Serological response of lambs vaccinated with a biological bacterin-toxoid type against *caseous lymphadenitis*

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ABSTRACT

Objective: To evaluate the serological response to somatic, secretory, and soluble antigens in sheep vaccinated and revaccinated with an experimental bacterin-toxoid biological against ovine caseous lymphadenitis.

Design/Methodology/Approach: The serological response to different antigens was assessed in sheep vaccinated with an experimental bacterin-toxoid biological against *caseous lymphadenitis*. The animals' sera were tested using indirect enzyme-linked immunosorbent assay (ELISA) with different antigens. The antigens were obtained from a wild strain isolated from an ovine abscess.

Results: The antigens with the strongest immunogenic response were those obtained by sonication and the supernatant rich in phospholipase D.

Study Limitations/Implications: In Mexico, no commercial vaccines are currently available for the prevention of *caseous lymphadenitis*. Therefore, conducting research to develop effective control strategies for this disease is of great importance.

Findings/Conclusions: The most effective antigens were those obtained by sonication and the supernatant rich in phospholipase D, which is significant since phospholipase D is the primary pathogenic factor of *Corynebacterium pseudotuberculosis*. Vaccination against caseous lymphadenitis using a bacterin-toxoid biological could serve as a promising alternative for disease prevention, given the strong antigenic response observed in this study.

Keywords: *caseous lymphadenitis*, bacterin-toxoid, sheep, antibodies.

INTRODUCTION

Caseous lymphadenitis (CL) is a chronic infectious disease that affects sheep and goats. It is caused by the Gram-positive bacterium *Corynebacterium pseudotuberculosis* and is characterized

by suppurative and necrotizing lesions in the lymph nodes, lungs, and other visceral organs, leading to economic losses due to its impact on carcass and wool quality (Sánchez *et al.*, 2021; Ruíz *et al.*, 2020; Windsor *et al.*, 2011). *Corynebacterium pseudotuberculosis* is a facultative intracellular pathogen capable of replicating and surviving within phagocytes. Its virulence is primarily attributed to cell wall lipids and the production of phospholipase D (PLD) (Guimarães *et al.*, 2011). PLD is a 31 kDa protein that hydrolyzes sphingomyelin in cell membranes, releasing choline and facilitating bacterial dissemination (permeability factor). This toxin exhibits cytotoxic activity, particularly affecting endothelial cells and causing erythrocyte lysis in sheep and cattle. Additionally, it exerts a cytotoxic effect on goat macrophages (Sánchez *et al.*, 2021; Ruíz *et al.*, 2020; Guimarães *et al.*, 2011; Delgado *et al.*, 2016). The lipid layer of *C. pseudotuberculosis* functions as a chemotactic agent for phagocytes, while also exhibiting leukotoxic properties, leading to cell degeneration and lysis. Furthermore, it contributes to abscess formation and enhances resistance to lysosomal lytic enzymes (Rodríguez *et al.*, 2022; De La Fuente, 2012). The primary mode of transmission occurs through skin lesions via direct contact with contaminated shearing equipment, animal handling tools, or environmental surfaces. Additional transmission routes include inhalation and ingestion (Rodríguez *et al.*, 2022). Following infection, *C. pseudotuberculosis* multiplies locally, forming microabscesses. The bacterium is subsequently phagocytosed by macrophages and neutrophils, yet remains viable, allowing it to be transported to regional lymph nodes, where it induces caseous abscess formation and necrosis. PLD inhibits chemotaxis, prevents phagocytic cell degranulation, and activates the complement system via the alternative pathway, resulting in lymph node necrosis and thrombosis. Additionally, the bacterium utilizes extracellular iron through its serine- and protease-related proteins, facilitating lesion development (Rodríguez *et al.*, 2022). Cellular immunity is considered the primary immune response involved in CL pathogenesis; however, *C. pseudotuberculosis* is a complex pathogen, and both humoral and cellular immune mechanisms play significant roles in disease progression (Miranda *et al.*, 2010). The control of *caseous lymphadenitis* is challenging, necessitating serological diagnostic tests to identify and eliminate infected animals. Additionally, hygiene measures, including disinfection of surgical instruments, shearing equipment, and animal facilities, are crucial for disease prevention (Sánchez *et al.*, 2021). In countries where CL is endemic, vaccination is recommended, and several biological formulations have been developed for disease prevention (Braga *et al.*, 2007). In Mexico, no commercial vaccines are available for CL prevention. Therefore, conducting research to develop effective control strategies is of paramount importance. The aim of this study was to evaluate the serological response to somatic, secretory, and soluble antigens in sheep vaccinated and revaccinated with an experimental bacterin-toxoid biological against ovine *caseous lymphadenitis*.

