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Evaluation of anti-*Moraxella bovis* pili immunoglobulin-A in tears following intranasal vaccination of cattle

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ABSTRACT

Infectious bovine keratoconjunctivitis (IBK) is a highly contagious ocular disease of cattle caused by *Moraxella bovis* (Mb). Parenterally administered immunogens used to prevent the disease do not offer complete protection possibly because they stimulate a poor ocular mucosal secretory response, in which locally secreted immunoglobulin-A (sIgA) is one of the main components. The principal aim of this study was to evaluate by an indirect enzyme linked immunosorbent assay (ELISA), the local ocular mucosal sIgA response against Mb purified pili, produced after intranasal inoculation of experimental vaccines. Pili were adjuvanted by several different adjuvants (QuilA, Marcol Arlacel, Marcol Span, microencapsulated pili with PLGA polymers). Results were compared to sIgA response produced by adjuvant placebo inoculations and by IBK natural infection. Significantly higher anti-pili IgA response ($p < 0.05$) was detected in calves vaccinated intranasally with pili QuilA and pili Marcol Span compared to control calves, although this specific immune response did not seem to be related to protection against Mb infection or typical IBK lesion development.

1. Introduction

Infectious bovine keratoconjunctivitis is a highly contagious ocular disease of cattle caused by *Moraxella bovis*. Even though it does not produce mortality, the economic impact on the livestock farmer by reduced weight gain and eye disfigurement is substantial. The morbidity rate can approach 80%, reaching its maximum in the third or fourth week after of the onset of clinical cases (Blood et al., 2002).

Several risk factors may predispose the bovine eye to MB colonization/ocular damage. The face fly (*Musca autumnalis*) acts as mechanical vector of the agent (Kopecky et al., 1986), transferring the organism from one animal to another. Ultraviolet radiation and animal stress favor the emergence and spread of this disease. Also, other factors are implicated such as season, breed, eye pigmentation and mechanical irritation (dust, grass, weeds, plant awns, etc.) (Snowder et al., 2005). Pathogens such infectious bovine rhinotracheitis virus, *Mycoplasma* spp. and *Moraxella bovoculi* are associated with IBK lesions (Angelos et al., 2007a).

Variable responses to experimental vaccines have been reported. Hughes and Pugh (1971) indicated that repeated intramuscular (IM) injection of viable Mb organisms reduced to some degree the incidence of ocular infections, but did not reduce the severity of the disease in cattle that develop keratitis. Following this early research, many other studies reported the use of different immunogens designed to prevent eye infection by Mb and the development of conjunctival and corneal lesions associated with IBK (Lepper and Moore, 1992; Funk et al., 2009; Angelos et al., 2004, 2007b). New kinds of vaccines against IBK were developed based on formulations containing piliated strains of Mb or purified Mb pili. It was reported that pili were organelles specialized on adherence of the bacterium to target tissues and the antibodies directed against them prevented attachment to the bovine corneal epithelium (Annuar and Wilcox, 1985). Pugh and Hughes (1975) reported that a vaccine prepared with purified Mb pili without adjuvant, inoculated subconjunctivally, induced an acceptable immune response in calves. Nayar and Saunders (1975) reported that in the lacrimal secretions of calves with severe IBK there were elevated levels of IgA against Mb antigens, suggesting that local (presumably ocular) vaccination might be beneficial.

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Studies in mice inoculated intranasally with antigens showed increased IgA, produced by B-cells from NALT (Nasal Associated Lymphoid Tissue) (Zuercher, 2003). The NALT located below the nasal pharyngeal epithelium, contains aggregates of lymphoid follicles (B cell areas), interfollicular areas (T cells), macrophages and dendritic cells. This system would be in close proximity to CALT (Lymphoid Tissue Associated with the Conjunctiva) and LDALT (Lacrimal Drainage Associated Lymphoid Tissue), so that stimulation of NALT or CALT could elicit a response, resulting in an increased sIgA in tears (Knop and Knop, 2000). It was hypothesized that the administration of antigens by an intrapalpebral route of inoculation could stimulate the CALT leading to the production of specific local ocular IgA (Knop and Knop, 2007).

