Clinical, humoral, and pathologic findings in adult alpacas with experimentally induced Corynebacterium pseudotuberculosis infection

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Objective—To experimentally infect adult alpacas by ID inoculation of Corynebacterium pseudotuberculosis, follow the clinical and pathologic course of disease, and study the humoral response to infection.

Animals—13 adult alpacas.

Procedures—9 alpacas were inoculated with 1.1 × 10^6 CFUs of C pseudotuberculosis from llama (n = 4) or alpaca (5) origin, and 4 alpacas were sham inoculated as controls. Alpacas were clinically observed after inoculation and euthanatized on days 16, 58, 93, or 128 after inoculation; necropsy examination and histologic evaluation were performed. An indirect ELISA, which made use of the C pseudotuberculosis cell wall as the antigen, was used to measure antibody titters in serum samples.

Results—Alpacas had a persistent febrile response, a local severe inflammatory response, and leucocytosis (> 30 × 10^3 WBCs/μL). Internal abscesses that localized mainly in the renal lymph node were observed. Corynebacterium pseudotuberculosis was recovered from the inoculation site 1 week after inoculation and from internal abscesses at 58 days after inoculation. Initial lesions were typical pyogranulomas with central caseous necrosis, whereas later lesions consisted of connective tissue, mononuclear cells, abundant neutrophils, and liquefactive necrosis. Infected alpacas had detectable serum antibody titers starting on day 16 that persisted until day 93 after inoculation. Sham-inoculated alpacas did not develop serum antibody titers, clinical signs of infection, or lesions.

Conclusions and Clinical Relevance—Alpacas inoculated with C pseudotuberculosis developed abscesses at the inoculation site and internally in the renal lymph nodes, without lung lesions. Corynebacterium pseudotuberculosis isolates from llama and alpaca origin were found to be pathogenically indistinct. (Am J Vet Res 2006;67:1570–1574)

Corynebacterium pseudotuberculosis is a gram-positive bacterium that causes CLA in sheep and goats. Caseous lymphadenitis in sheep is characterized by abscess formation in the superficial lymph nodes and internal organs such as the lungs and liver. The infection can result in reduced wool production and meat losses as a result of carcass condemnation. The natural route of transmission in sheep is considered to be the contamination of superficial skin wounds with purulent material from fistulae in abscessed lymph nodes. Prescapular lymph nodes are the most common site of superficial lesion development, and the lungs are the most commonly involved visceral organ. In sheep and goats experimentally infected with C pseudotuberculosis, the organism spreads to local lymph nodes and subsequently moves to the lungs and bronchial lymph nodes by hematogenous and lymphatic routes. Frequently, no specific clinical signs are associated with the visceral form of CLA in sheep.

In recent years, C pseudotuberculosis has been more frequently isolated from abscesses in alpacas, with much fewer instances in llamas. Abscesses in alpacas are localized mainly in renal lymph nodes in adult alpacas and in superficial lymph nodes in younger alpacas. Abscesses in the renal lymph nodes, those related to the sublumbar area and kidneys, oftentimes attain a diameter of 3 to 8 cm but may reach up to 15 cm in diameter. No evidence of lesions primarily localized in the lungs is normally observed in sheep or goats. Consequences of the disease in alpacas include debilitation and decreased fiber production, but the main economic loss is caused by condemnation of affected carcasses.

Although C pseudotuberculosis is a common pathogen in alpacas, its primary pathogenic role has not been established. Moreover, few studies have examined antibody responses to C pseudotuberculosis in infected small ruminants, and to our knowledge, no studies have been done in South American camelids. The purposes of the study reported here were to experimentally infect adult alpacas by ID inoculation of C pseudotuberculosis of alpaca and llama origin, follow the clinical and pathologic course of disease, and study the humoral response to infection by use of an ELISA.

Materials and Methods

Animals—Thirteen 2-year-old male alpacas that originated from healthy flocks in which CLA had never been detected...
that had negative ELISA results for a *C pseudotuberculosis* infection were used in this study. For 6 weeks prior to experimentally induced infection, alpacas were housed together. Alpacas were then randomly placed in 1 of 3 groups, group A (n = 4; alpacas 1 through 4), group B (5; alpacas 5 through 9), or group C (4; alpacas 10 through 13), and fed alfalfa hay-rye grass and provided water ad libitum. For euthanasia, first a state of anesthesia was induced by administration of a xylazine-ketamine combination (0.5 and 5 mg/kg IM, respectively) followed by exsanguination. The study was approved by the Institutional Ethical Committee of the San Marcos University School of Veterinary Medicine.

