10^9 CFU in 100 μ L (determined by retrospective assessment) of *B. ovis* PA given by both conjunctival (50 μ L) and preputial (50 μ L) routes, and then clinically examined at weekly intervals. Eight weeks after challenge, they were euthanized for bacteriological examination. For this, large portions of spleen and epididymides, the whole vesicular glands and ampullae, and the whole cranial (submaxillary, parotid and retropharyngeal), iliac, scrotal, prefemoral and superficial cervical lymph nodes were collected and submitted to culture on a suitable selective medium (see below). Briefly, each organ sample was degreased, superficially sterilized by gentle burning and homogenized in minimal

amount of BSS (1 mL per 10 g of tissue, approx.) using a stomacher. Each tissue homogenate was cultured by plating 1 mL in each of duplicate plates of CITA medium [42]. Cultures were examined under a stereomicroscope after 5–7 days of incubation at 37 °C in a 10% CO₂ atmosphere, and suspicious colonies streaked on BAB-S plates for identification by both standard procedures [40] and Bruce-ladder multiplex PCR [43]. A ram was classified as infected if at least one *B. ovis* PA CFU was isolated from any of the organs or lymph nodes sampled. The infection level of each sample was semi-quantitatively scored as follows: level 1, 1–5 CFU/plate; level 2, 6–25 CFU/plate; level 3, 26–125 CFU/plate; level 4, 126–250 CFU/plate; and level 5, > 250 CFU/plate (samples with level equal or higher than 3 were considered as severely infected, and rams showing at least one severely infected organ were counted as severely infected animals). Statistical comparisons of numbers of *B. ovis* infected (and severely infected) animals were made by Chi-square test (Fisher-Yates correction was applied when required). Numbers of infected (and severely infected) organs per animal were compared by STEPBOOT MULTTEST (5.0, SAS Institute Inc. Copyright©).

For serological studies, blood samples were taken before vaccination, one week after vaccination and every two or three weeks (including one week after challenge) until the end of the experiment.

Serological tests

The Rose Bengal (RBT) and the complement fixation (CFT) tests for *S. Brucella* were performed according to standard methods [34], and the S-LPS iELISA was performed as described before [44] using gold standard sera (from 46 *B. melitensis* culture positive and 78 brucellosis-free sheep) to determine the appropriate cut-off. The agar gel immunodiffusion (AGID) test for the serodiagnosis of *B. ovis* [45] was performed in 3 mm thick gels of 1% Noble Agar (Difco, USA) in borate buffer (0.1 M, pH 8.3)-10% NaCl using the recommended *B. ovis* REO 198 hot saline (HS) extract (rich in outer membrane proteins and R-LPS [46]) as antigen, and presence of precipitation bands was examined after 48 h of incubation at room temperature in a humid chamber. In addition, depending on the vaccine candidate (see “Results”), the antibody response was also examined by indirect ELISA (iELISA) using the appropriate antigen as described below.

The iELISA with HS extract of either *B. ovis* PA or Bov::CA Δ wadB was performed on standard 96-well polystyrene plates (Costar®, USA). Optimal coating conditions (antigen at 2.5 μ g/mL in carbonate buffer [0.06 M, pH 9.6] at 37 °C overnight), serum dilution (1/100) and conjugate (recombinant Protein G-HRP, Thermo Fisher Scientific, USA at 0.2 μ g/mL) were determined

by previous titration with gold-standard sera from 46 *B. ovis* culture-positive and 78 brucellosis-free sheep. A 0.1% solution of 2, 2-azinobis, 3-ethyl-benzothiazoline sulfonic acid diammonium salt (ABTS; Sigma Chemical Co., USA) with 0.004% H₂O₂ in citrate buffer (0.05 M, pH 4) was used as the substrate, and optical density readings (OD_{405nm}, Labsystems Multiskan RC) obtained after 15 min of incubation at room temperature. Results were expressed as the percentage of OD_{405nm} with respect to a positive control serum. Under these conditions, sera showing % OD_{405nm} above 40% (cut-off resulting in 100% specificity and maximum [98%] sensitivity with the gold standard sera) were considered positive.

An iELISA with N-acetyl-perosamine O-PS was performed using a LPS rich extract of the previously described *B. abortus* 2308 W *wbdRΔwbkC* construct [19]. This *wbdRΔwbkC*-iELISA was optimized using the same panel of gold-standard sera employed in the standard S-LPS ELISA (see above). Costar® plates were coated with 2.5 µg/mL of antigen in carbonate buffer (pH 9.6) at 37 °C overnight. Sera were tested at 1/100 dilution and the Protein-G-HRP (0.2 µg/mL) and the ABTS-H₂O₂ mixture described above were used as conjugate and substrate, respectively. The OD_{405nm} readings and % OD_{405nm} were obtained as described above and sera showing %OD_{405nm} ≥ 50% (optimal cut-off) were scored as positive.

