



Mucosal immunization with polymeric antigen BLSOmp31 using alternative delivery systems against *Brucella ovis* in rams

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ARTICLE INFO

Keywords:

BLSOmp31-Chitosan microspheres
BLSOmp31-Poloxamer 407-Chitosan gel
Mucosal immunization
Lams
Immunogenicity
Partial protection
Brucella ovis

ABSTRACT

Subcellular vaccines against ovine contagious epididymitis due *Brucella ovis* can solve some shortcomings associated with the use of *Brucella melitensis* Rev 1. We have demonstrated that the parenteral immunization with polymeric antigen BLSOmp31 emulsified in oil adjuvant conferred significant protection against *B. ovis* in rams. In our previous studies, we have characterized chitosan microspheres (ChMs) and a thermoresponsive and mucoadhesive *in situ* gel (Poloxamer 407-Ch) as two novel formulation strategies for the delivery of BLSOmp31 in nasal as well as conjunctival mucosa. In the present work, we evaluated the immunogenicity and protection conferred by the intranasal and conjunctival immunization with these two mucosal delivery systems against *B. ovis* in rams. BLSOmp31-ChM administered by intranasal route and BLSOmp31-P407-Ch applied by intranasal or conjunctival routes induced systemic, local and preputial IgG and IgA antibody response. Neither formulation showed interference in the serological diagnosis. Thus, mucosal immunization using either formulation induced significant specific cellular immune responses (*in vitro* and *in vivo*) and it prevented the excretion of *B. ovis* in semen. Although these vaccines did not prevent infection in immunized rams, colonization reduction of infected organs and bacterial distribution differed significantly between vaccinated and unvaccinated rams.

1. Introduction

Brucella ovis causes a disease characterized by epididymitis and subfertility in rams, and occasional abortions in ewes as well as neonatal death, leading to significant economic loss worldwide. Vaccination is recognized as the most suitable tool for control of the disease in countries with moderate to high prevalence (Blasco, 1990). Nowadays, the live *B. melitensis* Rev. 1 strain is considered the most effective available vaccine against *B. ovis* (Blasco, 1997). However, this vaccine displays a large number of shortcomings, including residual virulence, antibiotic resistance, pathogenicity for humans and, interferences with serodiagnosis which limit its global widespread use

(Moriyón et al., 2004). Consequently, research efforts have been focusing on the development of novel vaccines for controlling *B. ovis* infection. Recently, a mutant *B. ovis* strain lacking a putative ATP-binding cassette transporter ($\Delta abcBA$) and encapsulated with alginate was found to confer protection against *B. ovis* in mice (Silva et al., 2015a). It proved to be safe and also conferred protection against this pathogen in rams but, its use could interfere in serodiagnosis of ovine brucellosis (Silva et al., 2015b).

Therefore, subcellular preparations would offer a safer alternative for controlling *B. ovis* infection (Menzies, 2012). Different studies have previously demonstrated that outer membrane complex preparations of *B. ovis* (Hot Saline extracts (HS)) incorporated in selected adjuvants

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<https://doi.org/10.1016/j.vetimm.2019.02.005>

Received 2 June 2018; Received in revised form 5 February 2019; Accepted 9 February 2019

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conferred a similar degree of protection against *B. ovis* in mice and rams as *B. melitensis* Rev. 1 (Blasco et al., 1993; Muñoz et al., 2006; Da Costa Martins et al., 2012).

Recombinant proteins and DNA vaccines have been explored for their value as potential protective immunogens against *B. ovis* in the mouse model (Estein et al., 2003; Cassataro et al., 2007; Pasquevich et al., 2010, 2011). Among them, a polymeric antigen BLSOmp31 constructed by *Brucella* Lumazina Synthetase (BLS) decorated with a protective epitope of *Brucella* Outer Membrane Protein Omp31 is considered an attractive vaccine candidate against ovine brucellosis (Cassataro et al., 2007; Estein et al., 2009). In fact, parenteral immunization with this immunogen emulsified in oil adjuvant (Incomplete Freund Adjuvant (IFA)) elicited a similar protective immunity against *B. ovis* in mice than *B. melitensis* Rev. 1 (Cassataro et al., 2007). Furthermore, immunization of lambs with this antigen conferred protection against *B. ovis* and it did not interfere in routine serological tests for rough (R) or smooth (S) *Brucella*, suggesting that immunization with BLSOmp31 could be compatible with an eradication brucellosis program (Estein et al., 2009; Díaz et al., 2013).

Since *B. ovis* can invade hosts through the mucosal membranes, it is desirable to obtain a local immune response to block both colonization and disease development. In this regard, mucosal immunization with an appropriate vaccine delivery vehicle and adjuvant has been shown to induce both protective mucosal and systemic immune responses in the absence of needles (Yuki and Kiyono, 2009; Chadwick et al., 2010). Interestingly, there is evidence of a significant degree of compartmentalization within the common mucosal immune system. The use of one mucosal route of immunization will trigger the immune responses locally, systemically, and in at least one distal mucosal site (Wilson and Obradovic, 2015).

Relatively few studies have been carried out using anti-*Brucella* subunit vaccine administered by oral, IN or CONJ routes as an alternative to obtain immunity against this pathogen in mice or guinea pigs with variable results (Bhattacharjee et al., 2002, 2006; Pasquevich et al., 2011; da Costa Martins et al., 2012). As regards, Senevirathne et al. (2019) demonstrated that a cocktail of five conserved antigens of *Brucella* spp. administered by IN in mice conferred significant protection against the *B. abortus* nasal challenge. However, although laboratory animals are a valuable tool to evaluate vaccines in brucellosis, finding in these models cannot easily be extrapolated to susceptible hosts (Silva et al., 2011).

