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Immune response induced by conjunctival immunization with polymeric antigen BLSOmp31 using a thermoresponsive and mucoadhesive *in situ* gel as vaccine delivery system for prevention of ovine brucellosis

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Abstract

Control of ovine brucellosis with subcellular vaccines can solve some drawbacks associated with the use of *Brucella melitensis* Rev.1. Previous studies have demonstrated that the polymeric antigen BLSOmp31 administered by parenteral route was immunogenic and conferred significant protection against *B. ovis* in rams. Immunization with BLSOmp31 by conjunctival route could be efficient for the induction of mucosal and systemic immune responses. In this work, we evaluated the conjunctival immunization using a thermoresponsive and mucoadhesive *in situ* gel composed of Poloxamer 407 (P407) and chitosan (Ch) as vaccine delivery system for BLSOmp31 in rams. Serum samples, saliva, lacrimal, preputial and nasal secretions were analyzed to measure specific IgG and IgA antibodies. Cellular immune response was evaluated *in vivo* and *in vitro.* Immunization with BLSOmp31-P407-Ch induced high IgG antibody levels in serum and preputial secretions which remained at similar levels until the end of the experiment. Levels of IgG in saliva, lacrimal and nasal secretions were also higher compared to unvaccinated control group but decreased more rapidly. IgA antibodies were only detected in nasal and preputial secretions. BLSOmp31-P407-Ch stimulated a significant cellular immune response *in vivo* and *in vitro*. The induction of systemic and local immune responses indicates a promising potential of P407-Ch for the delivery of BLSOmp31 by conjunctival route.

Keywords: BLSOmp31-Poloxamer407-Chitosan formulation; conjunctival immunization; lambs; immunogenicity; Brucella ovis

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1. Introduction

Brucella ovis (*B. ovis*) causes genital disease in sheep characterized by epididymitis and reduced fertility in rams, and by occasional abortions in ewes. Infection by *B. ovis* is a major cause of economic loss in flocks worldwide and vaccination is recognized as the most suitable tool for control of the disease in endemic countries (Blasco 1990). Nowadays, the live attenuated vaccine *B. melitensis* Rev.1 is the most effective available vaccine against ovine brucellosis administered by subcutaneous or conjunctival routes (Blasco, 1997). Nevertheless, among all the drawbacks of the attenuated strain, Rev.1 displays high residual virulence for both animals and humans and it is resistant to streptomycin. Another inconvenience in the use of this vaccine is the interference with the serological diagnosis, avoiding the differentiation between infected and vaccinated animals (Blasco, 1997). In addition, vaccination with Rev.1 is not allowed in countries officially free from *B. melitensis*. Consequently, there is a need for development of new vaccines and subcellular preparations would offer a safer alternative.

Polymeric antigen BLSOmp31 formulated in oil adjuvant administered by parenteral route conferred significant protection against *B. ovis* in mice and rams (Cassataro et al., 2007; Estein et al., 2009; Díaz et al., 2013). Moreover, lack of interference in the diagnostic tests, the intrinsic adjuvanticity properties and innocuousness have converted BLSOmp31 into an attractive anti-*Brucella* vaccine candidate (Estein et al., 2009, Clausse et al., 2013).

Although subunit vaccines are generally regarded as safe, they often need adjuvants or delivery systems to enhance the immune response (Petrovsky and Aguilar, 2004). Sustained release depot systems have been widely investigated for their potential to improve the efficacy of subunit vaccines. However, most of them have been studied to be used for loading and controlling release of various therapeutic agents (Myschik et al., 2009). Among them, thermally induced gelling systems such as Poloxamers have gained enormous attention over the last decade (Ricci et al., 2005; Dumortier et al., 2006).