Results

Characterization of vaccine candidates

Table 1 summarizes the relevant characteristics of the vaccine candidates investigated. Analyses by SDS-PAGE and Western blot with monoclonal antibody A68/24G12/A08 (which discriminates intact and truncated *Brucella* LPS core oligosaccharides [36]) and polyclonal sera of the appropriate specificity [39], confirmed that the three candidates displayed the phenotype predicted according to previous works with homologous or heterologous *Brucella* mutants and constructs: (i) H38Δ*wbkF* carried a R-LPS with a complete core; (ii) Bov::CAΔ*wadB* R-LPS

showed the molecular weight shift and epitope defect that correspond to a truncated LPS core lateral branch; and (iii) Rev1::Tn7*wbdRΔwbkC* displayed the shorter O-PS and the epitopic changes associated with the presence of N-acetyl-perosamine and absence of N-formyl-perosamine in the O-PS (Additional file 3).

Residual virulence and protection in mice

These studies showed that the three vaccine candidates have attenuated virulence profiles with respect to their virulent counterparts (Figure 1). Bov::CAΔ*wadB* (Figure 1A) showed significantly lower CFU/spleen numbers than *B. ovis* PA virulent strains at weeks 3 (expected peak of multiplication for *B. ovis*) and 8 after inoculation. Attenuation with respect to the parental strain was clearly observed also for Rev1::*wbdRΔwbkC* (Figure 1B, from [19]) at post-inoculation weeks 1 (peak of multiplication for *B. melitensis*) and 5. H38Δ*wbkF* (Figure 1C) yielded similar CFU/spleen numbers to the parental strain at week 1, but the mutant attenuation was evident at later stages (week 5).

Using this model, we tested all vaccine candidates for protection against *B. ovis* in two successive experiments. As can be seen in Table 2, the results obtained with all vaccine candidates were not statistically different from those obtained with the Rev 1 vaccine.

Protection in rams

No relevant clinical signs were evidenced after vaccination. The mean rectal temperature remained normal in the animals inoculated with the three vaccine candidates. Some Rev 1 vaccinated rams showed a slight increase in body temperature and an inflammatory reaction at the inoculation site that was resolved in a few weeks. Upon challenge, no vaccinated ram developed clinical testicular lesions but these were observed in two unvaccinated rams.

Table 1 Vaccine candidates.

Vaccine	Genetic characteristics	Relevant phenotype	References
Rev1:: <i>wbdRΔwbkC</i>	Rev 1 <i>wbkC</i> (formyl-transferase gene) deleted mutant carrying a mini-Tn7 insert with <i>E. coli</i> O157:H7 <i>wbdR</i> (acetyl-transferase)	S-LPS with N-acetyl-perosamine O-PS	[19]
H38Δ <i>wbkF</i>	<i>B. melitensis</i> H38 <i>wbkF</i> (bactoprenol priming for O-PS polymerization gene) deleted mutant	R-LPS with intact core oligosaccharide	This work
Bov::CAΔ <i>wadB</i>	<i>B. ovis</i> PA <i>wadB</i> (core sugar glycosyl transferase gene) deleted mutant carrying a mini-Tn7 insert with the carbonic anhydrases (CAI and CAII) genes of <i>B. abortus</i> 2308 W	CO ₂ -independent; R-LPS with a truncated core oligosaccharide	This work. Δ <i>wadB</i> and::CA single mutants in [27] and [30], respectively

