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Research Article

Study of the Immune Response Induced by an Attenuated Strain of *Corynebacterium pseudotuberculosis* in Sheep Reared Under Extensive System

Danielle Lima[1], Míriam Rebouças[1], Bruno Bastos[1], Marcos Costa-Silva[1], José Tadeu Raynal[1], Paulo Carvalho-Filho[1],Vera Vale[2], Lília Moura-Costa[1], Soraya Trindade[1,3]*, Ricardo Portela[1] and Roberto Meyer[1]

[1]Department of Biointeraction, Immunology and Molecular Biology Laboratory, Health Sciences Institute (ICS), Federal University of Bahia (UFBA), Salvador, Bahia, Brazil

[2]Department of Physical and Earth Sciences (DCET), State University of Bahia (UNEB), Alagoinhas, Bahia, Brazil

[3]Department of Health, Feira de Santana State University (UEFS), Feira de Santana, Bahia, Brazil

***Corresponding Author**: Soraya Trindade, Immunology and Molecular Biology Laboratory – Health Sciences Institute (ICS)–Federal University of Bahia (UFBA); Av. Reitor Miguel Calmon, s/n; Vale do Canela, Salvador, Bahia, Brazil.

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Abstract

Caseous Lymphadenitis (CL) is an infectious chronic disease that affects sheep and goats, worldwide distribution, caused by *Corynebacterium pseudotuberculosis* which leads to formation of granulomas in superficial lymph nodes and some internal organs. A naturally attenuated strain of *C. pseudotuberculosis*, called T1was recently isolated, showing considerable promise as likely immunogen in initial tests in mice and goats. To test this strain in sheep, twenty animals were divided into five experimental groups were inoculated with *C. pseudotuberculosis* T1 line into two separate doses, 2 x 10^5 and 2 x 10^7 CFU, and two groups received a booster after two months. The control group was inoculated only with saline. The animals were challenged with a wild strain of *C. pseudotuberculosis*, and then necropsied. An ELISA for identification of specific IgG, and IFN-γ dosage for evaluating the cellular response was used. The animals immunized with a dose 2 x 10^7 CFU had increased antibody production than the other groups before challenge, and this difference was not statistically significant. The IFN-γ in all groups were low before challenge with a virulent strain, but it was statistically higher after challenge in the groups inoculated with 2 x 10^5 CFU. The animals inoculated with the attenuated bacteria present number and injury pattern similar to those of the control group animals. The attenuated strain of *C. pseudotuberculosis* T1 has not shown a satisfactory induce immunoprotection in these animals against a challenge with a wild strain of *C. pseudotuberculosis* in sheep when tested in the field.

Keywords: *Corynebacterium pseudotuberculosis; Caseous Lymphadenitis; IFN-γ; Sheep; Vaccine*

Abbreviations

BHI: Brain-Heart Infusion Médium; BSA: Bovine Serum Albumin; CFU: Colony Forming Units; CL: Caseous Lymphadenitis; ELISA: Enzyme Linked Immuno Sorbent Assay; IL-12: Interleukin-12; IL-4: Interleukin-4; INF-γ: Interferon Gamma; MCP-1: Monocyte Chemoattractant Protein-1; PBS: Phosphate Buffered Saline; PBS-T20: Phosphate Buffered Saline with Tween 20; PLD: Phospholipase D; SPSS: Statistical Package for Social Sciences; TMB: Tetramethylbenzidine; TNF-α: Tumor Necrosis Factor Alpha; TPF: Three-Phase Fractionation Technique; TPP: Three Phase Partitioning

Introduction

Caseous lymphadenitis (CL) is a chronic infectious disease affecting sheep and goats worldwide. This disease is caused by the bacterium *Corynebacterium pseudotuberculosis*, and results in the formation of granulomas in the superficial lymph nodes and other organs,

such as the lungs, spleen and liver [1,2]. In goat and sheep livestock operations, this disease is of critical importance due to economic losses resulting from decreased milk production, depreciation of the animal's skin due to scarring, as well as costs incurred by drug purchases and labor in the treatment superficial granulomas. Livestock production losses are seen when the affected lymph nodes are located in specific areas, such as the mandible, crural region, and udders, impairing the animal's normal activities including chewing, locomotion while grazing at pasture, and lactation [3].