MATERIAL AND METHODS

Thirty-three lambs between three and six months of age, from sheep production units with no clinical history of *caseous lymphadenitis*, were used. The animals were divided into four groups. Groups 1 and 2 consisted of ten lambs each, which were vaccinated and revaccinated 15 days later with 2 mL of an experimental bacterin-toxoid vaccine against

Corynebacterium pseudotuberculosis-induced *caseous lymphadenitis*. This biological preparation contained a concentration of 1×10^9 CFU/mL of bacteria inactivated with 0.4% formalin and enriched with a 48-hour culture supernatant incubated at 37 °C. Group 3, serving as control group 1, consisted of ten lambs that received only sterile physiological saline solution administered subcutaneously. Group 4, control group 2, comprised three lambs that did not receive any treatment.

Blood samples were collected weekly before and after vaccination for ten weeks to obtain serum samples. In week 7, the vaccinated animals and control group 1 were challenged with a live strain of *C. pseudotuberculosis* to assess the protective capacity of the biological; however, these results are not included in this publication. Serum samples were analyzed using indirect ELISA to detect antibodies against somatic, secretory, and soluble antigens. The antigens used included a crude supernatant from a liquid culture of *C. pseudotuberculosis* rich in phospholipase D (PLD), a protein extract from the bacterial cell wall obtained through chemical denaturation and heat (DCPE), a protein extract obtained solely through heat treatment (BCPE), and an antigen extracted using fluent steam.

All antigens were obtained from a wild *C. pseudotuberculosis* strain isolated from an abscess in a three-year-old ram. This strain was biochemically characterized and identified by polymerase chain reaction (PCR) and designated as the "ROBUS" strain. The BCPE antigen was obtained by culturing *C. pseudotuberculosis* on sheep blood agar at 37 °C under aerobic conditions for 48 hours, harvesting the colonies, suspending them in phosphate-buffered saline (PBS), and centrifuging at 3000 × g, repeating the process three times. The pellet was resuspended in PBS, heated at 100 °C for five minutes, centrifuged, and the resulting supernatant was used as the cell wall protein antigen. The DCPE antigen followed the same initial steps as BCPE but was resuspended in an antigen preparation buffer containing 0.5 M Tris pH 6.8, 5.2% SDS, and 8.7% 2-mercaptoethanol, boiled at 100 °C for five minutes, centrifuged at 3000 × g, and the supernatant was used as the antigen.

The fluent steam antigen was prepared by culturing *C. pseudotuberculosis* under the same conditions, harvesting the colonies, suspending them in physiological saline solution (SSF), and centrifuging at 3000 × g for 30 minutes. The supernatant was removed, the cells were washed, recentrifuged, and the final suspension was autoclaved in fluent steam (without pressure) for 30 minutes. After cooling for 30 minutes, it was centrifuged again for 30 minutes at 3000 × g, dialyzed with two changes of distilled water, centrifuged at 3000 × g for 50 minutes, repeated twice, and left to stand for 15 minutes. The final supernatant was filtered through 0.45 μm membranes.

The sonication antigen was obtained from *C. pseudotuberculosis* colonies cultured under the same conditions, suspended in 10 mL of SSF, and inactivated in a water bath at 90 °C for 15 minutes. The suspension was sonicated for ten cycles of 30 seconds, followed by 30 seconds of rest. After sonication, it was centrifuged at 3000 × g for 10 minutes, followed by a second centrifugation at 6000 × g for six minutes. The final supernatant, containing all cell fractions, was designated as the somatic antigen for ELISA testing.