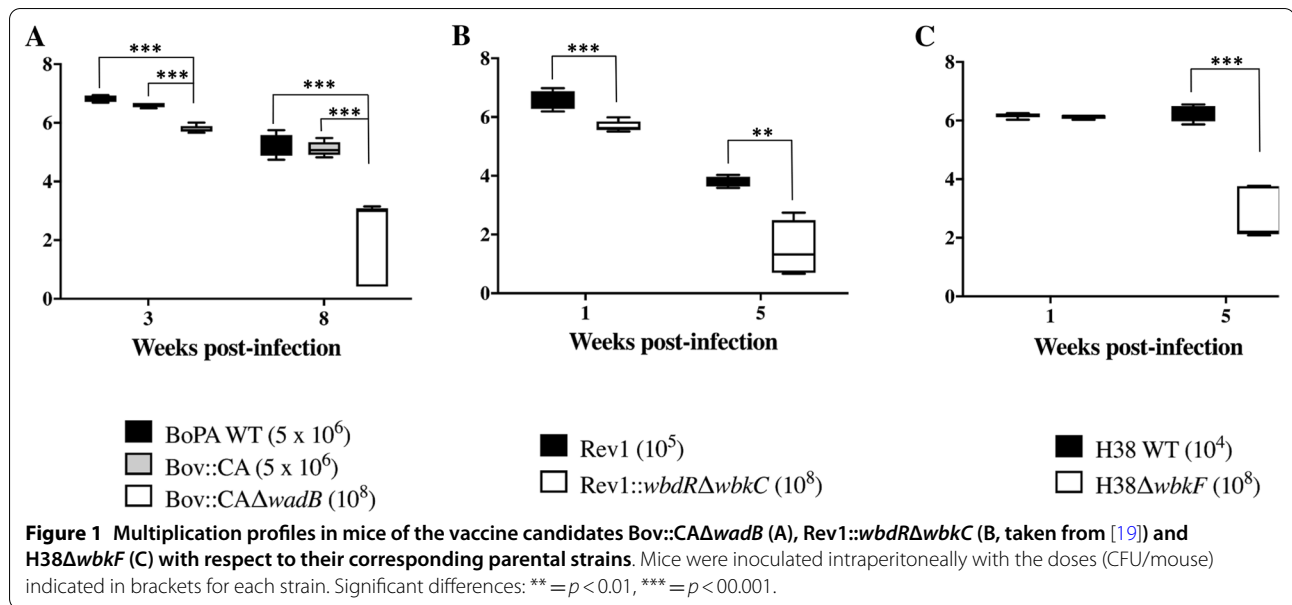


Table 2 Protection against *B. ovis* PA-Km^R in mice.

Experiment	Vaccine (CFU dose)	<i>B. ovis</i> PA-Km ^R (mean ± SD of log ₁₀ CFU/spleen)	Units of protection ^a
1	Bov::CAΔwadB (10 ⁸)	2.46 ± 1.68 ^{b,c}	3.6
	H38ΔwbkF (10 ⁸)	3.42 ± 0.62 ^{b,c}	2.6
	Rev 1 (10 ⁵)	2.50 ± 1.49 ^b	3.5
	Placebo (BSS)	6.04 ± 0.46	0
2	Rev1::wbdRΔwbkC (10 ⁸)	2.16 ± 0.95 ^{b,c}	4.1
	Rev 1 (10 ⁵)	2.45 ± 1.35 ^b	3.8
	Placebo (BSS)	6.26 ± 0.16	0

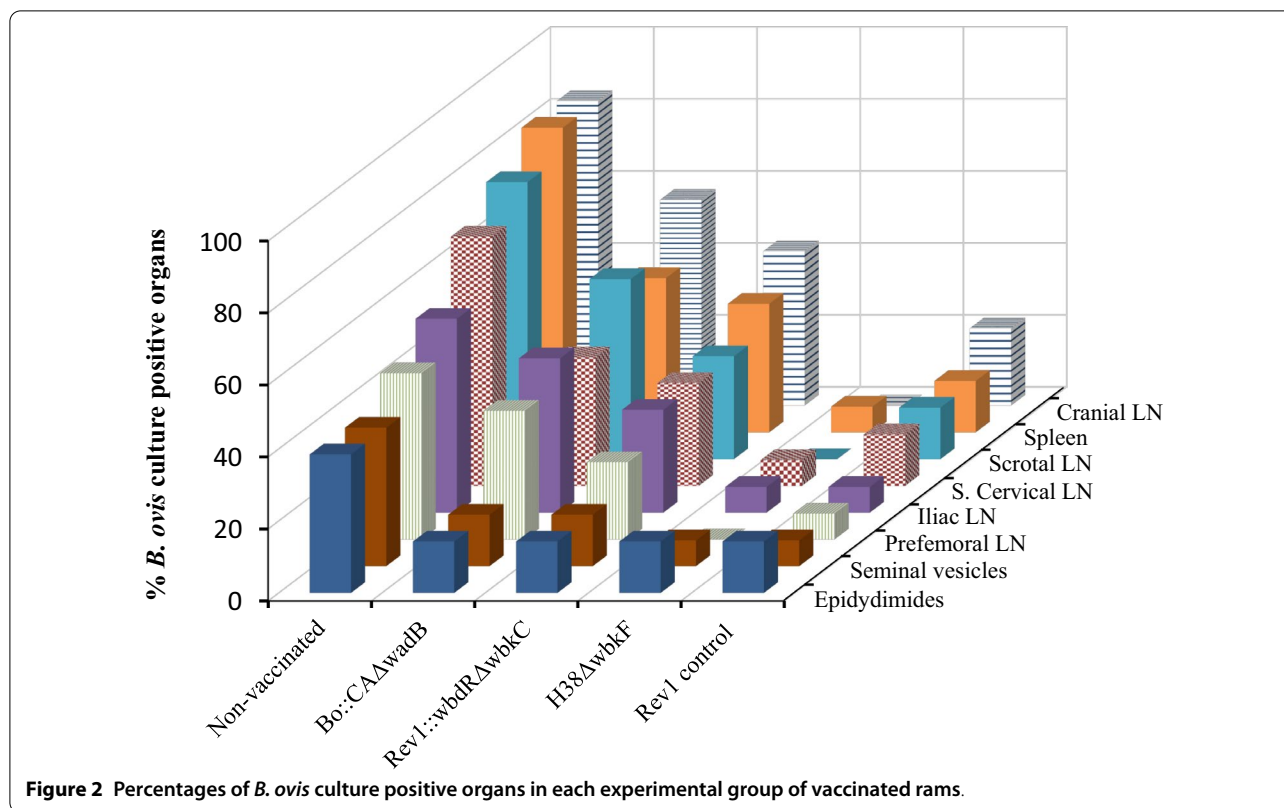
^a Units of protection: average log₁₀ CFU of challenge strain in the spleens of placebo (BSS) controls minus average of log₁₀ CFU of the challenge strain in the spleens of vaccinated mice. ^b Significant differences ($P < 0.01$) versus the placebo (BSS) control group (ANOVA and Fisher's PSLD test); ^c No significant differences versus the Rev 1 vaccinated group (ANOVA and Fisher's PSLD test).