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Poloxamer block copolymers have been used for many different pharmaceutical applications including parenteral, nasal, ophthalmic and topical formulations because of its emulsifying, solubilizing, and stabilizing properties (Dumortier et al., 2006). Poloxamer is a linear triblock polymer consisting of a polypropylene oxide block (PPO) between two polyethylene oxide blocks (PEO). Poloxamer 407 (P407) has a molecular weight of approximately 12,600 (9,840–14,600) and the lengths of PEO and PPO are 95–105 and 54–60 monomeric units respectively (Kabanov et al., 2002). Given that the PPO is less hydrophilic than the PEO, the copolymer easily self-assembles forming micelles. If this solution is sufficiently concentrated, the micellization is followed by gelation (Cabana et al., 1997. Additionally, P407 presents the property of reverse thermogelation in which aqueous solutions are free-flowing liquids at room temperature and form stable gels at physiological temperature. Therefore, P407 can be administered in liquid form and it provides a long-term sustained release of drugs at body temperature (Dumortier et al., 2006; Kabanov et al., 2002). In addition, some hydrophilic polymers such as chitosan (Ch) (Gratieri et al., 2010; Gratieri et al., 2011) have been incorporated into Poloxamer gels as auxiliary agents for controlled drug release and/or improvement of their capability of adhesion to the mucosal epithelium. These hydrogels are also biodegradable, non-toxic and stable, therefore they could be suitable for use as a controlled release delivery system for vaccine immunogens (Singh-Joy et al., 2008, Kojarunchitt et al., 2015).

Mucosal immunization routes possess certain advantages over the parenteral route including activation of dual immunity i.e. at mucosal sites and serum. Evidence from many studies has confirmed that the mucosal system is inter-connected. It is called common mucosa immune system because the stimulation at one mucosal site can lead to the generation of effector immune cells in local as well as distal mucosal surfaces (Pulendran et al., 2011). In addition to these immunological reasons, mucosal vaccination can also be

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safer and easier to dispense than traditional (parenteral) vaccines (Dietrich et al., 2003, Chadwick et al., 2010).

Mucosal surfaces, mostly the subepithelial regions, are enriched in immunocompetent B and T lymphocytes, as well as antigen-presenting cells (APCs). These cells are organized into the mucosa-associated lymphoid tissue (MALT) found in various sites of the body (Pulendran et al., 2011). In the eye, the conjunctiva, the palpebral area and the eye lachrymal drainage system are provided with an associated lymphoid tissue (termed CALT, conjunctiva-associated lymphoid tissue) (Petris et al., 2007) containing the specialized antigen sampling microfold-cells (M cells) (Liu et al., 2005) present at other mucosa.

Regarding the behavior of *B. ovis* during the infection and colonization processes, the delivery of an immunogen such as BLSOmp31 through mucosal surfaces, for instance conjunctival mucosa, is of remarkable interest in order to generate effective immunity at the major portals of entry for this microorganism.

The aim of this study was to evaluate the immunogenicity induced by the conjunctival immunization with polymeric antigen BLSOmp31 using a thermoresponsive and mucoadhesive *in situ* gel as a vaccine delivery system against *B. ovis* in rams.

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2. Materials and methods

2.1. Animals

Sixteen Corriedale 5-month-old lambs were obtained from a brucellosis-free herd. All experimental protocols in sheep were approved by The Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Facultad de Ciencias Veterinarias, (Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina; http://www.vet.unicen.edu.ar/index.php/facultad/comite-bienestar-animal).

2.2. Production of the chimera BLSOmp31

Recombinant chimeric protein based on the addition to the N-termini of BLS of a 27-mer peptide containing the exposed loop epitope of Omp31 (BLSOmp31) was expressed in *Escherichia coli* BL21 (Stratagene, La Jolla, CA), and was purified as previously described by Laplagne et al., 2004.

2.3. Preparation of BLSOmp31-P407-Ch gel vaccine

Poloxamer 407 (16% w/v) (BASF, Ludwigshafen, Germany) was added to an aqueous solution of BLSOmp31. To enhance the mucoadhesive capability of the formulation, chitosan (Ch) (0.25 % w/v) dissolved in 0.5% acetic acid solution (Parafarm, Argentina) was incorporated into the preparation. All reagents were dispersed at room temperature and then mixed on a magnetic stirrer at 4ºC until a homogeneous solution was obtained.

2.4. Characterization of BLSOmp31-P407-Ch in situ gelling formulation

2.4.1. Rheological analysis

The solid–gel transition temperature (T sol/gel) was measured using a Rheoplus MCR301 Anton Paar (Germany) with a stainless steel cone, plate geometry (50 mm diameter, 2° angle and a gap of 50 µm between the cone and plate) and a temperature ramp step

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oscillation procedure. Samples were carefully located onto the plate of the rheometer, thus ensuring that formulation shearing was minimized, and left to equilibrate for at least 10 min prior to analysis.