All sera were analyzed using the indirect ELISA technique. The standardization method known as "Chessboard Titrations" (CBT) or the "Checkerboard Method" was

used to determine the optimal antigen and serum concentrations. For ELISA, the plates were sensitized with the respective antigens, incubated for 24 hours at 4 °C, and washed four times with PBS-Tween 20. The plates were blocked with 2.0% skim milk, incubated for one hour at 37 °C, washed again, and dried. The primary antibody (test serum) was added and incubated for one hour at 37 °C, followed by four washes with PBS-Tween 20. The secondary antibody (anti-ovine IgG conjugated with peroxidase) was added at a 1:1000 dilution, incubated for one hour at 37 °C, and washed again. The substrate 2,2'-Azino diethylbenzothiazoline sulfonic acid (ABTS) was added and left to react for 15 minutes. The optical density was measured at a wavelength of 450 nm. Statistical analysis was performed using an analysis of variance (ANOVA) test, and differences between means were established using the Tukey test.

RESULTS

The animals immunized with the experimental bacterin-toxoid biological for the prevention of *caseous lymphadenitis* exhibited a significant increase ($p<0.05$) in their serological response to the different antigens used in the ELISA test. This difference was observed only in the immunized animals, compared to the non-immunized group, while no significant differences were detected in the serological response among the different evaluated antigens (BCPE, DCPE, somatic, and PLD). Figure 1 illustrates the serological response to somatic antigens (sonicated antigen) of *C. pseudotuberculosis*. As observed, from the second week, antibody levels began to increase in the two immunized groups. Following the fourth week, corresponding to revaccination, a pronounced rise in antibody levels was detected in the vaccinated groups, markedly distinguishing them from the control groups.

After the seventh week, when both vaccinated groups and control group 1 were challenged, a similar increase in antibody levels was observed among these three groups, with a significant difference compared to control group 2. The Figure 2 presents the

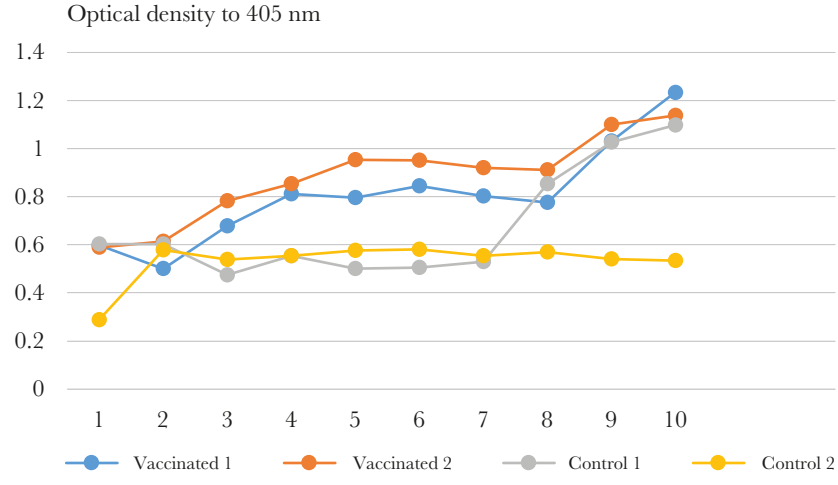

Figure 1. Serological response to somatic antigens of *Corynebacterium pseudotuberculosis*. Vaccinated groups 1 and 2 exhibited a notable increase in antibody levels following vaccination against somatic antigens, with this response becoming more pronounced after the challenge. Meanwhile, control group 1 showed a gradual increase in antibody levels only until the challenge.