Although the diagnosis and control of caseous lymphadenitis is not yet satisfactory, vaccination is understood as the most significant manner of reducing infection [4]. The search for a comprehensive vaccine capable of inducing significant immunoprotection has been long and arduous [5-7]. Several attempts have been made using a variety of antigens, such as whole, live, killed, and cell wall bacteria [6,8,9], and adjuvants have also been employed to enhance immunity [10]. Vaccination against lymphadenitis works in a prophylactic fashion, requiring the stimulus of phagocytosis by the host's monocytes using an adjuvant that allows for lower doses of an immunogen without compromising the degree of protection provided [11]. The present study investigated the immune response induced by immunizing sheep with an attenuated strain of *Corynebacterium pseudotuberculosis* in typical pasture-based livestock operation conditions.

Materials and Methods

Bacterial strains

The bacterial strains used for experimentation purposes were obtained from the Microorganisms Collection of the Microbiology Laboratory, located at the Health Sciences Institute of the Federal University of Bahia (Salvador, Brazil).

The strain denominated "T1" was isolated from the lymph node of a goat with granulomatous lesions, obtained from the Santa Luz region in the state of Bahia. The wild-type strain cataloged as "VD57" was isolated from another goat lymph node in the region of Juazeiro, Bahia. Both of these strains were grown in brain heart infusion (BHI) medium and cultivated at 37°C for 72h for use in experimental infection.

Bacterial culturing for immunization

Animals in experimental groups were inoculated with a bacterial suspension, cultured as follows: a colony of T1 strain *Corynebacterium pseudotuberculosis* was cultured in BHI agar for 48h at 37°C, then transferred to 5 mL of BHI broth. After an additional 48h at 37°C, the 5mL culture was subsequently transferred to 250 mL of BHI broth and stored at 37°C for 72h. The bacterial mass was then centrifuged under refrigeration for 30 min at 8000 rpm. Next, the bacterial mass was resuspended in 10 mL of sterile saline solution. Bacteria counts were made by performing 10x serial dilutions in PBS, which were grown by dissemination on BHI agar plates. Bacterial colonies were counted after 48h of incubation at 37ºC and concentrations were adjusted to 2×10^5 and 2×10^7 CFU/mL. After gram staining, the cultures were plated on BHI agar, blood agar and Sabouraud agar to ensure the absence of contamination.

Bacterial challenge inoculant

Wild-type *C. pseudotuberculosis* VD57 was cultivated in BHI broth at 37ºC in aerobic conditions for 72h. The bacterial mass was centrifuged at 3000g and washed twice in sterile PBS. Bacterial counts were subsequently made using serial decimal dilutions in PBS plated on BHI-agar. Counting was performed after 48h of culturing following inoculum adjustment to 10^5 CFU/mL.

C. pseudotuberculosis antigens

Attenuated T1 strain antigen secretion

The attenuated *C. pseudotuberculosis* T1 strain was grown in BHI medium and then cultured at 37°C for 72h. After culturing, the samples were for 30 min at 10,000g. The supernatant was then collected, aliquoted and subsequently frozen at -20°C until time of use in an ELISA assay.

Obtainment of secreted antigen concentrate using three-phase fractionation (TFT)

A three-phase fractionation technique (TPF) was used to obtain secreted antigen concentrate. Bacterial culture supernatants were saturated with 30% ammonium sulfate at room temperature under gentle mixing. Following pH adjustment, n-butanol was added in identical proportion to the culture medium volume. After vortex mixing for 1 min and 1h of resting, the interface proteins were collected and centrifuged at 1350g for 10 min. The interface precipitate was collected and dissolved in PBS buffer (ratio: 500 μL of extract to 5 mL of PBS).The quantification of protein was determined by the Lowry modified method by using a commercial detergent compatible protein assay kit [12].