Experimentally induced infection—Two isolates of *C pseudotuberculosis*, 1 of llama origin (23GM) and 1 of alpaca origin (7P), were used. Organisms preserved in Kliger iron agar at 5°C were reconstituted and grown for 48 hours in BHI agar supplemented with 5% ovine defibrinated blood and 0.05% Tween 80. Before experimental inoculation, bacteria were suspended in PBS solution (pH, 7.2) to a final volume of 0.5 mL/dose. Bacterial density was estimated first by use of a McFarland standard to determine equivalent turbidity and confirmed after 48 hours of culture in BHI blood agar. Bacterial counts revealed that inocula contained 1.1 × 10⁶ CFUs of *C pseudotuberculosis*. The 4 alpacas from group A were ID inoculated in the left flank with isolate 23GM, whereas the 5 alpacas from group B were ID inoculated in the left flank with isolate 7P. Alpacas from group C were ID sham inoculated with 0.5 mL of PBS solution.

Clinical observations and sample collection—On day 0 before experimental inoculation, alpacas were weighed and blood samples were collected. After inoculation, alpacas were observed for 128 days with rectal temperatures recorded daily for the first week and body weights measured monthly. Blood samples for hemotologic evaluation were collected daily from days 1 to 4 after inoculation, then weekly for 1 month. Blood samples in EDTA were used for determination of CBC, Hct, hemoglobin concentration, and WBC count by use of capillary tubes, a spectrophotometer, and a hemacytometer. Serum for an ELISA was obtained on days 0, 16, 30, 58, 93, and 128 after inoculation. For bacterial culture evaluation, swab specimens were taken from suppurative intra-dermal inoculation sites. Swab specimens were preserved in Cary-Blair transport medium for ≤20 hours and inoculated onto BHI blood agar. All *C pseudotuberculosis* isolates were identified by use of a commercial system.

Gross, histologic, and bacterial culture evaluation—One alpaca from each group was euthanatized on days 16, 58, 93, and 128 after inoculation, except on day 58 after inoculation, when 2 alpacas from group B were euthanatized. Alpacas from infected and control groups were observed at necropsy, and tissue specimens from organs with or without gross lesions were collected and processed for bacterial culture evaluation and histologic evaluation. For histologic evaluation, tissue specimens were fixed in 20% formalin or 3% glutaraldehyde, embedded in paraffin, sectioned, and stained with H&E. Selected sections were stained by a Steiner technique and toluidine blue to highlight the presence of bacterial organisms.

Preparation of antigen for the ELISA—The cell wall of *C pseudotuberculosis* from isolate 7P was used as the antigen for the ELISA. Two colonies, grown for 48 hours at 37°C on BHI blood agar, were transferred to 1 L of BHI broth containing 0.1% Tween 80. The broth was incubated for 72 hours at 37°C on a magnetic stirrer and then stored overnight at 4°C. The culture was centrifuged at 4,000 × g for 15 minutes at 4°C. Each pellet was washed 3 times in PBS solution (pH, 7.4) and centrifuged at 12,000 × g for 15 minutes at 4°C. Thereafter, pellets were resuspended in aliquots of 1.5 mL of PBS solution (pH, 7.4) and stored at 4°C for 24 hours. Then, cells were sonically disrupted at 60% capacity 2 times for 10 minutes each at 4°C, extracted with diethyl ether for 3 hours, and sonically disrupted 1 more time for 10 minutes at 4°C. The protein concentration was determined by the Bradford method by use of a protein assay kit.

ELISA procedures—An ELISA was performed in polystyrene flat-bottomed microplates as described. Optimal dilutions of reagents were determined by block titrations. Negative control sera were obtained from an alpaca flock without a history of CLA and that had never been raised with sheep. In addition, these alpacas did not contain any gross lesions at slaughter and tissue specimens of principal lymph nodes and viscera had negative results for *C pseudotuberculosis*. Positive control serum was obtained from an alpaca inoculated IM in both hind limbs with *C pseudotuberculosis* cell wall (0.219 mg/mL) and Freund complete adjuvant. On day 35, booster injections were administered in the same way but the antigen was mixed with Freund incomplete adjuvant.

