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Assessment of the immunogenicity of the leptospiral LipL32, LigAni, and LigBrep recombinant proteins in the sheep model

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ABSTRACT

Veterinary leptospirosis vaccines are composed of bacterins and present limitations, for example, the need for bacteriological culture and serovar-dependent immunity. Recombinant antigens represent a promising alternative. LigAni, LigBrep, and LipL32 proteins have been shown to promote a protective immune response against the homologous challenge in hamsters. Therefore, the next step is to evaluate the immunological properties of these immunogens in the actual hosts, as ruminants, which has never been performed before. The objective of this study was to evaluate the immunogenicity and potential adverse effects of the recombinant proteins LigAni, LigBrep, and LipL32 in the ovine model. For this, 16 Santa Inês sheep were allocated into three groups: two experimental (Groups A and B) and one control group (Group C). Group A was inoculated with a formulation containing the recombinant proteins in combination with the aluminum hydroxide adjuvant; Group B was inoculated with a formulation containing the recombinant proteins in combination with the Montanide adjuvant; and Group C was inoculated with adjuvants only. The results revealed that formulations containing the recombinant proteins induced total IgG seroconversion and led to a significant increase in antibody titers in the sheep model. Besides, there were no clinical changes or adverse effects. Thus, LigAni, LigBrep, and LipL32 proteins elicited a significant humoral immune response with elevated serum IgG levels, demonstrating that they possess the immunogenic and safety characteristics necessary to sustain their potential use as leptospirosis vaccines in the ruminant model.

1. Introduction

Leptospirosis is a zoonotic disease that is caused by pathogenic strains of the bacterium *Leptospira* spp. [1]. In livestock, leptospirosis causes economic losses due to the high rates of reproductive failures, such as abortion, stillbirth, infertility, and reduced milk production [2,3]. Immunization is the cheapest method of control and represents an essential measure for the control of leptospirosis; as such, its adoption is strongly recommended [4]. Vaccines currently available are composed of bacterins (inactivated cultures) and involve fastidious microorganisms. In addition, the immunity is mainly directed to the serovars antigenically related to those present in the vaccine formulation [5,6]. As such, recombinant vaccines often represent a more attractive alternative to bacterins since they eliminate time-consuming and costly cultivation and, in theory, may confer protection against several serovars [7]. To this end, recombinant vaccines have been developed as a means of overcoming the deficiencies of the bacterin-containing vaccines [8,9]. In order to develop these vaccines, researchers are seeking recombinant antigens that offer an optimal antigenic and immunogenic profile.

Previous studies using hamsters as an experimental model have demonstrated the potential of LipL32, fragments of LigA (the non-identical to LigB carboxy-terminal region, named LigAni), and LigB (residues 131–645, named LigBrep) recombinant proteins [10–12]. LipL32 is found exclusively in pathogenic leptospires, highly conserved, expressed in vivo, highly immunogenic, and has been able to confer protective immune response in some experiments [13,14]. LigAni is not present in all pathogenic species, however, this antigen has consistently been shown to induce protective immunity in the hamster model against homologous challenge [7,11]. LigBrep is present in all pathogenic species and a sterilizing immune protection has been demonstrated when this antigen was assessed as a single recombinant antigen vaccine in the hamster model [12].

Despite the evidences that the three recombinant antigens can

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integrate a novel recombinant vaccine against animal leptospirosis, the behavior of these immunogens in the actual hosts, i.e., the ruminants, is unknown. Thus, this study aimed to bridge that gap by evaluating the immunogenicity of the LipL32, LigAni, and LigBrep recombinant proteins and identifying any possible adverse effects of the use of such antigens in an experimental sheep model.