Recently, we reported the physical and chemical properties of two novel chimera BLSOmp31 mucosal delivery systems. In fact, Chitosan Microspheres (ChMs) (Díaz et al., 2016a) and the thermo-responsive and mucoadhesive *in situ* gel composed of Poloxamer 407 and Chitosan (P407-Ch) (Díaz et al., 2016b) were evaluated in lambs. BLSOmp31 adsorbed onto ChMs (BLSOmp31-ChM) administered in nasal mucosa induced systemic IgG and local IgG and IgA specific antibodies. Thus, BLSOmp31-P407-Ch gel formulation induced systemic and local specific antibodies and cellular immune response when administered via conjunctival route (CONJ) (Díaz et al., 2016b).

Based on these findings, ChMs and P407-Ch appear as promising potential delivery systems for BLSOmp31. In this study, we compared the immune responses induced by both formulations using the intranasal (IN) route and BLSOmp31-P407-Ch administered by CONJ route and the protection conferred in rams against experimental challenge with *B. ovis* reference strain.

2. Materials and methods

2.1. Bacteria

B. ovis PA-76250 was used as the challenge strain. Bacterial growth and inoculum preparation were performed as previously described (Estein et al., 2003, 2009).

2.2. Animals

Forty Romney Marsh-Corriedale 6-month-old lambs seronegative to *Brucella ovis* were purchased from a brucellosis-free herd. Lambs remained in BL3 facilities until the end of the studies (Campo Experimental de SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria)), Azul, Buenos Aires, Argentina). All the experiment was carried out following ethical 101 guidelines of the Animal Welfare Committee of the Faculty of Veterinary Medicine, 102 Universidad Nacional del Centro de la Provincia de Buenos Aires (Internal 120 Protocol: 03/2014; Approval date: 28,05, 2014).

2.3. BLSOmp31 production

Recombinant BLSOmp31 was expressed in *Escherichia coli* BL21 (Stratagene, USA), and was purified according to the procedure previously developed by Laplagne et al. (2004).

2.4. Preparation of vaccines

All vaccines had the same concentration of BLSOmp31 (500 µg/animal/dose). BLSOmp31-P407-Ch gel vaccine was prepared and characterized as previously described (Díaz et al., 2016a). Briefly, Poloxamer 407 (16% w/v) (BASF, Germany) was added to an aqueous solution of BLSOmp31. Chitosan (0.25% w/v) and was dissolved in a 0.5% acetic acid solution (Parafarm, Argentina).

ChMs was prepared using the spray-drying technique and BLSOmp31 was adsorbed by ionic interaction to the ChMs surface. As a control vaccine, we used BLSOmp31-IFA as previously described by Estein et al. (2009).

2.5. Experimental design

Scheme of experimental design is summarized in Fig. 1.

2.5.1. Vaccination schedule

Five groups of 8 lambs were immunized as follows: G1) BLSOmp31-ChM (0.5 mL/nostril), G2) BLSOmp31-P407-Ch gel by IN route (0.5 mL/nostril), G3) BLSOmp31-P407-Ch gel by CONJ via (0.05 mL/sac), G4) BLSOmp31-IFA administered by IM route (2 mL/dose) (SIGMA, USA) and G5) an unvaccinated group injected with saline solution by IM. Each lamb was immunized at days 0, 21 and 42 with 500 µg of BLSOmp31/animal.

2.5.2. Clinical inspections

All rams were clinically examined on inoculation day and at days 30 and 70 after challenge. Genital organs were carefully observed and palpated to detect the presence of alterations in epididymis, testis and/or scrotum.

2.5.3. Collection of samples and immunological studies

2.5.3.1. Serum, secretions and seminal plasma. Samples of blood without anticoagulant, nasal, preputial and lacrimal secretions were obtained throughout the study at different intervals to determine IgG and IgA specific antibodies (days 0, 10, 20, 30, 40, 60, 70, 90, 100, 135, 155, 190, 210, 222 (before challenge) and 255, 285, 302 (after challenge)). All samples were obtained as previously described (Estein et al., 2004; Díaz et al., 2016a, 2016b). Seminal plasma was collected after centrifugation of semen samples at days 0, 60, 190 (before challenge) and 255 and 285 (after challenge). All samples were clarified by centrifugation at 6000 rpm at 4 °C for 10 min and supernatants were kept at -20 °C until assayed.