Animals

A total of 20 ewes, crossbred from Dorper and Santa Inês stock, were obtained from a private farm in the municipality of Ipirá (Bahia, Brazil). All animals, ranging between three to 18 months of age, were kept in conditions typical of pasture-based livestock operation. Pathogen screening was performed on all sheep using a previously standardized indirect ELISA technique from the Laboratory of Immunology at the Health Sciences Institute of the Federal University of Bahia. Those found to be seronegative for *C. pseudotuberculosis* were included.

Experimental groups

A total of 20 selected ewes were divided into five experimental groups and inoculated in the right paralumbar fossa region with a range of doses of attenuated *C. pseudotuberculosis* T1 strain. Groups I and II were subcutaneously inoculated with 1 mL at a concentration of 2 x 10^5 CFU, while Groups III and IV were inoculated with 1 mL of 2 x 10^7 CFU. Groups I and III received a second dose, identical to the first, 60 days after the first inoculation. Group V, the control group, was inoculated only with sterile saline, the same suspension used as a delivery vehicle for the bacterial inoculant. At 180 days, all animals were challenged with a wild-type strain of *C. pseudotuberculosis* (denomination: VD57) containing 1mL of 2 x 10^5 CFU. The animals were accompanied for 90 days after receiving the bacterial challenge and then necropsied for anatomopathological investigation.

Clinical examination of animals

All animals inoculated with the T1 strain were clinically examined both before and after inoculation, as well as at monthly intervals, and inspections of superficial lymph nodes at multiple locations were consistently performed.

Immunological monitoring

The ewes' humoral and cellular immune responses were monitored on a monthly basis using an ELISA technique (antibody response) previously standardized at the Immunology Laboratory of the Health Sciences Institute, Federal University of Bahia [13].

Indirect ELISA to detect Anti-*C. pseudotuberculosis* IgG

Polystyrene flat bottom wells (Costar, USA) were sensitized with the culture supernatant of *C. pseudotuberculosis* diluted at 1:100 in 0.05M of carbonate-bicarbonate buffer, pH 9.6, and incubated for 12h at 4°C. After washing twice with phosphate saline containing 0.05% Tween-20 (PBS-T20), the plates were blocked with 200 μL/well of PBS-T20 containing 5% skim milk for two hours at 37°C. Next, plates were incubated with 100 μL/well of test sera, while control samples were diluted at 1:100 in PBS-T20 containing 1% skim milk for one hour at 37°C. After five repeated washing cycles in PBS-T20, 100 μL/well of rabbit anti-sheep IgG was added to the plates, conjugated to peroxidase (Dako Cat. No. P 0163, USA) and diluted at 1:20,000 in PBS-T20. Next, plates were incubated for 45 minutes at 37°C, washed again five times with PBS-T20, then reincubated with 50 μl/well of a solution containing H_2O_2 and tetramethylbenzidine (Moss Inc., Pasadena, MD)for 20 minutes at room temperature in a dark chamber. The reaction was stopped by adding 25 μL/well of 2N H_2SO_4 solution. Optical density readings were performed between 450 and 630nm using an automatic ELISA photo colorimeter. The previously standardized cutoff point of 0.250 was used to distinguish between healthy and affected animals [12]. Each serum sample was tested in duplicate and interplate correction was performed using optical density (OD) readings from a standardized positive test pool [13].