2. Methodology

2.1. Expression and purification of recombinant proteins

The LigAni, LigBrep, and LipL32 recombinant proteins were produced as described by Hartwig et al. [10], Hartwig et al. [13], and Grassmann et al. [14]. Briefly, pAE vectors containing *ligAni*, *ligBrep*, and *lipL32* genes were used to transform the *E. coli* TOP10F' and BL21 (DE3) Star expression strains respectively. The strains transformed with pAE recombinant vectors were cultured in 500 mL of LB medium until they reached the OD_{600} 0.5 - 0.7 at 37 °C. They were then induced with 1 mM isopropyl-β-1-D thiogalactopyranoside (IPTG) for 4 h. The cells were collected by centrifugation (7000 x *g*, 4 °C, 15 min), suspended in a solubilization buffer (200 mM NaH_2 PO 4, 0.5 M NaCl, 5 mM imidazole, pH 8.0), and incubated via orbital shaking at 60 rpm for 16 h at room temperature. Purification was performed by affinity chromatography using nickel-loaded HisTrap sepharose columns. To evaluate the expression of the recombinant proteins, the samples were examined using the SDS-PAGE (12% acrylamide) and Western blot using anti-6xHis monoclonal antibody (Sigma-Aldrich, Brazil). The purified recombinant proteins were dialyzed with 1X phosphate buffered saline (PBS) in 5 steps for 5 days at 4 °C. The concentration was determined by the BCA Protein Assay kit (Pierce, USA) and with the aid of TotalLab Quant software. The recombinant proteins were stored at −20 °C.

2.2. Preparation of formulations

Two formulations, composed of three recombinant antigens, LigAni, LigBrep, and LipL32, in combination with two adjuvants, Alhydrogel or Montanide ISA 50V2, were prepared. For this, 100 μg/mL of each recombinant antigen and 2 mg/mL Alhydrogel or the Montanide 50/50 (volume/volume) adjuvant was used. To achieve emulsification of the Alhydrogel-containing formulation, the formulation was lightly stirred for 16 h at 4 °C. In the case of the formulation containing Montanide ISA 50V2, an emulsifier was used. Thereafter, the vaccines were fractionated in a 9 mL volume. At the end of the procedure, the product was stored under refrigeration at 4 °C.

2.3. Experimental groups

Sixteen female, twelve-month-old Santa Inês breed sheep which had not previously been vaccinated against leptospirosis were used. The animals were randomly allocated into three groups (A, B, and C), with Groups A and B each consisting of five animals and Group C, the control group, of six animals. In addition to the hematobiochemical and clinical analyses, three consecutive days before immunization all the sheep were evaluated by serology (MAT) and urine PCR (targeting the *lipL32* gene), according to the methodology described by Rocha et al. [15]. The experiment was conducted at the Experimental Research Unit in Goats and Sheep (UniPECO-UFF), a Biological Safety Level 2 facility.

Each experimental group was inoculated with two doses of different formulations, intramuscularly at intervals of 21 days. The animals in Group A were inoculated with a formulation containing the LigAni, LigBrep, and LipL32 recombinant antigens plus 2 mg/mL Alhydrogel 2%. Those in Group B were inoculated with a formulation containing the LigAni, LigBrep, and LipL32 recombinant antigens plus Montanide ISA 50V2, 50/50 (volume/volume), and Group C animals were inoculated with formulations that contained only adjuvants. Three of the animals in Group C received Alhydrogel (2 mg/mL) and three received Montanide (volume/volume). The study was conducted with the full approval of the Ethics Committee of Universidade Federal Fluminense, Brazil (number 814/2016).

2.4. Sampling

To evaluate the humoral immune response and hematobiochemical changes, blood samples were collected by jugular vein puncture at days 0 (day of inoculation), 7, 14, 21, 28, 35, and 42 post-inoculation (p.i.), using sterile needles (40 x 12 mm), and stored in two vacuum tubes (Vacutainer®, BD, São Paulo, SP, Brazil). Anticoagulant (EDTA) was used for hemogram, and no anticoagulant was used for serology and biochemistry. Serum aliquots were stored in duplicate in 1.5 mL microtubes at a temperature of −20 °C until processing. Whole blood samples were processed immediately after collection.

2.5. Evaluation of the humoral immune response

The humoral immune response of the sheep was evaluated using the indirect Enzyme Linked ImmunonoSorbent Assay (ELISA). Each antigen was tested separately. Therefore, for the recombinant protein, plate sensitization was performed with 100 ng of antigen diluted in Carbonate-Bicarbonate buffer (pH 9.6) for 16 h at 4 °C. The plates were washed three times with phosphate-buffered saline pH 7.6 with 0.05% Tween 20 (PBSt) before the serum of the animals was evaluated at a dilution of 1:500 in a volume of 100 μL and incubated at 37 °C for 1 h.