2.5.3.2. Specific antibody detection. Specific IgG and IgA anti-BLSOmp31 levels in serum, and secretions were analyzed by indirect ELISA as described previously (Díaz et al., 2016a, 2016b). Antibody

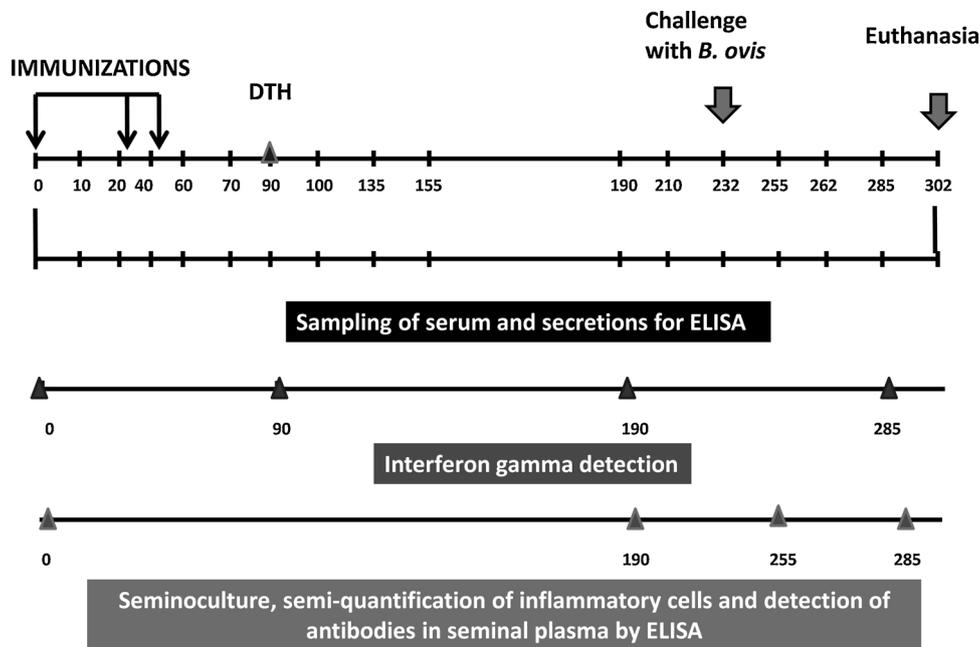


Fig. 1. Schematic representation of experimental design.

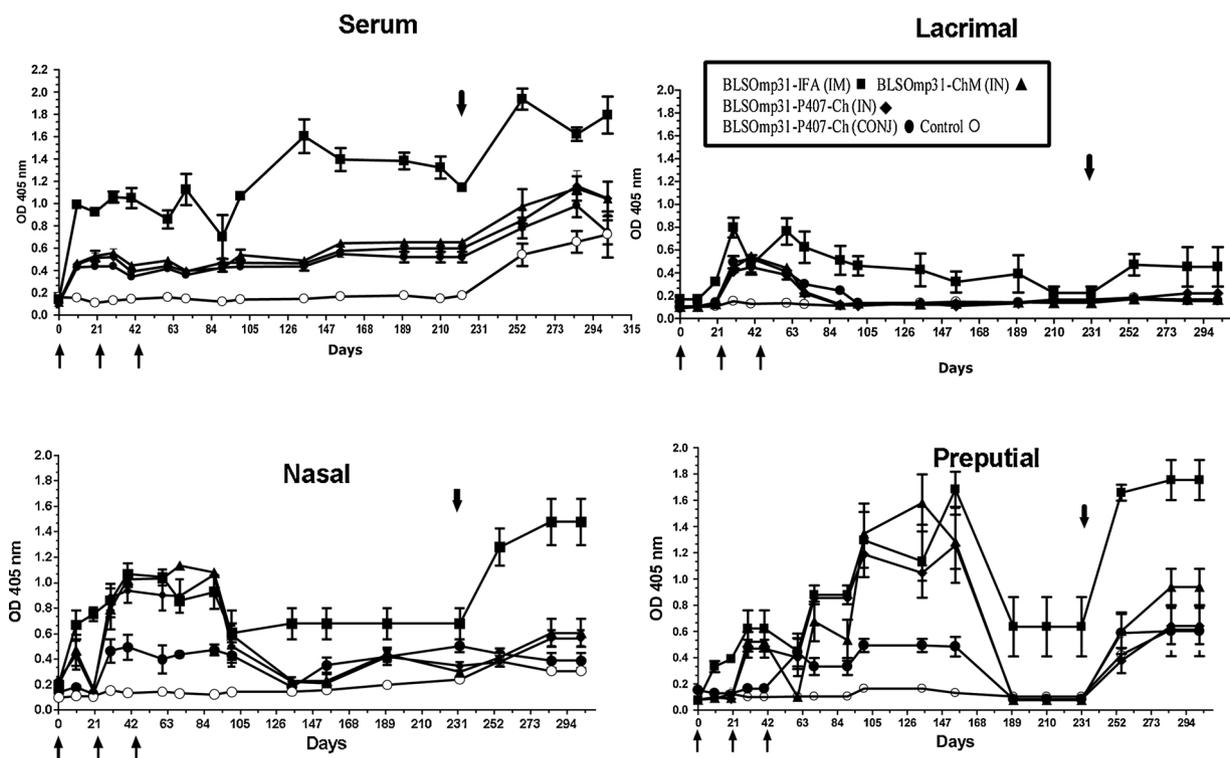


Fig. 2. Kinetics of IgG antibodies in serum, and lacrimal, nasal and preputial secretions induced by IN, CONJ and IM immunization routes using BLSOmp31 as immunogen. IFA: Incomplete Freund Adjuvant, ChMs: Chitosan microspheres and P407-Ch: Poloxamer 407-Chitosan (mucoadhesive *in situ* gel). Rams were immunized as indicated by the three arrows at the bottom. The top arrow indicates the time of *B. ovis* infection. Specific antibodies against BLSOmp31 were evaluated by indirect ELISA. Each value represents the mean \pm SEM of animals per group.

levels were expressed as the arithmetic mean \pm standard errors (SEM) of the OD obtained for rams included in each group.