Interferon-gamma quantification

Interferon gamma was measured in the supernatant of whole blood cultures at each inoculation interval, beginning with the first inoculation (time zero). Heparinized blood from each animal was plated in 24-well cellular culture plates (Costar, USA). After plating, 80 µg of TPP antigen was added to a single sample for each animal, while 5 µg of mitogen was added to a second sample (positive controls) and a third sample consisted of whole blood incubated only with PBS (negative controls). All plates were incubated for 48 hours at 37ºC in a humidified atmosphere of 5% CO_2. Culture supernatant was collected following centrifugation and the production of INF-γ was then evaluated using a commercially available enzyme immunoassay kit (Ovine INF-γ Mabtech).

Bacterial challenge protocol

A 1mL challenge corresponding to 2×10^5 CFU of wild strain VD57 was injected intradermally in each animal's right groin using an insulin syringe.

Necropsy

Ninety days after receiving the bacterial challenge, all animals were transported to the Baby Bode refrigerated slaughterhouse located in the city of Feira de Santana (Bahia, Brazil) where sheep lymph nodes, lungs, livers, spleens, as well as other tissues, such as the kidneys and mesentery, were inspected. Those presenting lesions were either aseptically placed into sterile collectors for subsequent bacterial identification, or stored in collectors with formalin for histological analysis.

Bacteria identification

Unfixed collected material samples were cultured in BHI-blood agar. Bacteria identification was performed using colony morphology, gram staining, catalase testing, urease testing or by glucose, lactose, sucrose or maltose carbohydrate fermentation.

Statistical analysis

All data were analyzed using SPSS (Statistical Package for Social Sciences) version 9.0 for Windows. The results of the ELISA for quantification of IFN-γ and determination of specific IgG against *C. pseudotuberculosis* were screened by variance analysis, and when statistical significance was found, the mean correlation was made through of non-parametric Mann-Whitney test used to compare each experimental group to controls.

Results

Anti-*C. pseudotuberculosis* IgG antibody production measured by indirect ELISA

The results of the experimental groups inoculated with the T1 strain demonstrate a similar pattern of humoral response and indicate that this strain did not significantly stimulate antibody production throughout the post-immunization period (Figure 1 and 2).

A comparison of group means reveals that the group III exhibited a higher production of IgG antibodies before the challenge in comparison to the other groups, yet this difference was not statistically significant. After being challenged with a virulent strain, the group III showed a decrease in antibody production, lower than levels observed in the other groups. According to the Mann-Whitney test results, at no time during the experiment did the control group produce antibodies at quantities significantly greater than time zero (Figure 3).

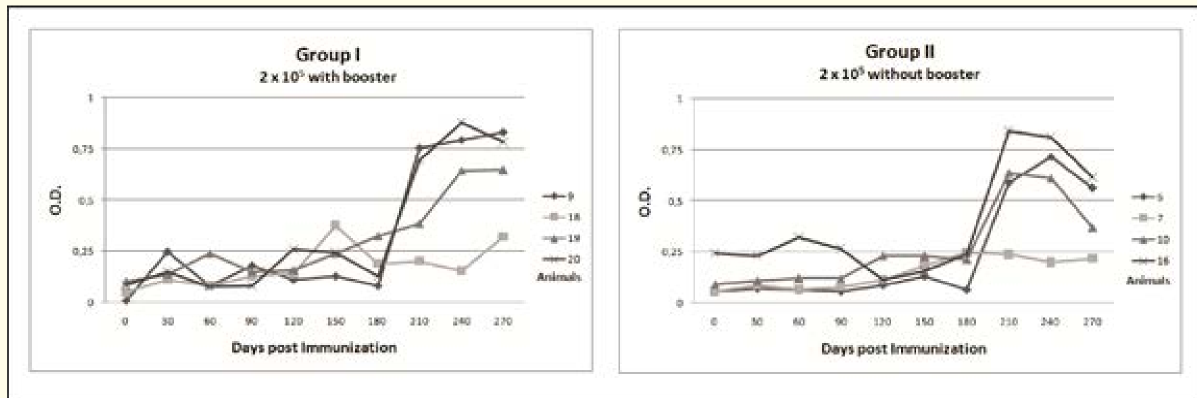

Figure 1: *A plot of the individual values of IgG optical density in the 2x10*^{5} *groups, with and without reinforcement, post-immunization and post-challenge. Arrows indicate the time of immunization with the T1 strain and stars indicate the time of challenge.*