After the primary serum was incubated overnight, the plates were washed again with PBSt, and the peroxidase-conjugated sheep anti-IgG antiserum was added at a dilution of 1:10,000 for 1 h at 37 °C in a volume of 100 μL. After the incubation time, the plates were again washed with PBSt. Finally, the reaction was developed using Orto Phenyl Diamine (3.4 mg in 10 mL of citrate phosphate buffer) with the addition of 10 μL of 30 vol of hydrogen peroxide in a volume of 100 μL per well. Fifteen minutes after adding the developing solution, an optical density reading was performed at 450 nm using an ELISA reader (Dynatech MR 700, Germany).

2.6. Hematobiochemical analysis and clinical signs

The complete blood count was performed according to the instrumental techniques described by Thrall [16], and the values were compared to those previously determined for the Santa Inês breed [17].

Concentration of urea and creatinine; serum alanine aminotransferase (ALT) activity; alkaline phosphatase (FAL) and gammaglutamyltransferase (GGT); protein and total fractions (albumin and globulin); and total, direct, and indirect bilirubin were analyzed spectrophotometrically using commercial kits (Labtest®, Labtest Diagnostica AS, Minas Gerais, Brazil) in an automatic biochemical analyzer (LabMax 240 premium®, Labtest Diagnostica AS, Minas Gerais, Brazil). The experimental animals were clinically evaluated on a daily basis by a team of veterinarians who were suitably qualified and specialized in the area. Different factors that may indicate the side effects of the inoculation, such as pyrexia, prostration, jaundice, hematuria, dyspnea, polypnea, anorexia, dehydration, fecal changes, and adverse reactions at the inoculation site, were evaluated.

2.7. Statistical analysis

Optical density data were analyzed using ANOVA for the different proteins and adjuvants using the IBM SPSS 22 software, with a 95% confidence interval. In addition, the Tukey HSD test was used to compare the mean values of the readings for each protein.

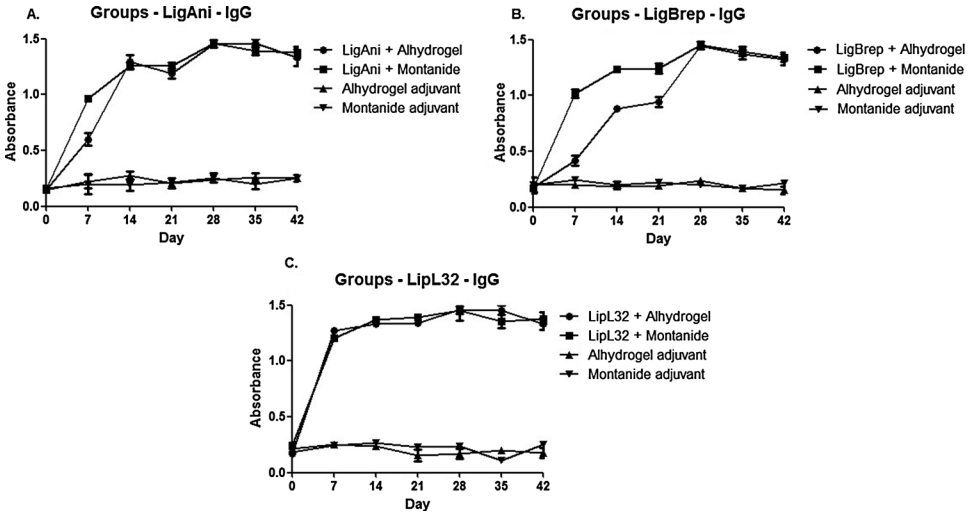

Fig. 1. Evaluation of the humoral Immune response obtained by the experimental group against each recombinant antigen. A. Humoral immune response against LigAni protein only. B. Humoral immune response against the LigBrep protein only. C. Humoral response against LimL32 proteins Only.