In addition, to determine the serological interference induced against *B. ovis*, serum samples were assayed in the Agar Gel Immunodiffusion Test (HS-AGID) using HS antigen from *B. ovis* following OIE instructions (OIE, 2015). The ELISA with HS antigen from *B. canis* (HS-ELISA) was performed with the same purpose, and as described previously (López et al., 2005).

2.5.3.2.1. Semen collection. Semen samples were obtained by electroejaculation with EE Electrojac V® stimulator (Ideal instruments, Neogen Company, Lansing MI, USA) at days 0, 60, 190 (before challenge) and 255, 285 (after challenge). Samples were processed as follow:

2.5.3.3. Semi-quantification of inflammatory cells. The presence of white blood cells (WBC) were evaluated by staining smears of semen with

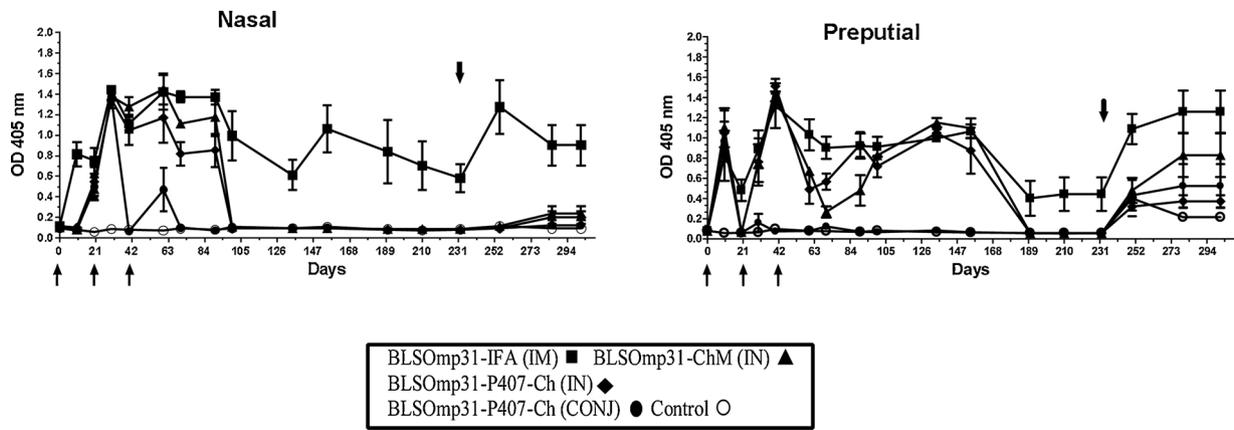


Fig. 3. Evolution of IgA antibody levels in nasal and preputial secretions elicited by IN, CONJ and IM immunization routes using BLSOmp31 as immunogen. IFA: Incomplete Freund Adjuvant, ChMs: Chitosan microspheres and P407-Ch: Poloxamer 407-Chitosan (mucoadhesive *in situ* gel). Rams were immunized three times (bottom arrows). The top arrow shows the time of *B. ovis* infection. Specific antibodies against BLSOmp31 were evaluated by indirect ELISA. Each value represents the mean \pm SEM of animals per group.