Antigen (0.9 μg) was diluted in 50 μL of carbonate buffer (pH, 9.6) and dispensed in the odd wells of the polystyrene plates. Carbonate buffer (pH, 9.6) was dispensed into even wells as a control. After incubation for 1 hour at 37°C in a shaker, plates were washed 3 times with PBS solution supplemented with Tween 20 and then 100 μL of carbonate buffer with 3% nonfat milk was added to all wells except for 2, which were used as controls. After an incubation of 1 hour at 37°C, plates were washed as described and serum samples were dispensed at a dilution of 1:1,000 in PBS solution plus 3% nonfat milk. After an additional 1-hour incubation and washes, peroxidase-labeled protein A diluted 1:500 in PBS solution plus 3% nonfat milk was used as conjugate. After 1 hour of incubation and washes as described, tetramethylbenzidine peroxidase solution (50 μL/well) was added as substrate. The color reaction was stopped with 2M H₂SO₄. The OD was read by use of a spectrophotometer set at 450 nm. The cutoff value of the ELISA for each antigen was determined as the corrected mean OD at 450 nm of the total number of serum samples from alpacas used as controls plus 2 times the value of the SD. A pool of serum obtained from alpacas on day 30 after inoculation was used to correct the readings from the subsequent plates by use of the following formula:

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\text{OD corrected} = \frac{\text{OD sample} \times \text{OD pool of standard plate}}{\text{OD pool of observed plate}}
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Statistical analysis—The study was conducted as a completely randomized design in a factorial arrangement of treatments (groups). The model statements were day, treatment, and the interaction day × treatment. Dependent variables rectal temperature, WBC count, percentage of neutrophils, and antibody OD were analyzed by use of a software program. The experimental unit was the alpaca (n = 13), and the covariance structure selected was variance component. A probability difference of <0.05 (P < 0.05) was considered significant.

Results

Clinical findings—Alpacas inoculated ID with 1.1 × 10⁶ CFUs of *C pseudotuberculosis* of alpaca (group A) or llama origin (group B) had a febrile response with significant differences with respect to the sham-inoculated (group C) alpacas lasting up to day 16 after inoculation (Figure 1). At 96 hours after inoculation, skin of infected alpacas was warm and swollen in an area ranging from 5 to 13 cm in diameter. By the end of the first week, inflammation at these sites was character-
ized by purulent exudate through a fistula in the necrotic skin. No signs of disease were found in alpacas from group C, and no significant differences in body weight were found among the 3 groups.

Except for group B alpacas on day 3 after inoculation (data not shown), infected alpacas had variations in Hct and hemoglobin concentrations with no significant differences with respect to group C alpacas. Infected alpacas had leucocytosis ranging from 19 \( \times 10^3 \) WBCs/\( \mu L \) to 30 \( \times 10^3 \) WBCs/\( \mu L \) (Figure 2). Results of CBC analysis revealed that leucocytosis was mainly the result of an increase in the percentage of neutrophils in group A and B alpacas with significant differences with respect to group C (Figure 3). Alpacas from group C did not have significant variations in WBC counts; they remained between 14 \( \times 10^3 \) WBCs/\( \mu L \) and 17 \( \times 10^3 \) WBCs/\( \mu L \), similar to those considered as reference values in alpacas.15,16

**Bacterial culture results**—*Corynebacterium pseudotuberculosis* was recovered from the ID inoculation sites in alpacas from groups A and B within the first week after inoculation. In alpacas euthanatized on day 16 after inoculation, bacteria were not recovered from visceral organs or lymph nodes but bacteria were isolated from the inoculation site. *Corynebacterium pseudotuberculosis* was recovered from internal abscesses on days 58, 93, and 128 after inoculation in alpacas from groups A and B, except 1 alpaca (No. 9) from group B that was euthanatized on day 128 after inoculation and did not have internal lesions.

**Gross and histologic findings**—Gross lesions consisted of focal inflammation and cicatrization in infected alpacas at the inoculation site. One group A alpaca (No. 1) that was euthanatized on day 16 after inoculation had an abscess in the subcutaneous tissue at the inoculation site. Larger abscesses were seen in 1 alpaca (No. 2) from group A and 2 alpacas (Nos. 6 and 8) from group B in which the abscesses had a diameter from 3 to 5 cm, exuding a white-yellowish purulent material, and were localized in the renal lymph node and in the liver. Several small abscesses from 1 to 3 mm in diameter were seen on a surface cut in a renal lymph node of 1 alpaca (No. 3) from group A.