The objective of this study was to evaluate the ability of different pilin-adjuvant combinations to stimulate anti-pilin sIgA in bovine lacrimal fluid from beef calves exposed to natural infection with Mb and differentiate this immune response from antibodies produced by IBK natural infection.

2. Material and methods

2.1. Vaccines elaboration

2.1.1. Strain

M. bovis strain 1194-04, pili serotype G (Zielinski et al., 1998) was used. It belongs to the Strain Bank of the Laboratory of Bacteriology (EEA-INTA Ms. Juárez). Pili expression was identified by the formation of characteristic agar corroding colonies (''pitting factor'') on blood agar (Ruehl et al., 1988).

2.1.2. Purification of *M. bovis* pili

Pili were purified following a previously published procedure (Ruehl et al., 1988). Briefly, *M. bovis* was inoculated onto 5% equine blood agar plates, being incubated aerobically at 37 °C for 18 h. Once the lawn was developed, organisms were harvested and suspended in 0.15 M ethanolamine buffer (Eth), being homogenized at 30,000 rpm for 10 min in an ice bath. After two washings at 10,000 and 12,000*g* at 4 °C for 30 and 60 min, respectively, the pellet was discarded and pili in supernatant were slowly precipitated with ammonium sulphate at 10% saturation. The pili were collected by centrifugation at 12,000*g* for 60 min, the pellet was dissolved in the Eth buffer and insoluble contaminants were removed by centrifugation at 12,000*g* for 60 min. The supernatant was dialyzed against 0.15 M NaCl containing 0.05 M Tris–HCl, pH 8.0 at 8 °C for 24 h. The pilus aggregates that formed were separated from soluble contaminants by centrifugation at 12,000*g* for 60 min and the pellet dissolved in a small amount of PBS buffer pH 7.4.

Protein concentration was measured through the method reported by Lowry et al. (1951). To evaluate the purity of purified pili, a SDS–PAGE was performed using 15% of polyacrylamide as described by Laemmli (1970).

2.1.3. Vaccine formulation

2.1.3.1. Chemicals. Saponin from Quillaga bark (QuilA®), Mineral oil (Marcol®), Sorbitan Sesquioleate (Arlacel®), Sorbitan monooleate (Span 80®) were purchased from Sigma–Aldrich Chemical Co (St. Louis, MO, USA). Polyoxyethylene (20) sorbitan monooleate (Tween 80®) Fluka was obtained from Sigma–Aldrich Chemical Co (St. Louis, MO, USA. The Poly(D,L-Lactide-co-Glycolide) 50:50, Resomer® RG 503H, was purchased from Boehringer Ingelheim (Ingelheim, Germany).

2.1.3.2. Adjuvanting of . Four different adjuvants were utilized: three surfactants blends and microspheres.

2.1.3.2.1. Surfactants blends. Adyuvant ''QuilA'': a 10 mg/ml aqueous stock solution of QuilA was mixed with PBS at 7% (v/v); Adyuvant ''Marcol Arlacel'' consisted in a mixture of Marcol 85%, Arlacel 80 13% and Tween 80 2% (% v/v); Adyuvant ''Marcol Span'' consisted in Marcol 94 5%, Span 80 3.5% and Tween 80 2% (% v/v).

Vaccines were prepared emulsifying the aqueous suspension of pili (200 μg/ml) on the adjuvants at 40:60 proportions v/v at room temperature. Placebos consisted in an emulsion of PBS buffer (without antigens) on the three different adjuvants at the same proportions as used for vaccines.