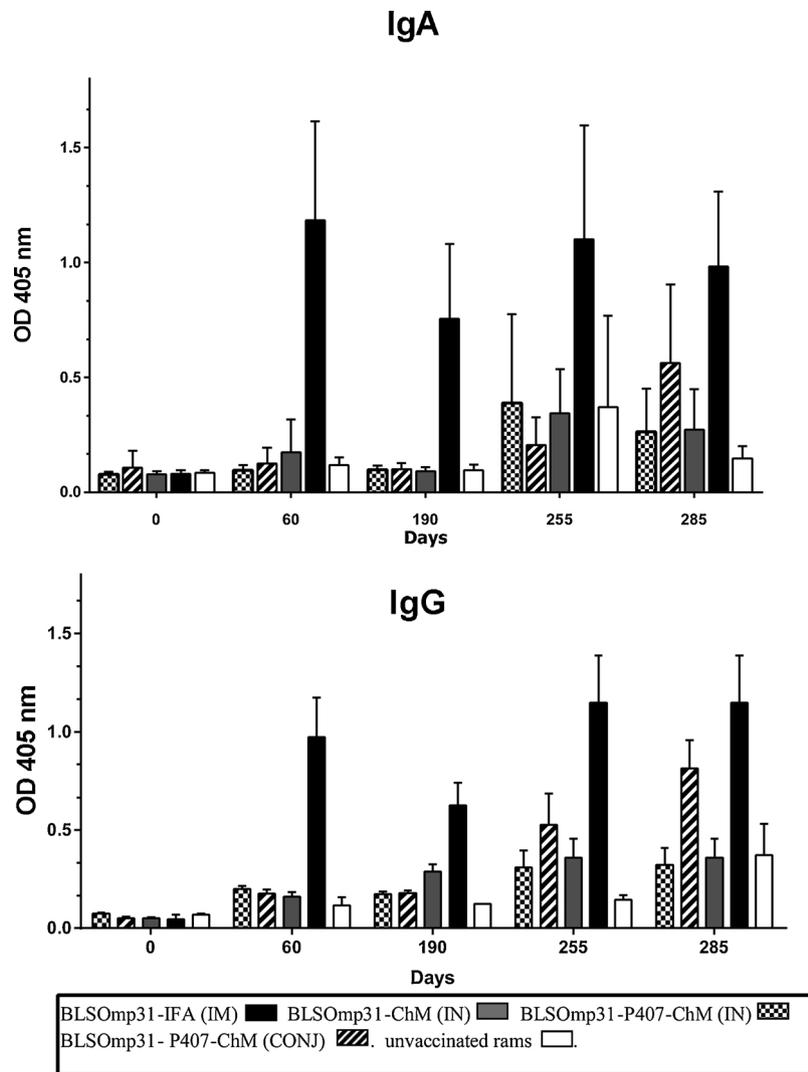


Fig. 4. IgA and IgG antibody levels in seminal plasma. Samples of semen were taken at days 0, 60, 190 (before challenge) 255 and 285 (after challenge). IFA: Incomplete Freund Adjuvant, ChMs: Chitosan microspheres and P407-Ch: Poloxamer 407-Chitosan (mucoadhesive *in situ* gel). Each value represents the mean \pm SEM of animals per group.

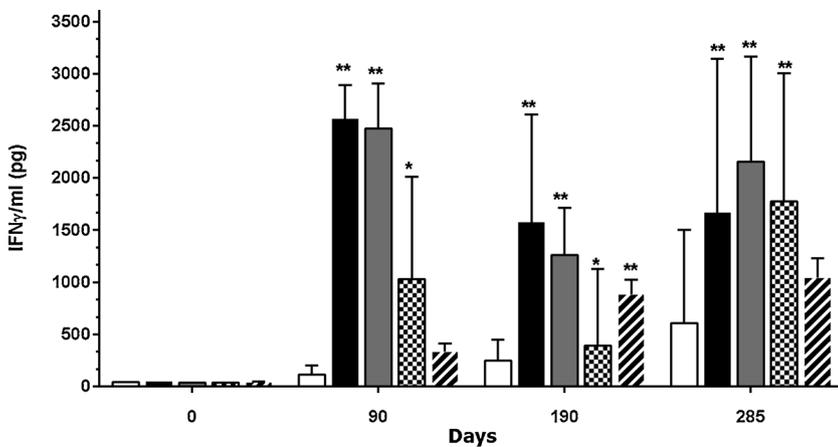


Fig. 5. Cellular immune responses against BLSOmp31: A) *in vitro* and B) *in vivo*. A) Antigen-specific IFN- γ response of whole-blood cells from lambs immunized with different strategies of vaccination. Samples from all rams were taken at days 0, 90, 190 (before the challenge) and 285 (after the challenge). Results are expressed as OD values and error bars indicate SEM from the mean. B) Increase in the skin thickness following an intradermal injection with BLSOmp31 at day 90 (before the challenge). Measures were taken 72 h after BLSOmp31 injection. Values expressed in mm represent the mean differences between skin thickness after and before injection in the same animal. Values significantly different from unvaccinated control group are indicated: * $p < 0.05$; ** $p < 0.01$.

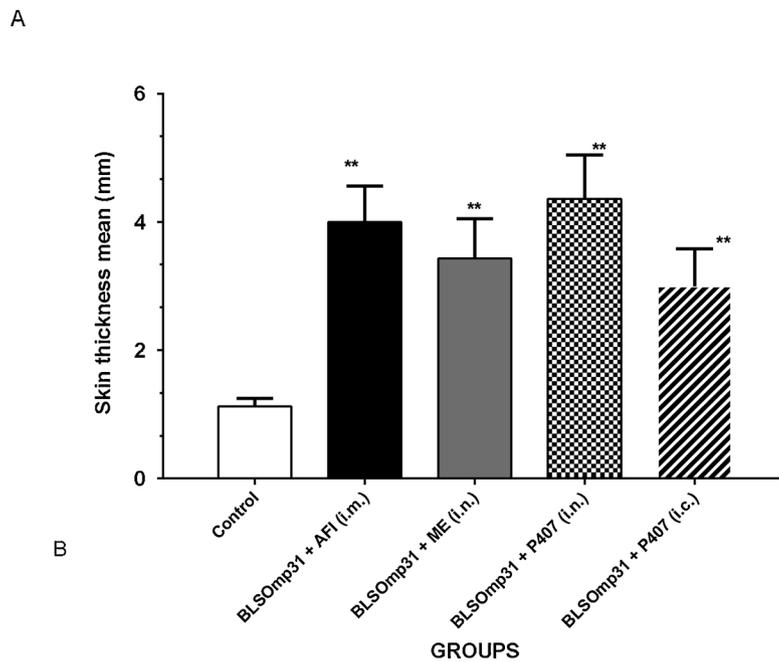


Table 1
Seminoculture and presence of inflammatory cells in semen of rams per group after challenge with *B. ovis* throughout the experiment.

GROUPS OF IMMUNIZATION	SEMINOCULTURE POSITIVE RAMS/GROUP (%)	INFLAMMATORY CELLS IN EYACULATE RAMS/GROUP (%)		
		ABSCENCE	MILD	INTENSE
CONTROL (n = 6)	4/6 (67%)	0 (0%)	0 (0%)	6/6 (100%)
BLSOmp31-IFA (IM) (n = 6)	2/6 (33%)	4 (67%)	2 (33%)	–
BLSOmp31-ChM (IN) (n = 7)	3/7 (43%)	4 (57%)	3 (43%)	–
BLSOmp31-P407-ChM (IN) (n = 8)	5/8 (83%)	4 (50%)	4 (50%)	–
BLSOmp31-P407-ChM (CONJ) (n = 6)	4/6 (67%)	3 (50%)	3 (50%)	–

May Grünwald -Giemsa stain and scoring stained smears as: absence when inflammatory cells were completely absent, mild or intense when the number of inflammatory cells were 0–10 (mild) or greater than 10 WBC (intense) per high-power microscopic field (400x), respectively (Kott et al., 1988).