The results of the thorough bacteriological search after necropsy are summarized in Table 3 and Figure 2. Taken together, the results of control groups show that the experimental conditions resulted in values suitable for statistical comparisons (84.6% vs. 21.4% infected rams in the unvaccinated and Rev 1 vaccinated groups, respectively; $P < 0.05$). The protection conferred by Bov::CAΔwadB was manifestly not significant (78.6% of infections) and that of the Rev1::wbdRΔwbkC candidate (50% of infections) not statistically different from that of the unvaccinated control group. On the other hand, H38ΔwbkF conferred a level of protection (16.7% of infections) similar to that obtained with Rev 1 (Table 3), with slightly lower percentages of infected organs and no isolation from cranial, scrotal or prefemoral lymph nodes (Figure 2). These results were paralleled in the numbers of infected organs (Table 3) (5.4% and 10.7% for H38ΔwbkF and Rev 1, respectively) and of severely infected animals/organs (7.1%/1.8% and 7.1%/0.9% for H38ΔwbkF and Rev 1, respectively).

Table 3 Protective efficacy of vaccine candidates against a *B. ovis* PA experimental challenge in rams.

Vaccine (CFU dose)	No. infected animals/total (%) ^a	No. infected organs/total (%) ^b	No. severely infected animals (%) ^{a,c} /no. severely infected organs (%) ^b
Bov::CAΔwadB (1 × 10 ¹⁰)	11/14 (78.6)	41/112 (36.6)	4 (28.6) ^d /11 (9.8) ^d
Rev1::wbdRΔwbkC (2.5 × 10 ¹⁰)	7/14 (50.0)	30/112 (26.8) ^d	5 (35.7)/13 (11.6) ^d
H38ΔwbkF (2.5 × 10 ¹⁰)	2/14 (16.7) ^{e,f}	6/112 (5.4) ^{e,f}	1 (7.1) ^{e,f} /2 (1.8) ^{e,f}
Rev 1 (2 × 10 ⁹)	3/14 (21.4) ^d	12/112 (10.7) ^e	1 (7.1) ^e /1 (0.9) ^e
Unvaccinated	11/13 (84.6)	64/104 (61.5)	9 (69.2)/30 (28.8)

^a Statistical comparisons by Chi-square test (with Fisher-Yates correction when required). ^b Statistical comparisons by STEPBOOT MULTTEST (SAS); ^c severely infected animals were those showing at least one organ sample with an infection level ≥ 3 (≥ 26 CFU/plate). ^d Significant difference ($P < 0.05$) versus unvaccinated control; ^e High significant difference ($P < 0.001$) vs. unvaccinated control; ^f no significant ($P > 0.05$) versus Rev 1 vaccinated group.