2.1.3.2.2. Microspheres. The microspheres containing purified pili were prepared as follows. PLGA (polylactic-coglicolyc acid) microspheres were obtained following the procedure of double emulsion and solvent evaporation method as described by Igartua et al. (1998). Briefly, purified pili purification was used as the internal phase (W1). The organic phase (O), consisting in 25 ml of a solution of PLGA 50:50 in diclorometane (O) was emulsified with 5 ml of W1 using a high shear homogeinizer (Heidolph® 900X), with or without 0.5% PVA (polyvinilic alcohol) for the preparation of Pili 17-PLGA or Pili 18-PLGA microspheres, respectively. The resulting emulsion (W1/O) was poured into 300 ml of the external phase (W2), consisting on 0.5% PVA being emulsified for 5 min at 22,000 rpm. Evaporation of solvent was carried out by vigorous agitation of emulsion W1/O/W2 at room temperature. The microspheres obtained were collected by centrifugation (10.000*g*; 15 °C; 15 min) washed, lyophilized and stored at 4 °C.

The protein content of microspheres was determined by a previously reported method. It involved alkaline hydrolysis of the microspheres and determination of the protein recovered using the Micro BCA Protein assay kit (Pierce®, Protein Research Products) (Blanco and Alonso, 1998). Briefly, 200 mg of lyophilized microspheres were shaken with 2 ml of a 5% (w/v) sodium dodecyl sulfate (SDS) in 0.1 M NaOH solution (SDS/NaOH) for 24 h at room temperature. Following centrifugation (10.000*g*; 15 °C, 15 min), the protein content was determined in the supernatant. Results are presented as 'encapsulation percentage' values (μg protein/100 mg microspheres).

2.2. Experimental design

2.2.1. Animals

Recently weaned 6–8 month old Angus calves were used in this study. The animals belonged to the bovine herd of the Experiment Station of Marcos Juárez, National Institute of Agriculture Technology (Marcos Juárez, Argentina). They were maintained on pasture with concentrated feed, and managed in a single group. This herd was never vaccinated against IBK but usually presents high prevalence of clinical cases of IBK annually. Calves received no other vaccination than mandatory foot and mouth disease inactivated viral vaccine. Animals were managed according to usual outdoors, open field livestock management practices, not being treated under painful or cruel procedures.

Prior to vaccination, the animals were bled and an eye swab was collected using a sterile cotton swab rolled over the dorsal and ventral surfaces of the conjunctiva for bacteriological Mb studies. Swabs were inoculated onto 5% equine blood agar and incubated aerobically at 37 °C. Suspected Mb colonies were isolated and inoculated onto a new blood agar plate for purification. On suspected cultures, organisms were studied for Gram reaction, oxidase, catalase, Kliegler iron agar, sugar attack and gelatinase (Pugh and Hughes, 1975). Only culture negative animal with no clinical signs of IBK were included in the experiment.

2.2.2. Experimental treatments

The calves were randomly divided into nine experimental groups of five animals per treatment, according to kind of experi-

Table 1
Experimental groups and route of administration.

Experimental group	Antigen	Adjuvant	Route of administration
1	Pilin	PLGA 17	Intranasal
2	Pilin	PLGA 18	Intranasal
3	Pilin	QuilA	Intranasal
4	Pilin	Marcol Arlacel	Intranasal
5	Pilin	Marcol Span	Intranasal
6	Placebo	QuilA	Intranasal
7	Placebo	Marcol Arlacel	Intranasal
8	Placebo	Marcol Span	Intranasal
9	Control	–	–

mental vaccine or placebo received, being individually ear tagged (Table 1). During each sampling animals were eye cultured for isolation of Mb and observed for lesions compatible with IBK. Animals culture positive for Mb that concomitantly showed typical IBK lesions (epiphora, keratitis, corneal ulcerations, keratoconus) were classified as IBK+.

2.3. Doses and routes of inoculation

Calves were immobilized in a squeeze chute for vaccine inoculation. A device was designed for inoculation of experimental vaccines as spray into the nostrils. It consisted in a canula used for oral administration of parasiticides connected to an automatic syringe in one side and to the top of a device developed by B&D for administration of intranasal vaccine in humans that shooted spray-form injections, on the other side. For microencapsulated pili, a total of 6 ml of the experimental vaccine were inoculated (3 ml/nostrail) with a total of 210 μg of antigen/nostrail. Respect to the other experimental vaccines, each animal received intranasally 6 ml of inoculums (3 ml/nostrail) with a total of 500 μg of antigen/animal.