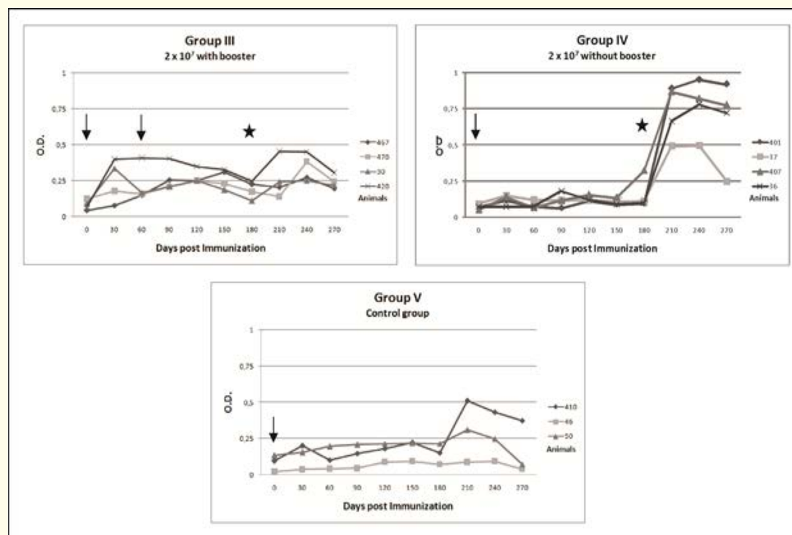

Figure 2: *A plot of the individual values of IgG optical density in groups 2x10*^{7} *with and without reinforcement, and the control group post-immunization and post-challenge. Arrows indicate the time of inoculation with the T1 strain and stars indicate the time of challenge.*

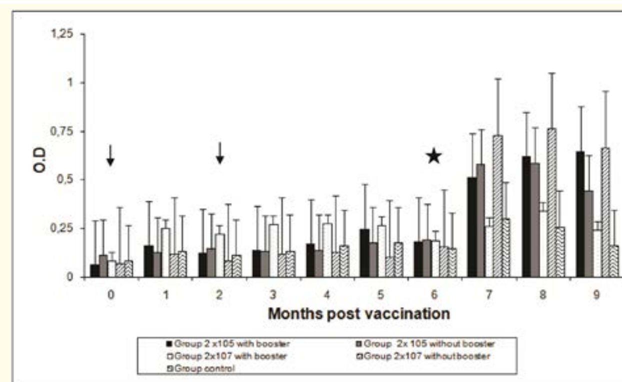

Figure 3: *Average IgG Optical Density values as measured by ELISA in serum samples from animals inoculated with different doses of T1 strain of Corynebacterium pseudotuberculosis, as well as control animals. Arrows indicate the time of inoculation with the T1 strain, and the star indicates the time of challenge.*

Interferon gamma production

A comparison of IFN-γ production among experimental groups reveals that the 2 x 10^5 Groups without reinforcement and 2 x 10^7 with reinforcement exhibited higher levels of production of this cytokine before the challenge, no statistical differences were observed between these groups. After the challenge, all four groups inoculated with the T1 strain showed an increase in IFN-γ production, which was higher in the groups inoculated with 2 x 10^5 CFU of T1 with and without reinforcement in the second month post-challenge. Statistical significance was observed in comparison to the other groups during this same month (Figure 4).