2.5.3.3.1. Seminoculture. Aliquots of semen were also seeded in modified Skirrow’s medium and incubated at 37 °C in a 5% CO₂ atmosphere for ten days.

2.5.3.3.2. Specific antibodies in seminal plasma. Specific IgG and IgA anti-BLSOmp31 levels were analyzed by indirect ELISA and were expressed as explained for other secretions.

2.5.4. Whole blood

To measure T cell- responses *in vitro*, sterile heparinized blood samples were collected by jugular venipuncture prior to immunization and at days 0, 90, 190 (before challenge) and 285 (after challenge).

2.5.5. IFN- γ production

Whole-blood samples were stimulated with BLSOmp31 (20 μ g/well), Pokeweed mitogen (10 μ g/well) (Sigma, USA), or medium alone. Cultures were incubated for 48 h at 37 °C in a 5% CO₂ atmosphere. Supernatants were collected after 48 h of culture and were assayed for ovine IFN- γ by using a commercial sandwich ELISA test (Bovigam TM, USA).

Table 2

Comparison of distribution and level of *B. ovis* PA infection in rams vaccinated with experimental vaccines and challenge with this bacteria. Groups of rams were immunized three times each 21 days with BLSOmp31-IFA by IM route, BLSOmp31-ChM by IN route, BLSOmp31-P407-Ch by IN route and BLSOmp31-P407-Ch by CONJ route. An unvaccinated control group was included. One hundred ninety days after last immunization, rams were inoculated with *B. ovis* PA (1.09×10^9 Colony Forming Unit (CFU)) and were necropsied 80 days later. Results of different genital and extragenital organs were shown.

Groups of immunization	Epididymides Heads	Tails	Testes	Ampullae	Vesicular glands	Spleen	Liver	Lymph Nodes
Control (n = 6)	5 83%	6 100%	6 100%	4 67%	5 83%	3 50%	3 50%	3 50%
BLSOmp31-IFA (IM) (n = 6) FET (p value)	1 17% 0.05	1 17% 0.01	1 17% 0.02	0 0% 0.05	0 0% 0.01	1 17% 0.54	1 17% 0.54	1 17% 0.54
BLSOmp31-ChM (IN) (n = 7) FET (p value)	1 15% 0.04	2 33% 0.05	2 33% 0.04	0 0% 0.02	0 0% 0.02	0 0% 0.07	1 14% 0.07	1 14% 0.07
BLSOmp31-P407-ChM (IN) (n = 8) FET (p value)	1 13% 0.03	3 38% 0.03	6 75% 0.03	1 13% 0.09	0 0% 0.01	0 0% 0.05	1 13% 0.05	1 13% 0.05
BLSOmp31-P407-ChM (CONJ) (n = 6) FET (p value)	0 0% 0.01	1 17% 0.01	2 33% 0.03	1 17% 0.24	3 50% 0.01	3 50% 0.05	2 33% 0.05	3 50% 0.05
Global FET (p value)	0.0027	0.02	0.03	0.05	0.048	0.02	0.56	0.37

FET: Fisher's Exact Test. Fisher's exact statistical differences with respect to unvaccinated control group (FET) and between groups were calculated (Global FET). Significant p values were remarked in bold letter (p < 0.05).

^aVesicular glands.

Total of genital or extragenital organs per group from which *Brucella ovis* PA was isolated.

Homogenates of whole genital and extragenital organs (lobe of liver and portion of spleen) were seeded in Skirrow's medium.

^{as}described in materials and methods.

2.5.5.1. Delayed-type hypersensitivity response to BLSOmp31. On day 90, intradermoreaction test to BLSOmp31 was performed as previously described (Díaz et al., 2013) to evaluate the specific cellular immune responses. The skin thickness was measured with a digital Vernier caliper just before injection (initial skin thickness) and 72 h later.

2.6. Challenge

Challenge inoculation with *B. ovis* PA was performed 190 days after the last immunization. Each ram received 1.09×10^9 Colony Forming Units (CFU) (as was shown by viable counts made on the day of inoculation) in a total volume of 120 μ L both conjunctivally (30 μ L/sac) and preputially (60 μ L). After challenge, each group of rams was allotted to separate pens without contact between groups.

2.7. Bacteriological examination

Animals were euthanized 80 days after challenge and bilateral samples of epididymides (head and tail), testes, ampullae, vesicular glands and cranial (retropharyngeal) lymph nodes were taken for bacteriological studies. In addition, a portion of spleen and a lobe of liver were taken. In total 14 samples were taken for each animal. Whole organs were homogenized after adding sterile saline by using a Stomacher (Stomacher 80 Biomaster Seward, Norfolk, UK). One mL of each homogenate was seeded of modified Skirrow's medium (duplicate) and incubated using the same procedures used for semen culture. A ram was classified as infected when *B. ovis* was isolated from any of the 14 samples taken at necropsy. An organ was considered as infected when at least one *B. ovis* CFU (Colony Forming Units) was isolated from any of the samples seeded (Estein et al., 2009). All samples were tested by culture on individual samples. Isolates were identified using routine methods (Alton et al., 1988).