2.4.2. In vitro release of BLSOmp31 from P407-Ch formulation

To evaluate the diffusion of the protein out of the gel in aqueous media, a tube with 1 mL of the formulation was cooled at 39°C in a thermo-controlled water bath. When the temperature inside the tube reached 39°C and gelation occurred, a buffer solution (3.5 mL, PBS, pH 6.85) was incorporated with mild magnetic stirring. Samples of 250 µL were withdrawn at selected time intervals (between 0 to 270 minutes) and were replaced by the same volume of fresh buffer. Each experiment was performed in triplicate. The amount of BLSOmp31 released from the gel was determined by a capture ELISA designed for this purpose.

2.5. Experimental design

2.5.1. Immunization protocol

Animals were randomly distributed into two experimental groups: G1 (n=8) vaccinated with BLSOmp31-P407-Ch gel by conjunctival route with a syringe (0.05 mL in each sac) three times at 0, 21 and 42 days with 500 µg of BLSOmp31/dose and G2 (n=8) unvaccinated group (control). After conjunctival immunizations, animals were inspected for local irritation symptoms.

2.6. Sample collection

2.6.1. Serum and whole blood

Heparinised whole blood and blood samples without anticoagulant were collected by jugular venipuncture using adequate tubes (BD Vacutainer) prior to immunization and at

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different intervals throughout the course of the study. Serum was separated and conserved at -20° until used.

2.6.2. Secretions

Samples of saliva and nasal, preputial and lacrimal secretions were obtained throughout the study at different intervals. Saliva and nasal secretions were collected by using synthetic soft sponges held by forceps which were rubbed onto the corresponding mucosal surfaces (Stanley et al., 2004; Díaz et al., 2016).

Preputial and lacrimal secretions were obtained by placing a cotton swab onto the preputial mucosa and in the conjunctival sacculus, respectively. Proteins were recovered from the swabs by overnight diffusion at 4°C in 0.75 mL of PBS with 0.01% sodium azide. All samples were clarified by centrifugation and supernatants were kept at -80ºC until assayed following the method described by Estein et al., 2004.

2.7. Immunological studies

2.7.1. Analysis of humoral immune response

2.7.1.1. Detection of specific antibody levels in samples of serum and secretions

Specific IgG and IgA anti-BLSOmp31 levels in serum and secretions were analyzed by indirect ELISA as described previously (Estein et al. 2009; Díaz et al., 2013; Díaz et al., 2016). Briefly, polystyrene microplates (NUNC Maxisorp, Denmark) were coated with BLSOmp31 and were incubated with diluted samples (1/200 (serum) and 1/50 (secretions)). Bound antibodies were detected by adding a donkey anti-sheep IgG (whole-molecule) conjugated to horseradish peroxidase (Sigma, St. Louis, MO) or a rabbit anti-sheep IgA conjugated to horseradish peroxidase (AbD Serotec). Reaction was developed with ABTS-H_2O_2. Absorbance was read at 405 nm in automatic ELISA reader (Titertek,

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Multiskan EX, Labsystems). Antibody levels (IgG and IgA) were expressed as the arithmetic mean ± SD of the O.D obtained for rams included in each group.

2.7.1.2 Agar Gel Immunodiffusion (AGID) test

All serum samples were assayed in AGID test to determine the serological interference induced against *B. ovis.* This test was done following OIE instructions (OIE, 2015). The heat-extracted *B. ovis* antigen (HS) was provided by SENASA (Servicio Nacional de Sanidad y Calidad Agroalimentaria, Argentina). Interpretation of results was made after incubation at room temperature for 48 h in a humid chamber.

2.7.2. Analysis of cellular immune response

2.7.2.1. γ–IFN production

Blood samples were collected in heparinised tubes (BD Vacutainer®) at days 55 and 180. Whole-blood cultures were incubated with BLSOmp31 (20 μg/well) in a 5% CO_2 atmosphere as previously described (Estein et al., 2009). Control cultures were incubated with Pokeweed mitogen (Sigma) (10 μg/well) and PBS. Supernatants were harvested after 48 h and were assayed for ovine γ–IFN by using a sandwich ELISA kit (Mabtech, Sweden). Concentration of ovine γ–IFN (pg/mL) was calculated according to the manufacturer's instructions.

2.7.2.2. Intradermal reaction to BLSOmp31

Ninety days after the first immunization, rams were injected intradermally into a wool-free region of the inside thigh with BLSOmp31 (Díaz et al., 2013). The skin thickness was measured before injection and 72 h later by using a Vernier caliper.