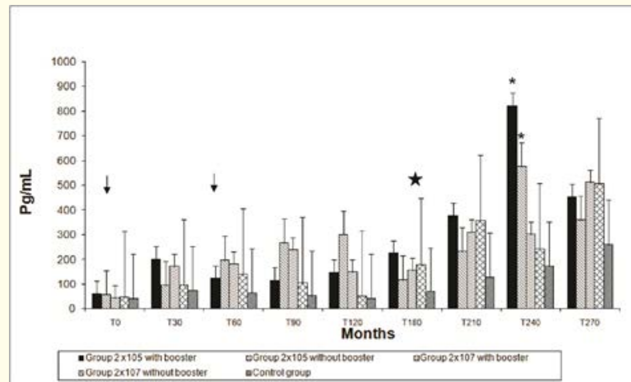

Figure 4: *Group means of IFN-γ production at pre-and post-challenge time points. Arrows indicate the time of inoculation with the T1 strain and the star indicates the time of challenge. Asterisks indicate significance, $p < 0.05$.*

Necropsy

All experimental animals were sent to a refrigerated slaughterhouse located in the city of Feira de Santana - BA where they were slaughtered. The organs evaluated were lungs, liver, spleen, and the internal anterior and posterior lymph nodes were separated, using the animal's midline as a parameter. Lesions were found at variable proportions in the experimental groups. The non-reinforced 2 x 10^7 group had a higher number of lesions in the posterior lymph nodes than other groups. However, this same group had no lesions in anterior lymph nodes, indicating that bacteria did not spread to the anterior region. The 2 x 10^5 group with reinforcement presented similar characteristics. The reinforced 2 x 10^7 group presented a lower numbers of lesions than other groups, yet this finding was not statistically significant. None of the groups had lung or spleen injuries, and just one animal from the 2 x 10^5 reinforced group presented a granulomatous lesion in the liver, in which the presence of *C. pseudotuberculosis* was confirmed by bacteriological examination (Table 1).

Groups	No. of Animals	LLD	Lung	Liver	Spleen	Anterior Lymph Nodes	Posterior Lymph Nodes
2x10^5 reinforced	4	4/4	0	1/4	0	0	2/4
2x10^5 unreinforced	4	4/4	0	0	0	1/4	2/4
2x10^7 reinforced	4	3/4	0	0	0	1/4	1/4
2x10^7 unreinforced	4	4/4	0	0	0	0	3/4
Control	3	3/3	0	0	0	1/3	1/3

Table 1: *Summary of necropsy findings showing compromised tissues post-challenge*

LLD: Lesion found at site of challenge inoculation

Bacteria identification

The presence of caseous material suggestive of *C. pseudotuberculosis* infection was detected in 30 samples. Colony morphology, Gram staining, catalase and urease testing, and carbohydrate fermentation: glucose, lactose, sucrose and mannose, identified all samples as consistent with *C. pseudotuberculosis*. Synergistic hemolysis was performed on some of the collected samples and the hemolytic pattern was compared to the two experimental strains used (T1 and VD 57). The hemolytic characteristic was determined to be consistent with the wild-type strain.

Discussion

The present study aimed to evaluate humoral and cellular responses in sheep inoculated with different doses of attenuated *C. pseudotuberculosis* T1 strain raised in pasture-based livestock operation conditions. We observed low humoral responses after immunization in all four groups, with values similar to those observed in the control group. These results are consistent with those that tested the humoral and cellular responses of the T1 strain at a dose of 2×10^6 CFU in goats [7]. The group that received inoculations with T1 showed increases in antibody production in the post-challenge period, similar to results published in a previous study [7]. The variable responses of different animal groups are probably the result of differences among animals within each group, especially due to the animals lacking a defined breed.

Previous studies, such as one involving the live vaccine *C. pseudotuberculosis* strain 1002, and another employing a formalin-killed vaccine produced from this same strain, indicate that the killed vaccine induces greater antibody production than the live vaccine, probably due to the presence of an adjuvant [14]. Tests conducted with the live strain 1002 and lyophilized in doses of 10^{10} and 10^{11} CFU, using different vaccination regimens, showed higher levels of induced antibodies, as determined by ELISA, than those observed in a control group (Meyer., *et al*. 2008).