Calves were inoculated by the corresponding vaccine or placebo at day 0 and day 15. After the booster vaccination animals were observed and sampled every 30 days during 5 months. (Fig. 1).

2.4. Sample collection

At each sampling, samples of tears (1–2 ml) were taken by manual stimulation of conjunctiva and collected with a plastic dropper. These samples were frozen at −20 °C until used in the ELISA assay. Calves were also observed for ocular lesions and conjunctivally swabbed for bacteriological studies. Individual animals showing IBK lesions were recorded as well as animals culture positive to Mb in each sampling.

2.5. Serological evaluation

The concentration of anti-Mb pili secretory IgA in tears was evaluated by an indirect-ELISA test based on the methodology described by Dominguez et al. (2002) and Moore and Rutter (1987) with modifications, using purified pili as antigen adsorbed onto a 96-well plastic plate (Costar, Corning). Tears of an animal with high titers of IgA anti-*M. bovis* selected in previous studies and Fetal Calf Serum (Sigma F2442) were used as positive and negative controls respectively. Briefly, 96-well plates (Corning) were coated with 1 μg/well Mb purified pili in coating buffer (0.5 M carbonate bicarbonate buffer, pH 9.6). After overnight incubation at 37 °C the plates were incubated with blocking buffer (PBS-Tween Ovalbumin 1%). After 1 h at 37 °C the plates were washed with PBS-T. Calves tears diluted 1:25 in blocking buffer were added to individual wells in duplicate. After 2 h at 37 °C the plates were washed three times and 100 μl/well of sheep anti-bovine IgA HRP conjugated (Bethyl Laboratories), diluted in PBS-T OVA 1% (1/1500) was added. Following 3 h incubation at 37 °C plates were washed with PBS-T. Then 100 μl/well of chromogen (*o*-Phenylenediamine dihydrochloride, Sigma P6662) was added according to the manufacturer's instructions. The reaction was stopped with 50 μl/well of 5 $N{\cdot}H_2SO_4$ after 30 min and read at 490 nm in a Labsystems Multiskan ELISA reader.

2.6. Statistical analysis

Results from each observation concerning optical density (OD) of IgA antibodies in tears were analyzed by Analysis of Variance (ANOVA) using SPSS software. The reaction cutoff point was determined by ELISA on paired samples by the T0–Tx (T0 = day 0, Tx = day 15, 45 or corresponding with the subsequent samplings). Tear samples were considered positive (antibodies conversion) when Tx showed double value of OD when compared to T0 (Dominguez et al., 2002; Zamorano et al., 2002; Villarreal-Ramos et al., 1998). Also, antibody levels produced after natural infection were evaluated by the Kruskal–Wallis and Mann Whitney test. The samples that were positive for Mb IgA specific antibodies were analyzed in relation to subsequent Mb isolation or lesions by the same tests. Differences were considered significant when p values were lower than 0.05.

3. Results

3.1. ELISA results of anti-pili Mb

Significant differences in OD values between groups inoculated with different vaccines ($p < 0.05$) and between samplings ($p < 0.05$) were detected. Mean differences between inoculated groups and their respective placebos are presented in Fig. 2. The ELISA test used showed that immunization with pili-QuilA and pili-M.Span was able to induce IgA specific antibodies in tears. For both experimental vaccines, treatments values of OD were higher ($p < 0.05$) than their respective placebos from day 45 to day 135 post immunization. The antibody titers generated by inoculation of pili-QuilA were higher than pili-M.Span.

In general, no significant differences were observed between OD values of pili-M.Span and pili-PLGA microencapsulated treatments with their placebos ($p > 0.05$). Only pili-17 PLGA and pili-18 PLGA showed significantly higher antibody titers than ($p < 0.05$) with their control groups in day 15 and day 45 post immunization (Fig. 2D).

3.2. Relationship between isolations, lesions and IBK+ with groups that presented IgA Mb specific conversion

Culture positive, lesioned and IBK+ calves were detected along the trial, although no differences in proportions were found between treatments ($p > 0.05$).