3. Results

3.1. Humoral immune response

As shown in Fig. 1, all recombinant proteins (LigAni, LigBrep, and LipL32) elicited the specific antibody titers in the ruminant model. A significant difference was observed in protein comparison ($p = 0.002$). This difference was observed in LigBrep (mean = 1.16) and LipL32 (mean = 1.37), triggering the humoral immune response. A significant IgG seroconversion was observed against the recombinant antigens between Day 0 and Day 7 p.i. In this period, LipL32 protein generated the highest serum levels of IgG on Day 7, remaining stable thereafter until Day 42. The other proteins started to generate high serum levels of IgG only on Day 14 p.i., and the seroconversion remained constant after the second dose of the formulation on Day 21. No seroconversion was observed in the control group. Furthermore, when proteins were compared in Groups A and B, significant differences were observed in Group A ($p = 0.006$) for LigBrep (mean = 1.05) and LipL32 (mean = 1.38). In contrast, no difference in mean was observed in Group B ($p = 0.153$). The results demonstrate that the ruminants in Group A and Group B developed a humoral immune response against the inoculated recombinant antigens (Fig. 2).

In terms of the antibody titers generated, the recombinant proteins responded differently to each other, as did the two experimental groups. The animals in Group A that were inoculated with a formulation containing the proteins plus the Alhydrogel adjuvant obtained antibody titers as follows: LigAni (1:51,200), LigBrep (1:25,600), and LipL32 (1:25,600). The animals in Group B, which were vaccinated with a formulation that contained the proteins plus the Montanide ISA 50V2 adjuvant, obtained LigAni (1:25,600), LigBrep (1:51,200), and LipL32 (1:102,400).

3.2. Adjuvant

There was generally no significant difference ($p = 0.084$) observed in terms of the effect of the adjuvant in serum conversion. Nevertheless, a non-significant difference was observed in the LigAni ($p = 0.705$) and LipL32 ($p = 0.605$) protein responses in both groups. The highest titers were observed for LigBrep ($p = 0.023$) in Group B (mean = 1.35).

3.3. Analysis of clinical signs and hematobiochemical alterations

A small inflammatory reaction followed by swelling at the inoculation site of the formulation was observed in the sheep in Group A. No other clinical signs or hematobiochemical changes were observed in any of the animals in the groups evaluated.

4. Discussion

This study aimed to analyze the humoral immune profile of sheep inoculated with recombinant proteins belonging to the genus *Leptospira*, which represent promising vaccine candidates for infection control. Several studies have evaluated the antigenic and immunogenic potential of recombinant proteins belonging to the genus *Leptospira* for the development of new vaccines [18,19]. However, the studies have been restricted to the use of the Golden Syrian Hamster experimental model, a vivarium model best suited for experiments involving leptospirosis [20]. Nonetheless, leptospirosis has a high incidence in livestock and leads to significant economic losses in those animals [3,21]. Thus, it is necessary and extremely important to evaluate the immunogenicity of the recombinant proteins in those animals. Although other studies have described how the immune system of ruminants responds to vaccines composed of bacterins [22,23], to the best of the authors' knowledge, the current study represents the first to perform analysis of recombinant vaccines in a ruminant model.

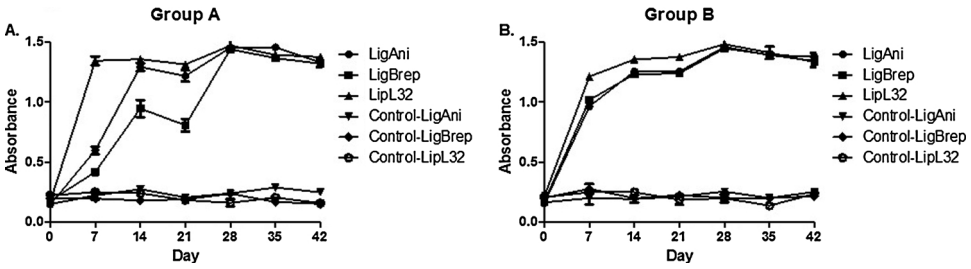

Fig. 2. Evaluation of humoral obtained by each of experimental group. Group A: Sheep inoculated with a formulation composed of 100 μg of the LipL32, LigAni and LigBrep proteins and adsorbed with the Alhydrogel adjuvant. Group B: Sheep inoculated whith a formulation composed of 100 μg the LipL32, LigAni and LigBrep proteins plus Montanide ISA 50V2.