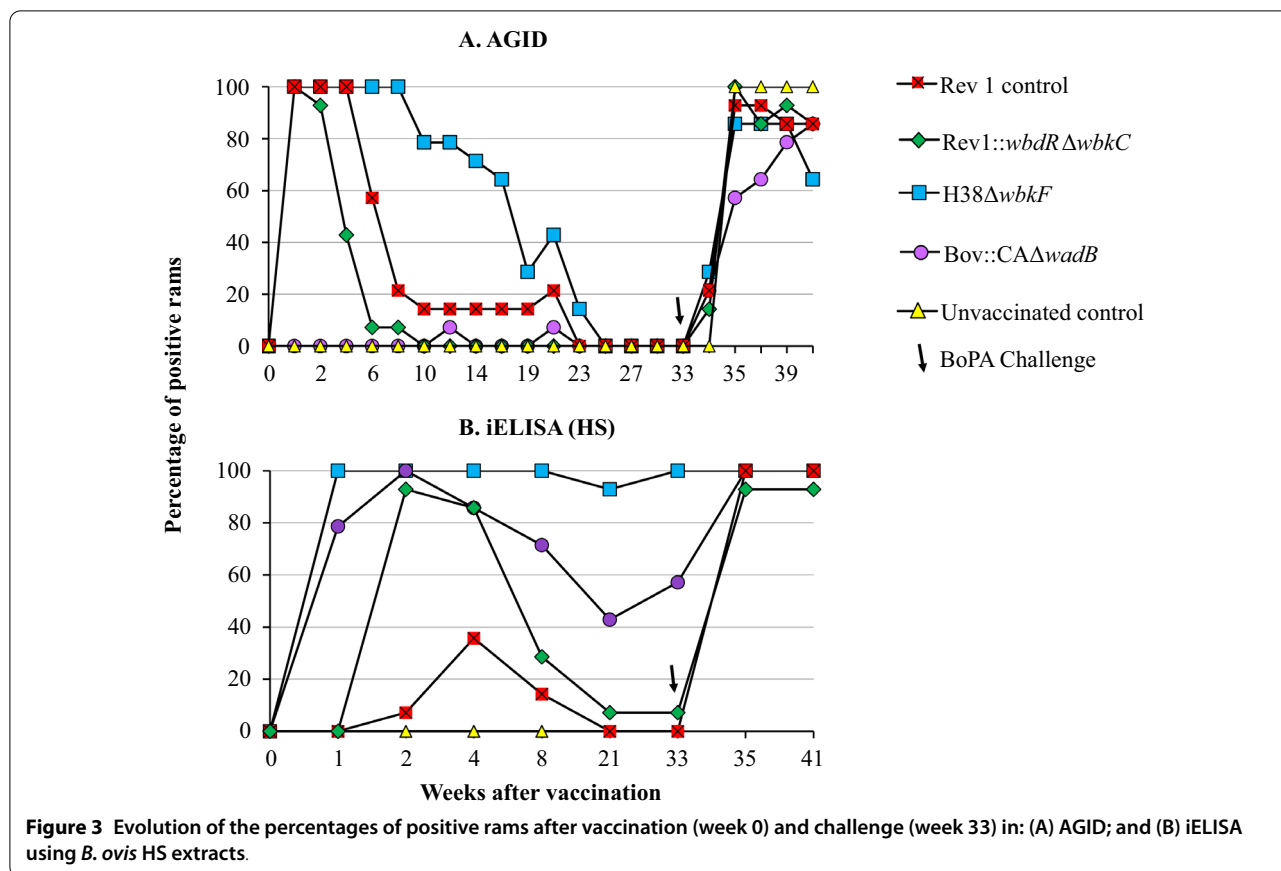


Serological responses in rams

Figure 3 presents the results of the antibody response to the *B. ovis* HS envelope antigens generated in vaccinated rams before and after challenge. All rams were positive in the AGID assay by week 1 after immunization, with the notable exception of those vaccinated with Bov::CAΔwadB among which very few positive responses were detected before challenge (Figure 3A). In the Rev1::wbdRΔwbkC and Rev 1 vaccinated groups, AGID-positive responses decreased rapidly and all animals became negative by weeks 10 and 23, respectively (Figure 3A). As judged by the percentage of AGID-positive animals, H38ΔwbkF elicited the most durable antibody response, with over 60% of positive animals until week 17 (Figure 3A). No matter the experimental group, all rams were AGID-negative before challenge (week 33). When assessed by iELISA (Figure 3B), the percentage of positive animals in the groups vaccinated with Bov::CAΔwadB and H38ΔwbkF were higher and more persistent than those observed in the AGID test and only the group vaccinated with Rev1::wbdRΔwbkC was totally negative by the time of challenge. Interestingly, when sera from Bov::CAΔwadB vaccinated rams were studied in the iELISA with a homologous HS antigen, 100% of them were positive from week 1 until the end of the experiment (not shown). Upon challenge,

most animals became both AGID and HS iELISA-positive, as expected.

Figure 4 presents the results obtained with serodiagnostic tests for *S. brucellae*. Unsurprisingly, all animals vaccinated with Rev 1 were positive in RBT, CFT and S-LPS iELISA by week 2 and a high percentage remained positive thereafter. Although Rev1::wbdRΔwbkC bears a modified O-PS, all rams vaccinated with this candidate were positive in both RBT and CFT during the first weeks but the reactions persisted less than those induced by Rev 1. However, only very few and transient reactions were detected in the S-LPS iELISA in these Rev1::wbdRΔwbkC vaccinated rams (Figure 4C), even though these rams showed a more intense and persistently positive response in the wbdRΔwbkC iELISA (Additional file 4). In contrast with Rev 1 and Rev1::wbdRΔwbkC vaccinates, no ram immunized with H38ΔwbkF or Bov::CAΔwadB showed antibodies detectable in either RBT or CFT, and only a few of those vaccinated with H38ΔwbkF showed a transiently positive and relatively early response in the S-LPS iELISA before challenge (Figure 4). As expected, the proportion of RBT and CFT positive animals did not increase after the challenge for any of the experimental groups (Figures 4A and B) but 50% and less than 20%, respectively, of H38ΔwbkF and Bov::CAΔwadB vaccinated rams reacted then in the S-LPS iELISA (Figure 4C).