Fig. 3 shows proportions of animals with Mb isolation, detected lesions, IBK+ and Mb pili IgA+. In Fig. 3A and B (groups inoculated with pili-QuilA and pili-M.Span) respectively, it can be observed that proportion of calves with IgA positive conversion were higher than the groups corresponding to placebos ($p < 0.05$) (Fig. 3C and D). However, percentages of animals with Mb isolation, lesions or IBK+ were not different ($p > 0.05$).

In Fig. 3A and B, it can be observed that anti Mb pili antibodies increased from day 15 to the end of the trial, while for placebo groups a small proportion of animals showed the same antibody response at the end of the samplings (Fig. 3C and D).

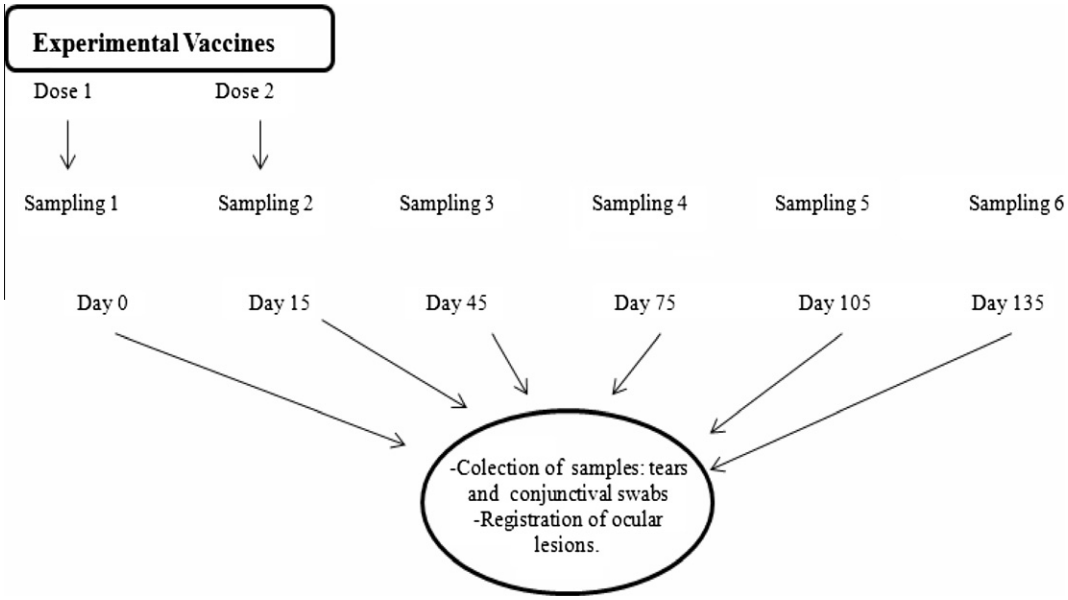

Fig. 1. Experimental design.