Despite the wide range of pathogenic species and strains, ruminants have been mainly infected by strains belonging to the serogroup Sejroe [24,25]. In this context, *L. borgpetersenii* serovar Hardjo (type Hardjobovis), *L. interrogans* serovar Hardjo (type Hardjoprajitno) and *L. santarosai* serovar Guaricura are the most representative of this serogroup worldwide [24,26]. It is important to highlight that two of antigens used in this study (LigBrep and LipL32) are conserved in these species. Thus, the demonstration of a humoral response elicited by these antigens may represent an encouragement to other studies focused on recombinant immunogens.

In terms of the adjuvants, the results indicated that there was no statistically significant difference in titer levels between the groups. However, the formulation containing Montanide ISA 50V2 as adjuvant (Group B) maintained the seroconversion of antibodies in a more homogenous manner throughout the experimental period. This result can be justified by taking into account the mechanism of action of the adjuvant. Montanide is considered to represent a deposit adjuvant that slowly releases the antigen at the site of inoculation. This entails that the antigen is exposed to the immune response for a longer period, potentiating the immune response, generating both a humoral (Th2) immune response and a cellular (Th1) immune response, with the most stimulated Th1 immune response [27,28]. In addition, the Montanide adjuvant has been specially developed for use in small and large ruminants (Manufacturer Information). Therefore, when the effect of the Montanide ISA 50V2 adjuvant was evaluated, it was observed that, in addition to a high seroconversion, there was no significant difference between the different recombinant antigens.

Conversely, the aluminum hydroxide adjuvant (Alhydrogel 2%) was also able to potentiate the immune response and aid the seroconversion through high serum titers of antibodies. Studies suggest that the mechanism of action of aluminum hydroxide can be attributed to the formation of antigen deposit and immunostimulation [29]. However, researchers have yet to develop a working understanding of the actual action of this adjuvant [30,31]. It is known that aluminum hydroxide mainly stimulates an associated humoral immune response (type th2), associated with IL-4, inducing a reduced cellular immune response [32]. However, studies based on ruminant populations have shown that the cellular immune response (Th1 type) is the most incident to a leptospirosis infection [22,23]. These findings reinforce the need for the use of an adjuvant that enhances this immune response.

The only side effect that was observed in the current study was a small inflammatory reaction at the site of application of the formulation, and this only occurred in the experimental animals in Group A. This reaction can be attributed to the mechanism of action of the aluminum hydroxide adjuvant, which induces inflammation and stimulates the local production of granuloma, subcutaneous nodules, and contact hypersensitivity [33,34]. Furthermore, no hematobiochemical changes were observed in any of the evaluated groups. Thus, it can be argued that the formulations studied are safe for use in sheep since no moderate or severe adverse effects were observed.

Over the last two decades, several studies provided convincing and reliable outcomes regarding the immunogenicity of recombinant proteins in hamster [7,10–14,18,20]. Based on those results we have taken a step further and tested recombinant antigens in a ruminant model. The antigens were able to induce a significant humoral immune response, without side effects, demonstrating that they possess the immunogenic and safety characteristics necessary to sustain their potential use as leptospirosis vaccines in the ruminant model. In this context, our results highlight the usefulness of sheep as an experimental model either for the evaluation of the efficacy of such antigens or for the screening of new vaccine targets.

Sheep have been characterized as being a good experimental model in several studies spanning, for example, cardiovascular, respiratory, reproductive, and mainly immunological domains [23,35]. They are relatively small in comparison to other ruminants, such as cattle, and can be easily allocated in smaller stalls and bays, facilitating the management of research and minimizing the costs of experimentation [36]. Moreover, cattle and sheep have a strong chromosomal homology, which may justify the similarities in the immune responses that they present [37]. This indicates that sheep may be a good experimental model for evaluating the immune response of ruminants.

The results obtained in this study, confirming the immunogenicity and safety of the recombinant antigens, prompt us to continue our studies and to conduct challenge experiments with virulent leptospires. The sheep model of leptospirosis could be the way forward in order to translate the immunoprotection results obtained in the hamster model of leptospirosis and the large ruminant species, particularly bovine, that would benefit from an improved vaccine formulation.

5. Conclusion

In conclusion, the recombinant proteins evaluated in this study (LipL32, LigAni, and LigBrep) elicited a significant humoral immune response in sheep, generating high serum immunoglobulin (IgG) titers. The encouraging results that were originally observed in hamsters can be successfully reproduced in the ruminant model without side effects, which indicates an important step forward towards the search for a recombinant vaccine for bovine leptospirosis.

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