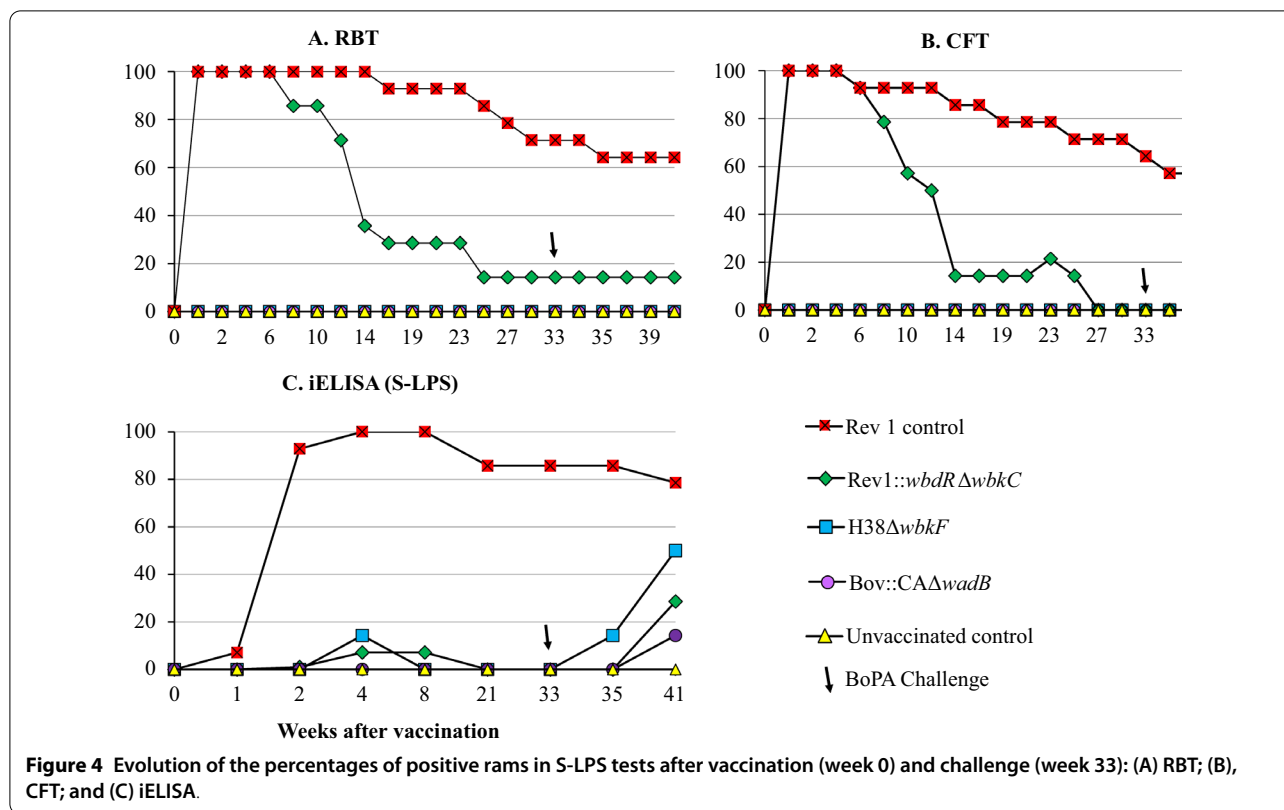
Discussion

As underlined in the Introduction, there is a need for a *B. ovis* vaccine to replace Rev 1, banned in areas where *B. melitensis* has been eradicated or has never been present. Following the evidence that live-attenuated vaccines are advantageous for triggering protective responses against *Brucella* [47], in this work we investigated three vaccine candidates of this kind.