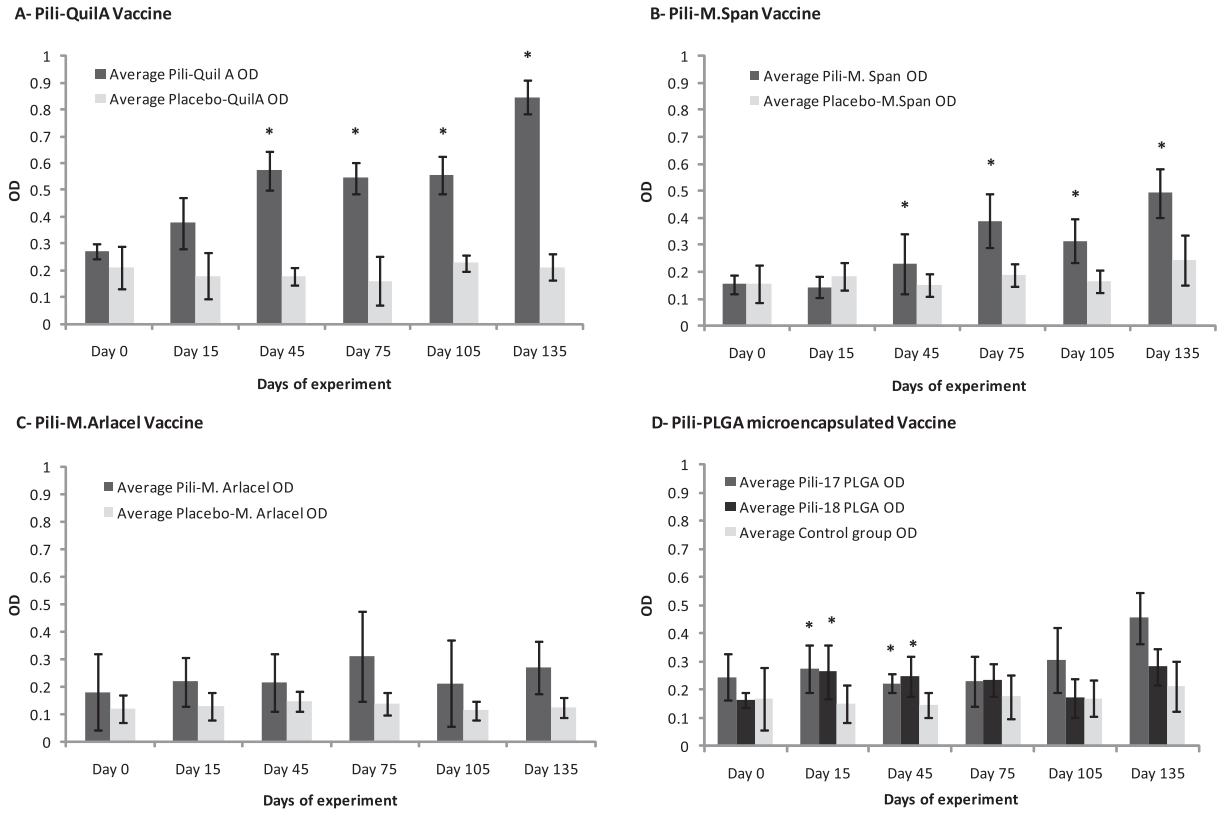

Fig. 2. Titers curves of average OD obtained for each experimental vaccine group. Each group inoculated with experimental vaccines was compared with its placebo. *Indicate significant differences.

4. Discussion

The main purpose of this work was to study the capability of several formulations of experimental vaccines containing purified Mb pili with four different adjuvants, inoculated intranasally, to induce a local mucosal immune response in calves measured as specific sIgA in tears that could suggest a protective response. Specific anti Mb antibodies in lacrimal secretions were predominantly of the IgA class, indicative of a local production. It is known the protective function of this antibody isotype in terms of toxin neutralization and attachment inhibition of antigens (Bishop et al., 1982; Pedersen, 1973; Mach and Pahud, 1971).

The method used to assess specific Mb pili antibody was an indirect ELISA, which was adapted to the conditions required by this experimental work. In previous trials conducted by Dominguez et al. (2002), the IgG response of commercial bacterins against