Attenuated vaccines are advantageous due to increased durability in the body, and of inducing immunity that is principally cellular in nature. Sheep that were experimentally infected with *C. pseudotuberculosis* and then challenged with a virulent strain demonstrated protection [3]. A group of researchers inactivated the PLD gene to serve as a basis for a live recombinant *C. pseudotuberculosis* vaccine. The degree of protection was tested by challenges with a virulent strain in which humoral and cellular responses were unremarkable. These authors concluded that the marked reduction in humoral and cellular responses was due to the complete removal of the PLD gene. We believe that the strain employed by the present study secretes low amounts of PLD, and thus induces nominal humoral responses [15]. These same authors later constructed a mutant of PLD in which the residue of histidine amino acid was replaced by tyrosine, thus producing a biologically inactive mutant PLD gene. The authors divided Experimental animals into two groups: animals vaccinated with the recombinant gene in which PLD was absent ("Toxminus") and another group vaccinated with inactivated PLD. The sheep vaccinated with inactivated PLD exhibited more protection than the other group [16].

A commercial kit was used to quantify the production of IFN-γ in whole blood cultures under stimulation by TPP secreted antigen. The study of cytokine profiles produced during the infection process provides important information regarding these protein mediators, which have a bearing on both natural and acquired immunity, providing information about essential mechanisms that enable a small number of specific lymphocytes, activating diverse effector mechanisms to eliminate pathogens [17]. Granuloma development may occur in the presence of Th1 and Th2 cells, as well as cytokines, such as IFN-γ, IL-12, IL-4, TNF-α e MCP-1, whose levels are elevated during the initial formation of granuloma, probably contributing to lesion development, which plays an important role in reducing the dissemination of bacteria [18]. Accordingly, the experiments performed herein were developed with the aim of better understanding the pattern of cell response in animals inoculated with the T1 strain of *C. pseudotuberculosis*.

An analysis of IFN-γ production under stimulation by secreted antigens of the T1 strain reveals low levels before the bacterial challenge was initiated in all experimental groups. Post-challenge, the induction of IFN-γ was higher in all groups, which is consistent with

data from a previous study that found high levels of IFN-γ induced by secreted TPP antigen, and reduced production when leukocytes were stimulated with a somatic antigen [19]. ELISA was used to measure IFN-γ as a diagnostic method in sheep inoculated with the commercially available vaccine Glanvac-6 (composed of an ultrafiltered solution of diverse antigens *C. pseudotuberculosis, Clostridium perfringens, Clostridium tetani, Clostridium novyi and Clostridium septicum*). These researchers found that vaccinated animals had lower IFN-γ production when compared to a group of naturally infected animals [20].

The results obtained from necropsy indicate that the group which received 2 x 10^7 CFU with reinforcement suffered the fewest number of lesions. This same group showed increased antibody production following immunization, although this declined post-challenge. We believe that, after receiving the second dose, the T1 strain probably remained at the site of inoculation longer in this group, which induced a continuous stimulus with respect to the production of antibodies. The protocols of dosage and inoculation route used in the bacterial challenge should stimulate natural infection, since, depending on the amount of bacteria used and the site of inoculation, the host immune response may be masked, hindering the protective effect of the vaccine being tested [6].

The experiments conducted herein utilizing the T1 strain suggest the need for further study of this strain, since previous work has already demonstrated a certain degree of protection in both murine and goat models [7]. The validation and certification of a strain for use in a vaccine candidate involves many attempts and discoveries. It is also worth mentioning that the animals used in this study were housed in a semi-natural environment, which implies variation and contact with other animals not foreseen at the outset of experimentation, different than the conditions afforded by other studies.

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Conflict of Interest

None of the authors has a financial relationship with other people or organizations that could inappropriately influence its findings.

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