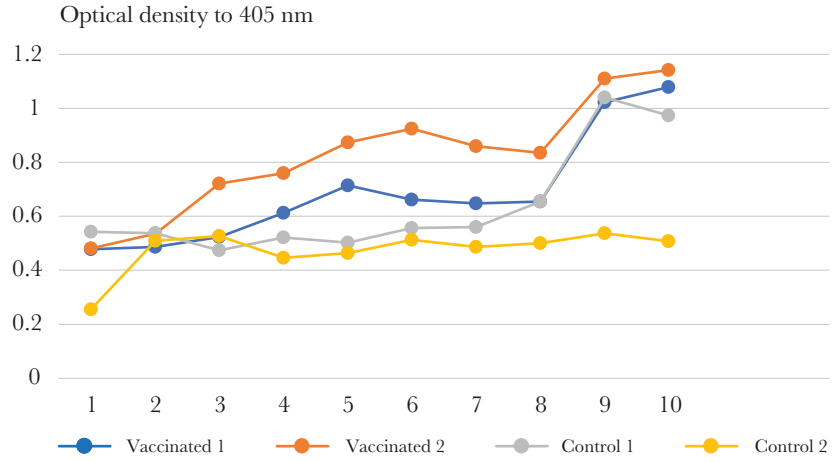

Figure 2. Serological response to heat-derived antigens (BCPE) of *C. pseudotuberculosis*. Vaccinated groups 1 and 2 showed increased antibody levels after vaccination against these antigens, this increase being more evident after challenge. Control group 1 increased its values until the challenge.

serological response to surface antigens of *Corynebacterium pseudotuberculosis* (BCPE). By the third week, antibody levels increased in vaccinated group 2, whereas vaccinated group 1 showed an increase in antibody levels by the fourth week. By week 7, the two immunized groups and control group 1 exhibited comparable antibody levels, whereas control group 2 maintained consistently low levels.

In the Figure 3 illustrates the antibody response to antigens obtained through heat and chemical denaturation. Antibody levels showed a gradual increase over time; however, by week 8, a notable rise was observed in both vaccinated groups compared to control group 1.

Figure 4 illustrates the serological response to *Corynebacterium pseudotuberculosis* toxin. Following immunization, a rise in antibody levels was observed in the vaccinated group,

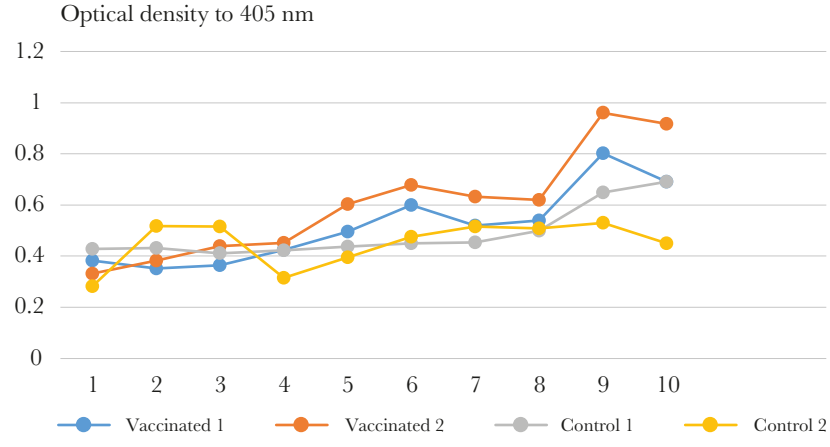

Figure 3. Serological response to *C. pseudotuberculosis* antigens obtained by heat and chemical denaturation (DCPE). Vaccinated groups 1 and 2 showed slightly increased antibody levels after vaccination against these antigens, this increase being more evident after challenge. Control group 1 increased its values until the challenge.