It is known that *Brucella* behaves as a stealthy parasite that avoids detection by innate immunity at the onset of infection, thus retarding the adaptive response and facilitating to reach intracellular niches [48]. This furtive behavior is related to the absence of marked pathogen-associated molecular patterns in envelope molecules, chiefly in the LPS [48]. Immunological, genetic and structural investigations have established that the core of *Brucella* LPS carries a pentasaccharide branch that hinders recognition of the lipid A-inner core by the TLR4-MD2 receptor system of innate immunity [27, 36, 49, 50]. Hence, mutations preventing the assembly of this pentasaccharide bolster Th1 immunoresponses, thereby causing attenuation [27, 36, 49, 50]. Consequently, disruption of the core lateral branch was proposed as a strategy to develop brucellosis vaccines [51] and previous

work with *B. ovis* shows that the core glycosyltransferase gene *wadB* is an appropriate target for this purpose [27]. Yet, we found that, when combined with CA genes of *B. abortus* to overcome the practical limitations of CO₂-dependence, the *B. ovis wadB* mutant was totally ineffective as vaccine in rams even though it was protective in mice. These results mean that the mouse model failed utterly to predict a positive vaccine result in the natural host, and are in line with other data that strongly suggest that its usefulness in the search for a brucellosis live vaccine is limited to screen out overattenuated or none protective candidates [32, 33, 52, 53].

Nevertheless, the total failure of Bov::CAΔwadB as a vaccine was unexpected and, although the explanation is far from obvious, it is worthwhile to speculate on the reasons. Noteworthy, proportions of Bov::CAΔwadB vaccinated rams positive in AGID and iELISA with *B. ovis* HS were respectively lower than those in any other vaccinated group. Since all rams remained positive in the iELISA with HS of the homologous Bov::CAΔwadB, and HS is rich in R-LPS [46], these results evidence the importance of the epitopes of the core branch in the antibody response to *B. ovis* LPS. In mice, a role for antibodies in *B. ovis* protective immunity has been shown in passive



transfer assays with monoclonal antibodies to envelope antigens and with sera from mice immunized with RB51 (reviewed in [35]). However, vaccination with HS in sheep does not show a correlation between protection and the intensity of the antibody response to these antigens [20, 35]. On the other hand, experiments with the same antigens in several adjuvant formulations provide clear evidence that a strong Th1 cell-mediated immunoreponse is important in protection in sheep [21, 35].

Therefore, although the lack of antibodies to the full core may play a role, we favor the hypothesis that the failure of Bov::CAΔwadB to protect against *B. ovis* was caused by an inappropriate Th1 response. This may seem paradoxical because the LPS core defect has been shown to increase recognition by LPS receptors (see above), thereby bolstering pro-inflammatory cytokine production and initiating the Th1 response at earlier times than the wild-type [49]. However, this comparatively early Th1 response accelerates clearance of *Brucella* core mutants in mice [49] and, as discussed below, a given permanence in the host is necessary for a vaccine to generate protective immunity. Thus, it could be that Bov::CAΔwadB clearance was too rapid to generate such a response. Investigation of these hypotheses could help to clarify the virulence mechanisms of *B. ovis* and the immunity to this comparatively less studied species, knowledge with

which it may be possible to develop an homologous *B. ovis* vaccine.

We also examined the Rev1::wbdRΔwbkC construct. Based on the results in mice [19], the premises were that this candidate could protect sheep against *B. ovis* and elicit antibodies that, while not interfering in standard S-LPS tests, could be useful to identify vaccinated animals. Concerning protection, we found that it was insufficient, even though in this case the differences with the results in mice were not as dramatic as those of Bov::CAΔwadB. It is worth pointing out that since the first studies with vaccine RB51 a much larger dose has been found necessary to show that some R mutants match the protection obtained with S19 or Rev 1 (reference OIE S vaccines) in mice [16, 32, 52, 53], and this is also true for Rev1::wbdRΔwbkC (Table 2 and [19]). However, to reproduce in the natural host the 3 log dose increase applied in the mouse model is not feasible (doses over 10¹⁰ CFU are not practical in sheep). Therefore, we used a dose equivalent to that of R vaccines (1–5 × 10¹⁰, [33]). Thus, the contrasting results obtained with Rev1::wbdRΔwbkC in rams and in mice are likely to reflect the laboratory bias in dose introduced for screening purposes and the limitations of the mouse model.

Why Rev1::wbdRΔwbkC being smooth could require a dose larger than Rev 1 in the mouse model may reflect

a defect in cell entry and/or control of intracellular trafficking caused by the O-PS modification because both are critical processes in the biology of *S. Brucella* in which the wild-type O-PS plays an important role [54, 55]. Concerning the specificity of the antibodies, our results show that the change in O-PS epitopic structure of Rev1::wbdRΔwbkC is insufficient to fully abrogate the interference in standard S-LPS tests (Figure 4) or to differentiate vaccinated and infected rams (Additional file 4). These results, which are reminiscent of the reactivity of IgG of infected cattle with the O-PS of the *E. hermannii* serotypes that carry N-acetyl-perosamine [56] set another difference with the mouse model [19] and discourage a practical use of this vaccine epitopic tagging.