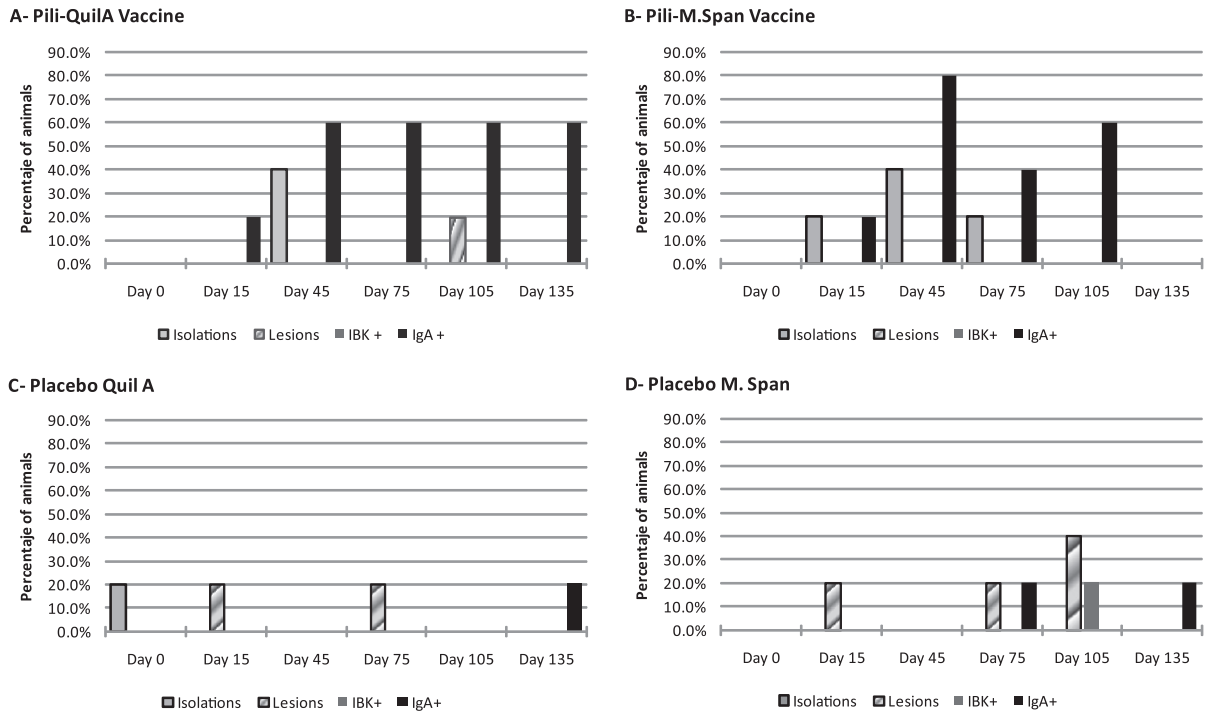

Fig. 3. Percentage of animals that showed positive isolations for Mb, ocular lesions, IBK+ and seroconvertion of specific IgA (IgA+) in Pili-QuilA and Pili-M.Span groups. Black line indicates the trend of specific antibodies.

M. bovis was measured, utilizing as antigen inactivated soma of Mb adsorbed onto ELISA plates. In contrast, for this work purified Mb pili was adsorbed onto ELISA plates and experimental vaccines were used to stimulate a specific immune response. In previous studies, it was observed that ELISA was a suitable test to investigate antibody response directed to Mb antigens in cattle sera and lacrimal secretions as well (Angelos et al., 2004; Bishop et al., 1982).

This trial allowed us to demonstrate that intranasally inoculated Mb pili was able to stimulate a specific immune response measured as locally secreted IgA. Since specific IgA antibodies were almost not detected either in placebo inoculated animals nor in uninoculated controls, it can be assumed that titers found in pili inoculated treatments were produced by experimental inocula.

The experimental groups inoculated with PLGA microencapsulated pili (Pili17 and Pili18) did not generate high titers of IgA (Fig. 2D), contradicting results obtained with the same type of antigen presenting system (PLGA microcapsules), although prepared with different type of antigens (Wyatt and Frederick, 2006; Sinha and Aman, 2003; O'Brien et al., 1996). These unexpected results could be attributed to several issues. One could be that the inoculation was not correctly sprayed; therefore, the microspheres did not reach the nasal mucosa. Additionally, the microspheres size could not have been sufficiently small, so that their uptake by antigen presenting cells could have been affected. The immune response is dependent on the size of the particles presented to antigen presenting cells, the smaller the particle, the higher the immune response (Vila et al., 2005). Several authors (O'Brien et al., 1996; Eldridge et al., 1991) demonstrated that successful mucosal immunization required microspheres smaller than 10 μm that could be ingested by antigen-presenting cells (Meclean et al., 1998). In our case, although there were microspheres smaller than 10 μm in the preparations measured by scanning electron microscopy (SEM) (data not shown) most of the particles were of a larger size and presented aggregates that could be seen in the SEM preparations.