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2.8. Statistical analysis

Data from serological and cellular immune responses were analyzed using ANOVA by ANOVA followed by Tukey *post hoc* tests. The analysis was performed using SAS v 9.3 (2012).

3. Results and discussion

It has been previously demonstrated that the polymeric antigen BLSOmp31 emulsified in Incomplete Freund Adjuvant is able to stimulate an effective immune response (Estein et al., 2009, Díaz et al., 2013) and to confer a significant level of protection against *B. ovis* in rams (Estein et al., 2009). However, these studies were based on the use of BLSOmp31 injected by the parenteral route. Since *B. ovis* gains entry through mucosal tissues such as the reproductive and gastrointestinal tracts or the respiratory airways and/or the surface of the eye (Alton et al., 1988; Bulgin, 1990) it would be desirable to obtain a local mucosal immune response to block both infection (*i.e.* colonization) and disease development.

Recently, we have characterized spray dried chitosan microspheres as a carrier of BLSOmp31. Intranasal immunization of rams with this formulation induced systemic IgG and local IgG and IgA antibodies showing that chitosan microspheres are a promising mucosal vaccine delivery system of BLSOmp31 against ovine brucellosis (Díaz et al., 2016). Thus, the aim of the present work was to develop and evaluate the immunogenicity of another novel vaccine formulation for conjunctival administration against *B. ovis* in rams. This alternative is based on the use of thermoresponsive and mucoadhesive *in situ* gel composed of P407 and chitosan as a vaccine delivery system for BLSOmp31.

Poloxamer 407 and the cationic polysaccharide chitosan have been used as a delivery vehicle of different antigens by parenteral or mucosal routes of administration (Westerink et al., 2002; Barisani-Asenbauer et al., 2013). In our study, BLSOmp31 was directly mixed into an aqueous polymer solution and no antigen was lost during vaccine preparation,

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which can further cut costs compared to other delivery methods. BLSOmp31-P407-Ch formulation showed a reverse thermogelation in which the aqueous solution was free-flowing liquid at low temperature and formed a stable gel at physiological temperature (39ºC). Fast sol-gel transition is an important factor for a short term depot system that allows a sustained release of antigen (Kojarunchitt et al., 2011). The diffusion of BLSOmp31 from the P407-Ch gel formulation was confirmed *in vitro*. Release of BLSOmp31 from P407-Ch gel was slow at the beginning and then it began to increase. Approximately 50% of BLSOmp31 was released after 210 min and about 65% of BLSOmp31 was detected in PBS at the end of the assay (270 min) (data not shown). Sustained release showed that BLSOmp31 was tightly bound to the P407-Ch gel. Therefore, this formulation is able to deliver immunogen that is structurally intact and could enhance the immune response when administered *in vivo* on the conjunctival mucosa. In addition, formulation was well tolerated; there was no evidence of specific local reactions during the experiment.

Different studies have demonstrated that adjuvant system composed of P407-Ch contribute to enhance the specific IgG antibody response when this depot system was administered by parenteral (Coheshott et al., 2004) or by intranasal route in mice (Westerink et al., 2001) but there are not studies with this adjuvant formulation in sheep. In our study, immunization with BLSOmp31-P407-Ch elicited specific IgG response with significant differences respect to unvaccinated control group ($p<0.01$) in all serum samples taken throughout the study (Figure 1).

Conjunctiva-associated lymphoid tissue has the typical components of a physiologically protective mucosal immune system (Petris et al., 2007). In the ocular-mucosal immune system the lacrimal gland is the principal effector site where secretory IgA antibodies are produced (O'Sullivan et al., 2015). The protective mechanisms involved could be the bacterial agglutination by IgA, which abounds in secretions and the activation of alternative

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complement pathway by the IgA–antigen complex (Butler, 1983). In the present study, conjunctival immunization of lambs with BLSOmp31-P407-Ch induced higher levels of mucosal specific IgA after first and second immunization in lacrimal ($p<0.05$), preputial or nasal secretions ($p<0.01$) (Figure 2A; 2B; 2C) but levels of antibodies declined rapidly and remained without significant differences with respect to unvaccinated rams ($p>0.05$) while no IgA antibodies were detected in serum (data not shown) or saliva (Figure 2D).