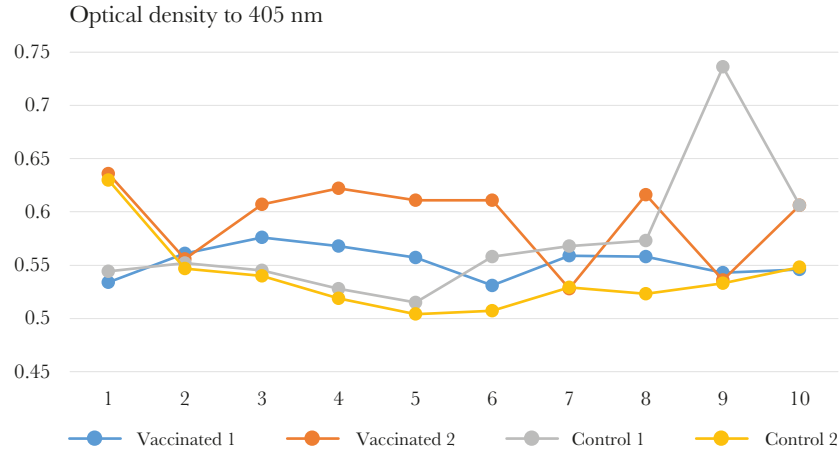

Figure 4. Serological response to *C. pseudotuberculosis* phospholipase D. Vaccinated groups 1 and 2 showed increased antibody levels after vaccination against this antigen. Control group 1 considerably increased its values up to the challenge.

followed by a subsequent decline. However, after the challenge, control group 1 exhibited a considerable increase in antibody levels.

Finally, Figure 5 shows the behaviour of the serological response of vaccinated and unvaccinated sheep against the antigens obtained by fluent vapour. Vaccinated group 2 showed a significant increase in antibody levels compared to the other groups, both at vaccination, revaccination, and challenge.

Figure 5. Serological response to antigens obtained by fluent vapour of *C. pseudotuberculosis*. Only vaccinated group 2 showed increased antibody levels after vaccination against this antigen. The increase was more evident after the challenge.

DISCUSSION

This study evaluated the serological response induced by an experimental bacterin-toxoid vaccine against *Corynebacterium pseudotuberculosis*, focusing on different antigenic preparations. The findings demonstrated that antigens obtained through sonication and heat extraction (BCPE) elicited the strongest immune responses, making them valuable

tools for assessing immunogenicity. Similar results were reported in the United Kingdom, where the use of sonicated antigens in ELISA tests on sera from clinically affected sheep yielded a sensitivity of 83% and a specificity of 71%, confirming the reliability of this approach (Corona *et al.*, 2011; Farias *et al.*, 2020). Consistently, the present study revealed that vaccinated animals developed a robust antibody response against somatic and surface antigens, reinforcing the potential of these antigens for immunodiagnostic applications. A detailed analysis of heat-extracted antigens revealed that BCPE antigens were more effective in detecting surface antigens than those obtained through chemical denaturation plus heat (DCPE). This finding contrasts with a previous study (Corona *et al.*, 2011), which found DCPE antigens to be more effective in diagnosing *caseous lymphadenitis*. However, an important distinction between these studies lies in the immune status of the evaluated animals. While the earlier research focused on naturally infected animals, this study analyzed the immune response following vaccination. Given that *C. pseudotuberculosis* is a facultative intracellular pathogen, the immune response elicited by active infection differs significantly from that triggered by vaccination, which could explain the discrepancy in antigen performance (Corona *et al.*, 2011; Abbas *et al.*, 2022). Additionally, alternative heat extraction techniques have been explored in other studies, but results have been inconsistent, likely due to variations in temperature protocols and antigen stability (De La Fuente *et al.*, 2012). Beyond surface and somatic antigens, this study also assessed the immune response to secretory antigens, particularly phospholipase D (PLD), which is the primary virulence factor of *C. pseudotuberculosis*. A strong serological response to PLD was observed in vaccinated animals, aligning with previous findings that identified neutralizing anti-PLD antibodies as key indicators of protective immunity (Tachedjian *et al.*, 1995). This is particularly relevant given that Hoelzle *et al.* (2013) demonstrated that PLD-based ELISA detected 100% of infected goats but only 70% of infected sheep, emphasizing the need for multiple antigenic targets to improve diagnostic sensitivity. In fact, these authors suggested that combining immunodominant bacterial proteins (150, 74, 48, and 30 kDa) with PLD enhances diagnostic accuracy, a strategy that could further optimize serological tests for *caseous lymphadenitis*. Another antigenic preparation that demonstrated strong immunogenicity was the fluent steam-extracted antigen. This response may be attributed to the continuous heat and moderate pressure applied during extraction, which likely preserved a broader spectrum of bacterial antigens. The development of ELISA assays for *C. pseudotuberculosis* detection has been a global priority, yet many of these tests remain commercially unavailable. For instance, Dercksen *et al.* (2000) developed four ELISA protocols, with Double-Sandwich ELISA using purified PLD antigen and hyperimmune sera demonstrating a sensitivity of 79% and specificity of 99%, making it particularly suitable for disease eradication programs. In addition to diagnostics, ELISA has been widely employed for monitoring vaccine-induced serological responses. De Oliveira *et al.* (2022) followed up on sheep vaccinated with either a PLD-rich bacterin or a recombinant PLD vaccine, demonstrating that ELISA using sonicated and toxoid antigens effectively measured humoral immunity against PLD. Their findings showed that the formalin-inactivated PLD vaccine conferred 95% protection, whereas the recombinant PLD vaccine provided only 44% protection