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**Figure 1**—Least squares means estimates of rectal temperature (°C) versus days after inoculation in alpacas infected with *Corynebacterium pseudotuberculosis* from alpaca origin (group A, diamonds; \( n = 4 \)) and llama origin (group B, squares; \( 5 \)) and in sham-inoculated alpacas (group C, triangles; \( 4 \)). Bars indicate SE. *†‡§**Significantly (\( P < 0.05, 0.01, \) or 0.001, respectively) different from group C alpacas.

**Figure 2**—Least squares means estimates of WBC count (mean \( \times 10^3 \) WBCs/\( \mu L \)) versus days after inoculation in alpacas infected with *C. pseudotuberculosis* from alpaca and llama origin and in sham-inoculated alpacas. See Figure 1 for key.

**Figure 3**—Least squares means estimates of percentage of neutrophils versus days after inoculation in alpacas infected with *C. pseudotuberculosis* from alpaca and llama origin and in sham-inoculated alpacas. See Figure 1 for key.

**Figure 4**—Least squares means estimates of antibody OD measured by an ELISA (OD at 450 nm) versus days after inoculation in alpacas infected with *C. pseudotuberculosis* from alpaca and llama origin and in sham-inoculated alpacas. Estimates on day 128 represent only 1 alpaca/group. See Figure 1 for key.
Substantial histopathologic findings were seen only in alpacas from groups A and B. Microscopic lesions consisted of granulomatous lymphadenitis observed on day 58 after inoculation. Initial lesions were present in the cortex and consisted of a slight infiltration of macrophages, epithelioid cells, and neutrophils coming from the subcortical and paracortical areas. Lymphoid hyperplasia was observed in some follicles with the presence of microabscesses (1 to 3 mm in diameter) on day 58 after inoculation. Microabscesses consisted of a major infiltration of mononuclear cells, mainly macrophages, plasma cells, and epithelioid cells, surrounding a central mass of necrotic tissue (caseous necrosis). Larger abscesses in lymph nodes consisted of a central mass of dead tissue and degenerated neutrophils, surrounded by a slight layer of mononuclear cells and macrophages (ie, liquefactive necrosis), with a thick fibrous capsule.

**ELISA results**—Antibody (immunoglobulin G) concentrations directed against the cell wall antigen from *C. pseudotuberculosis* in alpaca sera were determined (Figure 4). Sham-inoculated alpacas did not develop serum antibodies. Compared with group C alpacas, a significant antibody response was detected on day 16 after inoculation in infected alpacas from groups A and B, with peaks on days 58 and 93 after inoculation, respectively. Serum antibody titers decreased by day 128 after inoculation. Serum antibody titers were correlated mainly with the presence of lesions and a positive culture result for *C. pseudotuberculosis*. Compared with other alpacas from groups A and B, a lower serum antibody titer was observed in the 1 alpaca (No. 9) from group B that did not have internal lesions or a positive culture result for *C. pseudotuberculosis* on day 128 after inoculation.

**Discussion**

Alpacas ID inoculated with live organisms developed abscesses localized mainly at the inoculation site and internally in the renal lymph nodes. Our study on experimentally induced *C. pseudotuberculosis* infections in adult alpacas represents a slight modification to a study in goats. The ID route and inoculum size were selected in alpacas in an attempt to imitate a transmission route produced by a skin wound and the approximate number of CFUs of the bacteria contained in 1 drop of pus, respectively. Contrary to other studies conducted in lambs and goats in which ID or IV inoculation resulted in mediastinal and lung abscesses, no thoracic lesions were found in our study. Our results are similar to those found under natural conditions, in which internal abscesses were mainly localized in the renal lymph node, without lung lesions.

Signs of acute disease were observed in all the inoculated alpacas without substantial differences between groups A and B. A persistent febrile response was followed by marked inflammatory reactions at the inoculation site that exuded purulent material by the end of the first week and from which *C. pseudotuberculosis* was isolated. These results differed from those observed in lambs and goats experimentally infected with *C. pseudotuberculosis* in 2 ways. First, significant differences in rectal temperature of approximately 0.9°C with respect to sham-inoculated alpacas were recorded >7 days after the ID inoculation. Second, superficial lesions were restricted to the inoculation site without secondary dissemination to regional lymph nodes and lungs as in other ruminants.