In contrast with the other candidates, H38ΔwbkF generated protection in rams similar to that obtained with reference Rev 1 vaccine. In previous works, we found that an intact LPS core is necessary for an R vaccine to provide maximal protection in at least the mouse model, and wbkF mutants meet this requisite [16, 32]. Also in keeping with the predicted function of WbkF (Table 1), they are unable to synthesize internal O-PS precursors that would elicit anti-O-PS antibodies, an undesirable effect in an R vaccine that occurs in wadA (formerly wa**) and wzm R mutants [32, 33, 57, 58]. Moreover, as indicated in the Introduction, this candidate was not selected only by the results in mice. In a previous work [33], we found that a *B. melitensis* H38wbkF::Tn5 Km^R mutant had an organ distribution and persistence in sheep like those of Rev 1, strongly suggesting similarly intense antigenic stimuli. All these characteristics made strain H38 and gene wbkF attractive for developing a *B. ovis* vaccine, and for a potential use we constructed the mutant by deletion (to avoid any potential reversibility) without antibiotic-resistance markers. Thus, although we did not evaluate again the kinetics of infection of the H38ΔwbkF mutant in rams, its almost total identity with the H38wbkF::Tn5 Km^R should result in a distribution and persistence similar to that of Rev 1 and, in fact, both elicited similarly persistent positive responses in the *B. ovis* HS-AGID.

As predicted, vaccination with H38ΔwbkF did not generate a serological interference in either RBT or CFT, tests that detect anti-O-PS antibodies [3]. However, transient reactions were observed in the S-LPS ELISA, and the interference in this assay (but not in the RBT and CFT) was evident after challenge. This was not unexpected since R vaccines and *B. ovis* induce antibodies to the core epitopes shared by the R and S-LPS that, while not accessible in tests like RBT and CFT, become exposed to antibodies in ELISA and in the fluorescence polarization assay [3]. Therefore, if H38ΔwbkF (or any other R vaccine) is employed in *B. melitensis*-free areas

where the latter two tests are used for surveillance, this cross-reactivity should be considered and the simple and inexpensive RBT implemented as a parallel test in doubtful cases. A more difficult practical problem is posed by the interference of H38ΔwbkF in *B. ovis* serodiagnostic tests. Although more persistent in iELISA than in AGID, in which all animals were negative 27 weeks after vaccination (Figure 3), this is a protracted response that may hamper implementation of test and slaughter combined with H38ΔwbkF vaccination for the eradication of *B. ovis* infection. More information is necessary to better assess the effects of route of vaccination, vaccine dose and the optimal age of sheep at vaccination on this diagnostic interference, as well as the efficacy of the H38ΔwbkF vaccine under field conditions and the potential effects that anamnestic responses after contacts with *B. ovis* field strains may have in vaccinated animals. Given the importance of ewes in the epidemiology of *B. ovis* [10], the vaccination of female sheep would be essential to a suitable control of infection. Like Rev 1, at least the *B. melitensis* B115 spontaneous wzm R mutant is highly abortifacient in pregnant sheep [58] and this and other potential untoward effects of the H38ΔwbkF vaccine in adult sheep need to be investigated. Research is in progress to further define the conditions of field use of H38ΔwbkF.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13567-022-01034-z>.

Additional file 1: Primers used for mutant construction.

Additional file 2: *E. coli* S17 λpir and plasmids used for mutant construction.

Additional file 3: LPS characterization of vaccine candidates by SDS-PAGE electrophoresis-silver staining and Western blot analyses of SDS-proteinase K extracts. Upper panels (from [19]): Analyses of (1) Rev 1, (2) Rev1::Tn7wbdR, and (3) Rev1::Tn7wbdRΔwbkC extracts. Lower panels: Analyses of (4) H38, (5) H38ΔwbkF, (6) Bov::CA and (7) Bov::CAΔwadB extracts.

Additional file 4: Reactivity (%OD) in iELISA using N-formyl-perosamine wild-type S-LPS (A) or N-acetyl-perosamine S-LPS from a wbdRΔwbkC construct (B) and 46 sera from *B. melitensis* culture positive (C+) sheep, 78 brucellosis-free (BF) sheep and rams immunized with Rev1::wbdRΔwbkC.

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Authors' contributions

PMM, RC-A, IM and JMB conceived and coordinated the study. MI supervised the genomic studies. AZ-R, BA-A, MS-B and EM-G participated in the mutant construction and characterization. NV contributed to preliminary experiments in laboratory models. MJM, SA-B, MB and PMM conducted the experiments in mice and rams. PM, JMB, RC-A and IM wrote the manuscript. All authors read and approved the final version of the manuscript.

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Declarations

Ethics approval and consent to participate

Procedures in mice and rams were performed in accordance with the current European (directive 86/609/EEC) and Spanish (RD 53/2013) legislations, supervised by the corresponding Ethical Committee for Animal Experimentation and authorized by the Aragón (reports No. 2014-20, 2014-21 and 2014-24) and Navarra (CEEA 045/12) Governments.

Competing interests

The authors declare that they have no competing interests.

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