2.8. Statistical analysis

Data from ELISA and IFN- γ assay were analyzed by ANOVA followed by Tuckey *post-hoc* test. Means were reported with standard errors (SEM). Global Fisher's Exact Test and Fisher's exact test were used

to analyzing bacteriological results (p values < 0.05 were considered significant). The analysis was performed using SAS v 9.3 (2012).

3. Results and discussion

Developing new vaccines alternative to the *B. melitensis* Rev 1 vaccine for the prevention of ovine brucellosis caused by *B. ovis* requires intensive research aimed at the discovery of novel effective antigens, optimal adjuvants, and vaccine delivery systems.

B. ovis is mainly venereally transmitted in rams (Bulgin, 1990), and the bacteria gains further access to the body by penetrating mucosal membranes lining the respiratory airways, gastrointestinal tract and/or conjunctiva. Importantly, it was demonstrated that *B. ovis* generates an early and protective immunity at the main portals of entry (da Costa Martins et al., 2012), an observation that provides a strong rationale for developing a vaccine strategy based on the delivery of immunogens to mucosal surfaces. In addition, previous studies in mice demonstrated that IN immunization can induce mucosal immunity in both the respiratory and the genital tracts and protection against female reproductive pathogens, such as *Chlamydia* spp. (Hickey et al., 2004; Olsen et al., 2010).

Subcellular vaccines are advantageous because they are safer than live vaccines, however, an important limitation of these vaccines is that they cannot elicit long lasting protective cell-mediated immunity because they are unable to replicate. Consequently, developing effective mucosal vaccines requires the identification of potent and appropriate adjuvant/s and/or delivery systems and the selection of an effective route of immunization in order to achieve potent protective immune responses against the pathogens responsible for mucosal infections.

In this study, we continue a series of previous investigations aimed at developing an effective and safe subcellular vaccine against *B. ovis* infection in rams. Previous results based on two controlled vaccine delivery systems for mucosal immunization with BLSOmp31 (BLSOmp31-ChM and BLSOmp31-P407-Ch) (Díaz et al., 2016a, 2016b) prompted us to evaluate and compare protective and immune responses elicited by these formulations against ovine contagious epididymitis caused by *B. ovis*.

We initially vaccinated four groups of 8 lambs at days 0, 21 and 42

with 500 µg of BLSOmp31/dose animal using the IN, CONJ and IM routes, as described in the M&M section. One unvaccinated group of 8 lambs was used as a control. Analysis of IgG antibody titers upon using distinct vaccination strategies in serum, nasal, seminal plasma, and preputial secretion is shown in Fig. 2 and Fig. 4. Overall, the data shown a significant but variable increase in IgG antibody titers against BLSOmp31 in serum, lacrimal, nasal and preputial secretions compared to the unvaccinated control after immunizations ($p < 0.05$) (Fig. 1). Interestingly, BLSOmp31-IFA by IM route induced the highest levels of IgG antibodies in all samples collected (Figs. 2 and 3). However, IgG antibody levels induced in lacrimal and nasal secretions using either formulation declined faster than preputial samples. In addition, preputial secretions of rams immunized by IN route showed similar kinetics of IgG levels than parenteral immunization. In contrast, CONJ immunization induced lower levels of IgG antibodies in preputial as well in lacrimal secretions than IN route (Fig. 2). However, *B. ovis* challenge stimulated a quick specific anamnestic antibody response in serum, nasal and preputial secretions that were maintained until the necropsy of animals in all groups.

We also evaluated levels of secretory IgA (s-IgA) isotype. The findings indicated that rams immunized by IN and by IM routes had significant levels of anti-BLSOmp31 specific antibodies in nasal and preputial secretions that increased significantly after the first immunization compared to unvaccinated rams ($p < 0.05$) (Fig. 3). Conversely, immunization through CONJ route only induced low level of s-IgA antibodies in lacrimal (data not shown) and nasal secretions (Fig. 3) which subsequently quickly declined. Interestingly, s-IgA anti-BLSOmp31 titers increased in preputial secretion and in seminal plasma after challenge (Figs. 3 and 4). Contrary to other studies (Neutra and Kozłowski, 2006), parenteral immunization induced significant levels of s-IgA antibodies in all secretions ($p < 0.05$) which increased significantly in preputial samples, and in seminal plasma after *B. ovis* inoculation ($p < 0.05$) (Figs. 3 and 4).

We previously demonstrated that both controlled vaccine release systems are able to protect BLSOmp31 structure, while also enhancing its delivery *in vitro* (Díaz et al., 20016a, Díaz et al., 2016b). These features improve the immunogen uptake via the M or Dendritic Cells in immune inductive sites, and also enhance specific immune response in Nasal or Conjunctival Associated Lymphoid Tissues (NALT or CALT, respectively) (Meeusen et al., 2004). Also, it has been reported that IN immunization can induce mucosal immunity in both the respiratory and the genital tracts in mice (Hickey et al., 2004; Olsen et al., 2010).

Although s-IgA is the main immunoglobulin isotype induced and produced at mucosal surfaces by activated B cells in Mucosa Associated Lymphoid Tissue germinal centers or lymph nodes, IgG isotype locally produced or derived by transudation from the circulation also contributed to the mucosal immune defense (Chentoufi et al., 2010). In our study, the presence of both specific isotypes in preputial secretions could be the result of a preferential migration pattern from NALT or CALT to genital mucosa of mucosally primed T and B lymphocytes, resulting in the generation of specific antibodies in preputial mucosa. On the other hand, levels of s-IgA and IgG antibodies increased significantly after challenge in rams immunized by CONJ route, suggesting that bacterial inoculation may recall antigen-specific memory cells eliciting a specific and anamnestic immune response in the genital tract (Fig. 4).