Conjunctiva is interconnected with the nasal mucosa via the tear ducts, therefore the administration of immunogens into the conjunctival sac would additionally drain to the nasal-associated lymphoid tissue (NALT) (Barisani-Asenbauer et al, 2013) and could induce an immune response in the nasal secretion. In our study, conjunctival immunization with BLSOmp31-407-Ch resulted in specific IgG levels in saliva and nasal, preputial, and lacrimal secretions that increased after a second immunization and decreased with different kinetics in each secretion (Figure 3). The specific antibody levels in lacrimal and nasal secretions and saliva were significantly higher ($p<0.01$) than those found in unvaccinated control rams until day 90 after first immunization (Figure 3A, 3B, 3C). In contrast, IgG specific antibodies in preputial secretions remained at high levels until the end of the experiment in vaccinated rams (Figure 3D). The presence of IgG antibodies in these secretions could reflect the transudation of serum antibodies as a result of a systemic immune response. In addition, IgG and/or IgA specific antibodies in preputial secretions could be the result of this interconnection since different studies in mice, rabbit and human models have demonstrated that intranasal immunization results in the generation of immune effectors cells in genital mucosa (Rudin et al., 1999, Chentoufi et al., 2010). Even though secretory IgA is the major immunoglobulin in secretions, IgG antibodies may also play a role in the defense against pathogens in ruminant species, at least in the reproductive tract (Foster et al., 1988) and the upper respiratory tract of sheep

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(Cripps et al., 1976). Both specific immunoglobulins could prevent *B. ovis* colonization and subsequent infection when this bacterium enters by the venereal or other route.

Conjunctival route has been exploited using live *B. melitensis* Rev. 1 attenuated vaccine which has been proved to be efficient against brucellosis in small ruminants (Blasco, 1997). To our knowledge, the immunogenicity of subcellular vaccines against brucellosis by conjunctival immunization has not been studied in sheep. There is only one published study that has evaluated the immunization by conjunctival route with a hot saline complex extracted from *B. ovis* (HS) encapsulated in mannosylated nanoparticles (MAN-NP-HS) in mice. This formulation induced mucosal IgA and elicited IL-2, IL-4 and γ-IFN levels that showed good correlation with the degree of protection against this bacterium (Da Costa Martins et al, 2009)

The ability of BLSOmp31-P407-Ch to stimulate cellular immune response was also examined. As the most important finding, we demonstrated that conjunctival immunization induced a significant cellular immune response. In fact, cells from vaccinated rams produced significant levels ($p<0.01$) of γ–IFN upon *in vitro* stimulation with BLSOmp31 at day 90 after the first immunization (Figure 4A) indicating that this formulation administered by conjunctival route induced a specific cellular immune response mediated by activation of macrophages, which are the main effector mechanism mediating the killing of *Brucella* spp. (Estein et al., 2009). Similarly, BLSOmp31 elicited an *in vivo* significantly higher hypersensitivity response with respect to the unvaccinated control group ($p<0.001$) after intradermal injection in immunized rams (Figure 4B).

4. Conclusion

In summary, the results obtained in the present work indicate that conjunctival immunization with BLSOmp31-P407-Ch gel induced local and systemic immune response in rams without interfering in the serological diagnosis of ovine brucellosis caused by *B.*

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ovis. The protective activity of this formulation in challenged sheep remains to be investigated.

Conflict of interest

There is no conflict of interest.

Authors' contributions

Conceived and designed the experiments: S.M.E and A.G.D. Performed the experiments: A.G.D. and D.A.Q. Analyzed the data: M.A.R. and S.M.E. Contributed reagents/materials/analysis tools: S.M.E., S.E.G., V.Z. Wrote the paper: S.M.E. and A.G.D. D.A.Q., S.D.P., V.Z., F.A.G. critically revised the manuscript. All authors read and approved the final manuscript.

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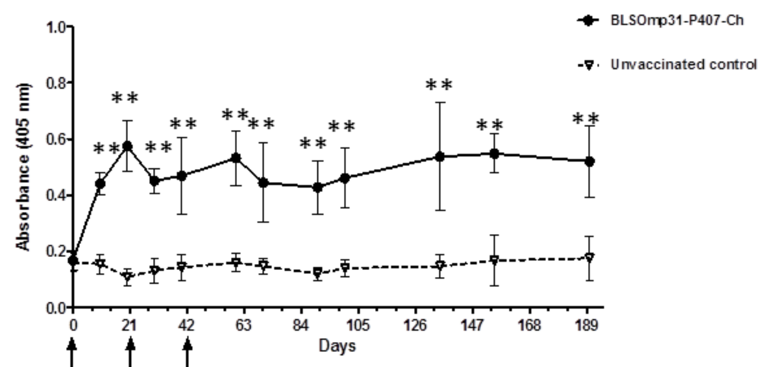

Figure 1. Anti-BLSOmp31 IgG induced in serum by conjunctival immunization with BLSOmp31 using P407-Ch *in situ* gel. Data are expressed as arithmetic mean ± SD absorbance units of animals per group (n=8). Arrows indicate immunizations.