(Farias *et al.*, 2020). These results reinforce the protective potential of PLD, which led to the development of the Glanvac22-24 toxoid vaccine in Australia (1984). This vaccine has demonstrated consistent efficacy in preventing *C. pseudotuberculosis* infections in multiple challenge studies (De Oliveira *et al.*, 2022). Further supporting the role of PLD in protective immunity, a study conducted in the United Kingdom evaluated different vaccination strategies, including a recombinant PLD derivative, a formalin-inactivated bacterin, and a bacterin containing recombinant PLD. ELISA-based serological follow-ups confirmed that PLD-containing vaccines provided statistically significant protection, prevented disease dissemination, and reduced the incidence of visceral forms of the disease (Fontaine *et al.*, 2006). In Mexico, Ibarra *et al.* (2016) tested an aroA mutant strain of *C. pseudotuberculosis* in cellular and murine models to evaluate attenuation and immunogenicity. Their study confirmed that the aroA mutant strain was significantly attenuated, yet failed to provide at least 80% protection, a threshold considered necessary for an effective immunogen. This highlights the challenges of developing attenuated vaccines and underscores the need to explore alternative antigenic targets (Ibarra *et al.*, 2016). The selection of bacterial strains for vaccine development is another critical factor. Parise *et al.* (2018) conducted phylogenomic analyses of *C. pseudotuberculosis* isolates from different hosts, revealing that genetic diversity in ovine strains is influenced more by animal transportation than host specificity. They also identified nrdF2, a gene previously described as a potential vaccine target, which could aid in developing novel diagnostics, vaccines, and therapeutics (Parise *et al.*, 2018). As highlighted in multiple studies, ELISA remains an essential tool for epidemiological surveillance and evaluation of vaccine efficacy. However, in Mexico and other countries, the lack of standardized ELISA tests for *caseous lymphadenitis* hinders diagnostic efforts. This has led researchers to explore alternative antigen extraction methods to enhance diagnostic accuracy and serological monitoring in vaccinated animals. Continued advancements in antigen selection and vaccine formulation will be crucial for improving disease control strategies and preventing the spread of *C. pseudotuberculosis* in sheep and goat populations.

CONCLUSIONS

Finally, this study successfully obtained *Corynebacterium pseudotuberculosis* antigens through various extraction methods, allowing for the evaluation of immune responses in vaccinated animals. Among the antigens tested, those obtained by sonication and the PLD-rich supernatant elicited the strongest immune responses in the vaccinated groups. Additionally, antigens derived from the native bacterial strain demonstrated acceptable immunoreactivity, indicating their potential for use in serological assessments. Given the robust antigenic response observed in this study, vaccination against *caseous lymphadenitis* using a bacterin-toxoid biological represents a promising alternative for the prevention and control of this disease.

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