It has been proposed that immunogens that are able to evoke a strong T cell-mediated immunity response are those of choice for vaccine development, since they are important for protection against intracellular pathogens such as *Brucella* spp. (Martirosyan et al., 2011). Specific IFN- γ secretion is critical for the activation of macrophages, a mechanism considered crucial for the development of strong protective immune responses against intracellular *Brucella* infection and also for inducing clearance of the bacteria from the infected host (Baldwin and Winter, 1994). In the present work, a robust and significant IFN- γ production cell-immune response was stimulated by vaccination.

Furthermore, this response was increased after challenge in groups immunized by parenteral or IN ($p < 0.01$) or by CONJ routes ($p < 0.05$) compared to control group (Fig. 5). Similarly, BLSOmp31 elicited significantly higher Th1 DTH reaction in all immunized rams after BLSOmp31 intradermal injection ($p < 0.01$) (Fig. 5). Taken together, these results show that, using either vaccine delivery system BLSOmp31-ChM or BLSOmp31-P407-Ch, the IN route stimulated both the innate and adaptive immunity resulting in an anamnestic humoral and cellular immune responses that account for the persistence of immune memory.

The WBC count in the semen is considered a good parameter to evaluate *B. ovis* infection in the genital tract of lambs. A previous investigation conducted in endemic flock showed that 45% of seropositive animals to *B. ovis* excrete this bacterium in semen while 100% of seropositive rams had higher number of neutrophils (Picard-Hagen et al., 2015). In our study, we were not able to find inflammatory cells in the semen samples from any rams prior to challenge. Interestingly, although all rams were positive after challenge, neutrophil counts were not present or were lower in animals immunized by parenteral, IN or CONJ routes compared to unvaccinated rams (Table 1).

Although forty rams were included in the study, seven rams from different groups died before the challenge for reasons not related to experiment. The infection rate obtained by *B. ovis* inoculation was considered acceptable since 67% of rams from the control group had positive seminal bacteriological culture throughout the study and specific anti-*B. ovis* antibodies were detected by specific tests in serum of all animals after the challenge (data not shown). However, significant testicular alterations were not detected during the clinical inspection. In fact, at necropsy, 80% of the unvaccinated rams showed macroscopic but not pathognomonic histopathological alterations such as testicular asymmetry, adhesion between the tunica albuginea and segments of the epididymis, congestion and edema in the tunica vaginalis (data not shown).

Bacteriological results obtained after the necropsy of experimental animals are summarized in Table 2. Although *B. ovis* was isolated from all control and immunized rams, IN immunization using either BLSOmp31-ChM or BLSOmp31-P407-Ch was able to significantly reduce the number of infected/total organs similarly to the control vaccine (BLSOmp31-IFA) ($p < 0.05$). In agreement with that, the number of *B. ovis* isolated from a majority of organs in rams immunized by IM and mucosal routes was low (≤ 15 CFU) while in control rams were high (≥ 250 CFU) (data not shown). When the proportion of infected organs was considered, significant differences were observed in groups immunized with BLSOmp31-IFA (IM), BLSOmp31-ChM (IN) and with BLSOmp31-P407-ChM (CONJ) with respect to unvaccinated animals. As shown in Table 2, these differences were observed mainly when heads and tails of epididymides, testes, ampullae and vesicular glands were compared.

In contrast to previous studies, rams vaccinated with our reference vaccine (BLSOmp31-IFA) resulted bacteriologically positive. We attributed the discrepancies in the degree and distribution of bacterial colonization to the euthanasia time. In fact, in this study, we changed the period challenge-euthanasia from 6 to 8 months to 2 months with the goal to shorten the length of the experiment, as published in other reports (Blasco et al., 1993; Muñoz et al., 2006; Silva et al., 2015b). Thus, we speculate that bacterial infection could have been solved by immune mechanisms stimulated by the different strategies of immunization if we had further prolonged the experiment.

4. Conclusion

In summary, our results indicate that mucosal immunization with BLSOmp31-ChM or BLSOmp31-P407-Ch gel could be promising strategies which induced both local and systemic immune responses. Although, these vaccines do not provide sterile immunity, they reduce *B. ovis* excretion significantly in semen. This is highly relevant since this

is the most important form of transmission of ovine brucellosis. In addition, the lack of serological interference and safety encourage us to further investigate these immunization strategies against *B. ovis*, on this occasion, using an extended time before performing euthanasia.

Conflict of interest

There is no conflict of interest.

Authors' contributions

S.M.E and A.G.D conceived and designed the experiments. A.G.D., D.A.Q. and MC performed the experiments. M.A.R. and S.M.E. analyzed the data. S.M.E., F.A.P., S.D.P., R.P.P., F.A.G. and V.Z. contributed with reagents, materials, analysis tools and with their expertise on the subject. S.M.E. and A.G.D wrote the paper. All authors reviewed, commented, and approved the manuscript.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina) (to S.M.E.). S.M.E., D.A.Q., V.Z., M.C. and F.A.G. are members of the Research Career of CONICET (Argentina). A.G.D. is recipient of a fellowship from CONICET (Argentina). The authors thank Dr. Carlos Suarez critical revision of the manuscript.

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