Increased WBC counts related to an increase in the percentage of neutrophils occurred in both inoculated groups. By day 3 after inoculation, WBC counts decreased, as has been reported for lambs. The decrease in the percentage of neutrophils by day 3 after inoculation was associated with an increased macrophage response and persistence of bacteria during experimental *C. pseudotuberculosis* infection in lambs. In our study, a massive infiltration of WBCs was seen at the inoculation site, characterized by purulent yellowish exudates and a fine capsule. Despite the presence of this superficial lesion, results of *C. pseudotuberculosis* isolation and histologic staining from the internal lymph nodes were negative for alpacas euthanatized on day 16 after inoculation. Nevertheless, in alpacas euthanatized on days 58 and 93 after inoculation, in addition to a severe purulent inflammatory response at the inoculation site, chronic lesions characterized by purulent abscesses were observed mainly in renal lymph nodes and *C. pseudotuberculosis* was isolated, observed in stained tissues, or both. This fact could be accounted for by an unsuccessful inflammatory response during the initial phase of infection, during which neutrophils often kill most of the bacteria; remaining bacteria may multiply, and a persistent infection may develop. In our study, 1 alpaca (No. 9) from group B did not have internal lesions or a positive culture result for *C. pseudotuberculosis* on day 128 after inoculation. Corynebacterium *pseudotuberculosis* had only been isolated from the inoculation site of this alpaca during the first week after inoculation. These findings could be accounted for by an individual's susceptibility to the bacteria and a quick and total release of pus from the inoculation site through fistulae, thereby leading to an unsuccessful establishment of an internal infection and low serum antibody titers.

Histopathologic lesions observed in renal lymph nodes on day 58 after inoculation were characterized by granulomatous lymphadenitis, as described for lambs. With the progression of the lesions, a central mass of caseous necrosis was observed in some instances. The development of pyogranuloma with central necrosis is regularly associated with spontaneous or experimentally induced infections of *C. pseudotuberculosis* in lambs. More advanced lesions contained a connective tissue capsule surrounding a zone of epithelioid cells and macrophages with a central core of neutrophils and liquefactive necrosis (ie, abscess). In a kinetic study by Pepin et al, the granulomatous lesion limited bacterial dissemination but also allowed the persistence of viable bacteria.

In our study, all alpacas infected with field strains of *C. pseudotuberculosis* of llama and alpaca origin seroconvert to the antigen used in the indirect ELISA. Serum immunoglobulin G titers were low in 1 alpaca (No. 9) of group B euthanatized on day 128 after inoculation, from which bacteria were not recovered.
and no lesions were found. In our study, the severity of the lesions was not correlated with the strength of the antibody response in infected alpacas, similar to finding in lambs with experimentally induced *C. pseudotuberculosis*. For example, 1 alpaca (No. 8) euthanatized on day 93 after inoculation had small abscesses (1 to 5 mm in diameter) in the renal lymph node but had a higher titer than another alpaca (No. 3) euthanatized on the same day that had larger abscesses (3 to 4 cm in diameter). However, serum antibody titers in infected alpacas were higher and persisted longer than the serum antibody titers in spontaneously and experimentally infected lambs. These serologic findings suggest that the ELISA, which is easy and convenient to perform on large groups of other small ruminants, could be a reliable test for evaluating antibody responses after experimentally induced *C. pseudotuberculosis* infections in alpacas. Moreover, seropositive alpacas were detected by day 16 after inoculation, indicating a higher sensitivity than the antihemolysin or synergistic-inhibition test. Some authors reported that the use of an ELISA with exotoxin from *C. pseudotuberculosis* as antigen gave better results than with the use of the cell wall or other cytoplasmic antigens. On the other hand, Ellis et al and Sutherland et al reported better results with the use of an ELISA with a cell wall antigen in naturally acquired CLA in lambs. In our study, the ELISA results were satisfactory since the assay detected all infected alpacas, whereas the control group did not develop antibodies. Nevertheless, to evaluate this ELISA with a cell wall antigen, field studies should be performed as well as a comparison with other tests or other antigens from this bacterium.

In conclusion, an experimentally induced *C. pseudotuberculosis* (from llama and alpaca origin) infection in alpacas was confirmed on the basis of clinical pathologic findings and gross and histologic lesions. The use of an ELISA, performed with cell wall antigen, resulted in detection of all infected alpacas from day 16 to 128 after inoculation. Serum antibody titers were not related to the stage of disease but to the presence of the bacterium. The experimentally induced *C. pseudotuberculosis* infection protocol used in our study produced lesions resembling those observed in alpacas under natural conditions and would be useful for evaluating a prevention method for lymphadenitis in alpacas.

References