In contrast to the results obtained with mucoadherent polymers, QuilA used as adjuvant produced a very different immune response in animals inoculated with antigens when compared to animals inoculated with placebo (Fig. 2A). This finding is interesting because experimental vaccines containing pili-QuilA produced high titers of specific sIgA anti Mb pili during four out of five samplings (120 days). Previous studies evaluated the effects of derivatives of *Quillaja saponaria* showing major increments of immunoproliferative cells (Chavali et al., 1987; Plohmann et al., 1997; Lacaille-Dubois et al., 1999). Animal trials also demonstrated that the use of QuilA as an adjuvant in veterinary formulations generated an excellent response from the immune system (Dalsgaard, 1987).

A specific immune response was also observed in the groups that received pilin in Marcol Span as adjuvant (Fig. 2B). While the conversion of specific IgA was observed on the fourth sampling (day 75), the magnitude of the increase was smaller than the one observed in the QuilA adjuvanted group. In contrast, in the group of animals inoculated with pilin in Marcol Arlacel, differences respect to control and placebo groups were observed, but the generation of specific antibodies titers was not high nor it was constant in time. The immunostimulatory properties of this adjuvant was reported for antigens inoculated subcutaneously (Garaicoechea et al., 2008; Snodgrass et al., 1982). In our experiment probably the route of administration was not adequate for this adjuvant, not allowing the contact between the antigen with the nasal mucosa, thus avoiding the stimulation of local immune response.

The Mb isolations and presence of lesions were variable and most of the times were not concomitantly found in the same animal. It is known that Mb can be isolated from healthy eyes and also that once IBK lesion are developed, chances to culture the organism diminish dramatically. In consequence, finding IBK+ animals (lesioned plus culture positive) is a rather rare event. It can be observed that, as long as sIgA titers increase along the trial for pili QuilA and pili M.Span vaccinated groups, the number of Mb isolates decrease (Fig. 3A and B). This phenomenon might be related

to protection induced by increase of specific sIgA titers, that could be caused by immunity generated by experimental vaccines. This should be proven, though, because animal in all groups comingled, being exposed to the same source of natural infection. However, a higher proportion of animals with IBK lesions were detected in placebo groups, although the differences were not significant ($p > 0.05$) (Fig. 3C and D).

The role of different antibody isotype (IgG–IgA) in lacrimal secretion deserves further studies. In a 5 month long study of natural IBK cases, Killinger et al. (1978) reported that three classes of Ig were detected in lacrimal secretions, being the IgG class the most persistent, but they could not attribute to immunoglobulins any role in prevention of clinical IBK. Also, Nayar and Saunders (1975) had determined that severely affected cattle with IBK developed a strong IgA response in tears, and that convalescent animals were no longer susceptible to reinfection, suggesting that IgA played a role in protection and that the use of locally administered Mb vaccines deserved further investigation. Recently, Angelos et al. (2004) reported that a reduction in the cumulative proportion of ulcerated corneas was observed in calves that received a recombinant Mb cytotoxin ISCOM matrix adjuvanted vaccine, discussing the role of systemic versus local ocular immune response in protecting cattle against IBK. Based on their findings, the authors suggested that subcutaneous vaccination using conventional adjuvants could be a reasonable choice for IBK vaccine development, but could not disregard the possibility that local response plays an important role in IBK protection. Furthermore, they suggested that the benefit of augmented local ocular IgA mediated protection required further study, since it could be the way to reduce local immune mediated ocular injury following attraction of neutrophils to the site of infection, that occurs during IBK lesion development.

In our study, statistical analysis found no relationship between Mb isolates/lesions and antibody conversion, indicating that increase in IgA titers were probably due to experimental vaccines. If so, this new ability to elicit an augmented local immune response, sIgA mediated, should be tested in further works using experimental and natural infection models with known Mb strains as challenge.

5. Conclusions

The method used in this study (ELISA) to measure immunoglobulin-A in tears in calves demonstrated the ability to detect the immune stimulation after the inoculation of experimental vaccines intranasally.

Considering these results, immunization of cattle with pili-QuilA and pili-M.Span have a good potential for increasing specific IgA in tears and could be useful as a preventive measure against the disease. Therefore, it would be necessary to conduct further experiments to evaluate potential protective capacity against *M. bovis* infection.

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