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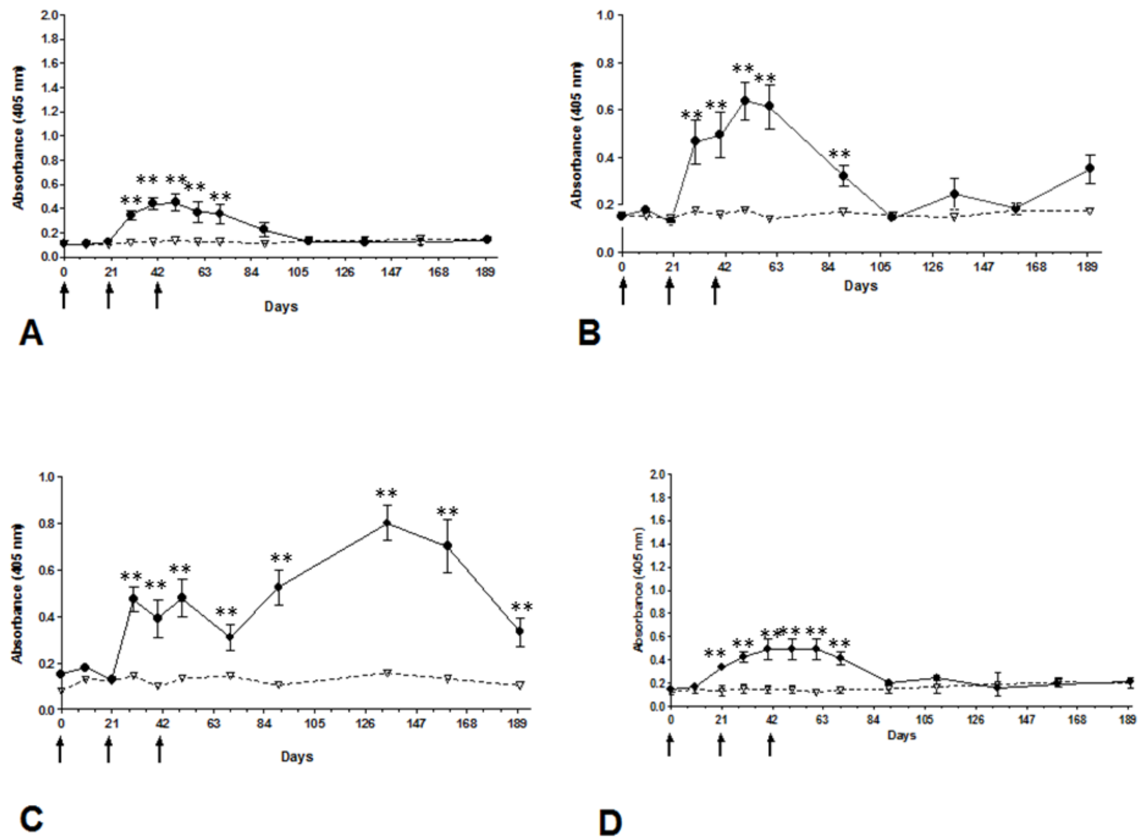

Figure 2. Anti-BLSOmp31 IgA induced in A) lacrimal, B) nasal, C) preputial secretions and D) saliva induced by conjunctival immunization with BLSOmp31 using P407-Ch *in situ* gel. Data are expressed as arithmetic mean ± SD absorbance units of animals per group (n=8). Arrows indicate immunizations. References: Black circles: vaccinated animals, empty triangles: unvaccinated animals.

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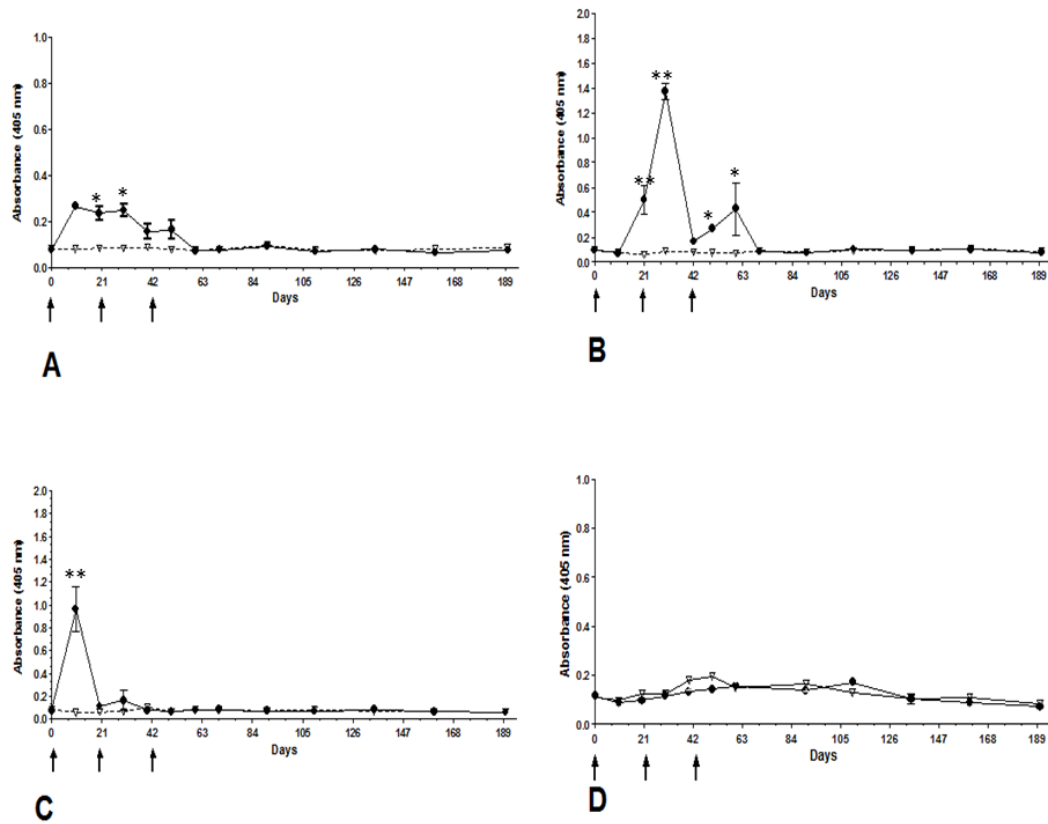

Figure 3. Anti-BLSOmp31 IgG induced in A) lacrimal, B) nasal, C) preputial secretions and D) saliva induced by conjunctival immunization with BLSOmp31 using P407-Ch *in situ* gel. Data are expressed as arithmetic mean ± SD absorbance units of animals per group (n=8). Arrows indicate immunizations. References: Black circles: vaccinated animals, empty triangles: unvaccinated animals.

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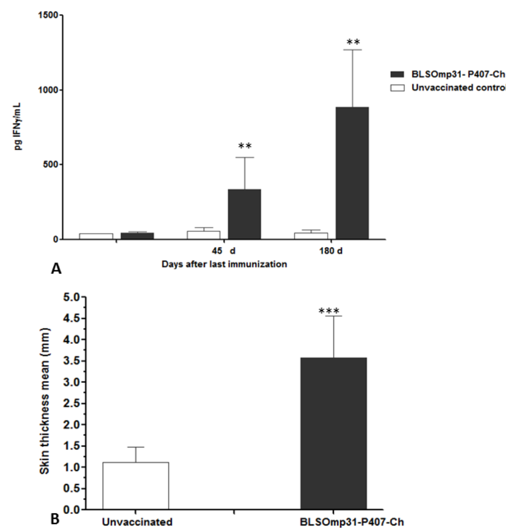

Figure 4. Cellular immune response A) *in vivo* and B) *in vitro* induced by conjunctival immunization with BLSOmp31 using P407-Ch *in situ* gel. A) γ–IFN response in whole-blood cell cultures stimulated with BLSOmp31 from vaccinated (n=8) and control rams (n=8). Samples were taken at days 0, 55 and 180 after the first immunization. Results are expressed as pg γ-IFN/mL and error bars indicate SD from the mean. B) Increase in the skin thickness following an intradermal injection with BLSOmp31 at day 90 after the first immunization. Measures were taken at 72 h after the injection. The increase was calculated as the difference between skin thickness after and before injection for each animal. The bars represent the mean ± SD for each group (n=8).